ISSN 2218-6204 (online)

World Journal of *Hematology*

2014 Bound Volume 3 Issue 1-4: 1-137

Published by Baishideng Publishing Group Inc

World Journal of Hematology

A peer-reviewed, online, open-access journal of hematology

Editorial Board

2012-2016

The *World Journal of Hematology* Editorial Board consists of 123 members, representing a team of worldwide experts in hematology. They are from 29 countries, including Argentina (2), Austria (1), Belgium (1), Brazil (1), Canada (1), China (4), Croatian (1), Denmark (1), France (6), Germany (4), Greece (4), India (1), Iran (1), Ireland (1), Israel (2), Italy (11), Japan (9), Luxembourg (1), Mexico (1), Netherlands (7), Norway (2), Romania (1), Singapore (1), South Korea (2), Spain (6), Thailand (1), Turkey (5), United Kingdom (9), and United States (36).

Masao Seto, *Nagoya*
Toshiki Shimizu, *Moriguchi*
Masafumi Takahashi, *Shimotsuke*

Luxembourg

Jacques Zimmer, *Luxembourg*

Mexico

Agustin Avilés, *Mexico City*

Netherlands

Miranda Buitenhuis, *Rotterdam*
Roland P Kuiper, *Nijmegen*
Jan Jacques Michiels, *Erasmus*
Gerry AF Nicolaes, *Maastricht*
Pieter Sonneveld, *Rotterdam*
Arnold Spek, *Amsterdam*
Ruurd Torensma, *Nijmegen*

Norway

Brynjar Foss, *Stavanger*
Mikhail Sovershaev, *Tromsø*

Romania

Adriana Georgescu, *Bucharest*

Singapore

Jerry Chan, *Singapore*

South Korea

Jung Weon Lee, *Seoul*
Myung-Geun Shin, *Gwangju*

Spain

Matilde Canelles, *Granada*
Slaven Erceg, *Seville*
Pedro Cosme Redondo Liberal, *Cáceres*
Julian Pardo, *Zaragoza*
Josep-Maria Ribera, *Badalona*
Juan M Zapata, *Madrid*

Thailand

Chirayu Udomsakdi Auewarakul, *Bangkok*

Turkey

Mutay Aslan, *Antalya*
Murat Biteker, *Istanbul*
Taner Demirer, *Ankara*
Selami Kocak Toprak, *Ankara*
Ertan Yetkin, *Mersin*

United Kingdom

Dominique Bonnet, *London*
Olga Tura Ceide, *Edinburgh*
Helen A Ireland, *London*
Charles Henderson Lawrie, *Oxford*
Drew Provan, *London*
Dipak Purshottam Ramji, *Cardiff*
Sabrina Tosi, *Uxbridge*
Shao-An Xue, *London*
Jian guo Zhuang, *Liverpool*

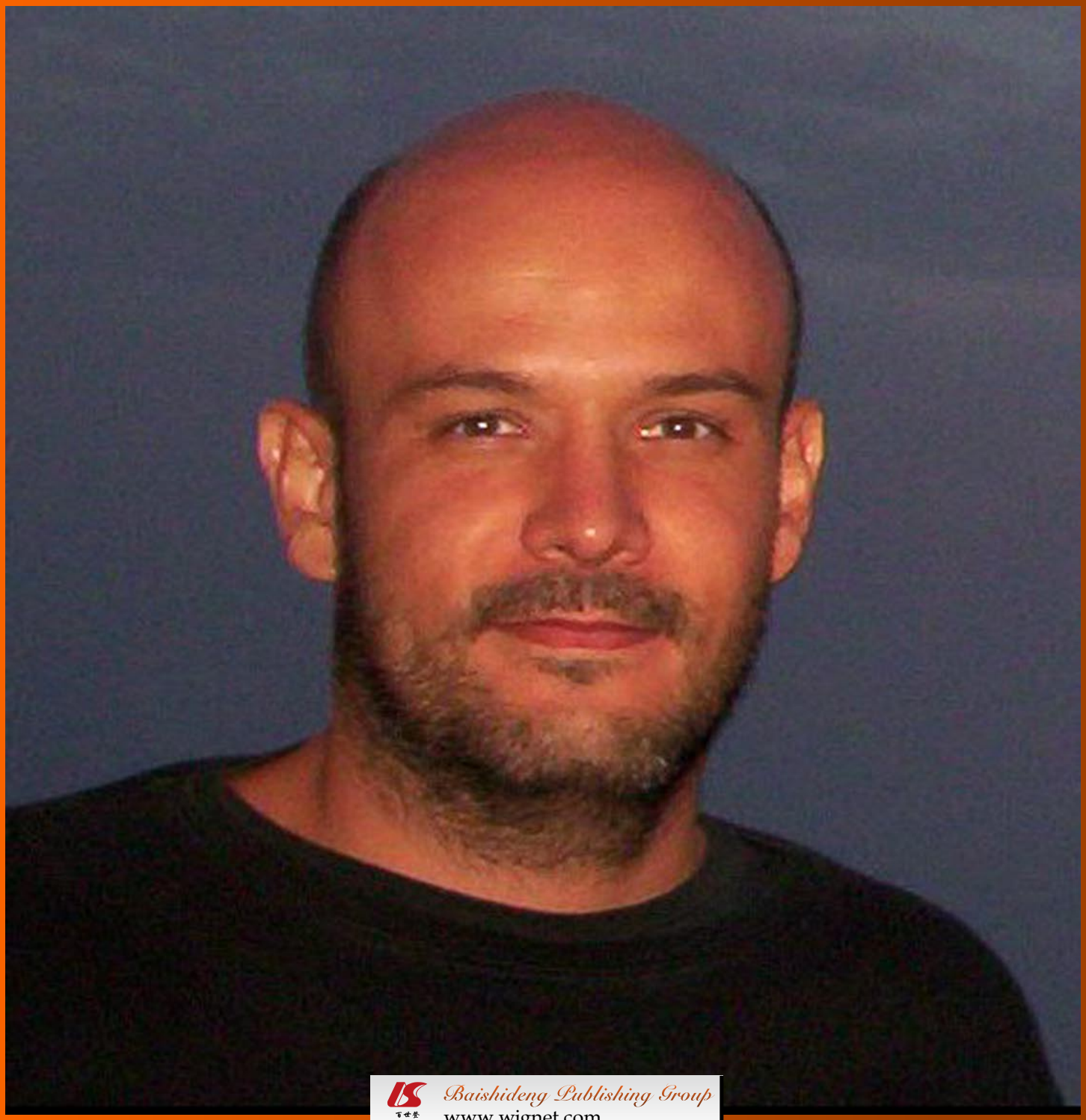
United States

Ivo Abraham, *Tucson*
Julia E Brittain, *Chapel Hill*
Chung-Che Chang, *Houston*
Edward Alan Copelan, *Cleveland*
Zeev Estrov, *Houston*
Steve Fiering, *Lebanon*
Suzanne T Ildstad, *Louisville*
Elias Jabbour, *Houston*
Ming Jiang, *Nashville*
Katsuhiro Kita, *Galveston*
Robert G Lerner, *Valhalla*
Shaoguang Li, *Worcester*
Dazhi Liu, *Sacramento*
Ming-Lin Liu, *Philadelphia*
Surya Nauli, *Toledo*
Steffan Nawrocki, *San Antonio*
Xuyang Peng, *Nashville*
Manuel L Penichet, *Los Angeles*
Luis Francisco Porrata, *Rochester*
Rehan Qayyum, *Baltimore*
L Vijaya Mohan Rao, *San Diego*
Guangwen Ren, *New Brunswick*
Xiaoping Ren, *Cincinnati*
Jatin J Shah, *Houston*
Angus M Sinclair, *Thousand Oaks*
Ali A Sovari, *Chicago*
Christopher A Tormey, *New Haven*
Olga Volpert, *Chicago*
Zack Zhengyu Wang, *Scarborough*
EllenLori Weisberg, *Boston*
Wen-Shu Wu, *Scarborough*
Yi Wu, *Newark*
Feng-Chun Yang, *Indiana*
Karina Yazdanbakhsh, *New York*
Xin-Fu Zhou, *Adelaide*

ISSN 2218-6204 (online)

World Journal of *Hematology*

World J Hematol 2014 February 6; 3(1): 1-17



Baishideng Publishing Group
www.wjgnet.com

WJH

World Journal of Hematology

Contents

Quarterly Volume 3 Number 1 February 6, 2014

World Journal of Hematology
Volume 3 Number 1 February 6, 2014

Contents

NAME OF JOURNAL
World Journal of Hematology

ISSN
ISSN 2218-6204 (online)

LAUNCH DATE
June 6, 2012

FREQUENCY
Quarterly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Hematology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjhematol@wjgnet.com
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
Hong Kong, China
Fax: +852-6555-7188
Telephone: +852-3177-9906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
February 6, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/2218-6204/g_info_20100722173604.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5315/wjh.v3.i1.1

World J Hematol 2014 February 6; 3(1): 1-17
ISSN 2218-6204 (online)

REVIEW

A concise, practical guide to diagnostic assessment for mast cell activation disease

Lawrence B Afrin, Gerhard J Molderings

Lawrence B Afrin, Division of Hematology/Oncology, Medical University of South Carolina, Charleston, SC 29425-6350, United States
Gerhard J Molderings, Institute of Human Genetics, University Hospital of Bonn, D-53127 Bonn, Germany
Author contributions: Afrin LB wrote the initial draft; Afrin LB and Molderings GJ contributed equally to the final revision processes; and Molderings GJ subsequent edits.
Correspondence to: Lawrence B Afrin, MD, BSB103, MSC635, Division of Hematology/Oncology, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425-6350, United States. afrinl@musc.edu
Telephone: +1-843-7924271 Fax: +1-843-7920644
Received: September 5, 2013 Revised: October 6, 2013
Accepted: November 15, 2013
Published online: February 6, 2014

Abstract

As recognition of mast cell (MC) involvement in a range of chronic inflammatory disorders has increased, diagnosticians' suspicions of MC activation disease (MCAD) in their chronically mysteriously inflamed patients have similarly increased. It is now understood that the various forms of systemic mastocytosis - diseases of inappropriate activation and proliferation of MCs seemingly driven by a small set of rare, usually constitutively activating mutations in assorted MC regulatory elements - comprise merely the tip of the MCAD iceberg, whereas the far larger and far more clinically heterogeneous (and thus more difficult to recognize) bulk of the iceberg consists of assorted forms of MC activation syndrome (MCAS) which manifest little to no abnormal MC proliferation and may originate from a far more heterogeneous set of MC mutations. It is reasonable to suspect MCAD when symptoms and signs of MC activation are present and no other diagnosis better accounting for the full range of findings is present. Initial laboratory assessment should include not only routine blood counts and serum chemistries but also a serum total tryptase level, which helps direct further evaluation for mastocytosis *vs* MCAS. Appropriate tissue examinations are needed to diagnose mastocytosis, while elevated levels of relatively specific mast cell mediators are sought to support diagnosis of MCAS. Whether assessing for mastocytosis or MCAS, testing is fraught with potential pitfalls which can easily yield false negatives leading to erroneous rejection of diagnostic consideration of MCAD in spite of a clinical history highly consistent with MCAD. Efforts at accurate diagnosis of MCAD are worthwhile, as many patients then respond well to appropriately directed therapeutic efforts.

Key words: Mast cell activation disease; Mastocytosis; Mast cell activation syndrome; Mast cell mediators; Tryptase; KIT mutations

Core tip: Mast cell activation disease (MCAD) is characterized by accumulation of genetically altered mast cells and/or abnormal release of these cells' mediators, affecting functions in potentially every organ system, often without causing abnormalities in routine laboratory or radiologic testing. Recent data suggest a high prevalence of MCAD. Thus, MCAD should be considered routinely in the differential diagnosis of patients with chronic multisystem polymorbidity of a generally inflammatory theme or patients in whom established diagnoses do not well account for the patient's presentation of symptoms consistent with mast cell mediator release. Mediator testing can be challenging but typically is manageable. Diagnostic efforts are worthwhile, as diagnosis often leads to effective therapy.

Afrin LB, Molderings GJ. A concise, practical guide to diagnostic assessment for mast cell activation disease. *World J Hematol* 2014; 3(1): 1-17 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i1/1.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i1.1

INTRODUCTION

Of hematopoietic origin, mast cells (MCs) are found in all human tissues, especially at the environmental interfaces and perivascular/perineural sites[1]. They serve largely as sentinels of environmental change and bodily insults and respond by releasing large and variable assortments of molecular mediators which directly and indirectly influence behavior in other local and distant cells and tissues to respond to changes and insults so as to maintain, or restore, homeostasis.

MCs and the related rare cutaneous disease urticaria pigmentosa (UP) were first discovered in the latter half of the 19th century[2,3], and the existence of seemingly even rarer systemic MC disease first became apparent in the middle of the 20th century[4,5]. For several decades it was thought that virtually all MC diseases were neoplastic, with symptoms resulting principally from accompanying inappropriate mediator release. Nearly a quarter century ago, the recurring somatic D816V mutation was discovered[6] in the dominant MC regulatory element, transmembrane tyrosine kinase receptor KIT[7], in a high proportion of patients with systemic mastocytosis (SM)[8,9]. This constitutively activating mutation has since been found to drive many features of SM including aberrant aggregation and spindled morphology of MCs in SM, tryptase and histamine overexpression, and aberrant MC surface co-expression of CD25[10-12]. Several other KIT mutations, also quite rare, have since been found in other cases of SM, mostly in KIT's kinase domain 1 at or near codon 816 or in the juxtamembrane region of KIT[11]. Recently, evidence has emerged that the KIT^{D816V} mutation induces the above-mentioned immunohistochemical and morphological changes in affected MCs but seems not to be solely responsible for the clinical symptoms of the MC disease[13,14]. This finding fits well with previous reports in healthy people bearing the KIT^{D816V} mutation in their peripheral blood leukocytes[15].

Around the same time as the discovery of KIT^{D816V}, though, the notion was first advanced that some portion of MC disease might be due to inappropriate mediator release with little to no accompanying MC proliferation[16]. This theory appeared validated when the first recognized cases of what is now called MC activation syndrome (MCAS) were published recently[17,18]. Although not yet independently confirmed, there soon followed provocative, repeated findings from one group of a very wide array of (presumably mostly constitutively activating) mutations scattered across all domains of KIT in small cohorts of MCAS patients[19,20]. Many of these patients appeared to bear multiple mutations in MC KIT, with no apparent recurring patterns. Similar mutational complexity has been found, too, across the spectrum of chronic myeloproliferative neoplasms (MPNs) within which the MC disorders reside[21], and in advanced mastocytosis itself[22].

In recognition of the fact that all MC disease is, first and foremost, disease of inappropriate MC activation, Akin *et al*[23] have proposed a new umbrella term of MC activation disease (MCAD) to describe the full spectrum of MC disease (Figure 1). MCAS is estimated to be more prevalent[13,24], but also more difficult to recognize, than other diseases traditionally ascribed to MC dysfunction. It also has been proposed that the various systemic MCAD variants and clinical phenotypes represent not distinct disease entities but rather varying presentations of a common generic root process of mast cell dysfunction[13]. The various forms of mastocytosis (principally cutaneous and systemic) may be the tip of a proverbial MCAD iceberg, fairly readily recognizable (in spite of their rarity) because of their defined unique immunohistochemical and relatively uniform clinical presentations, while the bulk of the iceberg - hidden below the waterline of easy clinical recognizability - may be a far larger, and far more heterogeneous, collection of variants of MCAS, some specifically named (*e.g.*, idiopathic anaphylaxis[18], cryopyrin-associated periodic syndrome[25]) but most not[23,26]. It seems logical that marked mutational heterogeneity would drive the marked heterogeneity of aberrant mediator expression and clinical presentation which are observed in MCAD and which can easily confound the diagnostician. For example, although MCAD can readily impact any or all systems in the body, cases have been described of MCAS causing hematologic presentations as diverse as pure red cell aplasia[27], erythrocytosis[28], and agranulocytosis[29].

Limited familial studies performed to date interestingly show high familial loading of MCAD despite a typical absence of germline mutations[24]; instead, most of the few families carefully studied show distinct sets of somatic mutations in the affected members with correspondingly varying clinical presentations. Clinical correlation suggests acquisition of the initial disease-causing mutations occurs relatively early in life, but additional mutations may develop in subclones over time. Efforts to reconcile the apparent dichotomy of high familial loading of MCAD with absence of apparent germline genetic mutations in affected families have led to thoughts that certain epigenetic alterations (GJM, unpublished data) may confer a state of genetic fragility which, upon interaction with varying biochemical milieus induced by assorted stressors (*e.g.*, infection), in turn

Figure 1 Spectrum of mast cell disease. SM-AHNMD: Systemic mastocytosis with associated clonal hematologic non-mast-cell-lineage disorder.

Form of disease		Features
Cutaneous mastocytosis		Usually found in childhood; prevalence in the general population of approximately 0.01%[116,117]; various forms such as urticaria pigmentosa or telangiectasia macularis eruptiva perstans; diagnosed by skin biopsy
SM		Usually emerges in middle age; prevalence in the general population of approximately 0.0003%[13]
	Indolent SM	Survival equal to that of the general population[118]
	Smoldering SM	Proposed category
	SM with associated clonal hematologic non-MC-lineage disorder	*e.g.*, SM with lymphoma or leukemia; outcomes are best if the SM and the AHNMD are treated concurrently[38]
	Aggressive SM	SM with end-organ failure such as hepatic fibrosis, portal hypertension, malabsorption, or cytopenias
	Mast cell leukemia	Rare; the most lethal and most therapeutically challenging of all mast cell diseases
Mast cell sarcoma		Rare
Extracutaneous mastocytoma		Rare

Figure 2 Diagnostic classification of mastocytosis[33]. SM: Systemic mastocytosis; AHNMD: Associated clonal hematologic non-mast-cell-lineage disorder.

induces MCAD-causing mutations in MC regulatory elements.

Diagnosticians willing to pursue the possibility of MCAD in their patients whose chronic multisystem polymorbidity (often, but not always, of a generally inflammatory theme) has defied extensive prior diagnostic efforts (typically focused on subspecialty-oriented symptom subsets rather than broad multisystem considerations) currently face many challenges including controversies in precise diagnostic criteria (particularly for MCAS), uncertainty regarding the utility of various diagnostic tests, and difficulties properly managing specimens for such testing. Whether assessing for mastocytosis or MCAS, testing is fraught with potential pitfalls which can easily yield erroneous conclusions that MCAD is not present and thereby potentially add yet much more time and cost to the very long and expensive path most patients with MCAD (particularly MCAS) require to establish diagnosis, let alone effective therapy. We describe here in detail our thoughts regarding current proposals for diagnostic criteria for MCAD and our approach to diagnostic testing for MCAD, *i.e.*, both mastocytosis and MCAS.

DIAGNOSTIC CRITERIA FOR MCAD

Classification systems and diagnostic criteria for mastocytosis were first proposed in the 1980s[30,31]. Since 2001 the World Health Organization (WHO) consensus criteria have guided classification and diagnosis globally[32]. The latest revision of these criteria, published in 2008[33], divide mastocytosis broadly into cutaneous and systemic forms as well as the even rarer solid MC tumors of MC sarcoma and extracutaneous mastocytoma (Figure 2).

The WHO 2008 consensus diagnostic criteria for SM are shown in Figure 2. Given that it is now understood that MC KIT codon 816 mutations (a minor criterion) drive MC aggregation (a major criterion) and certain other minor criteria including MC spindling, tryptase overexpression, and CD25 co-expression, reorganization of the WHO diagnostic criteria for SM may be in order.

Recognition of the need for an alternative, MCAS-like diagnosis came about in part because of the discovery of patients whose clinical presentation was highly consistent with SM but who did not satisfy the major SM diagnostic criterion and only satisfied one or two of the minor criteria. In particular, many patients significantly affected by aberrant MC activation previously have been denied diagnosis of, and treatment for, MCAD because of a serum tryptase level < 20 ng/mL. Presently there are two principal proposals for diagnostic criteria for MCAS (Figure 3). Potential problems with the Valent *et al*[34] criteria include (1) non-recognition of many of the symptoms[28] that can result from MC activation; (2) lack of published validation that the described tryptase increase reliably distinguishes ordinary baseline fluctuation of tryptase from fluctuation induced by aberrant MC activation; (3) practical difficulties in providing/obtaining a specimen for serum total tryptase within 4 h of onset of an exacerbation of symptoms; and (4) practical difficulties in many patients in finding - in such a heterogeneous disease - MC-targeting agents that can effect at least partial response. Thus, for example, patients whose aberrant MC activation causes substantial muscle/joint/bone aching, constipation and abdominal pain, paresthesias, adenitis, and cognitive dysfunction but not the symptoms listed in these criteria would not qualify for the diagnosis. Similarly, a rise in serum tryptase (whose normal range typically is approximately 0.5 to 11 ng/mL) from 2.0 ng/mL to 4.2 ng/mL would be considered "20% + 2 ng/mL" evidence of aberrant MC activation, but a rise to 4.1 ng/mL would not. Furthermore, patients often are sufficiently disabled during a flare of symptoms that they cannot easily get to a medical center, and those who do travel to an urgent care facility or emergency department often encounter providers resistant to pursuing tests not needed for immediate care of the presenting symptoms.

WHO 2008 diagnostic criteria for systemic mastocytosis[33]

Major criterion:

Multifocal, dense aggregates of MCs (15 or more) in sections of bone marrow or other extracutaneous tissues and confirmed by tryptase immunohistochemistry or other special stains

Minor criteria:

1 Atypical or spindled appearance of at least 25% of the MCs in the diagnostic biopsy
2 Expression of CD2 and/or CD25 by MCs in marrow, blood, or extracutaneous organs
3 KIT codon 816 mutation in marrow, blood or extracutaneous organs
4 Persistent elevation of serum total tryptase > 20 ng/mL

Diagnosis of SM made by either (1) major criterion + any one or more minor criteria; or (2) any three minor criteria

Proposed diagnostic criteria for MCAS: Valent *et al*[34] criteria

1 Chronic/recurrent symptoms (flushing, pruritus, urticaria, angioedema, nasal congestion or pruritus, wheezing, throat swelling, headache, hypotension, and/or diarrhea) consistent with aberrant MC mediator release
2 Absence of any other known disorder that can better account for these symptoms
3 Increase in serum total tryptase of 20% above baseline plus 2 ng/mL during or within 4 h after a symptomatic period
4 Response of symptoms to histamine H_1 and/or H_2 receptor antagonists or other "MC-targeting" agents such as cromolyn

Proposed diagnostic criteria for MCAS: Molderings *et al*[26] criteria

Major criteria:

1 Multifocal MC aggregates as per WHO major criterion for SM
2 Clinical history consistent with chronic/recurrent aberrant MC mediator release (symptoms per Table 4 in[28])

Minor criteria:

1 Abnormal MC morphology as per WHO SM minor criterion 1
2 CD2 and/or CD25 expression as per WHO SM minor criterion 2
3 Detection of known constitutively activating mutations in MCs in blood, marrow, or extracutaneous organs
4 Elevation in serum tryptase or chromogranin A, plasma heparin or histamine, urinary N-methylhistamine, and/or other MC-specific mediators such as (but not limited to) relevant leukotrienes (B4, C4, D4, E4) or PGD2 or its metabolite 11-β-PGF2α

Diagnosis of MCAS made by either (1) both major criteria, or (2) the second major criterion plus any one of the minor criteria, or (3) any three minor criteria

Figure 3 Diagnostic criteria for systemic mastocytosis and mast cell activation syndrome. WHO: World Health Organization; MCs: Mast cells; MCAS: Mast cell activation syndrome; SM: Systemic mastocytosis PG: Prostaglandin.

Such resistance often persists even when the patient presents a prescription from a MC disease specialist specifically requesting MC mediator testing at times of such flares. Finally, in concert with the observed marked heterogeneity of the clinical presentation of MCAS (perhaps due to underlying marked mutational heterogeneity), some MCAS patients benefit little from the first few or several MC-targeting therapies tried, risking premature rejection of the diagnosis.

A potential problem with the Molderings *et al*[26] criteria for diagnosis of MCAS is the lack of mention of excluding other diagnoses (including mastocytosis) better accounting for the full range of findings in the patient, but such an exclusion would seem to be implicit. In practice, this scheme most often leads to diagnosis of MCAS by pairing of the second major criterion with the last of the minor criteria, and it appears to permit applying the diagnosis of MCAS - and therefore also pursuing therapy for MCAS - in a wider population of otherwise mysteriously chronically multi systemically ill patients, possibly increasing risk of misdiagnosis.

There have been no studies of the diagnostic accuracy or efficiency of these two diagnostic schemes. Also, at present there are no "gold standards" distinguishing levels of MC mediators seen in normal MC activation/reaction from levels seen in aberrant MC activation. We believe such distinctions will be difficult to develop, therefore also making it difficult to perform comparative studies of accuracy of different diagnostic schemes. Routine use of MC whole exome/genome sequencing and mutational analysis in patients with clinical histories suspicious for MCAD may become the most efficient route to definitively diagnosing MCAD, but currently the specific polymerase chain reaction (PCR) assay for KIT^{D816V} (found often in SM but seldom in MCAS) is the only mutational analysis routinely available in most clinics. At present, though, it is worth noting that in view of the demonstrated great mutational complexity across the MPN spectrum, it may be premature to declare any given MCAS patient's disease as "non-clonal" based on negative clonality evaluations less complete than whole KIT sequencing. The term "uncertain clonality" may be more accurate.

Given the very limited mutational and clonality testing for MCAD presently available in most clinics, laboratory evaluation for MCAS will continue, at least for the near future, to depend far more on demonstration of elevated levels of MC-specific mediators.

DIAGNOSIS OF MCAD

Establishing suspicion

Accurate diagnosis of any condition begins with inclusion of the correct diagnosis in the considered differential diagnosis. Despite its rarity, development of clinical suspicion for mastocytosis is somewhat easier than for MCAS given the often flagrant nature of the clinical presentation, either with classic appearance of UP or telangiectasia macularis eruptiva perstans (TMEP) in cutaneous mastocytosis or with classic appearance of recurrent unprovoked flushing and/or anaphylaxis in systemic mastocytosis.

Initial suspicion of MCAS is more challenging due to its heterogeneity and, often, lack of flagrant acute presentation. Diagnosticians need to be cognizant that MCAD can affect every system, usually affects multiple systems, and usually manifests symptoms in a subacute or chronic waxing/waning or episodic fashion, though episodes also can arise acutely, *i.e.*, the so-called "flares" or "attacks" or "spells" that many patients have of one symptom set or another. Many symptoms often are categorized as inflammatory in nature (*e.g.*, pain, diarrhea), though non-inflammatory symptoms (or at least symptoms not traditionally thought to be inflammatory in nature) are prevalent, too (*e.g.*, fatigue, paresthesias).

Another clue that MCAD may be present is simply the oddity or unexpectedness of certain clinical events or findings in the patient. Given the very large array of mediators normally produced and released by the MC[35] and therefore potentially abnormally expressed in MCAD, with each mediator causing a unique, and usually wide, range of direct and indirect, local and distant effects, the potential for the disease to manifest "odd", "weird", "strange", "inexplicable" and "bizarre" clinical presentations is substantial. Such descriptors often are found throughout MCAD patients' charts for years prior to diagnosis. Especially when seen in the context of pre-existing chronic multisystem inflammatory illness, the appearance in the patient of "unusual" new clinical phenomena should provoke consideration of MCAD. (Of course, the diagnostician is abetted in such recognition by remaining abreast of the steadily enlarging scope of the chronic inflammatory diseases. For example, diabetes mellitus type 2, obesity, and atherosclerotic vascular disease have been recognized as chronic inflammatory diseases only relatively recently.)

By the time MCAD is diagnosed, most patients have seen many providers, have undergone extensive testing (often frustratingly yielding normal or non-specifically/minimally abnormal results), and have been assigned many diagnoses (often with less than a full measure of confidence) which explain assorted subsets of findings but do not well account for the full range of findings, including chronicity often dating back decades. Treatments for these preliminary diagnoses sometimes help somewhat but often do not help at all, further perplexing the diagnostician. It is reasonable to suspect MCAD when at least several symptoms and signs of MC activation are present (Table 1)[26,28,36,37] and no other diagnosis better accounting for the full range of findings is present. In particular, presence of a definitively diagnosed condition (*e.g.*, lymphoma) which does not well account for all symptoms and findings (*e.g.*, presyncope, erythrocytosis, *etc.*), or poor response of a definitively diagnosed condition to standard treatment for that condition, should raise suspicion for the presence of either a significant independent diagnosis or an alternative underlying ailment better accounting for the full range of symptoms and findings. In such cases, definitive diagnosis of a comorbid (and potentially even underlying) MCAD permits dual-directed therapy[38] which may lead to improved outcomes. Standardized detection of a MC mediator release syndrome can be achieved by a validated questionnaire (Figure 4). Although routine complete blood counts (CBCs) and metabolic panels often are confoundingly normal in MCAD patients, it is also the case that abnormalities in these and other common blood tests in these patients are commonly seen and are typically modest and stable. Frequent, though not necessarily constant, relative or absolute monocytosis, eosinophilia, basophilia, and/or reactive lymphocytosis, typically to just modest degrees, can be seen along with similar patterns of abnormality in routine chemistries such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP) (LBA and GJM, unpublished data), hyperbilirubinemia, and diet-independent hypercholesterolemia and hypertriglyceridemia[39]. Viewed in encounter-specific isolation, such abnormalities often raise no concern and garner no further attention, but trend analysis made easy by electronic medical record systems can quickly highlight the persistence of these abnormalities and permit expansion of the considered differential diagnosis to include entities such as MCAD which can account for these additional findings.

Mastocytosis or MCAS? Initial laboratory assessment

It is now understood that the serum total tryptase level much more reflects the total body MC load than the total body MC activation state[40-42]. As such, serum tryptase is expected to be elevated in mastocytosis but usually is elevated little to none in MCAS. "Spillover" into blood circulation of tryptase and other MC mediators initially released in the tissues can be influenced by many factors. Hence, a normal value for a mediator in blood or other bodily fluid does not rule out its unregulated release in tissue. In fact, about 20% of SM patients present with serum tryptase < 20 ng/mL[37,43,44], but such patients rarely harbor the more advanced forms of SM [aggressive SM (ASM) and MC leukemia (MCL)] which require therapeutic approaches different from those used for the more common indolent SM and MCAS. Marrow aspiration/biopsy, an uncomfortable procedure for some patients, is warranted in cases of suspected mastocytosis but seldom yields diagnostic findings in cases of MCAS not already manifesting significant hematologic abnormalities (LBA and GJM, unpublished data). Therefore, a serum tryptase persistently elevated to > 20 ng/mL in two or more spec-

Table 1 Symptoms and findings in mast cell activation disease

System	Potential manifestations of MCAD (most are chronic, low-grade; some are persistent, but many are either episodic or fluctuant)
Constitutional	Fatigue, malaise, asthenia, "chronic fatigue syndrome", subjective and/or objective hyperthermia and/or hypothermia, "sense of feeling cold much of the time", sweats/diaphoresis (not always nocturnal), flushing, plethora or pallor, increased or decreased appetite, early satiety, weight gain or loss, pruritus, chemical and/or physical environmental sensitivities (often odd)
Dermatologic/ integument	Rashes and lesions of many sorts (classic urticaria pigmentosa, "freckles", telangiectatic/angiomatous lesions, xerosis, warts, tags, folliculitis, ulcers, dyshidrotic eczema, diffusely migratory but sometimes focally persistent patchy macular erythema), pruritus (often diffusely migratory, sometimes aquagenic), flushing, angioedema, striae, dermatographism, hair thinning and alopecia, onychodystrophy (brittle nails, longitudinal ridges), poor healing
Ophthalmologic	Irritated eyes, increased or decreased lacrimation, suffusion, conjunctivitis, episodic difficulty focusing, lid tremor/tic (blepharospasm), solar sensitivity, infectious or sterile inflammation
Otologic/osmic	Infectious or sterile otitis externa and/or media, hearing loss or hyperacusis, tinnitus, otosclerosis, dysosmia, coryza, congestion
Oral/oropharyngeal	Pain or irritation (sometimes "burning"), leukoplakia, fibrosis, lichen planus, ulcers, sores, angioedema, dental decay, dysgeusia, throat tickle/discomfort/irritation/pain, post-nasal drip
Lymphatic	Adenopathy, usually sub-pathologic and often waxing/waning in size, sometimes asymptomatic but not uncommonly tender, sometimes focal, sometimes migratory, pathology usually shows reactive lymphocytosis or sometimes an atypical non-specific lymphoproliferative disorder; left upper quadrant discomfort (likely from release of mediators from splenic mast cells with or without detectable splenomegaly)
Pulmonary	Rhinitis, sinusitis, pharyngitis, laryngitis, bronchitis, pneumonitis (often confused with infectious pneumonia), cough, dyspnea (often low-grade, inconstant, "I just can't catch a deep breath" despite normal pulmonary function tests), wheezing, obstructive sleep apnea, pulmonary hypertension
Cardiovascular	Presyncope (lightheadedness, weakness, dizziness, vertigo) and/or syncope (patients may have been diagnosed with postural orthostatic tachycardia syndrome or neurocardiogenic syncope), hypertension and/or hypotension, palpitations, dysrhythmias, chest discomfort or pain (usually non-anginal in character), coronary and peripheral arterial atherosclerosis/spasm/infarction, idiopathic acute or chronic heart failure (*e.g.*, takotsubo), aneurysms, hemorrhoids, varicosities, aberrant angiogenesis (hemangiomas, arteriovenous malformations, telangiectasias), migratory edema (often non-dependent and with normal cardiac and renal function)
Gastrointestinal	Aerophagia, angioedema in any segment of the luminal tract, dysphagia (often proximal, possibly due to pharyngeal angioedema), bloating/gas, pain/inflammation (often migratory) in one or more segments of the luminal tract (from esophagitis to proctitis) and/or one or more solid organs (*e.g.*, hepatitis, pancreatitis), queasiness, nausea, vomiting (sometimes "cyclical"), diarrhea and/or constipation (often alternating), malabsorption (more often selective micronutrient malabsorption than general protein-calorie malabsorption), ascites either from portal hypertension and/or peritoneal serositis; gastroesophageal reflux disease (often "treatment-refractory") and inflammatory/irritable bowel syndrome are common pre-existing diagnoses
Genitourinary	Inflammation (often migratory) in one or more segments of the luminal tracts (ureteritis, cystitis, urethritis, vaginitis, vestibulitis) and/or one or more solid organs (*e.g.*, nephritis, prostatitis), chronic kidney disease, endometriosis, chronic low back pain or flank pain or abdominal pain, hydronephrosis (likely from ureteral angioedema), infertility, erectile dysfunction, decreased libido; in the appropriate setting of multisystem morbidity, miscarriages should prompt consideration of antiphospholipid antibody syndrome potentially due to MCAD
Musculoskeletal	Clinical myositis, often diffusely migratory (fibromyalgia is a common pre-existing diagnosis), subclinical myositis (*i.e.*, asymptomatic elevated creatine kinase not otherwise explained), arthritis (typically migratory), joint laxity/hypermobility (patients may have been diagnosed with Ehlers-Danlos Syndrome Type Ⅲ), osteoporosis/osteopenia, osteosclerosis, sometimes mixed osteoporosis/osteopenia/osteosclerosis; MCAD-driven musculoskeletal pain not uncommonly is poorly responsive to non-steroidal anti-inflammatory drugs and narcotics
Neurologic	Headache (esp. migraine), presyncope and/or syncope, peripheral (usually distal) sensory and/or motor neuropathies including paresthesias, tics, tremors (typically resting), chronic inflammatory demyelinating polyneuropathy, seizure disorders (can be "treatment-refractory"), pseudoseizures, dysautonomia
Psychiatric	Mood disturbances (*e.g.*, anger, depression), bipolar affective disorder, attention deficit-hyperactivity disorder, post-traumatic stress disorder, anxiety and panic, psychoses, memory difficulties, word-finding difficulties, other cognitive dysfunction, wide variety of sleep disruptions
Endocrinologic/metabolic	Abnormal electrolytes (including magnesium) and liver function tests, delayed puberty, dysmenorrhea, endometriosis, osteosclerosis and/or osteoporosis, hypothyroidism, hyperthyroidism, dyslipidemia, hyperferritinemia, selective vitamin and/or other micronutrient deficiencies, weight change, possibly diabetes mellitus
Hematologic/coagulopathic	Polycythemia or anemia (may be macrocytic, normocytic, or microcytic), leukocytosis or leukopenia, chronic (usually mild) monocytosis or eosinophilia or basophilia, thrombocytosis or thrombocytopenia, arterial and/or venous thromboembolic disease, "easy" bruising/bleeding; in mast cell activation syndrome the marrow usually does not show increased (or even flow-cytometrically aberrant) mast cells; marrow histology often read as normal or as unspecified myelodysplastic/myeloproliferative syndrome; standard cytogenetic studies are almost always normal or show culture failure
Immunologic	Type Ⅰ, Ⅱ, Ⅲ and Ⅳ hypersensitivity reactions, increased risk for malignancy, autoimmunity, impaired healing, increased susceptibility to infection, elevated or decreased levels of one or more isotypes of immunoglobulin; modest monoclonal gammopathy of undetermined significance not uncommon

MCAD: Mast cell activation disease.

imens facilitates an initial decision whether to evaluate further for SM with biopsies as appropriate including at least marrow, and possibly also skin, upper and lower gastrointestinal (GI) tract mucosa, and potentially other tissues, especially those at the environmental interfaces. An initial serum tryptase < 20 ng/mL makes SM (especially ASM and MCL) much less likely, and since the prognosis of, and therapeutic approach toward, indolent SM and MCAS are presently indistinguishable, there appear to be no adverse consequences if the clinician initially misdiag-

Clinical signs

The patient complains about recurring or continuing burning and/or crampy abdominal pain of unknown cause and/or recurring or continuing diarrhea of unknown cause and/or frequently intense meteorism/gassiness (independent of the composition of diet) and/or about episodically occurring nausea. □ 1

The symptoms respond to treatment with H1-antihistamines. □ 1

The progression of the symptoms occurred in episodes □ 1
with symptom-free periods becoming shorter. □ 1

The patient complains about episodically occurring burning and/or choking chest pain attacks, which are often experienced as life-threatening. Electro cardiographic findings are without pathological signs. □ 1

The patient complains about occasional or continuing pain in the urinary bladder and/or pelvis accompanied by painful desire to void and/or blood in the urine. There is no bacteriuria. □ 1

The patient complains about occasional or continuing paresthesia (burning, pins and needles, numbness) and/or pain which does not respond to treatment with analgesics. □ 1

Gastroscopy and biopsies from the stomach and duodenum
are without pathological findings. □ 0
or
show minor signs of inflammation. □ 1
or
show *Helicobacter pylori*- and NSAID-negative erosion and/or ulcer. □ 3
or
show clusters of mast cells and/or a considerable number of spindle-shaped mast cells and/or CD25-positive mast cells. □10

Colonoscopy and intestinal biopsies
are without pathological findings. □ 0
or
show minor signs of inflammation. □ 1
or
show melanosis coli (abuse of anthra-cenediones ruled out). □ 1
or
show clusters of mast cells and/or a considerable number of spindle-shaped mast cells and/or CD25-positive mast cells. □10

The patient reports the following signs of episodically occurring symptoms of autonomic dysfunction:
tachycardia or
palpitation/dysrhythmia □ 1
flush (redness, feeling of heat) □ 2
hot flash, sweat □ 2
paroxysmal hypo/hypertension with
dizziness to the point of syncope □ 2

Although there are no pathological findings in routine laboratory parameters and imaging methods, the patient present with
a pronounced asthenia. □ 1
fatigue. □ 1
loss in weight. □ 1

During the symptomatic periods of the disorder the patient is
afflicted with anal pruritus and/or anal eczema. □ 1
Intestinal adhesions are present without prior history of abdominal surgery □ 1

Triggering factors

Deprivation of sleep □ 1
Fasting for 24 h □ 1
Histamine containing food (*e.g.*, red wine, cheese, tuna) □ 1

Laboratory parameters

The patient shows signs of a bleeding diathesis (*e.g.*, abnormal secondary bleeding or bruises after minimal trauma and/or lesions). □ 1
During the symptomatic periods of the disorder the patient showed, at least once, hyperbilirubinemia (up to 2.5 mg/dL), and/or an increase of trans amidases (up to twice their upper limits of normal) and/or diet-independent hypercholesterolemia (up to 300 mg/dL). □ 1

There are low titers of autoantibodies without clinical signs in the organs or tissues against which the autoantibodies are directed. □ 1

The serum total tryptase was normal. □ 0
or
was elevated > 11 and < 20 ng/mL. □ 3
or
was elevated more than 20 ng/mL. □10

The level of heparin in blood was normal. □ 0

or	
was elevated > 0.05 anti-Factor Xa units/mL.	□ 3
The level of N-methylhistamine in a 12-hurine collection was normal.	□ 0
or	
was marginally elevated.	□ 1
or	
was elevated up to tenfold of the reference value.	□ 5
or	
was elevated by more than tenfold of the reference value.	□10
Imaging methods	
The patient has splenomegaly and/or hepatomegaly.	□ 1
The patient has bone pain with signs of osteoporosis and/or osteopenia and/or osteosclerosis.	□ 1
Medical history	
The patient shows involvement of the skin in terms of	
brown-Reddish maculopapulous rash/eruption.	□ 2
angioedema of the lips, lids of the eye, infraorbital.	□ 2
pruritus (itching) without rash/eruption and/or disease-related folliculitis	□ 1
a clear increase in the number of telangiectasias.	□ 1
The patient reports sudden attacks of migraine-like headache.	□ 1
The patient reports memory loss (ability to remember names or words) and/or concentration difficulty and/or sleep disturbances.	□ 1
The patient reports tinnitus attacks and/or ocular discomfort (dry eyes, red eyes, stinging eyes) and/or rhinorrhea/chronic nasal congestion and/or stomatitis (score if two or more of these symptoms are present).	□ 1
The patient reports non-allergic respiratory ailments such as asthma, compulsion to clear the throat, titillating/ticklish feeling in the respiratory tract and/or shortness of breath during routine tasks.	□ 1
In the past, common viral infections of the upper respiratory tract were frequently complicated by bacterial superinfection.	□ 1
The patient can state precisely the date of the first clinical manifestation of the mast cell mediator release syndrome because it appears to him to be associated with an infectious disease.	□ 1

Figure 4 Validated questionnaire to recognize symptoms as part of a mast cell activation disease in a standardized manner (modified from[39,119]). The indicated values for those items acknowledged by or found in the patient are summed. A total score above 8 but less than 14 indicates a pathological activation of mast cells. At a total score of 14 and more, a systemic mast cell mediator release syndrome is clinically verified. NSAID: Non-steroidal anti-inflammatory drugs.

noses the uncommon low-tryptase SM as MCAS.

Tryptase has been well established as a highly specific MC mediator[45]. Its biology is complex; many isoforms with different behaviors and functions have been elucidated[46]. It is heat-labile[47,48] and has a relatively short half-life *in vivo* (from 6-8 min in healthy subjects to 1.5-2.3 h in patients with hypersensitivity reactions), longer (approximately 4 d) in separated serum[34,49]. The WHO 2008 diagnostic criteria for mastocytosis call only for the measurement of total serum tryptase, setting a threshold of 20 ng/mL as the minimum level consistent with a diagnosis of SM[33]. It is unclear whether measurement of specific isoforms of tryptase would be beneficial in diagnosing MCAD in any form, and thus at present only the measurement of total serum tryptase can be recommended in the evaluation of a patient suspected of having MCAD. Although the serum total tryptase usually is not elevated in MCAS[20,26], an elevated level (though almost always < 20 ng/mL) can be found in a minority of MCAS patients, and any elevation at all can help buttress a diagnostician's suspicions of the involvement of MC activation in the patient's illness.

If the serum tryptase persistently exceeds 20 ng/mL, or if other clinical features of the presentation are characteristic for SM (*e.g.*, initial onset of symptoms in middle age, MCs observed in the peripheral blood smear, *etc.*), then further evaluation for SM is warranted. Marrow aspiration/biopsy is a standard part of such evaluation, and given both the patchy distribution of the disease in involved tissues and the observation that unilateral marrow biopsies are non-diagnostic in one-sixth of patients ultimately diagnosed with SM[50], bilateral aspirations/biopsies are preferred. Core biopsies should undergo routine staining as well as MC-targeted immuno/histochemical staining (*e.g.*, CD117, CD25, CD2, tryptase, Giemsa, toluidine blue, Alcian blue, *etc.*). There is no particular subset of these stains which has been established as a standard initial assessment. It has been proposed that CD30, too, be routinely examined when assessing for MCAD[38]. Also, CD68 - more classically associated with macrophages - can be displayed by MCs and thus may be useful in diagnosing MCAD[51]. All of the above having been said, MCs express surface CD117 (the extracellular portion of *c-kit*) roughly an order of magnitude more brightly than any other CD117-expressing cell, so often only CD117 staining is needed to estimate MC density and to characterize aggregation. Each aspirate should be sent for standard cytogenetic

analysis as well as multi-color flow cytometric assessment for co-expression of cell-surface doublets CD117/CD25 and CD117/CD2; occasionally even the triplet of CD117/CD25/CD2 is seen[52]. These flow cytometric signatures are widely considered to be pathognomonic for monoclonal MC disease. Each aspirate should be subjected to the full extent of MC mutational analysis available at the time, though at present this typically is limited to PCR analysis for the KIT^{D816V} mutation. If leukocytosis, erythrocytosis, or thrombocytosis is present, assessment is also warranted for the Janus kinase 2 (JAK2) mutations often found in the MPNs (and perhaps the $MPL^{W515L/K}$ mutation, too, in cases of leukocytosis or thrombocytosis without erythrocytosis which might signal the presence of essential thrombocythemia or myelofibrosis). Similarly, if prominent eosinophilia is observed, tyrosine kinases platelet-derived growth factor receptor alpha (PDGFRα), PDGFRβ, and fibroblast growth factor receptor 1 should be assessed for mutations as recently reviewed elsewhere[53].

The presence of skin lesions characteristic of UP or TMEP warrants biopsy of one or more such lesions, to be immediately processed with routine staining and the above-noted immunohistochemical staining plus assessment for clonality with flow cytometry. As solid tissue flow cytometry requires fresh tissue but PCR mutation analysis does not, it may be reasonable to defer PCR of skin lesion biopsies until other testing finds at least some evidence suggestive of MC disease.

Spontaneously appearing/disappearing, diffusely migratory macular erythematous patchy rashes are common in MCAS, as are scatterings of small ulcerative lesions, too. In the authors' experience, though, biopsies of such sites rarely reveal increased or otherwise aberrant MC populations, suggesting such lesions may be merely the end result of aberrant release of mediators from distantly located MCs.

If clinical suspicion for SM persists (whether due to serum tryptase persistently > 20 ng/mL or other factors) but two or more marrow biopsies (and possibly also biopsies of suspect skin lesions) are negative, upper and lower endoscopic examination of the gastrointestinal tract is reasonable. Biopsies should be performed of any macroscopically apparent lesions, but blind biopsies of macroscopically normal mucosa should be pursued, too, at multiple points along the tract. All such biopsies should be processed as described above for skin biopsies. Several studies have reported that mast cell counts in GI or genitourinary (GU) tract mucosal biopsies normally number fewer than 20 per high power field[54-58]. However, cut-offs between "normal" and "abnormal" numbers of non-aggregated mast cells are not addressed in either of the two currently proposed diagnostic schemes for MCAS. Furthermore, it is important to remember that the diagnosis of any form of MCAD rests in meeting a set of criteria, so an isolated finding of "increased" tissue mast cells does not establish the diagnosis regardless of the cut-off used to define "increased", and especially when such cells are not tightly clustered. In the context of other data meeting diagnostic criteria, though, a finding of "increased" tissue mast cells lends additional credence to the diagnosis.

MCs are highly pleomorphic. On routine hematoxylin and eosin (H and E) staining, MCs can be indistinguishable from lymphocytes, plasma cells, macrophages, histiocytes, or spindle cells[59,60]. Thus, in MC disease, not all "lymphocytes" seen with H and E staining are necessarily lymphocytes, and immuno/histochemical staining is required in cases of suspected MCAD. Not infrequently, because of their chronic idiopathic (and often treatment-refractory) gastrointestinal symptoms, patients being evaluated for MCAD have previously undergone GI tract endoscopies with biopsies. Thus, simple reassessment of archived tissue with MC-targeted immuno/histochemical staining may be sufficient to demonstrate that the "benign mucosa" or "mild chronic inflammation" previously seen with H and E staining actually harbors increased MCs. As PCR testing can be performed on preserved tissue, positive immuno/histochemical findings on archived tissue should lead, if sufficient tissue remains, to mutation analysis as possible.

Clinical judgment is required as to whether respiratory or GU tract biopsies are warranted in particularly diagnostically challenging cases, but if pursued, such tissues should be processed as for skin and GI tract biopsies.

Newly or persistently pathologically enlarged (> 1 cm) lymph nodes require biopsy to rule out malignant lymphoproliferative and granulomatous disorders (regardless of whether such disorders can be proven to be independent of, or consequent to, MCAD). However, diffusely migratory, spontaneously waxing/waning adenopathy and/or adenitis is common in MCAD and far more often is a reactive rather than malignant process, though the reactive patterns seen on biopsy can be bizarre (*e.g.*, sinus histiocytosis) and can have the pathologist on the verge of declaring a malignancy. In patients diagnosed with MCAD (or being evaluated for suspected MCAD) who have a longstanding history of spontaneous enlargement of nodes to pathologic size soon followed by spontaneous regression of such adenopathy, the new appearance of a modestly pathologically enlarged lymph node can be watched closely without reflexively proceeding to biopsy, but persistence or progression of adenopathy beyond the range, or outside of the pattern, the patient has usually experienced may warrant biopsy. Node biopsies should be excisional whenever possible and should be submitted not only for the above-noted analyses for MC disease but also for routine analyses for lymphoproliferative disorders and perhaps even - in appropriate clinical settings - specific plasma cell dyscrasias or granulomatous diseases such as amyloidosis, Castleman's disease, or sarcoidosis.

In addition to checking a serum tryptase, other initial laboratory assessment for MCAD (whether suspected to be mastocytosis or MCAS) should include a variety of common tests as listed in Figure 5. As MCAD commonly causes osteolysis (and, less commonly, osteosclerosis - and occasionally even both processes at different

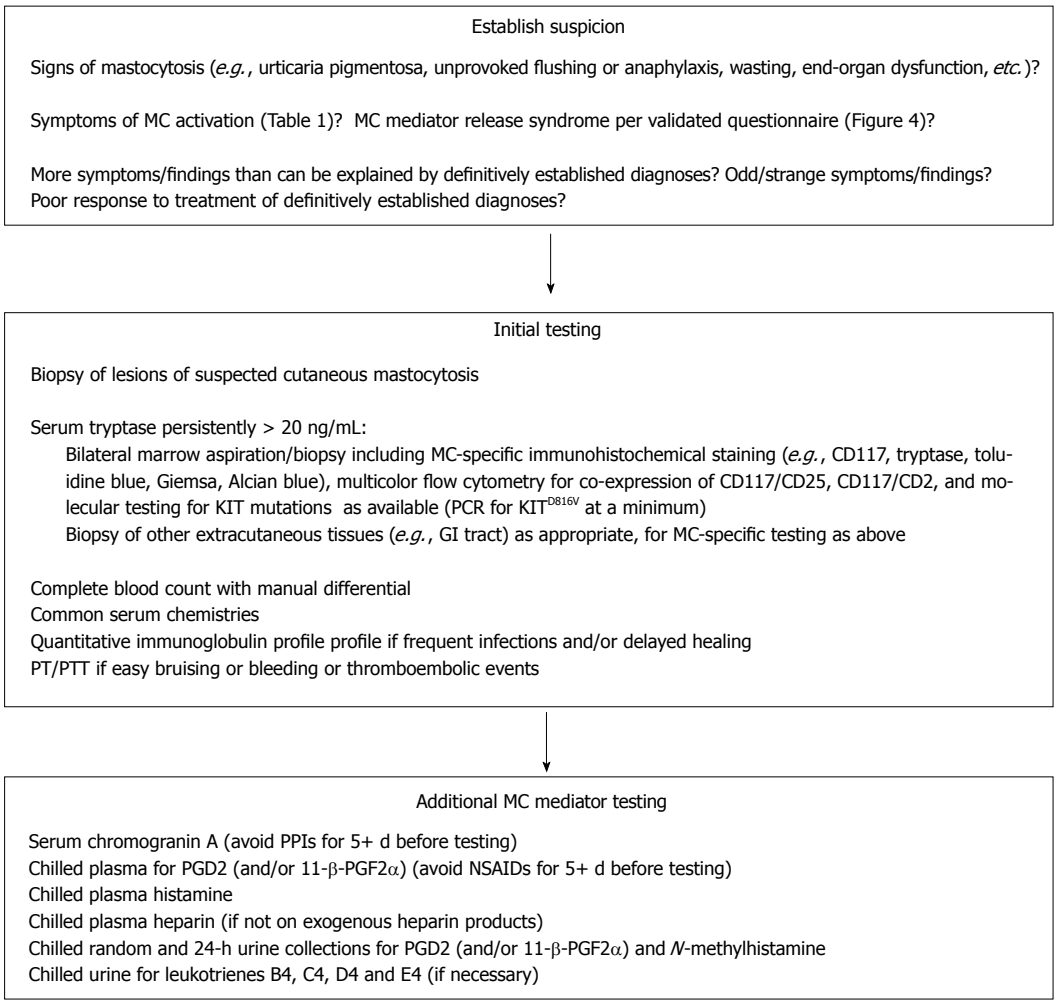


Figure 5 Diagnostic approach to mast cell activation disease. MCs: Mast cells; MCAS: Mast cell activation syndrome; GI: Gastrointestinal; PG: Prostaglandin; PCR: Polymerase chain reaction; NSAID: Non-steroidal anti-inflammatory drugs.

sites in the same patient), it is appropriate to establish a baseline for bone densitometry after the diagnosis of MCAD has been established, as the finding of osteopenia or osteoporosis has therapeutic implications. MCAD can drive erythropenia or erythrocytosis (either of which may be macrocytic, normocytic, or microcytic, engendering appropriate differential diagnostic considerations of secondary processes), leukopenia or leukocytosis, and/or thrombocytopenia or thrombocytosis, but there are no findings in the CBC that are specific for MCAD (other than significant numbers of circulating mast cells signaling mast cell leukemia). Also, as previously noted, certain subtle abnormalities are not uncommonly seen in the leukocyte differential, but often the differential is as normal as the CBC. Indeed, it is a confounding point for hematologists that this disease of fundamentally hematologic classification often presents with not a single abnormality in the CBC or differential. However, many MCAD patients - with either mastocytosis or MCAS - do manifest subtle to gross abnormalities in one or more elements of the CBC, providing the diagnostician important opportunities to consider alternative diagnoses. Of note, though, gross abnormalities in the CBC in advanced mastocytosis may be due more to marrow replacement by MCs, while gross abnormalities in the CBC in less advanced mastocytosis and MCAS are likely due more to MC mediator-driven interference with normal hematopoiesis.

Many MCAD patients also manifest abnormalities in the common serum chemistries, again typically modest in degree but providing the diagnostician opportunities to consider alternative diagnoses regardless of whether they may have risen independently of, or consequent to, MCAD.

Both hypergammaglobulinemia (usually a polyclonal inflammatory phenomenon but occasionally a monoclonal gammopathy of undetermined significance or more advanced clonal plasma cell dyscrasia) and hypogammaglobulinemia can be seen in MCAD[61]; patients sometimes have been previously diagnosed with common variable immunodeficiency. Markedly IgG-deficient patients (< 400 mg/dL) who have suffered major infections or substantial healing complications may be candidates for prophylactic immune globulin therapy.

Histories of "easy" bruising and/or bleeding are frequent amongst MCAD patients. Multiple mechanisms

are possible (and not necessarily mutually exclusive) including coagulation factor deficiencies, antiphospholipid antibodies, and/or MC release into the local tissues of (antithrombin-activating) heparin. However, fibrinolysis (as stimulated by heparin release[62] and other fibrinolytic mediators such as tissue plasminogen activator[63]) appears likely to be the dominant cause, explaining why antifibrinolytics such as tranexamic acid can be helpful in controlling MCAD-driven bleeding. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) remain reasonable screening tests in patients with suspected MCAD who report histories of easy bruising/bleeding, but the diagnostician should be aware that local MC release of mediators with sufficient direct or indirect anticoagulant effect to cause local bleeding usually do not provoke systemic anticoagulant effect sufficient to cause rises in PT or aPTT to abnormal levels. Thus, measurement of fibrinolytic mediators[63], as proximate to the onset of bleeding as possible, may be required to prove fibrinolysis if felt clinically important. Otherwise inexplicable prolongations in PT or aPTT should prompt appropriate further evaluation for clotting factor deficiencies. Also, assessment for antiphospholipid antibodies may not be unreasonable in suspected MCAD patients with otherwise inexplicable abnormalities (high or low) in either PT or aPTT and histories of not only easy bruising/bleeding but also thromboembolism or miscarriage.

If not already performed, a careful evaluation for known hypercoagulable disorders should be performed in the patient with suspected MCAD and a history of idiopathic thromboembolism. Such evaluation should include, among other considerations, determination of the plasma level of Factor Ⅷ (produced by various sources including the MC[64] and associated with hypercoagulability[65]) and, with a history of portal/splanchnic vein thrombosis, assessment for the JAK2^{V617F} mutation which might suggest the occult presence of an MPN[66]. Testing for inborn hypercoagulable disorders may not be warranted in cases of initial thromboembolism at ages above 40.

Most patients presenting for evaluation of MCAD have been chronically unwell and have undergone extensive diagnostic evaluation, but the diagnostician considering MCAD nevertheless must carefully review past evaluations and ensure other diagnostic considerations potentially fitting the patient's unique course have been excluded (*e.g.*, hypothyroidism, celiac sprue, Epstein-Barr virus disease, carcinoid, amyloidosis, porphyria, sarcoidosis, *etc.*). Even if the root cause of the patient's multisystem unwellness is MCAD, identification of specific secondary phenomena (*e.g.*, autoimmunity, infection, malignancy, *etc.*) may permit better outcomes via dual-directed therapy.

Laboratory assessment of other MC-specific mediators

If the serum tryptase is < 20 ng/mL and there are no other clinical hints that SM is more likely than MCAS, then an initial laboratory survey of other MC mediators should be pursued in patients clinically suspected of having MCAS. In the authors' opinion, such an initial mediator survey for evidence of MCAS should include serum chromogranin A; chilled plasma for prostaglandin D_2 (PGD_2) (and/or 11-β-$PGF_{2\alpha}$); chilled plasma histamine; chilled plasma heparin (in patients not on exogenous heparin products); and chilled random and 24-h urine collections for PGD_2 (and/or 11-β-$PGF_{2\alpha}$) and *N*-methylhistamine[26]. In some situations (see below), urinary levels of leukotrienes B4, C4, D4 and E4 may also be worth pursuing[26,67].

Although specimen collection as soon as possible following an acute flare of symptoms is ideal, there is no need to wait for such an event when initially assessing the patient with longstanding baseline symptoms consistent with aberrant MC mediator release. However, if the initial laboratory assessment in a patient with a history suspicious for MCAD is negative, repeat testing is usually warranted but preferably should be deferred until the presentation of an acute flare. If possible, hourly determinations of serum tryptase, plasma PGD_2 and histamine, and spot urinary PGD_2 and *N*-methylhistamine should be pursued at baseline and over the next 2-3 h as a flare evolves.

Chromogranin A (CgA) is another known MC product[35,68] which appears quite heat-stable[69] and was found elevated in the serum in 17% (21 out of 122) of a cohort of MCAD patients investigated (LBA, unpublished data). Elevated serum levels of CgA can be helpful toward diagnosing MCAS, but other potential causes of such elevations (heart failure, renal insufficiency, proton pump inhibitor (PPI) use, and neuroendocrine cancer) should be excluded. Changes in serum CgA level in response to initiation or cessation of PPI therapy are fairly reliable and rapid (within 5 d[70]) but highly variable in magnitude[71,72], likely due to multiple mechanisms including pharmacogenomic polymorphisms (largely in cytochrome p450 isoenzyme 2C19[73]) and increase in density of CgA-secreting enterochromaffin cells (especially in gastric tissue) in response to gastric acid reduction[74]. Cessation of PPI therapy at least 5 days prior to submission of a serum sample for CgA assessment should permit an accurate gauge of the baseline CgA level.

Although it was the first MC mediator to be discovered[75,76] and is a highly sensitive and specific indicator of MC activity[16,63], the plasma heparin level is difficult to measure accurately in the clinical setting. The metabolism of heparin is complex[77], but it is clear that it has a short half-life (generally less than one hour) and decomposes rapidly even at chilled temperatures[63]. Seidel *et al*[63] reported an upper limit of normal plasma heparin of 0.02 anti-Factor Xa units/mL, but the utility of assessing the plasma heparin level for evidence of MCAS may be constrained by the fact that most of the commercially available assays have a lower limit of detectability of 0.05 or 0.10 anti-Factor Xa units/mL since such assays are used far more to assess anticoagulant adequacy of therapeutic heparinization than to assess endogenous heparin levels. However, about 50% of MCAD patients

will manifest a plasma heparin level exceeding 0.05 anti-Factor Xa units/mL on commercially available assays (105 out of 202 MCAD patients in a current study; unpublished data), and in such patients whose detectable plasma heparin levels cannot be attributed to therapeutic heparin or to the only other known endogenous source of heparin in humans (basophils), MC activation almost certainly is the source.

Like heparin, histamine is slightly less specific for MC disease than tryptase in that histamine is also released by basophils. Unlike heparin, histamine is not particularly heat-labile[78] but nevertheless has a short half-life *in vivo*[79,80] (estimates have ranged from 1 min to 1 h and may vary based on increased histaminase activity during anaphylaxis[81]). Histamine appears to be stable in separated plasma at room temperature for at least 48 h[82]. However, because histamine assays often must be performed at distant reference laboratories (requiring specimen storage and transport exceeding 48 h), plasma specimens for histamine levels should be kept continuously chilled at all stages of handling. Histamine's major metabolite, *N*-methylhistamine, has a longer half-life *in vivo*[80] and may be a preferable measure over histamine, especially when a sample cannot be collected during a flare of symptoms or when other constraints preclude measurement of both molecules.

Although also produced in macrophages[83-86], Langerhans cells[87], liver endothelium[86], platelets[88], Th2 helper T cells[89], stimulated osteoblasts[90], and possibly adipocytes[91], PGD_2 appears to be far dominantly produced in MCs[92,93], yielding attractive specificity for clinical detection of MCAD. In patients with MC activation producing increases in urinary *N*-methylhistamine, the fold increase in urinary PGD_2 is substantially greater than seen for the histamine metabolite[94]. However, PGD_2 appears to have an even shorter half-life than histamine (on the order of 1-30 min in various studies)[95-97]. In fact, PGD_2 is metabolized so rapidly that its measured levels may substantially underestimate its total production[98]. Some PGD_2 metabolites, though, are more stable than the parent compound (*e.g.*, 9α,11β-$PGF_{2\alpha}$[92]), leading some to preferentially test such metabolites over PGD_2. Of note, though, far more $PGF_{2\alpha}$ comes from reduction of PGH_2, and even some from PGE_2, which are produced by a range of other types of cells[99], making the value of $PGF_{2\alpha}$ as a marker for MC activity less clear). Assays of levels of histamine and prostaglandin metabolites are further complicated by the need of many clinical laboratories to ship samples to a reference laboratory for measurement. Care must be taken by the patient and laboratory staff to maintain the samples (particularly for heparin and PGD_2) in chilled condition throughout collection, storage, and transport until final processing. It is unclear whether urinary PGD_2 is exclusively a product of the kidneys[100] or dominantly a filtering of plasma PGD_2[92]. If the former is true, a low urinary PGD_2 could be due to chronic kidney disease (CKD), though certainly patients with CKD due to activated MCs can manifest elevated urinary PGD_2 levels. Therefore, CKD is not a reason to forego testing of the urinary PGD_2 level.

Ideally, a 24-h urine collection is preferred over a spot/random collection given that the evanescence of mediator flares and the short half-lives of many mediators make spot urine collections and plasma assays less likely to catch elevated levels of mediators. However, from an efficiency perspective, testing both 24-h and random specimens at the same time may not be unreasonable in patients whose mysterious chronic illness has already consumed extensive resources.

Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenase and thus limit prostaglandin production, can result in low PGD_2 levels in the blood and urine. Similar to the precaution with PPI therapy when assessing for serum CgA elevation, the patient's history of use of NSAIDs, in both prescription and over-the-counter products, should be reviewed when planning the laboratory assessment for prostaglandin levels. Like most PPIs, some NSAIDs have long half-lives permitting daily dosing, and thus patients should be cautioned to abstain, if possible, from all NSAIDs for at least 5 d prior to specimen collection for assessment of prostaglandins.

Given the heterogeneity of MCAD and the above-noted challenges in detecting elevated levels of MC mediators, it should not be surprising that some patients present not only with histories which are classic for MCAS but also with initial screenings for the most sensitive and specific MC mediators which are normal. When repeated efforts to identify aberrant MC activation using the above-described screening approach all fail, consideration can be given to screening for aberrant expression of less specific MC mediators such as Factor Ⅷ[64,101], plasma free norepinephrine[102], tumor necrosis factor alpha[103-111] and interleukin-6[103]. Plasma free norepinephrine is often ordered as part of a plasma free catecholamine profile; in many MCAS patients a pattern of elevated norepinephrine, low-normal or low epinephrine, and sometimes elevated dopamine is seen (LBA and GJM, unpublished data).

Although commercial testing (whether through local or reference laboratories) is not yet widely available, levels of MC mediators leukotriene B4 and cysteinyl leukotrienes C4, D4 and E4 have been reported to be elevated in patients with SM as compared to healthy controls[67]. Plasma and urinary leukotrienes also increase with acute attacks of asthma[112], a disease increasingly suspected to involve aberrantly active MCs[113]. However, leukotriene expression has not been specifically studied in the MCAS population. Furthermore, leukotrienes are synthesized in a variety of myeloid cells (and epithelial cells as well)[114,115], so the specificity of elevated leukotriene metabolite levels for MCAD is unclear and may depend substantially on the presence of a clinical history compatible with MCAD. Whether elevated leukotriene levels are related more to MC load or to MC activity remains to be determined. However, if the clinical history is best explained by MC activation but abnormal levels

of the previously discussed biochemical markers cannot be identified, it would not be unreasonable to also examine levels of plasma and urinary leukotriene metabolites if commercial assays of such are accessible.

SPECIMEN HANDLING ISSUES

The short half-lives and thermolability of many MC mediators require continuous specimen chilling throughout collection, storage, and transport. Particularly with regard to 24-h urine collections for MC mediator testing, patients should be carefully educated to pre-chill the collection container overnight before beginning the collection and then to keep the container continuously chilled while following an otherwise standard 24-h urine collection protocol; the container should be removed from the refrigerator or ice chest only when imminently needed and should be returned to chilling as soon as possible. Patients should also be cautioned to maintain the container in a chilled environment throughout transport. We recommend the container be placed in a bag filled with ice and sealed, with the bag then placed in an ice chest filled with ice and sealed for transport to the accessioning lab, whereupon the bag can be removed from the chest and provided to the technician with a reminder of the criticality of keeping the specimen chilled.

Laboratory staff often are unfamiliar with MC mediator testing, previously a rarely undertaken endeavor. Consideration should be given by the diagnostician to sharing with laboratory personnel the ultimate clinical goal of such testing and the importance of maintaining thermal integrity of these specimens at all times, including at the time of initial accessioning as well as when packing specimens for transport to reference laboratories that may be thousands of miles distant, transits that may involve long periods sitting in unventilated cargo containers on hot tarmacs. Use of well-insulated containers, and liberal placement of cold packs in the insulated container, should be *de rigueur* when packing such specimens for long-distance transport.

If PGD_2 (or 11-β-$PGF_{2\alpha}$) levels below the lower limit of normal are determined and the patient denies any recent use of NSAIDs, or if "normal" MC mediator levels are repeatedly seen despite specimens being accessioned at particularly symptomatic times, it may be useful to ask the patient about his observations, while at the lab, as to whether the laboratory staff maintained the specimen in a chilled environment. Aside from NSAID use, loss of thermal integrity is the most common reason for low PGD_2 levels in the authors' experience.

CONCLUSION

With growing appreciation of the prevalence of MCAD (particularly MCAS), there is a growing need to pursue diagnostic evaluation for MCAD outside of the few centers specializing in this area. An understanding of the spectrum of MCAD and the similarities and differences amongst the various forms of the disease is helpful in guiding the approach to testing. Especially if MCAS is confirmed to usually be a clonal disease, the rise of personal genomics may come to obviate many of the present challenges in diagnosing MCAD, but with appropriate collaboration amongst the diagnostician, patient, other relevant clinicians, and laboratory staff, today's challenges can be surmounted. Especially given that the majority of the MCAD iceberg seems likely to be MCAS and that significantly helpful therapy can be found for most MCAD patients (including most MCAS patients), efforts to smooth the path toward diagnosis of MCAD are worthwhile.

REFERENCES

1 **Kalesnikoff J**, Galli SJ. New developments in mast cell biology. *Nat Immunol* 2008; **9**: 1215-1223 [PMID: 18936782 DOI: 10.1038/ni.f.216]
2 **von Recklinghausen F**. Ueber Eiter - und Bindegewebskörperchen. *Virchows Arch* 1863; **28**: 157-197 [DOI: 10.1007/BF01930779]
3 **Unna P**. Beiträge zur Anatomie und Pathogenese der Urticaria simplex und pigmentosa. *Monatschrift der praktischen Dermatologie* 1887; **6**: 9-18
4 **Ellis JM**. Urticaria pigmentosa; a report of a case with autopsy. *Arch Pathol* (Chic) 1949; **48**: 426-435 [PMID: 18149230]
5 **Efrati P**, Klajman A, Spitz H. Mast cell leukemia? Malignant mastocytosis with leukemia-like manifestations. *Blood* 1957; **12**: 869-882 [PMID: 13471655]
6 **Furitsu T**, Tsujimura T, Tono T, Ikeda H, Kitayama H, Koshimizu U, Sugahara H, Butterfield JH, Ashman LK, Kanayama Y. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest* 1993; **92**: 1736-1744 [PMID: 7691885 DOI: 10.1172/JCI116761]
7 **Akin C**, Metcalfe DD. The biology of Kit in disease and the application of pharmacogenetics. *J Allergy Clin Immunol* 2004; **114**: 13-19; quiz 20 [PMID: 15241338 DOI: 10.1016/j.jaci.2004.04.046]
8 **Nagata H**, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, Metcalfe DD. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci USA* 1995; **92**: 10560-10564 [PMID: 7479840]
9 **Garcia-Montero AC**, Jara-Acevedo M, Teodosio C, Sanchez ML, Nunez R, Prados A, Aldanondo I, Sanchez L, Dominguez M, Botana LM, Sanchez-Jimenez F, Sotlar K, Almeida J, Escribano L, Orfao A. KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. *Blood* 2006; **108**: 2366-2372 [PMID: 16741248 DOI: 10.1182/blood-2006-04-015545]
10 **D'Ambrosio C**, Akin C, Wu Y, Magnusson MK, Metcalfe DD. Gene expression analysis in mastocytosis reveals a highly consistent profile with candidate molecular markers. *J Allergy Clin Immunol* 2003; **112**: 1162-1170 [PMID: 14657877 DOI: 10.1016/j.jaci.2003.07.008]
11 **Orfao A**, Garcia-Montero AC, Sanchez L, Escribano L. Recent advances in the understanding of mastocytosis: the role of KIT mutations. *Br J Haematol* 2007; **138**: 12-30 [PMID: 17555444 DOI: 10.1111/j.1365-2141.2007.06619.x]
12 **Mayerhofer M**, Gleixner KV, Hoelbl A, Florian S, Hoermann G, Aichberger KJ, Bilban M, Esterbauer H, Krauth MT, Sperr WR, Longley JB, Kralovics R, Moriggl R, Zappulla J, Liblau RS, Schwarzinger I, Sexl V, Sillaber C, Valent P. Unique ef-

fects of KIT D816V in BaF3 cells: induction of cluster formation, histamine synthesis, and early mast cell differentiation antigens. *J Immunol* 2008; **180**: 5466-5476 [PMID: 18390729]
13 **Haenisch B**, Nöthen MM, Molderings GJ. Systemic mast cell activation disease: the role of molecular genetic alterations in pathogenesis, heritability and diagnostics. *Immunology* 2012; **137**: 197-205 [PMID: 22957768 DOI: 10.1111/j.1365-2567.2012.03627.x]
14 **Broesby-Olsen S**, Kristensen T, Vestergaard H, Brixen K, Møller MB, Bindslev-Jensen C. KIT D816V mutation burden does not correlate to clinical manifestations of indolent systemic mastocytosis. *J Allergy Clin Immunol* 2013; In press [DOI: 10.1016/j.jaci.2013.02.019]
15 **Lawley W**, Hird H, Mallinder P, McKenna S, Hargadon B, Murray A, Bradding P. Detection of an activating c-kit mutation by real-time PCR in patients with anaphylaxis. *Mutat Res* 2005; **572**: 1-13 [PMID: 15790486 DOI: 10.1016/j.mrfmmm.2004.08.015]
16 **Roberts LJ**, Oates JA. Biochemical diagnosis of systemic mast cell disorders. *J Invest Dermatol* 1991; **96**: 19S-24S; discussion 24S-25S; 60S-65S [PMID: 16799604 DOI: 10.1111/1523-1747.ep12468945]
17 **Sonneck K**, Florian S, Müllauer L, Wimazal F, Födinger M, Sperr WR, Valent P. Diagnostic and subdiagnostic accumulation of mast cells in the bone marrow of patients with anaphylaxis: Monoclonal mast cell activation syndrome. *Int Arch Allergy Immunol* 2007; **142**: 158-164 [PMID: 17057414 DOI: 10.1159/000096442]
18 **Akin C**, Scott LM, Kocabas CN, Kushnir-Sukhov N, Brittain E, Noel P, Metcalfe DD. Demonstration of an aberrant mast-cell population with clonal markers in a subset of patients with "idiopathic" anaphylaxis. *Blood* 2007; **110**: 2331-2333 [PMID: 17638853 DOI: 10.1182/blood-2006-06-028100]
19 **Molderings GJ**, Kolck UW, Scheurlen C, Brüss M, Homann J, Von Kügelgen I. Multiple novel alterations in Kit tyrosine kinase in patients with gastrointestinally pronounced systemic mast cell activation disorder. *Scand J Gastroenterol* 2007; **42**: 1045-1053 [PMID: 17710669 DOI: 10.1080/00365520701245744]
20 **Molderings GJ**, Meis K, Kolck UW, Homann J, Frieling T. Comparative analysis of mutation of tyrosine kinase kit in mast cells from patients with systemic mast cell activation syndrome and healthy subjects. *Immunogenetics* 2010; **62**: 721-727 [PMID: 20838788 DOI: 10.1007/s00251-010-0474-8]
21 **Kralovics R**. Genetic complexity of myeloproliferative neoplasms. *Leukemia* 2008; **22**: 1841-1848 [PMID: 18754034 DOI: 10.1038/leu.2008.233]
22 **Schwaab J**, Schnittger S, Sotlar K, Walz C, Fabarius A, Pfirrmann M, Kohlmann A, Grossmann V, Meggendorfer M, Horny HP, Valent P, Jawhar M, Teichmann M, Metzgeroth G, Erben P, Ernst T, Hochhaus A, Haferlach T, Hofmann WK, Cross NC, Reiter A. Comprehensive mutational profiling in advanced systemic mastocytosis. *Blood* 2013; **122**: 2460-2466 [PMID: 23958953 DOI: 10.1182/blood-2013-04-496448]
23 **Akin C**, Valent P, Metcalfe DD. Mast cell activation syndrome: Proposed diagnostic criteria. *J Allergy Clin Immunol* 2010; **126**: 1099-1104.e4 [PMID: 21035176 DOI: 10.1016/j.jaci.2010.08.035]
24 **Molderings GJ**, Haenisch B, Bogdanow M, Fimmers R, Nöthen MM. Familial occurrence of systemic mast cell activation disease. *PLoS One* 2013; **8**: e76241 [PMID: 24098785 DOI: 10.1371/journal.pone.0076241]
25 **Nakamura Y**, Kambe N, Saito M, Nishikomori R, Kim YG, Murakami M, Núñez G, Matsue H. Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria. *J Exp Med* 2009; **206**: 1037-1046 [PMID: 19364881 DOI: 10.1084/jem.20082179]
26 **Molderings GJ**, Brettner S, Homann J, Afrin LB. Mast cell activation disease: a concise practical guide for diagnostic workup and therapeutic options. *J Hematol Oncol* 2011; **4**: 10 [PMID: 21418662 DOI: 10.1186/1756-8722-4-10]
27 **Afrin LB**. Mast cell activation disorder masquerading as pure red cell aplasia. *Int J Hematol* 2010; **91**: 907-908 [PMID: 20526893 DOI: 10.1007/s12185-010-0605-x]
28 **Afrin LB**. Polycythemia from mast cell activation syndrome: lessons learned. *Am J Med Sci* 2011; **342**: 44-49 [PMID: 21642812 DOI: 10.1097/MAJ.0b013e31821d41dd]
29 **Afrin LB**. Mast cell activation syndrome masquerading as agranulocytosis. *Mil Med* 2012; **177**: 113-117 [PMID: 22338992]
30 **Travis WD**, Li CY, Bergstralh EJ, Yam LT, Swee RG. Systemic mast cell disease. Analysis of 58 cases and literature review. *Medicine* (Baltimore) 1988; **67**: 345-368 [PMID: 3054417]
31 **Travis WD**, Li CY, Yam LT, Bergstralh EJ, Swee RG. Significance of systemic mast cell disease with associated hematologic disorders. *Cancer* 1988; **62**: 965-972 [PMID: 3409177]
32 **Valent P**, Horny HP, Escribano L, Longley BJ, Li CY, Schwartz LB, Marone G, Nuñez R, Akin C, Sotlar K, Sperr WR, Wolff K, Brunning RD, Parwaresch RM, Austen KF, Lennert K, Metcalfe DD, Vardiman JW, Bennett JM. Diagnostic criteria and classification of mastocytosis: a consensus proposal. *Leuk Res* 2001; **25**: 603-625 [PMID: 11377686 DOI: 10.1016/S0145-2126(01)00038-8]
33 **Horny HP**, Metcalfe DD, Bennett J, Bain BJ, Akin C, Escribano L, Valent P. Mastocytosis. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW. WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues (4th edition.). Lyon, France: International Agency for Research and Cancer, 2008: 54–63
34 **Valent P**, Akin C, Arock M, Brockow K, Butterfield JH, Carter MC, Castells M, Escribano L, Hartmann K, Lieberman P, Nedoszytko B, Orfao A, Schwartz LB, Sotlar K, Sperr WR, Triggiani M, Valenta R, Horny HP, Metcalfe DD. Definitions, criteria and global classification of mast cell disorders with special reference to mast cell activation syndromes: a consensus proposal. *Int Arch Allergy Immunol* 2012; **157**: 215-225 [PMID: 22041891 DOI: 10.1159/000328760]
35 **Ibelgaufts H**. Mast Cells. In: COPE: Cytokines and Cells Online Pathfinder Encyclopaedia, 2013, Sept. 3. Avalible from: URL: http: //www.copewithcytokines.de/cope.cgi?key=mast cells
36 **Afrin L**. Presentation, Diagnosis, and Management of Mast Cell Activation Syndrome. In: Murray D. Mast Cells: Phenotypic Features, Biological Functions, and Role in Immunity. Happauge, NY: Nova Science Publishers, 2013: 155-231
37 **Hermine O**, Lortholary O, Leventhal PS, Catteau A, Soppelsa F, Baude C, Cohen-Akenine A, Palmérini F, Hanssens K, Yang Y, Sobol H, Fraytag S, Ghez D, Suarez F, Barete S, Casassus P, Sans B, Arock M, Kinet JP, Dubreuil P, Moussy A. Case-control cohort study of patients' perceptions of disability in mastocytosis. *PLoS One* 2008; **3**: e2266 [PMID: 18509466 DOI: 10.1371/journal.pone.0002266]
38 **Valent P**, Sperr WR, Akin C. How I treat patients with advanced systemic mastocytosis. *Blood* 2010; **116**: 5812-5817 [PMID: 20855864 DOI: 10.1182/blood-2010-08-292144]
39 **Alfter K**, von Kügelgen I, Haenisch B, Frieling T, Hülsdonk A, Haars U, Rolfs A, Noe G, Kolck UW, Homann J, Molderings GJ. New aspects of liver abnormalities as part of the systemic mast cell activation syndrome. *Liver Int* 2009; **29**: 181-186 [PMID: 18662284 DOI: 10.1111/j.1478-3231.2008.01839.x]
40 **Schwartz LB**, Sakai K, Bradford TR, Ren S, Zweiman B, Worobec AS, Metcalfe DD. The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. *J Clin Invest* 1995; **96**: 2702-2710 [PMID: 8675637 DOI: 10.1172/JCI118337]
41 **Borer-Reinhold M**, Haeberli G, Bitzenhofer M, Jandus P, Hausmann O, Fricker M, Helbling A, Müller U. An increase in serum tryptase even below 11.4 ng/mL may indicate a

mast cell-mediated hypersensitivity reaction: a prospective study in Hymenoptera venom allergic patients. *Clin Exp Allergy* 2011; **41**: 1777-1783 [PMID: 22092437 DOI: 10.1111/j.1365-2222.2011.03848.x]
42 **Sperr WR**, Jordan JH, Fiegl M, Escribano L, Bellas C, Dirnhofer S, Semper H, Simonitsch-Klupp I, Horny HP, Valent P. Serum tryptase levels in patients with mastocytosis: correlation with mast cell burden and implication for defining the category of disease. *Int Arch Allergy Immunol* 2002; **128**: 136-141 [PMID: 12065914 DOI: 10.1159/000059404]
43 **Alvarez-Twose I**, González de Olano D, Sánchez-Muñoz L, Matito A, Esteban-López MI, Vega A, Mateo MB, Alonso Díaz de Durana MD, de la Hoz B, Del Pozo Gil MD, Caballero T, Rosado A, Sánchez Matas I, Teodósio C, Jara-Acevedo M, Mollejo M, García-Montero A, Orfao A, Escribano L. Clinical, biological, and molecular characteristics of clonal mast cell disorders presenting with systemic mast cell activation symptoms. *J Allergy Clin Immunol* 2010; **125**: 1269-1278.e2 [PMID: 20434205 DOI: 10.1016/j.jaci.2010.02.019]
44 **van Doormaal JJ**, van der Veer E, van Voorst Vader PC, Kluin PM, Mulder AB, van der Heide S, Arends S, Kluin-Nelemans JC, Oude Elberink JN, de Monchy JG. Tryptase and histamine metabolites as diagnostic indicators of indolent systemic mastocytosis without skin lesions. *Allergy* 2012; **67**: 683-690 [PMID: 22435702 DOI: 10.1111/j.1398-9995.2012.02809.x]
45 **Schwartz LB**, Metcalfe DD, Miller JS, Earl H, Sullivan T. Tryptase levels as an indicator of mast-cell activation in systemic anaphylaxis and mastocytosis. *N Engl J Med* 1987; **316**: 1622-1626 [PMID: 3295549 DOI: 10.1056/NEJM198706253162603]
46 **Hallgren J**, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. *FEBS J* 2006; **273**: 1871-1895 [PMID: 16640553 DOI: 10.1111/j.1742-4658.2006.05211.x]
47 **Schwartz LB**, Bradford TR. Regulation of tryptase from human lung mast cells by heparin. Stabilization of the active tetramer. *J Biol Chem* 1986; **261**: 7372-7379 [PMID: 3519608]
48 **Fajardo I**, Pejler G. Human mast cell beta-tryptase is a gelatinase. *J Immunol* 2003; **171**: 1493-1499 [PMID: 12874242]
49 **Goldstein SM**, Wintroub BU. Mast cell proteases. In: Kaliner MA, Metcalfe DD. The Mast Cell in Health and Disease. New York: Marcel Dekker, 1993: 343-380
50 **Butterfield JH**, Li CY. Bone marrow biopsies for the diagnosis of systemic mastocytosis: is one biopsy sufficient? *Am J Clin Pathol* 2004; **121**: 264-267 [PMID: 14983941 DOI: 10.1309/2EWQKN00PG02JKY0]
51 **Horny HP**, Valent P. Diagnosis of mastocytosis: general histopathological aspects, morphological criteria, and immunohistochemical findings. *Leuk Res* 2001; **25**: 543-551 [PMID: 11377679 DOI: 10.1016/S0145-2126(01)00021-2]
52 **Escribano L**, Orfao A, Díaz-Agustin B, Villarrubia J, Cerveró C, López A, Marcos MA, Bellas C, Fernández-Cañadas S, Cuevas M, Sánchez A, Velasco JL, Navarro JL, Miguel JF. Indolent systemic mast cell disease in adults: immunophenotypic characterization of bone marrow mast cells and its diagnostic implications. *Blood* 1998; **91**: 2731-2736 [PMID: 9531582]
53 **Havelange V**, Demoulin JB. Review of current classification, molecular alterations, and tyrosine kinase inhibitor therapies in myeloproliferative disorders with hypereosinophilia. *J Blood Med* 2013; **4**: 111-121 [PMID: 23976869 DOI: 10.2147/JBM.S33142]
54 **Yeom JS**, Choi MB, Seo JH, Park JS, Lim JY, Park CH, Woo HO, Youn HS, Ko GH, Baik SC, Lee WK, Cho MJ, Rhee KH. Relationship between headache and mucosal mast cells in pediatric Helicobacter pylori-negative functional dyspepsia. *Cephalalgia* 2013; **33**: 323-329 [PMID: 23291287 DOI: 10.1177/0333102412472070]
55 **Zare-Mirzaie A**, Lotfi M, Sadeghipour A, Haghi-Ashtiani MT. Analysis of colonic mucosa mast cell count in patients with chronic diarrhea. *Saudi J Gastroenterol* 2012; **18**: 322-326 [PMID: 23006460 DOI: 10.4103/1319-3767.101128]
56 **Martínez C**, Lobo B, Pigrau M, Ramos L, González-Castro AM, Alonso C, Guilarte M, Guilá M, de Torres I, Azpiroz F, Santos J, Vicario M. Diarrhoea-predominant irritable bowel syndrome: an organic disorder with structural abnormalities in the jejunal epithelial barrier. *Gut* 2013; **62**: 1160-1168 [PMID: 22637702 DOI: 10.1136/gutjnl-2012-302093]
57 **Vivinus-Nébot M**, Dainese R, Anty R, Saint-Paul MC, Nano JL, Gonthier N, Marjoux S, Frin-Mathy G, Bernard G, Hébuterne X, Tran A, Theodorou V, Piche T. Combination of allergic factors can worsen diarrheic irritable bowel syndrome: role of barrier defects and mast cells. *Am J Gastroenterol* 2012; **107**: 75-81 [PMID: 21931380 DOI: 10.1038/ajg.2011.315]
58 **Bassotti G**, Villanacci V, Nascimbeni R, Cadei M, Manenti S, Sabatino G, Maurer CA, Cathomas G, Salerni B. Colonic mast cells in controls and slow transit constipation patients. *Aliment Pharmacol Ther* 2011; **34**: 92-99 [PMID: 21539589 DOI: 10.1111/j.1365-2036.2011.04684.x]
59 **Brunning RD**, McKenna RW, Rosai J, Parkin JL, Risdall R. Systemic mastocytosis. Extracutaneous manifestations. *Am J Surg Pathol* 1983; **7**: 425-438 [PMID: 6614308]
60 **Swieter M**, Lee TD, Stead RH, Fujimaki H, Befus D. Mast cell pleomorphism: properties of intestinal mast cells. *Adv Exp Med Biol* 1987; **216A**: 613-623 [PMID: 2446472]
61 **Worobec AS**, Semere T, Nagata H, Metcalfe DD. Clinical correlates of the presence of the Asp816Val c-kit mutation in the peripheral blood mononuclear cells of patients with mastocytosis. *Cancer* 1998; **83**: 2120-2129 [PMID: 9827716]
62 **Upchurch GR**, Valeri CR, Khuri SF, Rohrer MJ, Welch GN, MacGregor H, Ragno G, Francis S, Rodino LJ, Michelson AD, Loscalzo J. Effect of heparin on fibrinolytic activity and platelet function in vivo. *Am J Physiol* 1996; **271**: H528-H534 [PMID: 8770093]
63 **Seidel H**, Molderings GJ, Oldenburg J, Meis K, Kolck UW, Homann J, Hertfelder HJ. Bleeding diathesis in patients with mast cell activation disease. *Thromb Haemost* 2011; **106**: 987-989 [PMID: 21901238 DOI: 10.1160/TH11-05-0351]
64 **Kindblom LG**. Factor VIII related antigen and mast cells. *Acta Pathol Microbiol Immunol Scand A* 1982; **90**: 437-439 [PMID: 6187180]
65 **Kyrle PA**, Minar E, Hirschl M, Bialonczyk C, Stain M, Schneider B, Weltermann A, Speiser W, Lechner K, Eichinger S. High plasma levels of factor VIII and the risk of recurrent venous thromboembolism. *N Engl J Med* 2000; **343**: 457-462 [PMID: 10950667 DOI: 10.1056/NEJM200008173430702]
66 **Kiladjian JJ**, Cervantes F, Leebeek FW, Marzac C, Cassinat B, Chevret S, Cazals-Hatem D, Plessier A, Garcia-Pagan JC, Darwish Murad S, Raffa S, Janssen HL, Gardin C, Cereja S, Tonetti C, Giraudier S, Condat B, Casadevall N, Fenaux P, Valla DC. The impact of JAK2 and MPL mutations on diagnosis and prognosis of splanchnic vein thrombosis: a report on 241 cases. *Blood* 2008; **111**: 4922-4929 [PMID: 18250227 DOI: 10.1182/blood-2007-11-125328]
67 **Raithel M**, Zopf Y, Kimpel S, Naegel A, Molderings GJ, Buchwald F, Schultis HW, Kressel J, Hahn EG, Konturek P. The measurement of leukotrienes in urine as diagnostic option in systemic mastocytosis. *J Physiol Pharmacol* 2011; **62**: 469-472 [PMID: 22100848]
68 **Prasad P**, Yanagihara AA, Small-Howard AL, Turner H, Stokes AJ. Secretogranin III directs secretory vesicle biogenesis in mast cells in a manner dependent upon interaction with chromogranin A. *J Immunol* 2008; **181**: 5024-5034 [PMID: 18802106]
69 **Takiyyuddin MA**, Cervenka JH, Hsiao RJ, Barbosa JA, Parmer RJ, O'Connor DT. Chromogranin A. Storage and release in hypertension. *Hypertension* 1990; **15**: 237-246 [PMID: 2406199 DOI: 10.1161/01.HYP.15.3.237]
70 **Pregun I**, Herszényi L, Juhász M, Miheller P, Hritz I, Patócs A, Rácz K, Tulassay Z. Effect of proton-pump inhibitor therapy on serum chromogranin a level. *Digestion* 2011; **84**: 22-28

[PMID: 21304238 DOI: 10.1159/000321535]
71 **Sanduleanu S**, Stridsberg M, Jonkers D, Hameeteman W, Biemond I, Lundqvist G, Lamers C, Stockbrügger RW. Serum gastrin and chromogranin A during medium- and long-term acid suppressive therapy: a case-control study. *Aliment Pharmacol Ther* 1999; **13**: 145-153 [PMID: 10102943 DOI: 10.1046/j.1365-2036.1999.00466.x]
72 **Fossmark R**, Jianu CS, Martinsen TC, Qvigstad G, Syversen U, Waldum HL. Serum gastrin and chromogranin A levels in patients with fundic gland polyps caused by long-term proton-pump inhibition. *Scand J Gastroenterol* 2008; **43**: 20-24 [PMID: 18938772 DOI: 10.1080/00365520701561959]
73 **Furuta T**, Shirai N, Sugimoto M, Ohashi K, Ishizaki T. Pharmacogenomics of proton pump inhibitors. *Pharmacogenomics* 2004; **5**: 181-202 [PMID: 15016609 DOI: 10.1517/phgs.5.2.181.27483]
74 **Sanduleanu S**, De Bruïne A, Stridsberg M, Jonkers D, Biemond I, Hameeteman W, Lundqvist G, Stockbrügger RW. Serum chromogranin A as a screening test for gastric enterochromaffin-like cell hyperplasia during acid-suppressive therapy. *Eur J Clin Invest* 2001; **31**: 802-811 [PMID: 11589723 DOI: 10.1046/j.1365-2362.2001.00890.x]
75 **Holmgren H**, Wilander O. Beiträge zur Kenntnis der Chemie and Funktion der Ehrlichschen Mastzellen. *Z Mikroskop Anat Forsch* 1937; **42**: 242-278
76 Jorpes E, Holmgren H, Wilander O. Über das Vorkommen von Heparin in den Gefässwänden und in den Augen. *Z Mikroskop Anat Forsch* 1937; **42**: 279-300
77 **Hirsh J**, Anand SS, Halperin JL, Fuster V. Guide to anticoagulant therapy: Heparin : a statement for healthcare professionals from the American Heart Association. *Circulation* 2001; **103**: 2994-3018 [PMID: 11413093 DOI: 10.1161/01.CIR.103.24.2994]
78 **Emborg J**, Laursen BG, Dalgaard P. Significant histamine formation in tuna (Thunnus albacares) at 2 degrees C--effect of vacuum- and modified atmosphere-packaging on psychrotolerant bacteria. *Int J Food Microbiol* 2005; **101**: 263-279 [PMID: 15925710 DOI: 10.1016/j.ijfoodmicro.2004.12.001]
79 **Laroche D**, Vergnaud MC, Sillard B, Soufarapis H, Bricard H. Biochemical markers of anaphylactoid reactions to drugs. Comparison of plasma histamine and tryptase. *Anesthesiology* 1991; **75**: 945-949 [PMID: 1741515]
80 **Takeda J**, Ueda E, Takahashi J, Fukushima K. Plasma N-methylhistamine concentration as an indicator of histamine release by intravenous d-tubocurarine in humans: preliminary study in five patients by radioimmunoassay kits. *Anesth Analg* 1995; **80**: 1015-1017 [PMID: 7537026]
81 **Lake AM**, Kagey-Sobotka A, Jakubowicz T, Lichtenstein LM. Histamine release in acute anaphylactic enteropathy of the rat. *J Immunol* 1984; **133**: 1529-1534 [PMID: 6205084]
82 **Laroche D**, Dubois F, Gérard JL, Lefrançois C, André B, Vergnaud MC, Dubus L, Bricard H. Radioimmunoassay for plasma histamine: a study of false positive and false negative values. *Br J Anaesth* 1995; **74**: 430-437 [PMID: 7734264 DOI: 10.1093/bja/74.4.430]
83 **Meyers CD**, Liu P, Kamanna VS, Kashyap ML. Nicotinic acid induces secretion of prostaglandin D2 in human macrophages: an in vitro model of the niacin flush. *Atherosclerosis* 2007; **192**: 253-258 [PMID: 16945375 DOI: 10.1016/j.atherosclerosis.2006.07.014]
84 **Hsueh W**. Prostaglandin biosynthesis in pulmonary macrophages. *Am J Pathol* 1979; **97**: 137-148 [PMID: 495692]
85 **Decker K**. Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur J Biochem* 1990; **192**: 245-261 [PMID: 2170121 DOI: 10.1111/j.1432-1033.1990.tb19222.x]
86 **Kuiper J**, Zijlstra FJ, Kamps JA, van Berkel TJ. Identification of prostaglandin D2 as the major eicosanoid from liver endothelial and Kupffer cells. *Biochim Biophys Acta* 1988; **959**: 143-152 [PMID: 3126817]
87 **Maciejewski-Lenoir D**, Richman JG, Hakak Y, Gaidarov I, Behan DP, Connolly DT. Langerhans cells release prostaglandin D2 in response to nicotinic acid. *J Invest Dermatol* 2006; **126**: 2637-2646 [PMID: 17008871 DOI: 10.1038/sj.jid.5700586]
88 **Ali M**, Cerskus AL, Zamecnik J, McDonald JW. Synthesis of prostaglandin D2 and thromboxane B2 by human platelets. *Thromb Res* 1977; **11**: 485-496 [PMID: 918907 DOI: 10.1016/0049-3848(77)90202-X]
89 **Tanaka K**, Ogawa K, Sugamura K, Nakamura M, Takano S, Nagata K. Cutting edge: differential production of prostaglandin D2 by human helper T cell subsets. *J Immunol* 2000; **164**: 2277-2280 [PMID: 10679060]
90 **Gallant MA**, Samadfam R, Hackett JA, Antoniou J, Parent JL, de Brum-Fernandes AJ. Production of prostaglandin D(2) by human osteoblasts and modulation of osteoprotegerin, RANKL, and cellular migration by DP and CRTH2 receptors. *J Bone Miner Res* 2005; **20**: 672-681 [PMID: 15765187 DOI: 10.1359/JBMR.041211]
91 **Jowsey IR**, Murdock PR, Moore GB, Murphy GJ, Smith SA, Hayes JD. Prostaglandin D2 synthase enzymes and PPAR-gamma are co-expressed in mouse 3T3-L1 adipocytes and human tissues. *Prostaglandins Other Lipid Mediat* 2003; **70**: 267-284 [PMID: 12611492]
92 **Bochenek G**, Nizankowska E, Gielicz A, Swierczyńska M, Szczeklik A. Plasma 9alpha,11beta-PGF2, a PGD2 metabolite, as a sensitive marker of mast cell activation by allergen in bronchial asthma. *Thorax* 2004; **59**: 459-464 [PMID: 15170023 DOI: 10.1136/thx.2003.013573]
93 **Dahlén SE**, Kumlin M. Monitoring mast cell activation by prostaglandin D2 in vivo. *Thorax* 2004; **59**: 453-455 [PMID: 15170020 DOI: 10.1136/thx.2004.026641]
94 **Morrow JD**, Guzzo C, Lazarus G, Oates JA, Roberts LJ. Improved diagnosis of mastocytosis by measurement of the major urinary metabolite of prostaglandin D2. *J Invest Dermatol* 1995; **104**: 937-940 [PMID: 7769262]
95 **Suzuki F**, Hayashi H, Hayaishi O. Transport of prostaglandin D2 into brain. *Brain Res* 1986; **385**: 321-328 [PMID: 3465420 DOI: 10.1016/0006-8993(86)91079-6]
96 **Maclouf J**, Corvazier E, Wang ZY. Development of a radioimmunoassay for prostaglandin D2 using an antiserum against 11-methoxime prostaglandin D2. *Prostaglandins* 1986; **31**: 123-132 [PMID: 3456623]
97 **Schuligoi R**, Schmidt R, Geisslinger G, Kollroser M, Peskar BA, Heinemann A. PGD2 metabolism in plasma: kinetics and relationship with bioactivity on DP1 and CRTH2 receptors. *Biochem Pharmacol* 2007; **74**: 107-117 [PMID: 17452035 DOI: 10.1016/j.bcp.2007.03.023]
98 **Haberl C**, Hültner L, Flügel A, Falk M, Geuenich S, Wilmanns W, Denzlinger C. Release of prostaglandin D2 by murine mast cells: importance of metabolite formation for antiproliferative activity. *Mediators Inflamm* 1998; **7**: 79-84 [PMID: 9836493 DOI: 10.1080/09629359891216]
99 **Zhang J**, Gong Y, Yu Y. PG F(2α) Receptor: A Promising Therapeutic Target for Cardiovascular Disease. *Front Pharmacol* 2010; **1**: 116 [PMID: 21607067 DOI: 10.3389/fphar.2010.00116]
100 **Liston TE**, Roberts LJ. Metabolic fate of radiolabeled prostaglandin D2 in a normal human male volunteer. *J Biol Chem* 1985; **260**: 13172-13180 [PMID: 3863815]
101 **Akiyama M**, Watanabe Y, Nishikawa T. Immunohistochemical characterization of human cutaneous mast cells in urticaria pigmentosa (cutaneous mastocytosis). *Acta Pathol Jpn* 1991; **41**: 344-349 [PMID: 1651041]
102 **Freeman JG**, Ryan JJ, Shelburne CP, Bailey DP, Bouton LA, Narasimhachari N, Domen J, Siméon N, Couderc F, Stewart JK. Catecholamines in murine bone marrow derived mast cells. *J Neuroimmunol* 2001; **119**: 231-238 [PMID: 11585626 DOI: 10.1016/S0165-5728(01)00384-8]
103 **Azzolina A**, Bongiovanni A, Lampiasi N. Substance P induces TNF-alpha and IL-6 production through NF kappa B in peritoneal mast cells. *Biochim Biophys Acta* 2003; **1643**: 75-83

[PMID: 14654230 DOI: 10.1016/j.bbamcr.2003.09.003]
104 **Gordon JR**, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature* 1990; **346**: 274-276 [PMID: 2374592 DOI: 10.1038/346274a0]
105 **Echtenacher B**, Männel DN, Hültner L. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* 1996; **381**: 75-77 [PMID: 8609992 DOI: 10.1038/381075a0]
106 **Kaartinen M**, Penttilä A, Kovanen PT. Mast cells in rupture-prone areas of human coronary atheromas produce and store TNF-alpha. *Circulation* 1996; **94**: 2787-2792 [PMID: 8941103 DOI: 10.1161/01.CIR.94.11.2787]
107 **Suto H**, Nakae S, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cell-associated TNF promotes dendritic cell migration. *J Immunol* 2006; **176**: 4102-4112 [PMID: 16547246]
108 **Nakae S**, Suto H, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cells enhance T cell activation: Importance of mast cell-derived TNF. *Proc Natl Acad Sci USA* 2005; **102**: 6467-6472 [PMID: 15840716 DOI: 10.1073/pnas.0501912102]
109 **Bradding P**, Mediwake R, Feather IH, Madden J, Church MK, Holgate ST, Howarth PH. TNF alpha is localized to nasal mucosal mast cells and is released in acute allergic rhinitis. *Clin Exp Allergy* 1995; **25**: 406-415 [PMID: 7553243]
110 **Walsh LJ**, Trinchieri G, Waldorf HA, Whitaker D, Murphy GF. Human dermal mast cells contain and release tumor necrosis factor alpha, which induces endothelial leukocyte adhesion molecule 1. *Proc Natl Acad Sci USA* 1991; **88**: 4220-4224 [PMID: 1709737 DOI: 10.1073/pnas.88.10.4220]
111 **Nakae S**, Ho LH, Yu M, Monteforte R, Iikura M, Suto H, Galli SJ. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J Allergy Clin Immunol* 2007; **120**: 48-55 [PMID: 17482668 DOI: 10.1016/j.jaci.2007.02.046]
112 **Sampson AP**, Castling DP, Green CP, Price JF. Persistent increase in plasma and urinary leukotrienes after acute asthma. *Arch Dis Child* 1995; **73**: 221-225 [PMID: 7492159]
113 **Brightling CE**, Bradding P. The re-emergence of the mast cell as a pivotal cell in asthma pathogenesis. *Curr Allergy Asthma Rep* 2005; **5**: 130-135 [PMID: 15683613 DOI: 10.1007/s11882-005-0086-9]
114 **Tanaka S**, Tanaka H, Abe S. High dose of inhaled fluticasone reduces high levels of urinary leukotriene E4 in the early morning in mild and moderate nocturnal asthma. *Chest* 2003; **124**: 1768-1773 [PMID: 14605047 DOI: 10.1378/chest.124.5.1768]
115 **Leukotriene B4**. Human metabolome database, 2013, May 29. Avalible from: URL: http: //www.hmdb.ca/metabolites/HMDB01085
116 **Longley J**, Duffy TP, Kohn S. The mast cell and mast cell disease. *J Am Acad Dermatol* 1995; **32**: 545-651; quiz 562-564 [PMID: 7896943 DOI: 10.1016/0190-9622(95)90336-4]
117 **Rosbotham JL**, Malik NM, Syrris P, Jeffery S, Bedlow A, Gharraie S, Murday VA, Holden CA, Carter ND. Lack of c-kit mutation in familial urticaria pigmentosa. *Br J Dermatol* 1999; **140**: 849-852 [PMID: 10354021 DOI: 10.1046/j.1365-2133.1999.02814.x]
118 **Lim KH**, Tefferi A, Lasho TL, Finke C, Patnaik M, Butterfield JH, McClure RF, Li CY, Pardanani A. Systemic mastocytosis in 342 consecutive adults: survival studies and prognostic factors. *Blood* 2009; **113**: 5727-5736 [PMID: 19363219 DOI: 10.1182/blood-2009-02-205237]
119 **Molderings GJ**, Kolck U, Scheurlen C, Brüss M, Frieling T, Raithel M, Homann J. Systemic mast cell disease with gastrointestinal symptoms--a diagnostic questionnaire. *Dtsch Med Wochenschr* 2006; **131**: 2095-2100 [PMID: 16981082 DOI: 10.1055/s-2006-951337]

P- Reviewers: Fozza C, Imashuku S, Takahashi M
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Wu HL

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
wjhematol@wjgnet.com
www.wjgnet.com

World J Hematol 2014 February 6; 3(1): I-V
ISSN 2218-6204 (online)

GENERAL INFORMATION

World Journal of Hematology (*World J Hematol*, *WJH*, online ISSN 2218-6204, DOI: 10.5315) is a peer-reviewed open access (OA) academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

Aim and scope

WJH covers topics concerning experimental, clinical, oncological and transplant hematology, transfusion science, hemostasis and thrombosis, evidence-based medicine, epidemiology and nursing. The current columns of *WJH* include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography. Priority publication will be given to articles concerning diagnosis and treatment of hematological diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

We encourage authors to submit their manuscripts to *WJH*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

WJH is edited and published by Baishideng Publishing Group (BPG). BPG has a strong professional editorial team composed of science editors, language editors and electronic editors. BPG currently publishes 42 OA clinical medical journals, including 41 in English, has a total of 15 471 editorial borad members or peer reivewers, and is a world first-class publisher.

Columns

The columns in the issues of *WJH* will include: (1) Editorial: The editorial board members are invited to make comments on an important topic in their field in terms of its current research status and future directions to lead the development of this discipline; (2) Frontier: The editorial board members are invited to select a highly cited cutting-edge original paper of his/her own to summarize major findings, the problems that have been resolved and remain to be resolved, and future research directions to help readers understand his/her important academic point of view and future research directions in the field; (3) Diagnostic Advances: The editorial board members are invited to write high-quality diagnostic advances in their field to improve the diagnostic skills of readers. The topic covers general clinical diagnosis, differential diagnosis, pathological diagnosis, laboratory diagnosis, imaging diagnosis, endoscopic diagnosis, biotechnological diagnosis, functional diagnosis, and physical diagnosis; (4) Therapeutics Advances: The editorial board members are invited to write high-quality therapeutic advances in their field to help improve the therapeutic skills of readers. The topic covers medication therapy, psychotherapy, physical therapy, replacement therapy, interventional therapy, minimally invasive therapy, endoscopic therapy, transplantation therapy, and surgical therapy; (5) Field of Vision: The editorial board members are invited to write commentaries on classic articles, hot topic articles, or latest articles to keep readers at the forefront of research and increase their levels of clinical research. Classic articles refer to papers that are included in Web of Knowledge and have received a large number of citations (ranking in the top 1%) after being published for more than years, reflecting the quality and impact of papers. Hot topic articles refer to papers that are included in Web of Knowledge and have received a large number of citations after being published for no more than 2 years, reflecting cutting-edge trends in scientific research. Latest articles refer to the latest published high-quality papers that are included in PubMed, reflecting the latest research trends. These commentary articles should focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions. Basic information about the article to be commented (including authors, article title, journal name, year, volume, and inclusive page numbers; (6) Minireviews: The editorial board members are invited to write short reviews on recent advances and trends in research of molecular biology, genomics, and related cutting-edge technologies to provide readers with the latest knowledge and help improve their diagnostic and therapeutic skills; (7) Review: To make a systematic review to focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions; (8) Topic Highlight: The editorial board members are invited to write a series of articles (7-10 articles) to comment and discuss a hot topic to help improve the diagnostic and therapeutic skills of readers; (9) Medical Ethics: The editorial board members are invited to write articles about medical ethics to increase readers' knowledge of medical ethics. The topic covers international ethics guidelines, animal studies, clinical trials, organ transplantation, *etc.*; (10) Clinical Case Conference or Clinicopathological Conference: The editorial board members are invited to contribute high-quality clinical case conference; (11) Original Articles: To report innovative and original findings in hematology; (12) Brief Articles: To briefly report the novel and innovative findings in hematology; (13) Meta-Analysis: To evaluate the clinical effectiveness in hematology by using data from two or more randomised control trials; (14) Case Report: To report a rare or typical case; (15) Letters to the Editor: To discuss and make reply to the contributions published in *WJH*, or to introduce and comment on a controversial issue of general interest; (16) Book Reviews: To introduce and comment on quality monographs of hematology; and (17) Autobiography: The editorial board members are invited to write their autobiography to provide readers with stories of success or failure in their scientific research career. The topic covers their basic personal information and information about when they started doing research work, where and how they did research work, what they have achieved, and their lessons from success or failure.

Name of journal

World Journal of Hematology

ISSN

ISSN 2218-6204 (online)

Launch date

June 6, 2012

Frequency

Quarterly

Editor-in-Chief

Xiaoyan Jiang, MD, PhD, Associate Professor, Medical Gen-

Editorial office
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Hematology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: wjhematol@wjgnet.com
http://www.wjgnet.com

Publisher
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza, 315-321 Lockhart Road,
Wan Chai, Hong Kong, China
Telephone: +852-58042046
Fax: +852-31158812
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

Production center
Beijing Baishideng BioMed Scientific Co., Limited
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381892
Fax: +86-10-85381893

Representative office
USA Office
8226 Regency Drive,
Pleasanton, CA 94588-3144, United States

Instructions to authors
Full instructions are available online at http://www.wjgnet.com/2218-6204/g_info_20100722173604.htm.

Indexed and Abstracted in
Digital Object Identifier.

SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing
Statistical review is performed after peer review. We invite an expert in Biomedical Statistics to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

Conflict-of-interest statement
In the interests of transparency and to help reviewers assess any potential bias, *WJH* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of informed consent
Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Statement of human and animal rights
When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now

available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://www.wjgnet.com/esps/. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/2218-6204/g_info_20100722173604.htm) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to wjhematol@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Title: Title should be less than 12 words.

Running title: A short running title of less than 6 words should be provided.

Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece

Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, *e.g.*, Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, *e.g.*, Telephone: +86-10-85381892 Fax: +86-10-85381893

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision on acceptance is made only when at least two experts recommend publication of an article. All peer-reviewers are acknowledged on Express Submission and Peer-review System website.

Abstract

There are unstructured abstracts (no less than 200 words) and structured abstracts. The specific requirements for structured abstracts are as follows:

An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, *e.g.*, 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Core tip

Please write a summary of less than 100 words to outline the most innovative and important arguments and core contents in your paper to attract readers.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc.* It is our principle to publish high resolution-figures for the E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P <$

0.05, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID and DOI

Pleased provide PubMed citation numbers to the reference list, *e.g.*, PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/2218-6204/g_info_20100723213202.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing

All types of articles' writing style and requirement will be found in the link: http://www.wjgnet.com/esps/NavigationInfo.aspx?id=15

RESUBMISSION OF THE REVISED MANUSCRIPTS

Authors must revise their manuscript carefully according to the revision policies of Baishideng Publishing Group Co., Limited. The revised version, along with the signed copyright transfer agreement, responses to the reviewers, and English language Grade A certificate (for non-native speakers of English), should be submitted to the online system *via* the link contained in the e-mail sent by the editor. If you have any questions about the revision, please send e-mail to esps@wjgnet.com.

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; and (4) Grade D: rejected. Revised articles should reach Grade A.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/2218-6204/g_info_20100723213049.htm.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/2218-6204/g_info_20100723212803.htm.

Proof of financial support

For papers supported by a foundation, authors should provide a copy of the approval document and serial number of the foundation.

Links to documents related to the manuscript

WJH will be initiating a platform to promote dynamic interactions between the editors, peer reviewers, readers and authors. After a manuscript is published online, links to the PDF version of the submitted manuscript, the peer-reviewers' report and the revised manuscript will be put on-line. Readers can make comments on the peer reviewer's report, authors' responses to peer reviewers, and the revised manuscript. We hope that authors will benefit from this feedback and be able to revise the manuscript accordingly in a timely manner.

Publication fee

WJH is an international, peer-reviewed, OA online journal. Articles published by this journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium and format, provided the original work is properly cited. The use is non-commercial and is otherwise in compliance with the license. Authors of accepted articles must pay a publication fee. Publication fee: 600 USD per article. All invited articles are published free of charge.

ISSN 2218-6204 (online)

World Journal of *Hematology*

World J Hematol 2014 May 6; 3(2): 18-48

Baishideng Publishing Group
www.wjgnet.com

World Journal of Hematology

Contents

Quarterly Volume 3 Number 2 May 6, 2014

Contents

NAME OF JOURNAL
World Journal of Hematology

ISSN
ISSN 2218-6204 (online)

LAUNCH DATE
June 6, 2012

FREQUENCY
Quarterly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Hematology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjhematol@wjgnet.com
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
Hong Kong, China
Fax: +852-6555-7188
Telephone: +852-3177-9906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
May 6, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/2218-6204/g_info_20100722173604.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5315/wjh.v3.i2.18

World J Hematol 2014 May 6; 3(2): 18-28
ISSN 2218-6204 (online)

EDITORIAL

Ex vivo expansion of hematopoietic stem and progenitor cells: Recent advances

Katsuhiro Kita, Fangming Xiu, Marc G Jeschke

Katsuhiro Kita, Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch, Galveston, TX 77555, United States
Fangming Xiu, Sunnybrook Research Institute, University of Toronto, Ontario, M5S 2W6, Canada
Marc G Jeschke, Ross Tilley Burn Centre, Sunny brook Health Science Centre and Sunnybrook Research Institute and Division of Plastic Surgery, Department of Surgery, University of Toronto, Toronto, M5S 2W6, Canada
Author contributions: All authors solely contributed to this paper.
Supported by Canadian Institutes of Health Research, No. 123336; CFI Leader's Opportunity Fund, No. 25407; Physicians' Services Incorporated Foundation - Health Research Grant Program (MGJ)
Correspondence to: Katsuhiro Kita, PhD, Research Scientist of Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555, United States. kakitatemp@yahoo.com
Telephone: +1-212-7460293 Fax: +1-212-7468866
Received: April 17, 2012 Revised: December 7, 2013
Accepted: January 17, 2014
Published online: May 6, 2014

Abstract

Hematopoietic stem cells (HSCs) have become the most extensively studied stem cells and HSC-based cellular therapy is promising for hematopoietic cancers and hereditary blood disorders. Successful treatment of patients with HSC cells depends on sufficient number of highly purified HSCs and progenitor cells. However, stem cells are a very rare population no matter where they come from. Thus, *ex vivo* amplification of these HSCs is essential. The heavy demands from more and more patients for HSCs also require industrial-scale expansion of HSCs with lower production cost and higher efficiency. Two main ways to reach that goal: (1) to find clinically applicable, simple and efficient methods (or reagents) to enrich HSCs; (2) to find new developmental regulators and chemical compounds in order to replace the currently used cytokine cocktails for HSCs amplification. In this Editorial review, we would like to introduce the current status of *ex vivo* expansion of HSCs, particularly focusing on enrichment and culture supplements.

Key words: Hematopoietic stem cell; *Ex vivo* expansion; Serum-free culture; Cell surface markers; Enrichment; Stem cell isolation

Kita K, Xiu F, Jeschke MG. *Ex vivo* expansion of hematopoietic stem and progenitor cells: Recent advances. *World J Hematol* 2014; 3(2): 18-28 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i2/18.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i2.18

INTRODUCTION

The early evidence and definition of hematopoietic stem cells (HSCs) came from studies of people exposed to lethal doses of radiation in 1945 and the pioneering studies by Till and McCullock[1]. But it took until late 1980s and early 1990s for researchers to show the isolation and characterization of HSCs from human and rodents[2-4]. Since then, HSCs have become the most extensively studied stem cells (SCs), and HSC-based cellular therapy is promising to treat hematopoietic cancers, such as leukemia and lymphoma, and hereditary blood disorders such as inherited anemia.

HSCs are a small sub-set of SCs generated by the bone marrow (BM) niche which are essential for self-renewal and derivation of whole blood systems. Therefore, any deficiency of HSCs will cause serious, mostly fatal, outcomes as seen in patients of hematopoietic malignancies treated with high doses of chemotherapy and radiation. To treat patients with these complica-

tions, BM transplantation has been widely used since 1980s. Although whole BM transplantation used to be the only clinically viable option, increased numbers of *ex vivo*-expanded HSCs isolated from either the BM, or peripheral blood or cord blood cells are being used for current transplantation procedures in clinics. Currently, approximately 3000 clinical trials can be found in US with 1/3 of trials focused on the applications of HSCs for leukemia treatment (http://www.clinicaltrials.gov/). Hereafter we use the term HSCs to represent both HSCs and hematopoietic progenitor cells. We should emphasize that cells used in clinical settings are HSC-enriched but do contain progenitors. Thus, the term "HSCs" used throughout this review includes the $CD34^+$ fraction. HSCs only constitute a very minor fraction of the whole blood or BM cells, 1/10000 for BM SCs and 1/100000 for peripheral blood SCs. Thus, it is absolutely essential to develop large-scale culture methods for *ex vivo* expansion. Although HSC culture is the most advanced system among SC culture, further improvement of *ex vivo* culture for HSC expansion is important. Particularly, animal product (*e.g.*, serum)-free cell culture media will be one of the most important factors to be considered. In this short review, we would like to introduce the current status of *ex vivo* culture of HSCs and the most recently emerging reagents. Although culture devices (such as bioreactors) are another important component in *ex vivo* expansion, the main purpose of this Editorial review is to concisely summarize the new advances in enrichment of HSCs and *ex vivo* expansion of HSCs. For additional information on culture devices, we recommend reading Nielsen's excellent review[5]. We also suggest a few recent review articles that nicely and concisely summarized recent advances in the area of *ex vivo* expansion of hematopoietic stem/progenitor cells[6,7].

SOURCES OF HSC

There are potentially three main sources of haematopoietic stem cells for transplantation - BM, peripheral blood stem cells and umbilical cord blood stem cells. Table 1 summarizes advantages and disadvantages of 3 sources. Over the past 40 years, BM transplantation and stem cells isolated from BM has been widely used to treat numerous malignant and nonmalignant diseases. In spite of increasing therapeutic applications utilizing G-CSF to mobilize HSCs into circulation and $CD34^+$ cells are collected[8], BM cell isolation is still a widely accepted HSC source worldwide[9,10]). However, isolation of HSCs from BM is not a comfortable experience for donors, and it carries the risk of infection transmission. Increasing numbers of studies have used umbilical cord blood (UCB) as an alternative source to obtain HSCs. UCB has fewer ethical issues for obtaining cells, rapid availability and reduced stringency for HLA matching[11] make them another promising source of HSC for transplantation. Probably because of lower effective infused cell dose, slower engraftment of UCB-derived HSCs than BM-derived HSCs has been reported[12,13]. In addition, some progenitor cell populations (myeloid progenitors) in UCB were reported to be more chemoresistant[14], which could be beneficial when combined with high-dose chemotherapies. The most recent study describing the isolation of single HSC capable of long-term, multi-lineage engraftment derived from UCB, which suggests UCB cell may contain functionally most primitive HSCs[15]. Although UCB is likely to be more popular as a source to obtain HSCs, further research and careful examinations will certainly be necessary to determine the pros/cons, similarities/differences, and quantity/quality of HSCs isolated from both BM and UCB.

ENRICHMENT OF HSC

As previously mentioned, Weismann's group was the first group demonstrating isolation and purification of HSCs (from mouse BM)[2]. This first study used multiple HSC markers including Thy-1^{low}, Lin^- and Sca-1^+, a widely used criteria nowadays, to purify HSCs[2]. Note that Sca-1 is not found in human HSCs. Since HSCs are a very rare population in whole BM cells (1 in 10000-15000 BM cells), enrichment of HSCs certainly is a very important step for effective *ex vivo* expansion of HSCs. CD34 is a widely recognized cell surface marker to enrich HSCs (as reviewed in[16]). Immunoselection based on cell surface CD34 expression is well accepted in clinical settings, although it should be noted that the $CD34^+$ fraction does contain many progenitor cells such as endothelial progenitor cells[17]. Since a $CD34^+$ fraction still consists of crude cell populations, a $CD34^+CD38^-$ fraction may contain a higher percentage of primitive HSCs[18]. Therefore, a $CD34^+CD38^-Lin^-Sca^+$ selection criteria may be reasonable for selecting a highly enriched, primitive fraction of HSCs. c-Kit (stem cell factor receptor/CD117)[19,20] and CD133[21,22] are also the markers for the selection, although combination of too many antibodies may not be realistic in clinical settings because of a significant loss of HSCs during purification. We would like to note that these selections would certainly help enrich for higher quality HSCs; however, the above processes are still not enough to identify a nearly pure, HSC population. Most recently, John Dick's group isolated single hematopoietic stem cells[15] whose phenotype is $Lin^-CD34^+CD38^-Thy1^+CD45RA^-Rho^{low}CD49f^+$ can regenerate the entire hematopoietic system.

It should also be noted that there is evidence showing a considerable number of HSCs can be CD34 negative[23-25]. There are two possibilities accounting for why some studies have shown apparently different trends (cite examples of CD34 negative). First of all, CD34-positive and -negative cells may be interchangeable[26]. This means that HSCs may retain CD34 in intracellular compartments upon induction of cell surface CD34 molecules. Since the majority of anti-CD34 antibodies used for flow cytometric analyses recognize the extracellular domain of CD34, CD34 expression will appear to be negative when intracellular accumulation of CD34 occurs (unless the plasma membrane of the cells are

Table 1 Comparison of the sources for hematopoietic stem cells

	Advantages	Disadvantages
Bone marrow	1 The longest history as a source of HSC 2 Well established procedure	1 High stringency for HLA matching 2 Some complications associated with harvesting 3 Risk of GVHD
Umbilical cord blood	1 Off-the-shelf availability 2 Reduced stringency for HLA matching 3 Low risk of infection/transmission 4 Absence of donor risk	1 Delayed engraftment 2 Lower yield 3 Lack of additional immune cells
Peripheral blood	Compared to BM, 1 More comfortable for donors 2 Better yield with mobilization	1 Extremely low level of HSC (1/100000) without "mobilization" 2 Risk of GVHD

HSC: Hematopoietic stem cell; HLA: Human leukocyte antigen; BM: Bone marrow; GVHD: Graft-versus-host disease.

Table 2 Cell-surface makers of undifferentiated hematopoietic stem cells

Mouse	Human
$CD34^{low/-}$	$CD34^{low/-}$
$Sca\text{-}1^{+}$	$Sca\text{-}1^{+}$
$CD90/Thy\text{-}1^{+/low}$	$CD90/Thy\text{-}1^{+/low}$
$c\text{-}Kit^{+}$	$c\text{-}Kit^{+}$
$CD38^{+}$	$CD38^{-}$
$CD150^{+}$	$CD7^{-}$
Side population (high hoechst-efflux activity)	$CD49f^{+}$
$CD48^{-}$	Rhodamine 123^{low}
$CD244^{-}$	$CD133/AC133^{-}$
Lin^{-}	$CD45RA^{-}$
	Lin^{-}

permeabilized). The exact mechanism why $CD34^{+}$ HSCs and $CD34^{-}$ HSCs have interchangeable phenotypes remains unresolved. In mouse, the most primitive HSCs can be either $CD34^{-}$ or $CD34^{low}$. Nevertheless, human HSCs can be found in $CD34^{+}$ fraction[27] (Table 2). The other potential pitfall is a technical issue related to anti-mouse CD34 monoclonal antibodies (mAbs). There are several anti-mouse CD34 mAbs available to stain cell surface CD34. At least two clones widely used among researchers, clones MEC14.7[28] and HM34[29], have different characteristics in regard to staining. HM34 is reported to be unable to stain CD34 expression on marrow cells by some unknown mechanism. In addition, clone MEC14.7 is known to show relatively low affinity against CD34; therefore, it is recommended to incubate the mAb with samples for a prolonged period of 90-120 min (in contrast, the majority of commonly used protocols in flow cytometry suggest a 30-60 min incubation). Some of the murine HSC studies might not have carefully checked these technical tips requiring careful interpretation of studies reporting $CD34^{-}$ mouse HSCs.

It should likewise be noted that positive selection leaves antibodies attached on the cell surface which may not be favorable in terms of quality control issues. Furthermore, as most recently discussed by Lodish's group[30], the large amount of antibodies used for a clinical scale (10^{9} per unit for cord blood transplantation) will certainly raise the cost for purification of HSCs. Therefore, development of chemical or enzymatic activity-based substrates may be a great advantage to enrich HSCs in clinical settings. Chemical substrates could help lowering production costs, and simplifying quality control compared to mAbs. In addition, substrate-based selection may shorten the time and reduce the labors to prepare a HSC-rich fraction. Interestingly, hematopoietic progenitor cells have been shown to highly express cytosolic aldehyde dehydrogenase (ALDH)[31]. A fluorescent dye-conjugated ALDH substrate, developed by Storms and colleagues[32], enabled them to conduct a relatively simple, ALDH activity-based selection of HSCs and hematopoietic progenitor cells. Prior to this method, side populations selected based on influx of the DNA staining dye, Hoechst 33342 had been reported[33-36]. The combination of Hoechst dye efflux activity and ALDH activity was first shown by Pearce and Bonnet[37]. Their study showed that Hoechst side population (SP) only contained $CD34^{+}$ cells, thus, SP-based selection may not be suitable to enrich certain sub-populations such as $CD34^{-}CD38^{+}$ cells; which suggests that, at least in mouse, SP may not contain the most primitive HSC. Therefore, further studies need to confirm whether SP contains the primitive HSC population in humans. The authors concluded that Hoechst exclusion may not be appropriate for HSC isolation[37]. It was also reported that Hoechst 33342 is even more toxic than the other most commonly used DNA staining dye, 4',6-diamidino-2-phenyl indole dihydrochloride[38], by interrupting DNA topoisomerase I[39]. Topoisomerases play an essential role in cutting damaged DNA during DNA replication. Even temporary inhibition of a topoisomerase, might cause accumulation of unfavorable mutations during *ex vivo* expansion of HSCs; therefore, careful assessment on the long-term effects of the use of Hoechst 33342 must be assessed to pursue an application of Hoechst 33342 for HSC selection. Overall, currently ALDH-based selection may be the best option to sort/enrich HSCs.

Although it is very important to explore the best set of markers to purify the most primitive fraction of HSCs for *ex vivo* expansion and transplantation, significant technical hurdles exist. The combination of many surface antigens to purify HSCs causes a considerable reduction of the number of cells, which may limit the application in clinical settings. The other potentially impor-

tant issue is that maintaining the capacity of self-renewal while preserving the primitive capabilities of these HSCs during expansion of HSCs may require paracrine signals from other less primitive progenitor cells or differentiated leukocytes in culture. As we will describe in the following section, keeping the number of primitive HSCs in HSC-enriched (but still crude) fractions is certainly important. In fact, a simple $CD34^+$ fraction gives cells with enough *ex vivo* expansion capacity[40] to be used in clinical settings. In addition, the most "primitive" HSCs are quiescent, and rapid proliferation of these cells may cause the loss of primitive HSCs[41]. Therefore, rapid expansion of the small number of HSCs may often force HSCs to exit from the resting cycle and lead to HSC exhaustion. It is worthy of note that a nearly pure HSC fraction may not be suitable for large-scale expansion. Another critical issue needs to consider is the long-term repopulation capability of *ex vivo*-expanded fractions. This capability may depend on the number of primitive HSCs after *ex vivo* culture. Therefore, from the clinical stand point, the most important goal in *ex vivo* expansion of HSC may be to develop simple and efficient protocols to obtain HSC-enriched fractions containing a sufficient number of HSCs that would result in satisfactory *in vivo* engraftment after transplantation. Faster recovery of the number of neutrophils in the body seems to be an indicator of successful engraftment of transplanted cells[42], which again justifies the possible advantage of HSC-enriched fractions over nearly pure HSCs. In summary, even great efforts have been made to search the markers for HSCs, it is important to stress that none of the surface markers is entirely specific to the long-term HSCs.

EX VIVO CULTURE MEDIA FOR HSC EXPANSION

Since the limited number of HSCs is the major obstacle in clinical applications of *ex vivo*-expanded HSCs, successful *ex vivo* expansion of HSCs is one of the critical determinants emphasized throughout this review. This is particularly important for adult patients, who require increased units of HSCs in comparison to pediatric patients. The HSC field is relatively well established in comparison to the other SCs, and serum-free culture systems have already been used to expand HSCs. Development of serum-free culture is necessary to avoid the use of animal products such as bovine serum. As mentioned above, it is very important to avoid the use of animal products: (1) to prevent transmission of any possible diseases from animals, such as Creutzfeldt-Jabob disease (caused by Prion protein); (2) to achieve good quality control of expanded cells. In addition, accumulation of animal serum proteins in HSCs might increase the risk of host immune response upon transplantation of *ex vivo* cultured HSCs. The current research has focused on a cytokine cocktail-based culture to allow significant expansion of enriched HSCs.

Another major hurdle in current *ex vivo*-expanded HSCs (or HSC-enriched units) in their clinical applications is that some primitive HSCs are lost during *ex vivo* expansion of HSCs. Since the most primitive HSCs are thought to be in a resting state, the goals to achieve rapid expansion of HSCs and retain primitive HSCs stand at odds to one another. Therefore, to achieve better clinical outcomes (to reduce morbidity and mortality after transplantation of *ex vivo*-expanded cells), it is also essential to improve the number of long-term repopulating cells; *i.e.*, to maintain the number of primitive cells that retain self-renewal capacity. We will address this issue later in the section "Expansion versus *in vivo* reconstitution". Co-culture of feeder cells such as a monolayer of mesenchymal stem cells with HSCs may be a better method, since theoretically feeder cell layers should be able to provide a physiologically more relevant environment (stromal cell-HSC interaction and simultaneous feeding of sets of growth factors/cytokines that facilitate expansion of HSCs without unfavorable differentiation). However, co-culture with a feeder cell layer is not suitable for large-scale culture, and thus is difficult to translate into clinical settings. Most recently, an attempt to introduce computer simulations was made to help understand complicated paracrine mechanisms involving progenitor cells and differentiated cells in HSC culture[43]. This study may bring a paradigm shift in *ex vivo* cutulre of HSCs. In the following sub-sections, we would like to discuss emerging substrates as well as currently used soluble factors.

Soluble factors (i.e., growth factors and cytokines)

Soluble factors, such as Flt3/Flk2 ligand[44,45], stem cell factor (SCF)[46,47], interleukin-3 (IL-3)[48-50], IL-6[51], and thrombopoietin (Tpo)[52-54] are commonly used as culture supplements for HSCs. Flt3/Flk2 ligand was discovered as a factor promoting proliferation of primitive hematopoietic cells, and[55] is more effective than SCF[56], although SCF probably has the longest history as a supplement. IL-3's effect *in vitro* was first reported by Spivak *et al*[57] However, there is a report showing the suppression of the number of colony-forming cells by IL-3[58], thus, the effect of IL-3 may need careful re-evaluation. This study also showed that IL-3 reduced the reconstituting activity of HSCs in a mouse model system[58]. Other interleukins, such as IL-7 and IL-11, are also included as additional supplements in serum-free culture systems because of their potential to promote HSC proliferation. IL-7 was originally discovered as an interleukin that stimulated proliferation of B cell progenitors in mouse[59]. Although IL-7 alone did not show significant expansion of $Lin^-Sca\text{-}1^+$ HSCs, potent synergistic effect on proliferation of $Lin^-Sca\text{-}1^+$ HSCs was observed when IL-7 was combined with IL-3[60]. IL-11 was cloned as a gene product produced by BM-derived stromal cell lines in 1990[61], and the following study by Ogawa's group showed that IL-11 could also synergistically help expansion of primitive hematopoietic progenitor cells with either IL-3 or IL-4[62].

One interesting study showed that gp130 signaling

(*via* IL-6) synergistically enhanced the effect of Flt3 ligand[63] as well as SCF[64]. Interestingly, IL-6 alone was not enough to trigger this effect[48,63,64]. These findings suggest that gp130 signaling appears to be somewhat supportive in its role of potentiating the effects of SCF and Flt3 ligand as culture supplements. Commonly-used cytokine cocktails often contain IL-6. Sustained activation of the Janus kinase (JAK)-STAT signaling pathway may be an important element in *ex vivo* expansion of HSCs. It is noteworthy that thrombopoietin was reported to activate JAK-STAT signaling and thereby helping to protect $CD34^+$ cells from apoptosis in serum-free media[65]. Moreover, activation of c-Kit and gp130 was shown to synergistically induce thrombopoietin production by cord blood $CD34^+$ cells themselves[66]. It should note that there is a report describing impaired engraftment of murine BM cells cultured in the presence of IL-6[67]. Similar to IL-6, it was reported in the same year that IL-3 reduced the number of colony-forming cells[58], impaired engraftment might be mainly associated with IL-3 in the culture. It was surprising that SCF and some of the interleukins could have adverse effects upon transplantation[67]; although SCF is a well-accepted growth factor for serum-free culture of HSCs or HSC-enriched fractions. In addition to the previously mentioned cytokines, fibroblast growth factors (FGFs) were also found to be effective to help *in vitro* culture of whole blood cells or enriched hematopoietic cells[68,69]. The later study also showed that FGF receptors were not expressed on human $CD34^+$ cells[70]. Thus, the effect of FGF may be controversial.

Recent studies by Lodish have added three endothelial growth factors (angiopoietin-like 5, insulin-like growth factor-binding protein 2, and pleiotrophin) as potential soluble factors that may further help *ex vivo* expansion of HSCs[71].

Although great efforts has been made to improve the rate of HSC engraftment, *ex vivo* cytokine-based expansion protocols may have reached plateau. The use of cytokine cocktails can raise the cost for *ex vivo* expansion of HSCs, too. Therefore, there are a great need of additional factors/molecules in order to support HSC self-renewal and amplification *in vitro*.

Exposure to developmental regulators

It has been suggested recently that some developmentally conserved pathways or transcriptional factors are important in the regulation of the adult stem cell compartment, such as wingless-type (Wnt), Notch, Hox transcription factors and Sonic hedge hog Shh/BMP signaling[72]. Among them, Notch signaling is the most extensively studied. Notch ligand may be the most promising recombinant protein product to assist in *ex vivo* expansion of HSCs. In 1994, $CD34^+$ cells were shown to express high levels of the human homolog of *Drosophila* Notch[73], and this initial observation led to hypothesize the role of Notch signaling in maintenance of undifferentiated status of HSCs. Immobilized Notch ligand (Delta-1) was shown to dramatically increase (up to approximately 100-fold) the number of $CD34^+$ cells[74]. More importantly, the expanded cells enhanced repopulating ability of hematopoietic cells in NOD/SCID mice[74]. The effectiveness of Notch ligand was further confirmed by a follow-up study by the same group[75]. The outcome of their clinical trial is very encouraging, because preliminary data of a phase I clinical trial showed engraftment of the $CD34^+$ cord blood cells has significant advantage over the controls including shorter periods for neutrophil recovery after transplantation[75]. One potential side-effect of Delta-1 is that it has potential to cause density-dependent apoptosis of cells[76], and it is necessary to carefully control ligand density to minimize the loss of cells or unfavorable phenotypic changes during expansion. Interestingly, the most recent study by Rafii's group highlighted the critical role of Notch signaling in the BM microenvironment, where Notch activation by BM endothelium is essential for self-renewal of long-term HSCs[77]. This study clearly indicates that Notch signaling is a naturally-occurring, critical signal for maintenance of HSCs.

Besides Notch 1 ligand, TAT (HIV virus-derived cell permeable peptide)-tagged HOXB4 protein was shown to be effective to expand HSCs *in vitro*[78]. The advantage of TAT-HOXB4 protein over Notch ligands is the lack of a requirement to immobilize it onto the culture apparatus. Overexpression of HOXB4 had been shown to be one of the most potent stimulators of HSC expansion[79,80]; however, recently it was reported that there was high incidence of leukemia in large animals two years after receiving HOXB4-carrying retrovirus-introduced *HSC* gene therapy[81]. Therefore, the use of the plasma membrane-permeable recombinant HOXB4 protein may be a reasonable alternative for *ex vivo* expansion of HSCs. Although Krosl *et al*[78] study showed the effectiveness of recombinant HOXB4, the study did not show long-term repopulating activity of expanded cells. Therefore, further studies examining any potential safety issues caused by HOXB4 would be necessary. Overall, Notch ligands seem to be the most promising peptide-type supplements for HSC expansion at current moment.

Stromal support

In the BM niche, HSCs interact with stromal cells, and direct interaction of HSCs with stromal cells or extracellular matrices in the BM may help maintain small populations of primitive HSCs. Thus, it was quite reasonable to hypothesize that major extracellular matrices in the BM niche facilitated sustained hematopoiesis. In fact, a major extracellular matrix, fibronectin, was found to be perhaps the most important extracellular matrix protein to facilitate proliferation/self-renewal as well as adhesion of HSCs and progenitor cells in the BM niche[82,83]. The initial studies showed that stromal support increased not only gene transduction efficiency but also successfully preserved the ability of human $CD34^+$ cells to sustain long-term hematopoiesis in immune deficient mice[84]. This study also showed that a C-terminal fragment of

fibronectin could help successful long-term engraftment of human HSCs and progenitor cells to bnx/hu mice. This C-terminal fragment contains both CS-1 and RGD(S) domains. The CS-1 domain is known to interact with VLA-4 (integrin α4β1)[85], and interestingly, Verfaillie *et al*[82] reported that primitive progenitors bound to fibronectin CS-1 *via* VLA-4, but expression of VLA-4 was lost upon differentiation of the cells. Immobilized fibronectin peptides were also shown to help *ex vivo* expansion of human cord blood HSCs ($CD34^+$ fraction)[86]. In this study, the authors used several fibronectin peptides including those carrying mutations on binding domains. Although all peptides they used showed significant increase in colony forming units as well as expansion of cells, only the peptide containing the intact CS-1 domain could give successful long-term engraftment of transplanted cells and survival of NOD/SCID mice[86]. Thus, short C-terminal fragments of fibronectin containing the CS-1 domain may be a good coating material for *ex vivo* expansion of HSCs or progenitor cells.

The major disadvantage of these proteins/peptides, except TAT-HOXB4 protein, is their requirement of immobilization onto the surface of cell culture apparatuses. This will increase culture volume as well as cost of industry-scale production of cells; thus, development of suspension culture systems that allow both self-renewal of HSCs and a reasonable level of expansion would be desirable.

Small molecules

It would be desirable to use chemical compounds as supplements for serum-free culture systems, which may significantly help reducproduction costs of HSCs at the industrial scale and at the same time, facilitate efficient *ex vivo* expansion of HSCs.

Most recently, one study has shown that a chemical compound could also be used for *ex vivo* expansion of HSCs[87]. Chemical compounds would be superior to biological compounds because of potentially lower production costs and easier quality control.

These promising chemical compounds include the retinoic acid receptor agonist all-trans retinoic acid, copper chelator tetraethylenepentamine (TEPA), histone deacetylase inhibitors, acryl hydrocarbon receptor antagonist [referred to as StemRegenin1 (SR1)] and, PGE2[72]. Among them, TEPA are the most promising chemical to be used for expansion of human HSCs at current moment. TEPA-supplemented, *ex vivo* cultures of $CD34^+$ cord blood cells significantly increased the number of HSCs and enhanced NOD/SCID repopulating capacity[88]. A phase I/II clinical trial of TEPA-cultured cord blood cells showed the safety of this approach[89]. The efficiency of TEPA-cultured HSPC is currently under investigation in an ongoing phase Ⅱ/Ⅲ study.

Aryl hydrocarbon receptor antagonists (SR1) were identified as potential drug candidates for promoting *ex vivo* expansion of HSCs by microscopy-based high-throughput screening[87]. The antagonist was subsequently tested with a feedback culture system[43]. Aryl hydrocarbon receptor signaling emerged as an important element in HSC functions[87]. Currently, these compounds require the supplementation of cytokines, and thus further studies will be necessary to apply this strategy for large scale expansion of HSCs without animal serum. Nevertheless, this discovery may eventually pave a way for establishing industrial-scale production of HSCs with chemically defined culture media.

EXPANSION *VS IN VIVO* RECONSTITUTION

Although finding the optimum conditions for clinical-scale *ex vivo* expansion culture is an absolutely important determinant to use HSCs in clinical settings, another important factor in determining the clinical outcomes of HSC transplantation is engraftment and reconstitution of the hematopoietic system in a recipient. Successful engraftment requires repopulation of transplanted cells to irradiated BM, of which success may mainly rely on the ability of transplanted cells to migrate toward BM. (SDF-1/CXCR4 axis). Therefore, it is also essential to test repopulation and engraftment of expanded cells by immunologically incompetent small animal models such as severe combined immunodeficient (SCID) mice. On the other hand, reconstitution of the hematopoietic system requires *de novo* production of hematopoietic lineages from donor-derived HSCs. This function requires self-renewable, primitive HSCs in the BM after transplantation to prevent exhaustion of self-renewable HSCs in BM niche. The $CD34^+$ fraction, which is commonly used in the current clinical settings as a source of HSC-enriched cells, should be T cell-free to avoid graft-versus-host-disease. Successful engraftment is likewise likely to be associated positively with the number of neutrophils present in the body[42].

Before closing this section, we also would like to comment on the quiescence of stem cells under normal conditions. HSCs retain labeled thymidine, indicating replicative quiescence in their niche[41]. We speculate that this may be because HSCs need to maintain longevity and to minimize mutations concomitant with replication errors. Therefore, a line of studies may be necessary to confirm that rapid *ex vivo* expansion of HSCs does not generate and accumulate genetic mutations that can lead to the formation of cancerous cells. In addition, we should always keep in our mind that rapid *ex vivo* expansion of HSCs may often cause the loss of long-term engraftment capacity of HSCs or exhaustion of the most primitive HSC pool. It was demonstrated for more than a decade ago that HSCs proliferate slow *in vivo*[90,91]. Therefore, it is postulated that HSC quiescence is critical in maintaining the stem cell compartment. Weissman's group estimated that approximately 8% of long-term self-renewing HSCs enter the cell cycle per day[91]. However, contradictory results appeared later showing traditionally used DNA labeling dye, BrdU (5-bromo-2-deoxyuridine), may not be specific enough to label slow-dividing cells[92]. More recently, different labeling

strategies have been proposed as alternative methods to trace slowly dividing HSC fractions[93,94]. Proliferation of cells requires the entry to G_1 cycle, thus, it is quite natural to hypothesize that perturbation of cyclin dependent kinase inhibitors may initiate dramatic cell proliferation followed by the exhaustion of HSC pools. Scadden's group showed for the first time, $p21^{cip/waf1}$ deletion eventually causes exhaustion of HSCs in a mouse model system[95].

Since phosphatase and tensin homolog (PTEN)/Akt pathway plays a critical role in cell proliferation, survival and growth, it was predicted that mis-regulation of this pathway could cause problems on hematopoiesis. In fact, PTEN knockout mice study showed decreased numbers of HSCs[96]. PTEN knockout mice also developed leukemia, suggesting there is a critical factors suppressing cell cycle progression. The phenotype of Foxo3a knockout mice is quite interesting. Foxo3a-KO significantly decreased the number of colony forming cells in BM. Age-dependent decrease of HSC pool was also observed. However, Foxo3a-KO little effects on hematopoietic progenitor cells except a decreased number of erythrocytes[97]. More interestingly, distribution of Foxo3a in a cell further implies Foxo3a's specific role in maintaining primitive HSCs; Foxo3a accumulate in inside of a nucleus in a $CD34^-c\text{-}Kit^+Sca\text{-}1^+lineage^-$ cell, however, Foxo3a no longer accumulates in a nucleus and translocates to a nuclear envelop in a $CD34^+c\text{-}Kit^+Sca\text{-}1^+lineage^-$ cell, which is less primitive than $CD34^-c\text{-}Kit^+Sca\text{-}1^+lineage^-$ cells. Thus, it appears that Foxo3a may hold a key role in the maintenance of HSCs in BM.

Although c-Kit may be dispensable for extensive proliferation of HSCs, mice carrying c-Kit mutation (W41/W41) were reported to show approximately 2-fold reduction of long term-HSCs[98]. This study was further reinforced with the result that multipotent progenitor cells were less affected by this mutation, suggesting that c-Kit function may also be very important for maintenance of primitive HSCs for sustained hematopoiesis.

Thus, we should keep in our mind that our attempt to improve *ex vivo* expansion of HSCs by manipulating some signaling pathways could be a "double-edged sword".

CONCLUSION

HSC-based cellular therapy is promising for hematopoietic cancers and hereditary blood disorders. Adaptive transfer of sufficient number of enriched HSCs is the key for successful transplantation. Besides BM, peripheral blood and umbilical cord blood also are the sources of HSCs. Traditionally, BM is the main source of HSCs, but peripheral blood and UCB are increasingly used as sources due to non-invasive harvesting procedures. Soluble growth factors, signaling molecules, small molecules and extracellular matrix support are commonly-used methods to amplify isolated HSCs, but chemical compounds such as TEPA is promising because of its lower cost and easier quality control.

A rapidly increasing number of studies and clinical trials have demonstrated very promising applications of HSCs in clinical settings. Currently, applications of HSCs are often limited to children mostly due to restrictions in the quantity of HSCs that can be obtained. Increased demand of HSC therapies, including those for adult patients, is expected. Therefore, it is necessary to establish large-scale production of HSCs to supply enough units of HSCs in the near future. In addition to cytokine cocktails, most recent studies have added several other options such as recombinant proteins and chemical compounds for *ex vivo* expansion of HSCs. Chemical-based culture would be advantageous, however, it will take some time until it is ready for clinical application.

Using multiple cell surface markers, including CD34, CD38, Thy-1, CD45, c-kit, CD133, CD49f and Lineage cocktail, it is possible to isolate single HSCs with the full capacity to regenerate the entire hematopoietic system. However, it is worthy to note that purification of ultimately primitive HSCs may not be necessary for clinical-scale production of cells. Instead, "HSC-enriched" fractions would be useful because of the possible positive feedback by the other less primitive cells in the culture. Thus, the effective *ex vivo* expansion of HSCs or hematopoietic progenitor cells may require inclusion of precise feedback regulation (*i.e.*, autocrine and paracrine), since the hematopoietic system is a highly complicated system requiring maintenance of a highly ordered hierarchy. Further investigation should develop more promising methods which will substantially enhance generation of HSCs with high efficiency for clinical application.

ACKNOWLEDGMENTS

The authors really appreciate Kimberlee Burckart (Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch) for editing the manuscript.

REFERENCES

1 **TILL JE**, McCULLOCH EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 1961; **14**: 213-222 [PMID: 13776896 DOI: 10.2307/3570892]
2 **Spangrude GJ**, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science* 1988; **241**: 58-62 [PMID: 2898810 DOI: 10.1126/science.2898810]
3 **Siena S**, Bregni M, Brando B, Ravagnani F, Bonadonna G, Gianni AM. Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. *Blood* 1989; **74**: 1905-1914 [PMID: 2478216]
4 **Berardi AC**, Wang A, Levine JD, Lopez P, Scadden DT. Functional isolation and characterization of human hematopoietic stem cells. *Science* 1995; **267**: 104-108 [PMID: 7528940 DOI: 10.1126/science.7528940]
5 **Nielsen LK**. Bioreactors for hematopoietic cell culture. *Annu Rev Biomed Eng* 1999; **1**: 129-152 [PMID: 11701485 DOI: 10.1146/annurev.bioeng.1.1.129]

6 **Hai-Jiang W**, Xin-Na D, Hui-Jun D. Expansion of hematopoietic stem/progenitor cells. *Am J Hematol* 2008; **83**: 922-926 [PMID: 18839435 DOI: 10.1002/ajh.21262]

7 **Dahlberg A**, Delaney C, Bernstein ID. Ex vivo expansion of human hematopoietic stem and progenitor cells. *Blood* 2011; **117**: 6083-6090 [PMID: 21436068 DOI: 10.1182/blood-2011-01-283606]

8 **Matsunaga T**, Sakamaki S, Kohgo Y, Ohi S, Hirayama Y, Niitsu Y. Recombinant human granulocyte colony-stimulating factor can mobilize sufficient amounts of peripheral blood stem cells in healthy volunteers for allogeneic transplantation. *Bone Marrow Transplant* 1993; **11**: 103-108 [PMID: 7679596]

9 **Neben S**, Marcus K, Mauch P. Mobilization of hematopoietic stem and progenitor cell subpopulations from the marrow to the blood of mice following cyclophosphamide and/or granulocyte colony-stimulating factor. *Blood* 1993; **81**: 1960-1967 [PMID: 7681707]

10 **Bensinger WI**, Clift RA, Anasetti C, Appelbaum FA, Demirer T, Rowley S, Sandmaier BM, Torok-Storb B, Storb R, Buckner CD. Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony stimulating factor. *Stem Cells* 1996; **14**: 90-105 [PMID: 8820955 DOI: 10.1002/stem.140090]

11 **Harris DT**, Schumacher MJ, Locascio J, Besencon FJ, Olson GB, DeLuca D, Shenker L, Bard J, Boyse EA. Phenotypic and functional immaturity of human umbilical cord blood T lymphocytes. *Proc Natl Acad Sci USA* 1992; **89**: 10006-10010 [PMID: 1438190 DOI: 10.1073/pnas.89.21.10006]

12 **Wagner JE**, Kernan NA, Steinbuch M, Broxmeyer HE, Gluckman E. Allogeneic sibling umbilical-cord-blood transplantation in children with malignant and non-malignant disease. *Lancet* 1995; **346**: 214-219 [PMID: 7616801 DOI: 10.1016/S0140-6736(95)91268-1]

13 **Barker JN**, Davies SM, DeFor T, Ramsay NK, Weisdorf DJ, Wagner JE. Survival after transplantation of unrelated donor umbilical cord blood is comparable to that of human leukocyte antigen-matched unrelated donor bone marrow: results of a matched-pair analysis. *Blood* 2001; **97**: 2957-2961 [PMID: 11342417 DOI: 10.1182/blood.V97.10.2957]

14 **Toren A**, Einat M, Fabian I, Nagler A. Human umbilical cord blood myeloid progenitor cells are relatively chemoresistant: a potential model for autologous transplantations in HIV-infected newborns. *Am J Hematol* 1997; **56**: 161-167 [PMID: 9371528]

15 **Notta F**, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 2011; **333**: 218-221 [PMID: 21737740 DOI: 10.1126/science.1201219]

16 **Krause DS**, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. *Blood* 1996; **87**: 1-13 [PMID: 8547630]

17 **Asahara T**, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964-966 [DOI: 10.1126/science.275.5302.964]

18 **Sakabe H**, Yahata N, Kimura T, Zeng ZZ, Minamiguchi H, Kaneko H, Mori KJ, Ohyashiki K, Ohyashiki JH, Toyama K, Abe T, Sonoda Y. Human cord blood-derived primitive progenitors are enriched in CD34+c-kit- cells: correlation between long-term culture-initiating cells and telomerase expression. *Leukemia* 1998; **12**: 728-734 [PMID: 9593271 DOI: 10.1038/sj.leu.2401001]

19 **Ogawa M**, Matsuzaki Y, Nishikawa S, Hayashi S, Kunisada T, Sudo T, Kina T, Nakauchi H, Nishikawa S. Expression and function of c-kit in hemopoietic progenitor cells. *J Exp Med* 1991; **174**: 63-71 [PMID: 1711568 DOI: 10.1084/jem.174.1.63]

20 **Briddell RA**, Broudy VC, Bruno E, Brandt JE, Srour EF, Hoffman R. Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to the c-kit receptor. *Blood* 1992; **79**: 3159-3167 [PMID: 1375842]

21 **Yin AH**, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 1997; **90**: 5002-5012 [PMID: 9389720]

22 **Miraglia S**, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK, Buck DW. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 1997; **90**: 5013-5021 [PMID: 9389721]

23 **Jones RJ**, Collector MI, Barber JP, Vala MS, Fackler MJ, May WS, Griffin CA, Hawkins AL, Zehnbauer BA, Hilton J, Colvin OM, Sharkis SJ. Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. *Blood* 1996; **88**: 487-491 [PMID: 8695796]

24 **Osawa M**, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 1996; **273**: 242-245 [PMID: 8662508 DOI: 10.1126/science.273.5272.242]

25 **Goodell MA**, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, Grupp SA, Sieff CA, Mulligan RC, Johnson RP. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* 1997; **3**: 1337-1345 [PMID: 9396603 DOI: 10.1038/nm1297-1337]

26 **Donnelly DS**, Krause DS. Hematopoietic stem cells can be CD34+ or CD34-. *Leuk Lymphoma* 2001; **40**: 221-234 [PMID: 11426544 DOI: 10.3109/10428190109057921]

27 **Shizuru JA**, Negrin RS, Weissman IL. Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. *Annu Rev Med* 2005; **56**: 509-538 [PMID: 15660525 DOI: 10.1146/annurev.med.54.101601.152334]

28 **Garlanda C**, Berthier R, Garin J, Stoppacciaro A, Ruco L, Vittet D, Gulino D, Matteucci C, Mantovani A, Vecchi A, Dejana E. Characterization of MEC 14.7, a new monoclonal antibody recognizing mouse CD34: a useful reage for identifying and characterizing blood vessels and hematopoietic precursors. *Eur J Cell Biol* 1997; **73**: 368-377 [PMID: 9270880]

29 **Brown J**, Greaves MF, Molgaard HV. The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain, and embryonic fibroblasts. *Int Immunol* 1991; **3**: 175-184 [PMID: 1709048 DOI: 10.1093/intimm/3.2.175]

30 **Chou S**, Chu P, Hwang W, Lodish H. Expansion of human cord blood hematopoietic stem cells for transplantation. *Cell Stem Cell* 2010; **7**: 427-428 [PMID: 20887947 DOI: 10.1016/j.stem.2010.09.001]

31 **Kastan MB**, Schlaffer E, Russo JE, Colvin OM, Civin CI, Hilton J. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood* 1990; **75**: 1947-1950 [PMID: 2337669]

32 **Storms RW**, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, Smith C. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci U S A* 1999; **96**: 9118-9123 [PMID: 10430905 DOI: 10.1073/pnas.96.16.9118]

33 **McAlister I**, Wolf NS, Pietrzyk ME, Rabinovitch PS, Priestley G, Jaeger B. Transplantation of hematopoietic stem cells obtained by a combined dye method fractionation of murine bone marrow. *Blood* 1990; **75**: 1240-1246 [PMID: 1968771]

34 **Leemhuis T**, Yoder MC, Grigsby S, Agüero B, Eder P, Srour EF. Isolation of primitive human bone marrow hematopoietic progenitor cells using Hoechst 33342 and Rhodamine 123. *Exp Hematol* 1996; **24**: 1215-1224 [PMID: 8765497]

35 **Bertoncello I**, Williams B. Hematopoietic stem cell characterization by Hoechst 33342 and rhodamine 123 staining. *Methods Mol Biol* 2004; **263**: 181-200 [PMID: 14976367]

36 **Rossi L**, Challen GA, Sirin O, Lin KK, Goodell MA. Hematopoietic stem cell characterization and isolation. *Methods Mol*

Biol 2011; **750**: 47-59 [PMID: 21618082 DOI: 10.1007/978-1-61779-145-1_3]
37 **Pearce DJ**, Bonnet D. The combined use of Hoechst efflux ability and aldehyde dehydrogenase activity to identify murine and human hematopoietic stem cells. *Exp Hematol* 2007; **35**: 1437-1446 [PMID: 17656008 DOI: 10.1016/j.exphem.2007.06.002]
38 **Park CH**, Kimler BF, Smith TK. Comparison of the supravital DNA dyes Hoechst 33342 and DAPI for flow cytometry and clonogenicity studies of human leukemic marrow cells. *Exp Hematol* 1985; **13**: 1039-1043 [PMID: 4054243]
39 **Chen AY**, Yu C, Bodley A, Peng LF, Liu LF. A new mammalian DNA topoisomerase I poison Hoechst 33342: cytotoxicity and drug resistance in human cell cultures. *Cancer Res* 1993; **53**: 1332-1337 [PMID: 8383008]
40 **Briddell RA**, Kern BP, Zilm KL, Stoney GB, McNiece IK. Purification of CD34+ cells is essential for optimal ex vivo expansion of umbilical cord blood cells. *J Hematother* 1997; **6**: 145-150 [PMID: 9131444]
41 **Zhang J**, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 2003; **425**: 836-841 [PMID: 14574412 DOI: 10.1038/nature02041]
42 **Fernández MN**, Regidor C, Cabrera R, García-Marco JA, Forés R, Sanjuán I, Gayoso J, Gil S, Ruíz E, Little AM, McWhinnie A, Madrigal A. Unrelated umbilical cord blood transplants in adults: Early recovery of neutrophils by supportive co-transplantation of a low number of highly purified peripheral blood CD34+ cells from an HLA-haploidentical donor. *Exp Hematol* 2003; **31**: 535-544 [PMID: 12829030 DOI: 10.1016/S0301-472X(03)00067-5]
43 **Csaszar E**, Kirouac DC, Yu M, Wang W, Qiao W, Cooke MP, Boitano AE, Ito C, Zandstra PW. Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. *Cell Stem Cell* 2012; **10**: 218-229 [PMID: 22305571]
44 **McKenna HJ**, de Vries P, Brasel K, Lyman SD, Williams DE. Effect of flt3 ligand on the ex vivo expansion of human CD34+ hematopoietic progenitor cells. *Blood* 1995; **86**: 3413-3420 [PMID: 7579445]
45 **Conneally E**, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. *Proc Natl Acad Sci U S A* 1997; **94**: 9836-9841 [PMID: 9275212 DOI: 10.1073/pnas.94.18.9836]
46 **Gabutti V**, Timeus F, Ramenghi U, Crescenzio N, Marranca D, Miniero R, Cornaglia G, Bagnara GP. Expansion of cord blood progenitors and use for hemopoietic reconstitution. *Stem Cells* 1993; **11** Suppl 2: 105-112 [PMID: 7691315 DOI: 10.1002/stem.5530110818]
47 **Abboud MR**, Xu F, Payne A, Laver J. Effects of recombinant human Steel factor (c-kit ligand) on early cord blood hematopoietic precursors. *Exp Hematol* 1994; **22**: 388-392 [PMID: 7512047]
48 **Kyoizumi S**, Murray LJ, Namikawa R. Preclinical analysis of cytokine therapy in the SCID-hu mouse. *Blood* 1993; **81**: 1479-1488 [PMID: 7680919]
49 **Rossmanith T**, Schröder B, Bug G, Müller P, Klenner T, Knaus R, Hoelzer D, Ottmann OG. Interleukin 3 improves the ex vivo expansion of primitive human cord blood progenitor cells and maintains the engraftment potential of scid repopulating cells. *Stem Cells* 2001; **19**: 313-320 [PMID: 11463951 DOI: 10.1634/stemcells.19-4-313]
50 **Bryder D**, Jacobsen SE. Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. *Blood* 2000; **96**: 1748-1755 [PMID: 10961873]
51 **Tajima S**, Tsuji K, Ebihara Y, Sui X, Tanaka R, Muraoka K, Yoshida M, Yamada K, Yasukawa K, Taga T, Kishimoto T, Nakahata T. Analysis of interleukin 6 receptor and gp130 expressions and proliferative capability of human CD34+ cells. *J Exp Med* 1996; **184**: 1357-1364 [PMID: 8879208 DOI: 10.1084/jem.184.4.1357]
52 **Schipper LF**, Brand A, Reniers NC, Melief CJ, Willemze R, Fibbe WE. Effects of thrombopoietin on the proliferation and differentiation of primitive and mature haemopoietic progenitor cells in cord blood. *Br J Haematol* 1998; **101**: 425-435 [PMID: 9633882 DOI: 10.1046/j.1365-2141.1998.00737.x]
53 **Ohmizono Y**, Sakabe H, Kimura T, Tanimukai S, Matsumura T, Miyazaki H, Lyman SD, Sonoda Y. Thrombopoietin augments ex vivo expansion of human cord blood-derived hematopoietic progenitors in combination with stem cell factor and flt3 ligand. *Leukemia* 1997; **11**: 524-530 [DOI: 10.1038/sj.leu.2400588]
54 **Piacibello W**, Sanavio F, Garetto L, Severino A, Danè A, Gammaitoni L, Aglietta M. The role of c-Mpl ligands in the expansion of cord blood hematopoietic progenitors. *Stem Cells* 1998; **16** Suppl 2: 243-248 [PMID: 11012196 DOI: 10.1002/stem.5530160727]
55 **Lyman SD**, James L, Vanden Bos T, de Vries P, Brasel K, Gliniak B, Hollingsworth LT, Picha KS, McKenna HJ, Splett RR. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell* 1993; **75**: 1157-1167 [PMID: 7505204 DOI: 10.1016/0092-8674(93)90325-K]
56 **Shapiro F**, Pytowski B, Rafii S, Witte L, Hicklin DJ, Yao TJ, Moore MA. The effects of Flk-2/flt3 ligand as compared with c-kit ligand on short-term and long-term proliferation of CD34+ hematopoietic progenitors elicited from human fetal liver, umbilical cord blood, bone marrow, and mobilized peripheral blood. *J Hematother* 1996; **5**: 655-662 [PMID: 9117254 DOI: 10.1089/scd.1.1996.5.655]
57 **Spivak JL**, Smith RR, Ihle JN. Interleukin 3 promotes the in vitro proliferation of murine pluripotent hematopoietic stem cells. *J Clin Invest* 1985; **76**: 1613-1621 [PMID: 3932469 DOI: 10.1172/JCI112145]
58 **Yonemura Y**, Ku H, Hirayama F, Souza LM, Ogawa M. Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. *Proc Natl Acad Sci USA* 1996; **93**: 4040-4044 [PMID: 8633013 DOI: 10.1073/pnas.93.9.4040]
59 **Namen AE**, Lupton S, Hjerrild K, Wignall J, Mochizuki DY, Schmierer A, Mosley B, March CJ, Urdal D, Gillis S. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* 1988; **333**: 571-573 [PMID: 3259677 DOI: 10.1038/333571a0]
60 **Jacobsen FW**, Veiby OP, Skjønsberg C, Jacobsen SE. Novel role of interleukin 7 in myelopoiesis: stimulation of primitive murine hematopoietic progenitor cells. *J Exp Med* 1993; **178**: 1777-1782 [PMID: 7693856 DOI: 10.1084/jem.178.5.1777]
61 **Paul SR**, Bennett F, Calvetti JA, Kelleher K, Wood CR, O'Hara RM, Leary AC, Sibley B, Clark SC, Williams DA. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proc Natl Acad Sci USA* 1990; **87**: 7512-7516 [PMID: 2145578 DOI: 10.1073/pnas.87.19.7512]
62 **Musashi M**, Clark SC, Sudo T, Urdal DL, Ogawa M. Synergistic interactions between interleukin-11 and interleukin-4 in support of proliferation of primitive hematopoietic progenitors of mice. *Blood* 1991; **78**: 1448-1451 [PMID: 1832057]
63 **Ebihara Y**, Tsuji K, Lyman SD, Sui X, Yoshida M, Muraoka K, Yamada K, Tanaka R, Nakahata T. Synergistic action of Flt3 and gp130 signalings in human hematopoiesis. *Blood* 1997; **90**: 4363-4368 [PMID: 9373247]
64 **Sui X**, Tsuji K, Tanaka R, Tajima S, Muraoka K, Ebihara Y, Ikebuchi K, Yasukawa K, Taga T, Kishimoto T. gp130 and c-Kit signalings synergize for ex vivo expansion of human primitive hemopoietic progenitor cells. *Proc Natl Acad Sci USA* 1995; **92**: 2859-2863 [PMID: 7535932 DOI: 10.1073/

pnas.92.7.2859]
65 **Majka M**, Ratajczak J, Villaire G, Kubiczek K, Marquez LA, Janowska-Wieczorek A, Ratajczak MZ. Thrombopoietin, but not cytokines binding to gp130 protein-coupled receptors, activates MAPKp42/44, AKT, and STAT proteins in normal human CD34+ cells, megakaryocytes, and platelets. *Exp Hematol* 2002; **30**: 751-760 [PMID: 12135673 DOI: 10.1016/S0301-472X(02)00810-X]
66 **Matsui A**, Sato T, Maekawa T, Asano S, Nakahata T, Tsuji K. Glycoprotein 130 and c-kit signals synergistically induce thrombopoietin production by hematopoietic cells. *Int J Hematol* 2000; **72**: 455-462 [PMID: 11197211]
67 **Peters SO**, Kittler EL, Ramshaw HS, Quesenberry PJ. Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. *Blood* 1996; **87**: 30-37 [PMID: 8547656]
68 **de Haan G**, Weersing E, Dontje B, van Os R, Bystrykh LV, Vellenga E, Miller G. In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. *Dev Cell* 2003; **4**: 241-251 [PMID: 12586067 DOI: 10.1016/S1534-5807(03)00018-2]
69 **Yeoh JS**, van Os R, Weersing E, Ausema A, Dontje B, Vellenga E, de Haan G. Fibroblast growth factor-1 and -2 preserve long-term repopulating ability of hematopoietic stem cells in serum-free cultures. *Stem Cells* 2006; **24**: 1564-1572 [PMID: 16527900 DOI: 10.1634/stemcells.2005-0439]
70 **Ratajczak MZ**, Ratajczak J, Skorska M, Marlicz W, Calabretta B, Pletcher CH, Moore J, Gewirtz AM. Effect of basic (FGF-2) and acidic (FGF-1) fibroblast growth factors on early haemopoietic cell development. *Br J Haematol* 1996; **93**: 772-782 [PMID: 8703802 DOI: 10.1046/j.1365-2141.1996.d01-1736.x]
71 **Zhang CC**, Kaba M, Iizuka S, Huynh H, Lodish HF. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood* 2008; **111**: 3415-3423 [PMID: 18202223 DOI: 10.1182/blood-2007-11-122119]
72 **Walasek MA**, van Os R, de Haan G. Hematopoietic stem cell expansion: challenges and opportunities. *Ann N Y Acad Sci* 2012; **1266**: 138-150 [PMID: 22901265]
73 **Milner LA**, Kopan R, Martin DI, Bernstein ID. A human homologue of the Drosophila developmental gene, Notch, is expressed in CD34+ hematopoietic precursors. *Blood* 1994; **83**: 2057-2062 [PMID: 7512837]
74 **Ohishi K**, Varnum-Finney B, Bernstein ID. Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells. *J Clin Invest* 2002; **110**: 1165-1174 [PMID: 12393852 DOI: 10.1172/JCI200216167]
75 **Delaney C**, Heimfeld S, Brashem-Stein C, Voorhies H, Manger RL, Bernstein ID. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat Med* 2010; **16**: 232-236 [PMID: 20081862 DOI: 10.1038/nm.2080]
76 **Delaney C**, Varnum-Finney B, Aoyama K, Brashem-Stein C, Bernstein ID. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood* 2005; **106**: 2693-2699 [PMID: 15976178 DOI: 10.1182/blood-2005-03-1131]
77 **Butler JM**, Nolan DJ, Vertes EL, Varnum-Finney B, Kobayashi H, Hooper AT, Seandel M, Shido K, White IA, Kobayashi M, Witte L, May C, Shawber C, Kimura Y, Kitajewski J, Rosenwaks Z, Bernstein ID, Rafii S. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* 2010; **6**: 251-264 [PMID: 20207228 DOI: 10.1016/j.stem.2010.02.001]
78 **Krosl J**, Austin P, Beslu N, Kroon E, Humphries RK, Sauvageau G. In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat Med* 2003; **9**: 1428-1432 [PMID: 14578881 DOI: 10.1038/nm951]
79 **Antonchuk J**, Sauvageau G, Humphries RK. HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. *Exp Hematol* 2001; **29**: 1125-1134 [PMID: 11532354 DOI: 10.1016/S0301-472X(01)00681-6]
80 **Antonchuk J**, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* 2002; **109**: 39-45 [PMID: 11955445 DOI: 10.1016/S0092-8674(02)00697-9]
81 **Zhang XB**, Beard BC, Trobridge GD, Wood BL, Sale GE, Sud R, Humphries RK, Kiem HP. High incidence of leukemia in large animals after stem cell gene therapy with a HOXB4-expressing retroviral vector. *J Clin Invest* 2008; **118**: 1502-1510 [PMID: 18357342]
82 **Verfaillie CM**, McCarthy JB, McGlave PB. Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. *J Exp Med* 1991; **174**: 693-703 [PMID: 1875168 DOI: 10.1084/jem.174.3.693]
83 **Teixidó J**, Hemler ME, Greenberger JS, Anklesaria P. Role of beta 1 and beta 2 integrins in the adhesion of human CD34hi stem cells to bone marrow stroma. *J Clin Invest* 1992; **90**: 358-367 [PMID: 1379610 DOI: 10.1172/JCI115870]
84 **Nolta JA**, Smogorzewska EM, Kohn DB. Analysis of optimal conditions for retroviral-mediated transduction of primitive human hematopoietic cells. *Blood* 1995; **86**: 101-110 [PMID: 7795215]
85 **Williams DA**, Rios M, Stephens C, Patel VP. Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. *Nature* 1991; **352**: 438-441 [PMID: 1861722 DOI: 10.1038/352438a0]
86 **Jiang XS**, Chai C, Zhang Y, Zhuo RX, Mao HQ, Leong KW. Surface-immobilization of adhesion peptides on substrate for ex vivo expansion of cryopreserved umbilical cord blood CD34+ cells. *Biomaterials* 2006; **27**: 2723-2732 [PMID: 16376984 DOI: 10.1016/j.biomaterials.2005.12.001]
87 **Boitano AE**, Wang J, Romeo R, Bouchez LC, Parker AE, Sutton SE, Walker JR, Flaveny CA, Perdew GH, Denison MS, Schultz PG, Cooke MP. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science* 2010; **329**: 1345-1348 [PMID: 20688981 DOI: 10.1126/science.1191536]
88 **Peled T**, Landau E, Mandel J, Glukhman E, Goudsmid NR, Nagler A, Fibach E. Linear polyamine copper chelator tetraethylenepentamine augments long-term ex vivo expansion of cord blood-derived CD34+ cells and increases their engraftment potential in NOD/SCID mice. *Exp Hematol* 2004; **32**: 547-555 [PMID: 15183895]
89 **de Lima M**, McMannis J, Gee A, Komanduri K, Couriel D, Andersson BS, Hosing C, Khouri I, Jones R, Champlin R, Karandish S, Sadeghi T, Peled T, Grynspan F, Daniely Y, Nagler A, Shpall EJ. Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. *Bone Marrow Transplant* 2008; **41**: 771-778 [PMID: 18209724 DOI: 10.1038/sj.bmt.1705979]
90 **Bradford GB**, Williams B, Rossi R, Bertoncello I. Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Exp Hematol* 1997; **25**: 445-453 [PMID: 9168066]
91 **Cheshier SH**, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci USA* 1999; **96**: 3120-3125 [PMID: 10077647 DOI: 10.1073/pnas.96.6.3120]
92 **Kiel MJ**, He S, Ashkenazi R, Gentry SN, Teta M, Kushner JA, Jackson TL, Morrison SJ. Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. *Nature* 2007; **449**: 238-242 [PMID: 17728714 DOI: 10.1038/nature06115]
93 **Nygren JM**, Bryder D. A novel assay to trace proliferation history in vivo reveals that enhanced divisional kinetics ac-

company loss of hematopoietic stem cell self-renewal. *PLoS One* 2008; **3**: e3710 [PMID: 19002266 DOI: 10.1371/journal.pone.0003710]

94 **Foudi A**, Hochedlinger K, Van Buren D, Schindler JW, Jaenisch R, Carey V, Hock H. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol* 2009; **27**: 84-90 [PMID: 19060879 DOI: 10.1038/nbt.1517]

95 **Cheng T**, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden DT. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 2000; **287**: 1804-1808 [PMID: 10710306 DOI: 10.1126/science.287.5459.1804]

96 **Zhang J**, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, Haug JS, Rupp D, Porter-Westpfahl KS, Wiedemann LM, Wu H, Li L. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature* 2006; **441**: 518-522 [PMID: 16633340 DOI: 10.1038/nature04747]

97 **Miyamoto K**, Araki KY, Naka K, Arai F, Takubo K, Yamazaki S, Matsuoka S, Miyamoto T, Ito K, Ohmura M, Chen C, Hosokawa K, Nakauchi H, Nakayama K, Nakayama KI, Harada M, Motoyama N, Suda T, Hirao A. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. *Cell Stem Cell* 2007; **1**: 101-112 [PMID: 18371339 DOI: 10.1016/j.stem.2007.02.001]

98 **Thorén LA**, Liuba K, Bryder D, Nygren JM, Jensen CT, Qian H, Antonchuk J, Jacobsen SE. Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol* 2008; **180**: 2045-2053 [PMID: 18250409]

P- Reviewers: Buitenhuis M, Chang TM, Erceg S
S- Editor: Song XX **L- Editor**: A **E- Editor**: Wu HL

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5315/wjh.v3.i2.29

World J Hematol 2014 May 6; 3(2): 29-43
ISSN 2218-6204 (online)

REVIEW

Anti-CD20 monoclonal antibodies and associated viral hepatitis in hematological diseases

Shih-Hung Yang, Chiun Hsu, Ann-Lii Cheng, Sung-Hsin Kuo

Shih-Hung Yang, Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei 100, Taiwan
Shih-Hung Yang, Chiun Hsu, Ann-Lii Cheng, Sung-Hsin Kuo, Department of Oncology, National Taiwan University Hospital, Taipei 100, Taiwan
Shih-Hung Yang, Chiun Hsu, Ann-Lii Cheng, Sung-Hsin Kuo, Cancer Research Center, National Taiwan University College of Medicine, Taipei 100, Taiwan
Chiun Hsu, Ann-Lii Cheng, Sung-Hsin Kuo, Graduate Institute of Oncology, National Taiwan University College of Medicine, Taipei 100, Taiwan
Ann-Lii Cheng, Department of Internal Medicine, National Taiwan University Hospital, Taipei 100, Taiwan
Author contributions: Yang SH conducted the literature review and drafted the article; Hsu C and Cheng AL wrote the manuscript; Kuo SH revised the content and approved the final version for publication.
Supported by Research grants NSC 101-2321-B-002-032 and NSC 101-2314-B-002-157-MY3 from the National Science Council, Taiwan; NHRI-EX102-10239BI, and NHRI-EX102-10239BI from the National Health Research Institutes, Taiwan; and DOH100-TD-B-111-001 from the Department of Health, Taiwan
Correspondence to: Sung-Hsin Kuo, MD, PhD, Department of Oncology, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei 100, Taiwan. shkuo101@ntu.edu.tw
Telephone: +886-2-23123456 Fax: +886-2-23123456
Received: November 13, 2013 Revised: January 27, 2014
Accepted: March 17, 2014
Published online: May 6, 2014

Abstract

Over the past decade, the administration of anti-CD20 monoclonal antibodies such as rituximab has demonstrated various degrees of effectiveness and has improved patients' outcomes during the treatment of autoimmune hematological disorders and hematological malignancies. However, the depletion of B-cells, the distribution of T-cell populations, and the reconstruction of host immunity resulting from the use of anti-CD20 monoclonal antibodies potentially lead to severe viral infections, such as hepatitis B virus (HBV), hepatitis C virus (HCV), parvovirus B19, and herpes viruses, in patients who are undergoing immune therapy or immunochemotherapy. Of these infections, HBV- and HCV-related hepatitis are a great concern in endemic areas because of the high morbidity and mortality rates in untreated patients. As a result, prophylaxis against HBV infection is becoming a standard of care in these areas. Parvovirus B19, a widespread pathogen that causes red blood cell aplasia in immunocompromised hosts, also causes hepatitis in healthy individuals. Recently, its association with hepatitis was recognized in a patient treated with rituximab. In addition, adenovirus, varicella-zoster virus, hepatitis E virus, and rituximab itself have been linked to the occurrence of hepatitis during or after rituximab treatments. The epidemiologies and pathogeneses of these etiologies remain unknown. Because of the increasing use of anti-CD20 monoclonal antibodies for the treatment of hematological malignancies or autoimmune hematological disorders, it is imperative that physicians understand and balance the risks of hepatotropic virus-associated hepatitis against the benefits of using anti-CD20 monoclonal antibodies.

Key words: CD20; Monoclonal antibody; Hepatitis; Hepatitis B virus; Hepatitis C virus

Core tip: Anti-CD20 monoclonal antibodies are widely used for the treatment of hematological malignancies and autoimmune disorders. These agents produce prolonged B-cell depletion and significant immune suppression. In this review, we summarized the clinical use of anti-CD20 monoclonal antibodies and the reports of acute or chronic hepatitis associated with the use of these agents. Most of these hepatitis cases had viral etiologies. We discuss the mechanisms of the hepatitis caused by these drugs. These infections not only interrupted the immunotherapy but are also associated with high mortality and morbidity.

This review may prompt physicians to monitor patients' liver function more closely and to provide adequate prophylaxis while using these agents.

Yang SH, Hsu C, Cheng AL, Kuo SH. Anti-CD20 monoclonal antibodies and associated viral hepatitis in hematological diseases. *World J Hematol* 2014; 3(2): 29-43 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i2/29.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i2.29

INTRODUCTION

The nonglycosylated transmembrane phosphoprotein CD20 is a differentiation marker of B cells that was characterized over 30 years ago[1]. CD20 is expressed from early pre-B cells to mature B cells but not in plasma cells[2]. The majority of B-cell lymphomas variably express the CD20 surface marker[3]. CD20 can form multimeric complexes[4,5], interact with B-cell receptors in lipid rafts[5-7], mediate calcium influx[4,7], and regulate the cell cycle and apoptosis[8-11].

Because of the ubiquitous expression of CD20 in normal and malignant B cells, CD20 is an excellent target molecule for the treatment of B cell-related diseases. Rituximab, a genetically engineered chimeric murine/human monoclonal antibody (mAb)-targeting CD20, contains murine light- and heavy-chain variable regions and human constant regions[12]. Rituximab is used to exert cytotoxic effects on B cells by 3 mechanisms: antibody-dependent cell-mediated cytotoxicity (ADCC), complement-mediated lysis (CDC), and a direct apoptosis-inducing effect on $CD20^+$ cells[12,13].

Based on a pivotal trial, rituximab was first approved by the United States Food and Drug Administration in 1997 for relapsed or refractory follicular and low-grade B-cell non-Hodgkin's lymphoma (NHL)[14]. New anti-CD20 mAbs have been developed over the last few years, and these mAbs have been classified into 2 groups (type Ⅰ and type Ⅱ) according to their different activities in inducing CDC, ADCC, apoptosis, and lipid raft redistribution while binding to CD20[15,16]. Because anti-CD20 mAbs have optimized antibody structures and sometimes conjugated radioisotopes, they are used and indicated for the treatment of not only hematological malignancies but also autoimmune diseases[17]. However, following the widespread use of these agents, reports of infections related to B-cell depletion began to appear increasingly. Here, we discuss the clinical applications, the immunocompromising effects, and the association with hepatitis of anti-CD20 mAbs.

APPLICATIONS OF ANTI-CD20 MABS IN HEMATOLOGICAL DISEASES

Malignancies

Rituximab, the first mAb approved for the treatment of malignancies, was initially indicated for relapsed or refractory indolent B-cell NHL[14]. In this pivotal trial, the schedule consisted of 4 weekly doses of 375 mg/m^2. Nearly half of the 166 patients responded, with a projected median time-to-progression of 13 mo. Infusion reactions were the most frequently encountered acute adverse event[14]. The promising response and excellent tolerance to rituximab in indolent B-cell NHL cases were further demonstrated in clinical trials involving extended use and retreatment[18,19]. The excellent single-agent activity of rituximab was demonstrated not only in relapsed or refractory cases but also in newly diagnosed indolent B-cell NHL cases. In a phase Ⅱ trial of single-agent rituximab for patients with low-grade NHL, the initial response rate (RR) after 4 weekly doses of rituximab was 54%, and the RR improved to 64% when rituximab retreatment was administered[20]. In addition to rituximab monotherapy, several studies have demonstrated that the combination of rituximab and chemotherapeutic agents provided high RRs and long time-to-progression in relapsed, refractory, or newly diagnosed indolent B-cell NHL cases[21-25]. To prolong disease control after the initial treatment, maintenance therapies after initial chemotherapy alone[21,26] or rituximab in addition to chemotherapy[21,27,28] have been tested in relapsed, refractory, or newly diagnosed indolent B-cell NHL cases. All of these studies demonstrated considerable improvement in the response duration or progression-free survival[21,26-28]. Moreover, the clinical successes of rituximab were partially recapitulated in diffuse large B-cell lymphoma (DLBCL)[29-33]. The benefits of maintenance therapy were not observed after first-line chemotherapy with or without rituximab or autologous stem-cell transplantation used for treating cases of relapsed DLBCL[34,35]. The difference may reflect the distinct nature of the indolent and aggressive B-cell NHLs.

Because of the clinical benefits demonstrated in rituximab-based regimens, efforts have been made to improve the efficacy of rituximab through the development of new anti-CD20 mAbs or the conjugation of radioisotopes (Table 1). Overall, the administration of yttrium-90 ibritumomab tiuxetan or iodine-131 tositumomab radioimmunotherapy (RIT) has not consistently yielded improved efficacy and survival in relapsed, refractory, or newly diagnosed B-cell NHL cases[36-44]. However, the use of RIT, which offers the theoretical benefits of radiotherapy, can be a viable option for patients who have not responded to prior rituximab treatments or as an alternative or a supplemental method to stem-cell transplantation. Second- and third-generation humanized anti-CD20 mAbs were designed with improved binding affinities for CD20 or the FcγRⅢa receptor for enhanced CDC or ADCC. The efficacy of these agents was also investigated with or without chemotherapy in relapsed, refractory, or newly diagnosed B-cell NHL cases[45-59]. Details regarding these anti-CD20 mAbs are summarized in Table 1.

Autoimmune hematological diseases

The essential mechanism of autoimmune diseases is the

Table 1 Anti-CD20 monoclonal antibodies and associated hepatitis

Antibody	Structure	Clinical applications	Associated hepatitis	Ref.
Rituximab	IgG1, chimeric murine/human mAb	B-cell NHL, RA, SLE, MS, AIHA, TTP, ITP, acquired hemophilia, cryoglobulinemia	HBV, HCV, parvovirus B19, VZV, adenovirus, HEV, drug-related?	[14,18-35,60-65,71, 73,74,78,85,89-93,102-111, 140-147,172,174-179]
Y-90 ibritumomab tiuxetan	IgG1, mouse mAb, conjugated with tiuxetan to yttrium-90	B-cell NHL	HBV	[36-38,112]
I-131 tositumomab	IgG2, mouse mAb, covalently bound to iodine-131	B-cell NHL	None	[39-44]
Ofatumumab	IgG1, human mAb	CLL, B-cell NHL, RA, MS, AIHA	HBV or drug-related?	[45-50,68,114-116]
Veltuzumab	IgG1, humanized mAb	B-cell NHL, ITP	Drug-related?	[51,69]
Ocrelizumab	IgG1, humanized mAb	B-cell NHL, RA, SLE, MS	None	[52]
Obinutuzumab	IgG1, humanized mAb, modified Fc	CLL, B-cell NHL	Drug-related?	[53-57]
PRO131921	IgG1, humanized mAb, modified Fc	CLL, B-cell NHL	None	[58]
Ocaratuzumab	IgG1, humanized mAb, modified Fc	B-cell NHL	None	[59]

mAb: Monoclonal antibody; NHL: Non-Hodgkin lymphoma; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; MS: Multiple sclerosis; AIHA: Autoimmune hemolytic anemia; TTP: Thrombotic thrombocytopenic purpura; ITP: Immune thrombocytopenic purpura; DM: Diabetes mellitus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; VZV: Varicella-zoster virus; HEV: Hepatitis E virus; CLL: Chronic lymphocytic leukemia; None: No associated hepatitis reported in hematological autoimmune disorders or malignancies at the time of review (excluding non-hematological diseases).

loss of self-tolerance, which enables the immune system to evoke autoreactive humoral and cellular responses. Elimination of B cells with rituximab is effective in the treatment of autoimmune hematological diseases such as autoimmune hemolytic anemia (AIHA)[60], immune thrombocytopenic purpura (ITP)[61], acquired hemophilia[62], thrombotic thrombocytopenic purpura (TTP)[63], and cryoglobulinemia[64,65]. B cells produce autoantibodies and cytokines, act as antigen-presenting cells, promote naïve $CD4^+$ T-cell differentiation, and affect dendritic cell homeostasis[66]. However, the responses of these autoimmune diseases to the off-label use of rituximab varied widely[67]. The varied results may be partially attributed to the underlying heterogeneity in the etiologies and pathogeneses of these diseases.

The data about the use of non-rituximab anti-CD20 mAbs for the treatment of autoimmune diseases are extremely limited. RIT may also be a valuable option, but its use for the treatment of autoimmune hematological diseases has not been reported. Ofatumumab, approved by the US Food and Drug Administration for treating chronic lymphocytic leukemia (CLL) refractory to fludarabine and alemtuzumab, was demonstrated to be effective in CLL complicated with AIHA[68]. In a phase I trial, veltuzumab demonstrated a RR of 55% in relapsed ITP cases, and long durations of response were observed in some patients[69]. Second- and third-generation humanized anti-CD20 mAbs are being developed for treating nonhematological autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and multiple sclerosis.

HEPATITIS RELATED TO RITUXIMAB AND OTHER ANTI-CD20 MABS

Immune system changes after anti-CD20 mAb treatments

In general, peripheral blood B cells are depleted rapidly and effectively after anti-CD20 mAb treatment. The level of peripheral B cells remains extremely low and recovers gradually until 6 to 12 mo after the last dose of rituximab[61-65,70,71]. The depletion or recovery of B cells is not uniform among the various subsets and locations of B cells and may also depend on the baseline B-cell counts, the nature of the disease, and the dose, duration and type of anti-CD20 mAbs used[36,40,45,46,49-53,55,56,65,71,72]. The recovery of B cells starts with the immature or transitional B cells ($CD38^+$, $CD24^+$, $CD10^+$, $CD27^-$, IgD^+) followed by naïve B cells ($CD38^+$, $CD27^-$, IgD^+); however, memory B cells ($CD27^+$, $CD38^-$, IgD^+) may remain considerably depleted for at least 2 years[73-76]. The pattern of B-cell repopulation is similar between autoimmune disease and B-cell NHL cases[73-76]. The effects of B-cell depletion on plasma immunoglobulin levels and blunted responses to immunization are individualized and heterogeneous among the different diseases treated with rituximab[18,19,21,29,60-65,70,77-80]. Typically, the levels of complements do not change substantially[64,70], but a major increase of C4 levels in the serum was noted after rituximab treatment in type Ⅱ mixed cryoglobulinemia cases[65]. The levels, subsets, and functional status of T cells and natural killer (NK) cells after rituximab treatment are more complex because these factors depend on the expression of CD20 on T and NK cells[74] and the nature of the disease process[17,18,45,46,61,62,64,74,81-87]. Early and persistent reduction of peripheral $CD4^+/CD40L^+$ T cells was observed after treatment of SLE with rituximab[84]. The abnormalities of T-cell homeostasis can be reversed after rituximab administration[81-83,87], accompanied by increased $CD4^+CD25^+$ regulatory T cells[82,84,85,87], $CD8^+CD25^+$ T cells[85], or decreased autoreactive $CD4^+$ T cells[81]. In a mouse model, B-cell depletion inhibited $CD4^+$ but not $CD8^+$ T-cell activation and clonal expansion in response to new exogenous antigens. Therefore, adequate antigen-specific $CD4^+$ T-cell responses still

required the presence of B cells[88]. The limited data on T cell, NK cell, complement, and immunoglobulin levels following treatments with anti-CD20 mAbs other than rituximab were highly similar compared with those with rituximab[36,51-54,69]. Because of the immunodeficiency induced by anti-CD20 mAbs, hepatitis and other infections have become a growing concern since the approval of rituximab.

Hepatitis B virus

Acute hepatitis and even fulminant hepatic failure are a well-documented threat in patients receiving chemotherapy, particularly in lymphoma cases[89]. In a prospective study, 44% (34/78) of hepatitis B virus (HBV; hepatitis B surface antigen, $HBsAg^+$) carriers developed some form of hepatitis. Of these cases, 44% (15/34) were attributed to HBV reactivation[89]; 6 of these 15 were patients with lymphoma, and all of them had been treated with adriamycin, cyclophosphamide, vincristine, and prednisolone, also known as the CHOP regimen[89]. In addition, 4 of these 6 patients with lymphoma were seropositive for hepatitis B e antigen (HBeAg) at baseline and developed HBV reactivation sooner than the HBeAg-negative carriers[89]. In addition to those observations in lymphoma cases, HBV reactivation has been frequently reported in other hematological malignancies[90]. Corticosteroids, which are immunosuppressive agents frequently used in hematological malignancies and autoimmune diseases, may increase the risk of liver injury in HBV carriers[90,91]. Steroid-sparing regimens can be used to reduce the risk of HBV reactivation[92]. Additionally, adriamycin, a component of the CHOP regimen, can stimulate the replication of HBV[93]. Because the use of anti-CD20 mAb is common, designing a treatment plan that prevents the risk of HBV reactivation may be more complex than administrating chemotherapy for hematological malignancies or immunosuppressants for autoimmune diseases.

The natural history of HBV infection depends on the interaction of the host immunity, hepatocytes, and viral replication. Chronic HBV infections acquired early in life have 3 phases: the immune tolerance phase, the immune active phase, and the low-replication phase[94-96]. Some inactive HBV carriers (HBeAg seroconversion) can unexpectedly reenter the immune clearance phase and experience HBV reactivation with elevated HBV DNA and/or HBeAg reversion[97]. The incidence of HBV flares varies among studies and may depend on sex, HBV genotype, age at HBeAg seroconversion, and HBV DNA levels[96,97]. In adults, NK cells and type I interferon responses induced by HBV infection were observed before HBV-specific $CD4^+$ and $CD8^+$ T-cell responses[98]. In chronic HBV infections, HBV-specific $CD8^+$ T cells presented HBV antigens regardless of the status of antibody to hepatitis B core antigen (anti-HBc)[99]. Both anti-HBc^+ patients and inactive HBV carriers exhibited strong memory $CD8^+$ T-cell responses[99]. However, the levels of $FoxP3^+$, $CD25^+$, and $CD4^+$ regulatory T cells that inhibit HBcAg-specific responses were also higher in chronic HBV infection cases[100].

HBV replication within the liver and the subsequent spread of virions into the circulation may occur in cancer patients with immunosuppression induced by the diseases or treatments. Hepatitis flares can develop early or late, during immunosuppressive therapy or after its completion. The effects can range from asymptomatic elevation of HBV DNA to fulminant hepatitis. HBV reactivation has been reported not only in patients undergoing chemotherapy or steroid therapy but also in patients undergoing rituximab treatment, regardless of their HBV status[101-104]. In $HBsAg^+$ carriers with B-cell NHL, HBV reactivation occurred in 80% (8/10) of patients without prophylaxis[105]. Significantly fewer cases of HBV reactivation were consistently observed in $HBsAg^+$ carriers with prophylaxis[101]. In patients with resolved HBV ($HBsAg^-$/anti-HBc^+) and B-cell NHL, the rituximab-CHOP regimen led to more cases of HBV reactivation (reverse seroconversion or elevated HBV DNA) than the CHOP regimen alone did (23.8% *vs* 0%)[102]. The incidence rates of HBV reactivation and HBV hepatitis flare were reported as 10.4 and 6.4 per person-year in this group of patients, respectively[106]. The timing of HBV reactivation is closely associated with the lymphopenic state. The reconstruction of host immunity induced by rituximab and the recovery of B- and T-lymphocytes after rituximab may result in damage to HBV-infected hepatocytes by cytotoxic T-lymphocytes[90,104,106,107]. The impairment of the immune system appears to be more severe in patients treated with rituximab-CHOP than in those treated with rituximab alone[107].

Rituximab treatments, with or without steroids, were generally well-tolerated by patients with autoimmune hematological disorders such as AIHA[60,108], ITP[61,109], acquired hemophilia[62], TTP[63], and cryoglobulinemia[64,65]. No HBV reactivation was reported, most likely because of the exclusion of patients with positive HBV serology, a limited number of cases, or lesser immunosuppression in these autoimmune diseases compared with hematological malignancies. However, HBV reactivation is a major threat in patients with autoimmune disorders who are undergoing immunosuppressive therapy[110]; therefore, concurrent antiviral treatments are often prescribed to reduce the risk of reactivation[111].

Patients with chronic or resolved HBV receiving RIT or anti-CD20 mAbs are subject to the same or an even greater risk of HBV reactivation, and although these effects were not fully characterized in most prospective trials[36-59], scattered cases have been reported[111,112], both of which were successfully treated with lamivudine[112,113]. The United States Food and Drug Administration announced that physicians should be alert to potential HBV reactivation caused by ofatumumab[114]. Liver toxicities had been reported with ofatumumab with or without chemotherapy in hematological malignancies. However, the data on HBV reactivation in these cases were lacking[45,50,115,116].

HBV is endemic in the Asia-Pacific region with a prevalence of more than 10% in Taiwan, southern China, and certain areas of Southeast Asia[117]. In clinical prac-

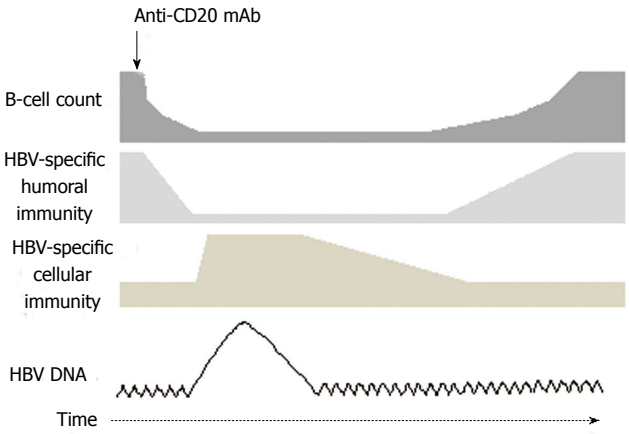


Figure 1 Anti-CD20 monoclonal antibodies and dynamic immunity in hepatitis B virus infection. This illustration summarizes the dynamic changes of hepatitis B virus (HBV) DNA and immune responses during HBV reactivation. Notably, the viral-specific T-cell immunity before and after the administration of anti-CD20 monoclonal antibody (mAb) depends on the carrier status, that is, hepatitis B surface antigen (HBsAg)$^+$ carrier or resolved HBV (anti-HBc$^+$/HBsAg$^-$, anti-HBs$^+$ or $^-$) status. HBV prophylaxis should be considered to maintain low level of HBV replication and prevent HBV-specific T-cell-mediated liver damage. The prophylaxis should be maintained for at least 6 mo after anti-CD20 mAb when HBV-specific T-cell immunity recovers.

tice, a greater than 10-fold increase above the baseline in serum HBV DNA levels, an absolute increase of more than 9 $\log_{10}$ copies/mL, a reappearance of HBV DNA in HBsAg, or a new onset of HBV DNA viremia in HBsAg-negative patients can be considered as an indication of HBV reactivation. In addition, an hepatitis flare is considered if the serum alanine transaminase (ALT) level is 3 times greater than the normal upper limit, or if an ALT level of more than 100 IU/L with a concomitantly increased HBV DNA level of more than 10 times the typical level is detected[89,118]. In high-risk endemic areas, HBV screening must be considered before implementing anti-CD20 mAb treatment[119-123]. In a cost-effectiveness study, screening for HBsAg in all patients about to receive rituximab-CHOP treatment considerably reduced the rate of HBV reactivation and cost the least[124]. The recommended tests for screening include HBsAg, anti-HBc, and anti-HBs (according to the CDC)[122], HBsAg and anti-HBc (AASLD and EASL)[121,125], and HBsAg with or without anti-HBc (ASCO)[123]. A more complex algorithm for screening in different at-risk populations was proposed[126]. Currently, oral agents approved for HBV treatment include lamivudine, adefovir, telbivudine, tenofovir, and entecavir. Based on research findings, the optimal start time and duration of HBV prophylaxis has not yet been determined[119-123]; however, initiating short-term antiviral therapy before starting anti-CD20 mAb treatment appears to be beneficial and safe. In a randomized trial involving HBsAg$^+$ patients with NHL, the prophylactic use of lamivudine reduced considerably the occurrence of HBV reactivation and hepatitis flares[127]. In a meta-analysis, all-cause mortality, HBV reactivation, HBV-related mortality, and interruption of anti-CD20 mAb therapy were considerably reduced with lamivudine prophylaxis[128]. One major problem of lamivudine, telbivudine, and adefovir is that drug resistance and hepatitis flares increase with continuous use[125,129]; therefore, the use of entecavir and tenofovir was suggested for longer duration of prophylaxis[125]. In addition to HBsAg$^+$ patients, HBV prophylaxis must be considered in patients with resolved HBV because the risk of reactivation remains (Figure 1)[106,130]. In a recent retrospective study of HBV reactivation by the Asia Lymphoma Study Group, the authors showed that patients receiving entecavir prophylaxis had a lesser incidence of HBV reactivation than those with lamivudine. Prospective studies to validate these findings are warranted.

Hepatitis C virus

The epidemiology of hepatitis C virus (HCV) differs from that of HBV, particularly because of the existence of a wide variation of HCV genotypes worldwide[131]. The most common types in Taiwan, China, Japan, and Korea are genotypes 1b and 2; by contrast, more diversity exists in North America and Europe[131]. The natural history of HCV infection consists of ramp-up and plateau phases in acute infections and various spontaneous clearances in chronic phases depending on several viral and host factors, including HCV genotypes, host immunity, and the genetic polymorphism of *IFNL3* (*IL-28B*)[131]. The treatment of chronic HCV consists primarily of interferon-α, ribavirin, and protease inhibitors that are accompanied by major toxicities[131].

During acute infections, HCV-specific T cells activated by CD8$^+$ and CD4$^+$ are generated readily against multiple epitopes within 10 wk[132]. In patients with chronic infections and persistent viremia, HCV-specific CD8$^+$ T cells are rarer and responsive to fewer epitopes than in those without viremia, where even HCV-specific CD8$^+$ T cell responses are mounted[132]. In addition, CD8$^+$ T cells are exhausted in chronic HCV with increased expression of programmed death-1 and cytotoxic T-lymphocyte-associated antigen-4[133]. HCV-specific CD8$^+$ T cells are suppressed by increased CD25$^+$ and CD4$^+$ regulatory T cells[134]. In addition to evading CD8$^+$ T-cell responses, HCV also evades CD4$^+$ T-cell responses and humoral immunity with escape mutants because of its error-prone RNA polymerase[135-137].

HCV itself is highly associated with the development of lymphoproliferative disorders, particularly with B-cell NHL with or without mixed cryoglobulinemia[138,139]. HCV-related fulminant hepatitis occurring after chemotherapy with or without corticosteroids was rare in patients with lymphoma[140,141]. Liver dysfunction after chemotherapy was less common in HCV than in HBV patients (18.2% *vs* 75.0%) with hematological malignancies[142]. The incidence of HCV-associated liver dysfunction appeared to be higher in rituximab-containing regimens[143,144]. In addition, increased HCV RNA levels were common during or after rituximab-based chemotherapy and were followed by hepatitis flares and decreased HCV RNA levels in various intervals[144-146]. In contrast to persistent B-cell depletion for several months after rituximab treatments, the HCV viral load and the number of

regulatory T cells were elevated initially, then decreased, indicating that B-cell depletion and HCV-specific T-cell responses participate in the mechanism of HCV reactivation in patients treated with rituximab-based regimens[64,147,148]. Although the elevation of HCV RNA load was not the major problem for lymphoma patients treated with standard courses of immunochemotherapy, the persistent elevation of HCV RNA load and subsequent liver cirrhosis can occur in follicular lymphoma patients treated with rituximab-maintenance therapy[147]. However, these studies evaluated HCV reactivation during and after rituximab regimens according to different criteria. The HCV RNA levels may increase up to 10 times above the baseline in chronic HCV infections[149]. In a retrospective study involving cancer patients, the acute exacerbation of chronic HCV was defined as a 3-fold or greater increase in ALT levels without tumor infiltration within the liver, without the use of hepatotoxic drugs or blood transfusion, and no concomitant systemic infections[150]. In addition, an at least 1 $\log_{10}$ IU/mL increase in HCV RNA levels after treatment with immunosuppressive agents was considered for HCV reactivation[150]. The same criteria may apply for HCV reactivation in anti-CD20 mAb treatments. A simple algorithm for monitoring ALT and HCV RNA in patients undergoing immunosuppressive therapy was proposed[151]. Although rituximab was also widely used in autoimmune hematological disorders such as HCV-related cryoglobulinemia, HCV reactivation with hepatitis flares was rare[65,152,153]. No data were available for RIT and non-rituximab anti-CD20 mAbs.

Traditionally, anti-HCV therapy is not considered in patients receiving immunosuppressive agents because of potential drug-drug interactions, the major side effects of anti-HCV therapy, and the rarity of severe HCV-related hepatitis flares. Currently, there is no consensus regarding the optimal strategy for the treatment and prevention of HCV reactivation in patients undergoing immunosuppressive therapy even though ribavirin with or without interferon-α has been successfully administered to patients with hematological malignancies[154,155].

Parvovirus B19

Parvovirus B19 infections are common infections that spread through respiratory droplets or blood, and seropositivity rates are increasing in people of all ages[156]. The disease spectrum can range from asymptomatic disease to hydrops fetalis, fifth disease, arthropathy, aplastic anemia, autoimmune disorders, meningitis, encephalitis, and even fulminant hepatitis[156,157]. This virus has a tropism for erythroid progenitors in the blood, bone marrow, and fetal liver[156]. In healthy adults, acute infections result in viremia within 2 wk, immediately followed by virus-specific IgM and IgG responses and clearance of the virus in the serum[158]. Humoral immunity appears to be critical for controlling this virus, and patients with immunodeficiency disorders can have chronic infections[159]. Virus-specific $CD8^+$ T cell responses toward multiple epitopes also develop soon after acute infection, and these striking $CD8^+$ T-cell responses may have long durations with continuous viremia[160]. In addition, interferon-γ-secreting, virus-specific $CD62L^+$ and $CD4^+$ T cells developed within 3 mo of acute infection[161].

Parvovirus B19 infection is a major problem in immunocompromised hosts[162-164]. The seropositivity rate was high in cancer patients receiving chemotherapy[162,165], and half of patients exhibited detectable viral DNA in their serum[165]. Several studies have demonstrated that acute parvovirus B19 infections are associated with fever, arthralgia, hepatitis, myocarditis, pneumonia, pancytopenia, and even graft dysfunction[162-164]. Most importantly, immunosuppressive therapies can impair humoral immunity, exposing patients to a high risk of parvovirus B19 infection[164,166]. Several case studies have reported that parvovirus B19 infection-related symptoms can develop in B-cell NHL or immune thrombocytopenia patients treated with rituximab-containing regimens or after being treated with rituximab-containing regimens[167-172]. The major parvovirus B19 infection-related symptom was cytopenia in the erythroid lineage, but neutropenia or thrombocytopenia without anemia occurred in some patients. The onset was preceded by fever and skin eruptions in 2 patients[169,171]. Our group identified the first case with acute hepatitis[172]. Most patients developed the clinical manifestations at least 2 mo after the initiation of rituximab. The patients in 2 cases recovered without treatment, and the others responded positively to intravenous immunoglobulin (IVIG). However, the hepatitis flare in our patient persisted for 7 mo and was paralleled with cytopenia, which was correlated with the recovery of B cells after rituximab treatment[172]. The effects of other anti-CD20 mAbs on parvovirus B19 are unknown.

The diagnosis of parvovirus B19 reactivation or infection is based on serology and viral DNA analysis[156]. However, conducting virus-specific serology may be problematic in immunocompromised hosts[164,166]. The pathogenesis of parvovirus B19-related hepatitis is largely unknown, and either direct cytopathic or indirect immunity-related mechanisms are possible. In addition, the pathology images obtained during liver biopsies are nonspecific. Although viral DNA or RNA can be detected in hepatocytes, the clinical significance remains to be defined[173]. The most reasonable diagnostic sequence may be to exclude the other common hepatotropic viruses first, such as HBV or HCV, and then to analyze serum serology and viral DNA if the tests for hepatotropic viruses are negative. Because liver biopsy is invasive, it must be considered last. The most effective treatment and prevention methods for parvovirus B19-related hepatitis remain unknown; however, IVIG can be used for treating severe cases because of the clinical success achieved in using it in other parvovirus B19-related diseases[156,166-169,171].

Other causes

In addition to HBV, HCV, and parvovirus B19, critical viral infections from cytomegalovirus, varicella-zoster virus (VZV), herpes simplex virus, echovirus, enterovirus,

influenza A virus, or BK/JC virus can occur in lymphoma patients treated with rituximab regimens[174]. Most of them did not induce hepatitis. However, there are reports of 2 cases of hepatitis associated with adenovirus, one case of hepatic necrosis associated with disseminated VZV, and one of chronic hepatitis E virus infection that developed after rituximab treatment[175-178]. These pathogens are rarely connected to the use of rituximab, and the underlying mechanisms of these pathogeneses associated with hepatitis are less characterized. However, the actual incidence rates of these uncommon viral infections in lymphoma patients treated with rituximab regimens may be underestimated. One patient with ITP experienced drug-induced acute hepatitis, and the pathogen was not identified; however, the patient recovered soon after rituximab treatment was stopped[179]. There were also scattered reports of anti-CD20 mAb-related liver function abnormalities in patients with hematological disorders. However, the etiology was most likely related to anti-CD20 mAb[45,50,51,55,69,115,116].

Future in vivo or in vitro studies

There are numerous reports of animal lymphoma models to test the preclinical activity of anti-CD20 mAbs[180-182]. However, the safety data in liver toxicities of these animal models are very limited[180-182]. In addition, no animal or in vitro models of viral hepatitis-induced by anti-CD20 mAbs has been established. It would be very difficult to establish the animal model with viral hepatitis reactivated by anti-CD20 mAbs because most of previous studies used tumor xenografts implanted into mice with severe combined immunodeficiency in their animal model. However, immune-mediated liver damage is important for anti-CD20 mAb-associated viral hepatitis. Most likely, this may work in evaluating the precise mechanism of hepatitis caused by the aforementioned viruses during and after anti-CD20 mAbs alone when using the immunocompetent animal model.

CONCLUSION

This review summarized the clinical use of anti-CD20 mAbs and reports of acute or chronic hepatitis associated with the use of these agents. Most data are from cases where rituximab was used to treat hematological malignancies. The majority of diseases studied were caused by viral infections. These infections must be clinically recognized soon after their occurrence because they not only interrupt immunotherapy but are also associated with high mortality and morbidity. Close monitoring of HBV and HCV infections before and during anti-CD20 mAb treatment is highly recommended in endemic areas. The prophylactic therapy for HBV has become standard of care, but patient selection and the optimal regimen or duration remain to be defined. Physicians must monitor patients being treated with rituximab-based regimens for the risks of hepatotropic viruses, including HBV, HCV, parvovirus B19, and other viruses. Nevertheless, this review has limitations. Some pathogens that rarely induce hepatitis may not be reported in the literature; therefore, the causal relation, epidemiology, and pathogenesis of these pathogens are not the most accurate. Occasionally, the causal association between hepatitis and rituximab is difficult to confirm because of the concurrent use of multiple immunosuppressants or hepatotoxic drugs. The data on non-rituximab anti-CD20 mAbs are limited, likely because most clinical trials employ strict case selection criteria and exclude patients with hepatitis. Therefore, in vivo or in vitro studies are warranted to establish the actual roles of these viruses in the pathogenesis of hepatitis during and after anti-CD20 mAb treatments.

REFERENCES

1 **Stashenko P**, Nadler LM, Hardy R, Schlossman SF. Characterization of a human B lymphocyte-specific antigen. *J Immunol* 1980; **125**: 1678-1685 [PMID: 6157744]

2 **Stashenko P**, Nadler LM, Hardy R, Schlossman SF. Expression of cell surface markers after human B lymphocyte activation. *Proc Natl Acad Sci USA* 1981; **78**: 3848-3852 [PMID: 6973760]

3 **Nadler LM**, Ritz J, Hardy R, Pesando JM, Schlossman SF, Stashenko P. A unique cell surface antigen identifying lymphoid malignancies of B cell origin. *J Clin Invest* 1981; **67**: 134-140 [PMID: 6969730]

4 **Bubien JK**, Zhou LJ, Bell PD, Frizzell RA, Tedder TF. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca2+ conductance found constitutively in B lymphocytes. *J Cell Biol* 1993; **121**: 1121-1132 [PMID: 7684739]

5 **Polyak MJ**, Li H, Shariat N, Deans JP. CD20 homo-oligomers physically associate with the B cell antigen receptor. Dissociation upon receptor engagement and recruitment of phosphoproteins and calmodulin-binding proteins. *J Biol Chem* 2008; **283**: 18545-18552 [PMID: 18474602 DOI: 10.1074/jbc.M800784200]

6 **Petrie RJ**, Deans JP. Colocalization of the B cell receptor and CD20 followed by activation-dependent dissociation in distinct lipid rafts. *J Immunol* 2002; **169**: 2886-2891 [PMID: 12218101]

7 **Li H**, Ayer LM, Lytton J, Deans JP. Store-operated cation entry mediated by CD20 in membrane rafts. *J Biol Chem* 2003; **278**: 42427-42434 [PMID: 12920111 DOI: 10.1074/jbc.M308802200]

8 **Golay JT**, Clark EA, Beverley PC. The CD20 (Bp35) antigen is involved in activation of B cells from the G0 to the G1 phase of the cell cycle. *J Immunol* 1985; **135**: 3795-3801 [PMID: 2415587]

9 **Tedder TF**, Forsgren A, Boyd AW, Nadler LM, Schlossman SF. Antibodies reactive with the B1 molecule inhibit cell cycle progression but not activation of human B lymphocytes. *Eur J Immunol* 1986; **16**: 881-887 [PMID: 3091375 DOI: 10.1002/eji.1830160802]

10 **Holder M**, Grafton G, MacDonald I, Finney M, Gordon J. Engagement of CD20 suppresses apoptosis in germinal center B cells. *Eur J Immunol* 1995; **25**: 3160-3164 [PMID: 7489758 DOI: 10.1002/eji.1830251126]

11 **Tedder TF**, Engel P. CD20: a regulator of cell-cycle progression of B lymphocytes. *Immunol Today* 1994; **15**: 450-454 [PMID: 7524522 DOI: 10.1016/0167-5699(94)90276-3]

12 **Reff ME**, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 1994; **83**: 435-445 [PMID: 7506951]

13 **Shan D**, Ledbetter JA, Press OW. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. *Blood* 1998; **91**: 1644-1652 [PMID: 9473230]

14 **McLaughlin P**, Grillo-López AJ, Link BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, Jain V, Ho AD, Lister J, Wey K, Shen D, Dallaire BK. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 1998; **16**: 2825-2833 [PMID: 9704735]

15 **Cragg MS**, Glennie MJ. Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. *Blood* 2004; **103**: 2738-2743 [PMID: 14551143 DOI: 10.1182/blood-2003-06-2031]

16 **Beers SA**, Chan CH, French RR, Cragg MS, Glennie MJ. CD20 as a target for therapeutic type I and II monoclonal antibodies. *Semin Hematol* 2010; **47**: 107-114 [PMID: 20350657 DOI: 10.1053/j.seminhematol.2010.01.001]

17 **Avivi I**, Stroopinsky D, Katz T. Anti-CD20 monoclonal antibodies: beyond B-cells. *Blood Rev* 2013; **27**: 217-223 [PMID: 23953071 DOI: 10.1016/j.blre.2013.07.002]

18 **Piro LD**, White CA, Grillo-López AJ, Janakiraman N, Saven A, Beck TM, Varns C, Shuey S, Czuczman M, Lynch JW, Kolitz JE, Jain V. Extended Rituximab (anti-CD20 monoclonal antibody) therapy for relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma. *Ann Oncol* 1999; **10**: 655-661 [PMID: 10442187]

19 **Davis TA**, Grillo-López AJ, White CA, McLaughlin P, Czuczman MS, Link BK, Maloney DG, Weaver RL, Rosenberg J, Levy R. Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of retreatment. *J Clin Oncol* 2000; **18**: 3135-3143 [PMID: 10963642]

20 **Hainsworth JD**, Burris HA, Morrissey LH, Litchy S, Scullin DC, Bearden JD, Richards P, Greco FA. Rituximab monoclonal antibody as initial systemic therapy for patients with low-grade non-Hodgkin lymphoma. *Blood* 2000; **95**: 3052-3056 [PMID: 10807768]

21 **van Oers MH**, Klasa R, Marcus RE, Wolf M, Kimby E, Gascoyne RD, Jack A, Van't Veer M, Vranovsky A, Holte H, van Glabbeke M, Teodorovic I, Rozewicz C, Hagenbeek A. Rituximab maintenance improves clinical outcome of relapsed/resistant follicular non-Hodgkin lymphoma in patients both with and without rituximab during induction: results of a prospective randomized phase 3 intergroup trial. *Blood* 2006; **108**: 3295-3301 [PMID: 16873669 DOI: 10.1182/blood-2006-05-021113]

22 **Forstpointner R**, Dreyling M, Repp R, Hermann S, Hänel A, Metzner B, Pott C, Hartmann F, Rothmann F, Rohrberg R, Böck HP, Wandt H, Unterhalt M, Hiddemann W. The addition of rituximab to a combination of fludarabine, cyclophosphamide, mitoxantrone (FCM) significantly increases the response rate and prolongs survival as compared with FCM alone in patients with relapsed and refractory follicular and mantle cell lymphomas: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood* 2004; **104**: 3064-3071 [PMID: 15284112 DOI: 10.1182/blood-2004-04-1323]

23 **Marcus R**, Imrie K, Belch A, Cunningham D, Flores E, Catalano J, Solal-Celigny P, Offner F, Walewski J, Raposo J, Jack A, Smith P. CVP chemotherapy plus rituximab compared with CVP as first-line treatment for advanced follicular lymphoma. *Blood* 2005; **105**: 1417-1423 [PMID: 15494430 DOI: 10.1182/blood-2004-08-3175]

24 **Hiddemann W**, Kneba M, Dreyling M, Schmitz N, Lengfelder E, Schmits R, Reiser M, Metzner B, Harder H, Hegewisch-Becker S, Fischer T, Kropff M, Reis HE, Freund M, Wörmann B, Fuchs R, Planker M, Schimke J, Eimermacher H, Trümper L, Aldaoud A, Parwaresch R, Unterhalt M. Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood* 2005; **106**: 3725-3732 [PMID: 16123223 DOI: 10.1182/blood-2005-01-0016]

25 **Herold M**, Haas A, Srock S, Neser S, Al-Ali KH, Neubauer A, Dölken G, Naumann R, Knauf W, Freund M, Rohrberg R, Höffken K, Franke A, Ittel T, Kettner E, Haak U, Mey U, Klinkenstein C, Assmann M, von Grünhagen U. Rituximab added to first-line mitoxantrone, chlorambucil, and prednisolone chemotherapy followed by interferon maintenance prolongs survival in patients with advanced follicular lymphoma: an East German Study Group Hematology and Oncology Study. *J Clin Oncol* 2007; **25**: 1986-1992 [PMID: 17420513 DOI: 10.1200/JCO.2006.06.4618]

26 **Hochster H**, Weller E, Gascoyne RD, Habermann TM, Gordon LI, Ryan T, Zhang L, Colocci N, Frankel S, Horning SJ. Maintenance rituximab after cyclophosphamide, vincristine, and prednisone prolongs progression-free survival in advanced indolent lymphoma: results of the randomized phase III ECOG1496 Study. *J Clin Oncol* 2009; **27**: 1607-1614 [PMID: 19255334 DOI: 10.1200/JCO.2008.17.1561]

27 **Forstpointner R**, Unterhalt M, Dreyling M, Böck HP, Repp R, Wandt H, Pott C, Seymour JF, Metzner B, Hänel A, Lehmann T, Hartmann F, Einsele H, Hiddemann W. Maintenance therapy with rituximab leads to a significant prolongation of response duration after salvage therapy with a combination of rituximab, fludarabine, cyclophosphamide, and mitoxantrone (R-FCM) in patients with recurring and refractory follicular and mantle cell lymphomas: Results of a prospective randomized study of the German Low Grade Lymphoma Study Group (GLSG). *Blood* 2006; **108**: 4003-4008 [PMID: 16946304 DOI: 10.1182/blood-2006-04-016725]

28 **Salles G**, Seymour JF, Offner F, López-Guillermo A, Belada D, Xerri L, Feugier P, Bouabdallah R, Catalano JV, Brice P, Caballero D, Haioun C, Pedersen LM, Delmer A, Simpson D, Leppa S, Soubeyran P, Hagenbeek A, Casasnovas O, Intragumtornchai T, Fermé C, da Silva MG, Sebban C, Lister A, Estell JA, Milone G, Sonet A, Mendila M, Coiffier B, Tilly H. Rituximab maintenance for 2 years in patients with high tumour burden follicular lymphoma responding to rituximab plus chemotherapy (PRIMA): a phase 3, randomised controlled trial. *Lancet* 2011; **377**: 42-51 [PMID: 21176949 DOI: 10.1016/S0140-6736(10)62175-7]

29 **Coiffier B**, Haioun C, Ketterer N, Engert A, Tilly H, Ma D, Johnson P, Lister A, Feuring-Buske M, Radford JA, Capdeville R, Diehl V, Reyes F. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study. *Blood* 1998; **92**: 1927-1932 [PMID: 9731049]

30 **Coiffier B**, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, Morel P, Van Den Neste E, Salles G, Gaulard P, Reyes F, Lederlin P, Gisselbrecht C. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med* 2002; **346**: 235-242 [PMID: 11807147 DOI: 10.1056/NEJMoa011795]

31 **Pfreundschuh M**, Trümper L, Osterborg A, Pettengell R, Trneny M, Imrie K, Ma D, Gill D, Walewski J, Zinzani PL, Stahel R, Kvaloy S, Shpilberg O, Jaeger U, Hansen M, Lehtinen T, López-Guillermo A, Corrado C, Scheliga A, Milpied N, Mendila M, Rashford M, Kuhnt E, Loeffler M. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. *Lancet Oncol* 2006; **7**: 379-391 [PMID: 16648042 DOI: 10.1016/S1470-2045(06)70664-7]

32 **Pfreundschuh M**, Schubert J, Ziepert M, Schmits R, Mohren

M, Lengfelder E, Reiser M, Nickenig C, Clemens M, Peter N, Bokemeyer C, Eimermacher H, Ho A, Hoffmann M, Mertelsmann R, Trümper L, Balleisen L, Liersch R, Metzner B, Hartmann F, Glass B, Poeschel V, Schmitz N, Ruebe C, Feller AC, Loeffler M. Six versus eight cycles of bi-weekly CHOP-14 with or without rituximab in elderly patients with aggressive CD20+ B-cell lymphomas: a randomised controlled trial (RICOVER-60). *Lancet Oncol* 2008; **9**: 105-116 [PMID: 18226581 DOI: 10.1016/S1470-2045(08)70002-0]

33 **Ketterer N**, Coiffier B, Thieblemont C, Fermé C, Brière J, Casasnovas O, Bologna S, Christian B, Connerotte T, Récher C, Bordessoule D, Fruchart C, Delarue R, Bonnet C, Morschhauser F, Anglaret B, Soussain C, Fabiani B, Tilly H, Haioun C. Phase III study of ACVBP versus ACVBP plus rituximab for patients with localized low-risk diffuse large B-cell lymphoma (LNH03-1B). *Ann Oncol* 2013; **24**: 1032-1037 [PMID: 23235801 DOI: 10.1093/annonc/mds600]

34 **Habermann TM**, Weller EA, Morrison VA, Gascoyne RD, Cassileth PA, Cohn JB, Dakhil SR, Woda B, Fisher RI, Peterson BA, Horning SJ. Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. *J Clin Oncol* 2006; **24**: 3121-3127 [PMID: 16754935 DOI: 10.1200/JCO.2005.05.1003]

35 **Gisselbrecht C**, Schmitz N, Mounier N, Singh Gill D, Linch DC, Trneny M, Bosly A, Milpied NJ, Radford J, Ketterer N, Shpilberg O, Dührsen U, Hagberg H, Ma DD, Viardot A, Lowenthal R, Brière J, Salles G, Moskowitz CH, Glass B. Rituximab maintenance therapy after autologous stem-cell transplantation in patients with relapsed CD20(+) diffuse large B-cell lymphoma: final analysis of the collaborative trial in relapsed aggressive lymphoma. *J Clin Oncol* 2012; **30**: 4462-4469 [PMID: 23091101 DOI: 10.1200/JCO.2012.41.9416]

36 **Witzig TE**, Gordon LI, Cabanillas F, Czuczman MS, Emmanouilides C, Joyce R, Pohlman BL, Bartlett NL, Wiseman GA, Padre N, Grillo-López AJ, Multani P, White CA. Randomized controlled trial of yttrium-90-labeled ibritumomab tiuxetan radioimmunotherapy versus rituximab immunotherapy for patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. *J Clin Oncol* 2002; **20**: 2453-2463 [PMID: 12011122 DOI: 10.1200/JCO.2002.11.076]

37 **Esmaeli B**, McLaughlin P, Pro B, Samaniego F, Gayed I, Hagemeister F, Romaguera J, Cabanillas F, Neelapu SS, Banay R, Fayad L, Wayne Saville M, Kwak LW. Prospective trial of targeted radioimmunotherapy with Y-90 ibritumomab tiuxetan (Zevalin) for front-line treatment of early-stage extranodal indolent ocular adnexal lymphoma. *Ann Oncol* 2009; **20**: 709-714 [PMID: 19150940 DOI: 10.1093/annonc/mdn692]

38 **Morschhauser F**, Radford J, Van Hoof A, Vitolo U, Soubeyran P, Tilly H, Huijgens PC, Kolstad A, d'Amore F, Gonzalez Diaz M, Petrini M, Sebban C, Zinzani PL, van Oers MH, van Putten W, Bischof-Delaloye A, Rohatiner A, Salles G, Kuhlmann J, Hagenbeek A. Phase III trial of consolidation therapy with yttrium-90-ibritumomab tiuxetan compared with no additional therapy after first remission in advanced follicular lymphoma. *J Clin Oncol* 2008; **26**: 5156-5164 [PMID: 18854568 DOI: 10.1200/JCO.2008.17.2015]

39 **Vose JM**, Wahl RL, Saleh M, Rohatiner AZ, Knox SJ, Radford JA, Zelenetz AD, Tidmarsh GF, Stagg RJ, Kaminski MS. Multicenter phase II study of iodine-131 tositumomab for chemotherapy-relapsed/refractory low-grade and transformed low-grade B-cell non-Hodgkin's lymphomas. *J Clin Oncol* 2000; **18**: 1316-1323 [PMID: 10715303]

40 **Kaminski MS**, Tuck M, Estes J, Kolstad A, Ross CW, Zasadny K, Regan D, Kison P, Fisher S, Kroll S, Wahl RL. 131I-tositumomab therapy as initial treatment for follicular lymphoma. *N Engl J Med* 2005; **352**: 441-449 [PMID: 15689582 DOI: 10.1056/NEJMoa041511]

41 **Kaminski MS**, Radford JA, Gregory SA, Leonard JP, Knox SJ, Kroll S, Wahl RL. Re-treatment with I-131 tositumomab in patients with non-Hodgkin's lymphoma who had previously responded to I-131 tositumomab. *J Clin Oncol* 2005; **23**: 7985-7993 [PMID: 16204016 DOI: 10.1200/JCO.2005.01.0892]

42 **Press OW**, Unger JM, Braziel RM, Maloney DG, Miller TP, LeBlanc M, Gaynor ER, Rivkin SE, Fisher RI. A phase 2 trial of CHOP chemotherapy followed by tositumomab/iodine I 131 tositumomab for previously untreated follicular non-Hodgkin lymphoma: Southwest Oncology Group Protocol S9911. *Blood* 2003; **102**: 1606-1612 [PMID: 12738671 DOI: 10.1182/blood-2003-01-0287]

43 **Press OW**, Unger JM, Rimsza LM, Friedberg JW, LeBlanc M, Czuczman MS, Kaminski M, Braziel RM, Spier C, Gopal AK, Maloney DG, Cheson BD, Dakhil SR, Miller TP, Fisher RI. Phase III randomized intergroup trial of CHOP plus rituximab compared with CHOP chemotherapy plus (131)iodine-tositumomab for previously untreated follicular non-Hodgkin lymphoma: SWOG S0016. *J Clin Oncol* 2013; **31**: 314-320 [PMID: 23233710 DOI: 10.1200/JCO.2012.42.4101]

44 **Vose JM**, Carter S, Burns LJ, Ayala E, Press OW, Moskowitz CH, Stadtmauer EA, Mineshi S, Ambinder R, Fenske T, Horowitz M, Fisher R, Tomblyn M. Phase III randomized study of rituximab/carmustine, etoposide, cytarabine, and melphalan (BEAM) compared with iodine-131 tositumomab/BEAM with autologous hematopoietic cell transplantation for relapsed diffuse large B-cell lymphoma: results from the BMT CTN 0401 trial. *J Clin Oncol* 2013; **31**: 1662-1668 [PMID: 23478060 DOI: 10.1200/JCO.2012.45.9453]

45 **Coiffier B**, Lepretre S, Pedersen LM, Gadeberg O, Fredriksen H, van Oers MH, Wooldridge J, Kloczko J, Holowiecki J, Hellmann A, Walewski J, Flensburg M, Petersen J, Robak T. Safety and efficacy of ofatumumab, a fully human monoclonal anti-CD20 antibody, in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: a phase 1-2 study. *Blood* 2008; **111**: 1094-1100 [PMID: 18003886 DOI: 10.1182/blood-2007-09-111781]

46 **Hagenbeek A**, Gadeberg O, Johnson P, Pedersen LM, Walewski J, Hellmann A, Link BK, Robak T, Wojtukiewicz M, Pfreundschuh M, Kneba M, Engert A, Sonneveld P, Flensburg M, Petersen J, Losic N, Radford J. First clinical use of ofatumumab, a novel fully human anti-CD20 monoclonal antibody in relapsed or refractory follicular lymphoma: results of a phase 1/2 trial. *Blood* 2008; **111**: 5486-5495 [PMID: 18390837 DOI: 10.1182/blood-2007-10-117671]

47 **Lemery SJ**, Zhang J, Rothmann MD, Yang J, Earp J, Zhao H, McDougal A, Pilaro A, Chiang R, Gootenberg JE, Keegan P, Pazdur R. U.S. Food and Drug Administration approval: ofatumumab for the treatment of patients with chronic lymphocytic leukemia refractory to fludarabine and alemtuzumab. *Clin Cancer Res* 2010; **16**: 4331-4338 [PMID: 20601446 DOI: 10.1158/1078-0432.CCR-10-0570]

48 **Wierda WG**, Kipps TJ, Dürig J, Griskevicius L, Stilgenbauer S, Mayer J, Smolej L, Hess G, Griniute R, Hernandez-Ilizaliturri FJ, Padmanabhan S, Gorczyca M, Chang CN, Chan G, Gupta I, Nielsen TG, Russell CA. Chemoimmunotherapy with O-FC in previously untreated patients with chronic lymphocytic leukemia. *Blood* 2011; **117**: 6450-6458 [PMID: 21498674 DOI: 10.1182/blood-2010-12-323980]

49 **Czuczman MS**, Fayad L, Delwail V, Cartron G, Jacobsen E, Kuliczkowski K, Link BK, Pinter-Brown L, Radford J, Hellmann A, Gallop-Evans E, DiRienzo CG, Goldstein N, Gupta I, Jewell RC, Lin TS, Lisby S, Schultz M, Russell CA, Hagenbeek A. Ofatumumab monotherapy in rituximab-refractory follicular lymphoma: results from a multicenter study. *Blood* 2012; **119**: 3698-3704 [PMID: 22389254 DOI: 10.1182/blood-2011-09-378323]

50 **Coiffier B**, Radford J, Bosly A, Martinelli G, Barca G, Davies A, Decaudin D, Gallop-Evans E, Padmanabhan-Iyer S, Van Eygen K, Wu KL, Gupta IV, Lin TS, Goldstein N, Jewell RC, Winter P, Lisby S. A multicentre, phase II trial of ofatu-

mumab monotherapy in relapsed/progressive diffuse large B-cell lymphoma. *Br J Haematol* 2013; **163**: 334-342 [PMID: 24032456 DOI: 10.1111/bjh.12537]
51 **Morschhauser F**, Leonard JP, Fayad L, Coiffier B, Petillon MO, Coleman M, Schuster SJ, Dyer MJ, Horne H, Teoh N, Wegener WA, Goldenberg DM. Humanized anti-CD20 antibody, veltuzumab, in refractory/recurrent non-Hodgkin's lymphoma: phase I/II results. *J Clin Oncol* 2009; **27**: 3346-3353 [PMID: 19451441 DOI: 10.1200/JCO.2008.19.9117]
52 **Morschhauser F**, Marlton P, Vitolo U, Lindén O, Seymour JF, Crump M, Coiffier B, Foà R, Wassner E, Burger HU, Brennan B, Mendila M. Results of a phase I/II study of ocrelizumab, a fully humanized anti-CD20 mAb, in patients with relapsed/refractory follicular lymphoma. *Ann Oncol* 2010; **21**: 1870-1876 [PMID: 20157180 DOI: 10.1093/annonc/mdq027]
53 **Salles G**, Morschhauser F, Lamy T, Milpied N, Thieblemont C, Tilly H, Bieska G, Asikanius E, Carlile D, Birkett J, Pisa P, Cartron G. Phase 1 study results of the type II glycoengineered humanized anti-CD20 monoclonal antibody obinutuzumab (GA101) in B-cell lymphoma patients. *Blood* 2012; **119**: 5126-5132 [PMID: 22431570 DOI: 10.1182/blood-2012-01-404368]
54 **Sehn LH**, Assouline SE, Stewart DA, Mangel J, Gascoyne RD, Fine G, Frances-Lasserre S, Carlile DJ, Crump M. A phase 1 study of obinutuzumab induction followed by 2 years of maintenance in patients with relapsed CD20-positive B-cell malignancies. *Blood* 2012; **119**: 5118-5125 [PMID: 22438256 DOI: 10.1182/blood-2012-02-408773]
55 **Salles GA**, Morschhauser F, Solal-Céligny P, Thieblemont C, Lamy T, Tilly H, Gyan E, Lei G, Wenger M, Wassner-Fritsch E, Cartron G. Obinutuzumab (GA101) in patients with relapsed/refractory indolent non-Hodgkin lymphoma: results from the phase II GAUGUIN study. *J Clin Oncol* 2013; **31**: 2920-2926 [PMID: 23835715 DOI: 10.1200/JCO.2012.46.9718]
56 **Morschhauser FA**, Cartron G, Thieblemont C, Solal-Céligny P, Haioun C, Bouabdallah R, Feugier P, Bouabdallah K, Asikanius E, Lei G, Wenger M, Wassner-Fritsch E, Salles GA. Obinutuzumab (GA101) monotherapy in relapsed/refractory diffuse large b-cell lymphoma or mantle-cell lymphoma: results from the phase II GAUGUIN study. *J Clin Oncol* 2013; **31**: 2912-2919 [PMID: 23835718 DOI: 10.1200/JCO.2012.46.9585]
57 **Radford J**, Davies A, Cartron G, Morschhauser F, Salles G, Marcus R, Wenger M, Lei G, Wassner-Fritsch E, Vitolo U. Obinutuzumab (GA101) plus CHOP or FC in relapsed/refractory follicular lymphoma: results of the GAUDI study (BO21000). *Blood* 2013; **122**: 1137-1143 [PMID: 23843495 DOI: 10.1182/blood-2013-01-481341]
58 **Friedberg JW**, Vose J, Kahl BS, Brunvand M, Goy A, Kasamon Y, Brington B, Li J, Ho W, Cheson BD. A Phase I Study of PRO131921, a Novel Anti-CD20 Monoclonal Antibody in Patients with Relapsed/Refractory CD20 Indolent NHL: Correlation Between Clinical Responses and AUC Pharmacokinetics. *ASH Annual Meeting Abstracts* 2009; **114**: 3472
59 **Wayne JL**, Ganjoo KN, Pohlman BL, De Vos S, Flinn IW, Dang NH, Mapara MY, Smith MR, O'Reilly AM, Marulappa SY, Jain VK. Efficacy of ocaratuzumab (AME-133v) in relapsed follicular lymphoma patients refractory to prior rituximab. *ASCO Meeting Abstracts* 2012; **30**: 8081
60 **Barcellini W**, Zaja F, Zaninoni A, Imperiali FG, Battista ML, Di Bona E, Fattizzo B, Consonni D, Cortelezzi A, Fanin R, Zanella A. Low-dose rituximab in adult patients with idiopathic autoimmune hemolytic anemia: clinical efficacy and biologic studies. *Blood* 2012; **119**: 3691-3697 [PMID: 22267606 DOI: 10.1182/blood-2011-06-363556]
61 **Stasi R**, Pagano A, Stipa E, Amadori S. Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura. *Blood* 2001; **98**: 952-957 [PMID: 11493438 DOI: 10.1182/blood.V98.4.952]
62 **Stasi R**, Brunetti M, Stipa E, Amadori S. Selective B-cell depletion with rituximab for the treatment of patients with acquired hemophilia. *Blood* 2004; **103**: 4424-4428 [PMID: 14996701 DOI: 10.1182/blood-2003-11-4075]
63 **Fakhouri F**, Vernant JP, Veyradier A, Wolf M, Kaplanski G, Binaut R, Rieger M, Scheiflinger F, Poullin P, Deroure B, Delarue R, Lesavre P, Vanhille P, Hermine O, Remuzzi G, Grünfeld JP. Efficiency of curative and prophylactic treatment with rituximab in ADAMTS13-deficient thrombotic thrombocytopenic purpura: a study of 11 cases. *Blood* 2005; **106**: 1932-1937 [PMID: 15933059 DOI: 10.1182/blood-2005-03-0848]
64 **Sansonno D**, De Re V, Lauletta G, Tucci FA, Boiocchi M, Dammacco F. Monoclonal antibody treatment of mixed cryoglobulinemia resistant to interferon alpha with an anti-CD20. *Blood* 2003; **101**: 3818-3826 [PMID: 12506023 DOI: 10.1182/blood-2002-10-3162]
65 **Zaja F**, De Vita S, Mazzaro C, Sacco S, Damiani D, De Marchi G, Michelutti A, Baccarani M, Fanin R, Ferraccioli G. Efficacy and safety of rituximab in type II mixed cryoglobulinemia. *Blood* 2003; **101**: 3827-3834 [PMID: 12560225 DOI: 10.1182/blood-2002-09-2856]
66 **LeBien TW**, Tedder TF. B lymphocytes: how they develop and function. *Blood* 2008; **112**: 1570-1580 [PMID: 18725575 DOI: 10.1182/blood-2008-02-078071]
67 **Barcellini W**, Zanella A. Rituximab therapy for autoimmune haematological diseases. *Eur J Intern Med* 2011; **22**: 220-229 [PMID: 21570637 DOI: 10.1016/j.ejim.2010.12.016]
68 **Nader K**, Patel M, Ferber A. Ofatumumab in rituximab-refractory autoimmune hemolytic anemia associated with chronic lymphocytic leukemia: a case report and review of literature. *Clin Lymphoma Myeloma Leuk* 2013; **13**: 511-513 [PMID: 23726016 DOI: 10.1016/j.clml.2013.02.022]
69 **Liebman HA**, Saleh MN, Bussel JB, Negrea OG, Horne H, Wegener WA, Goldenberg DM. Low-dose anti-CD20 veltuzumab given intravenously or subcutaneously is active in relapsed immune thrombocytopenia: a phase I study. *Br J Haematol* 2013; **162**: 693-701 [PMID: 23829485 DOI: 10.1111/bjh.12448]
70 **Maloney DG**, Grillo-López AJ, White CA, Bodkin D, Schilder RJ, Neidhart JA, Janakiraman N, Foon KA, Liles TM, Dallaire BK, Wey K, Royston I, Davis T, Levy R. IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood* 1997; **90**: 2188-2195 [PMID: 9310469]
71 **Nakou M**, Katsikas G, Sidiropoulos P, Bertsias G, Papadimitraki E, Raptopoulou A, Koutala H, Papadaki HA, Kritikos H, Boumpas DT. Rituximab therapy reduces activated B cells in both the peripheral blood and bone marrow of patients with rheumatoid arthritis: depletion of memory B cells correlates with clinical response. *Arthritis Res Ther* 2009; **11**: R131 [PMID: 19715572 DOI: 10.1186/ar2798]
72 **Kamburova EG**, Koenen HJ, Boon L, Hilbrands LB, effects of rituximab on the proliferation, activation and Joosten I. In vitro differentiation of human B cells. *Am J Transplant* 2012; **12**: 341-350 [PMID: 22070501 DOI: 10.1111/j.1600-6143.2011.03833.x]
73 **Roll P**, Palanichamy A, Kneitz C, Dorner T, Tony HP. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Arthritis Rheum* 2006; **54**: 2377-2386 [PMID: 16869000 DOI: 10.1002/art.22019]
74 **Leandro MJ**, Cambridge G, Ehrenstein MR, Edwards JC. Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis. *Arthritis Rheum* 2006; **54**: 613-620 [PMID: 16447239 DOI: 10.1002/art.21617]
75 **Anolik JH**, Friedberg JW, Zheng B, Barnard J, Owen T, Cushing E, Kelly J, Milner EC, Fisher RI, Sanz I. B cell recon-

stitution after rituximab treatment of lymphoma recapitulates B cell ontogeny. *Clin Immunol* 2007; **122**: 139-145 [PMID: 17008130 DOI: 10.1016/j.clim.2006.08.009]

76 **Abulayha AM**, Tabal SA, Shawesh EI, Elbasir MA, Elbanani AS, Lamami YM, Bredan A. Depletion of peripheral blood B cells with Rituximab and phenotype characterization of the recovering population in a patient with follicular lymphoma. *Leuk Res* 2010; **34**: 307-311 [PMID: 19580998 DOI: 10.1016/j.leukres.2009.06.005]

77 **Tsuda M**, Moritoki Y, Lian ZX, Zhang W, Yoshida K, Wakabayashi K, Yang GX, Nakatani T, Vierling J, Lindor K, Gershwin ME, Bowlus CL. Biochemical and immunologic effects of rituximab in patients with primary biliary cirrhosis and an incomplete response to ursodeoxycholic acid. *Hepatology* 2012; **55**: 512-521 [PMID: 22006563 DOI: 10.1002/hep.24748]

78 **Pescovitz MD**, Torgerson TR, Ochs HD, Ocheltree E, McGee P, Krause-Steinrauf H, Lachin JM, Canniff J, Greenbaum C, Herold KC, Skyler JS, Weinberg A. Effect of rituximab on human in vivo antibody immune responses. *J Allergy Clin Immunol* 2011; **128**: 1295-1302.e5 [PMID: 21908031 DOI: 10.1016/j.jaci.2011.08.008]

79 **Bedognetti D**, Zoppoli G, Massucco C, Zanardi E, Zupo S, Bruzzone A, Sertoli MR, Balleari E, Racchi O, Messina M, Caltabiano G, Icardi G, Durando P, Marincola FM, Boccardo F, Ferrarini M, Ansaldi F, De Maria A. Impaired response to influenza vaccine associated with persistent memory B cell depletion in non-Hodgkin's lymphoma patients treated with rituximab-containing regimens. *J Immunol* 2011; **186**: 6044-6055 [PMID: 21498665 DOI: 10.4049/jimmunol.1004095]

80 **van Vollenhoven RF**, Emery P, Bingham CO, Keystone EC, Fleischmann RM, Furst DE, Tyson N, Collinson N, Lehane PB. Long-term safety of rituximab in rheumatoid arthritis: 9.5-year follow-up of the global clinical trial programme with a focus on adverse events of interest in RA patients. *Ann Rheum Dis* 2013; **72**: 1496-1502 [PMID: 23136242 DOI: 10.1136/annrheumdis-2012-201956]

81 **Eming R**, Nagel A, Wolff-Franke S, Podstawa E, Debus D, Hertl M. Rituximab exerts a dual effect in pemphigus vulgaris. *J Invest Dermatol* 2008; **128**: 2850-2858 [PMID: 18563178 DOI: 10.1038/jid.2008.172]

82 **Stasi R**, Cooper N, Del Poeta G, Stipa E, Laura Evangelista M, Abruzzese E, Amadori S. Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. *Blood* 2008; **112**: 1147-1150 [PMID: 18375792 DOI: 10.1182/blood-2007-12-129262]

83 **Stasi R**, Del Poeta G, Stipa E, Evangelista ML, Trawinska MM, Cooper N, Amadori S. Response to B-cell depleting therapy with rituximab reverts the abnormalities of T-cell subsets in patients with idiopathic thrombocytopenic purpura. *Blood* 2007; **110**: 2924-2930 [PMID: 17548576 DOI: 10.1182/blood-2007-02-068999]

84 **Sfikakis PP**, Souliotis VL, Fragiadaki KG, Moutsopoulos HM, Boletis JN, Theofilopoulos AN. Increased expression of the FoxP3 functional marker of regulatory T cells following B cell depletion with rituximab in patients with lupus nephritis. *Clin Immunol* 2007; **123**: 66-73 [PMID: 17275413 DOI: 10.1016/j.clim.2006.12.006]

85 **Vallerskog T**, Gunnarsson I, Widhe M, Risselada A, Klareskog L, van Vollenhoven R, Malmström V, Trollmo C. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clin Immunol* 2007; **122**: 62-74 [PMID: 17046329 DOI: 10.1016/j.clim.2006.08.016]

86 **Feuchtenberger M**, Müller S, Roll P, Waschbisch A, Schäfer A, Kneitz C, Wiendl H, Tony HP. Frequency of regulatory T cells is not affected by transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Open Rheumatol J* 2008; **2**: 81-88 [PMID: 19156222 DOI: 10.2174/1874312900802010081]

87 **Saadoun D**, Rosenzwajg M, Landau D, Piette JC, Klatzmann D, Cacoub P. Restoration of peripheral immune homeostasis after rituximab in mixed cryoglobulinemia vasculitis. *Blood* 2008; **111**: 5334-5341 [PMID: 18292291 DOI: 10.1182/blood-2007-11-122713]

88 **Bouaziz JD**, Yanaba K, Venturi GM, Wang Y, Tisch RM, Poe JC, Tedder TF. Therapeutic B cell depletion impairs adaptive and autoreactive CD4+ T cell activation in mice. *Proc Natl Acad Sci USA* 2007; **104**: 20878-20883 [PMID: 18093919 DOI: 10.1073/pnas.0709205105]

89 **Yeo W**, Chan PK, Zhong S, Ho WM, Steinberg JL, Tam JS, Hui P, Leung NW, Zee B, Johnson PJ. Frequency of hepatitis B virus reactivation in cancer patients undergoing cytotoxic chemotherapy: a prospective study of 626 patients with identification of risk factors. *J Med Virol* 2000; **62**: 299-307 [PMID: 11055239 DOI: 10.1002/1096-9071(200011)]

90 **Takai S**, Tsurumi H, Ando K, Kasahara S, Sawada M, Yamada T, Hara T, Fukuno K, Takahashi T, Oyama M, Onishi H, Tomita E, Takami T, Imawari M, Moriwaki H. Prevalence of hepatitis B and C virus infection in haematological malignancies and liver injury following chemotherapy. *Eur J Haematol* 2005; **74**: 158-165 [PMID: 15654908 DOI: 10.1111/j.1600-0609.2004.00376.x]

91 **Tan J**, Zhou J, Zhao P, Wei J. Prospective study of HBV reactivation risk in rheumatoid arthritis patients who received conventional disease-modifying antirheumatic drugs. *Clin Rheumatol* 2012; **31**: 1169-1175 [PMID: 22544263 DOI: 10.1007/s10067-012-1988-2]

92 **Cheng AL**, Hsiung CA, Su IJ, Chen PJ, Chang MC, Tsao CJ, Kao WY, Uen WC, Hsu CH, Tien HF, Chao TY, Chen LT, Whang-Peng J. Steroid-free chemotherapy decreases risk of hepatitis B virus (HBV) reactivation in HBV-carriers with lymphoma. *Hepatology* 2003; **37**: 1320-1328 [PMID: 12774010 DOI: 10.1053/jhep.2003.50220]

93 **Hsu CH**, Hsu HC, Chen HL, Gao M, Yeh PY, Chen PJ, Cheng AL. Doxorubicin activates hepatitis B virus (HBV) replication in HBV-harboring hepatoblastoma cells. A possible novel mechanism of HBV reactivation in HBV carriers receiving systemic chemotherapy. *Anticancer Res* 2004; **24**: 3035-3040 [PMID: 15517913]

94 **Chu CM**, Karayiannis P, Fowler MJ, Monjardino J, Liaw YF, Thomas HC. Natural history of chronic hepatitis B virus infection in Taiwan: studies of hepatitis B virus DNA in serum. *Hepatology* 1985; **5**: 431-434 [PMID: 3997072]

95 **Ganem D**, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-1129 [PMID: 15014185 DOI: 10.1056/NEJMra031087]

96 **Liaw YF**, Chu CM. Hepatitis B virus infection. *Lancet* 2009; **373**: 582-592 [PMID: 19217993 DOI: 10.1016/S0140-6736(09)60207-5]

97 **Hsu YS**, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002; **35**: 1522-1527 [PMID: 12029639 DOI: 10.1053/jhep.2002.33638]

98 **Dunn C**, Peppa D, Khanna P, Nebbia G, Jones M, Brendish N, Lascar RM, Brown D, Gilson RJ, Tedder RJ, Dusheiko GM, Jacobs M, Klenerman P, Maini MK. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. *Gastroenterology* 2009; **137**: 1289-1300 [PMID: 19591831 DOI: 10.1053/j.gastro.2009.06.054]

99 **Zerbini A**, Pilli M, Boni C, Fisicaro P, Penna A, Di Vincenzo P, Giuberti T, Orlandini A, Raffa G, Pollicino T, Raimondo G, Ferrari C, Missale G. The characteristics of the cell-mediated immune response identify different profiles of occult hepatitis B virus infection. *Gastroenterology* 2008; **134**: 1470-1481 [PMID: 18355815 DOI: 10.1053/j.gastro.2008.02.017]

100 **Stoop JN**, van der Molen RG, Baan CC, van der Laan LJ,

Kuipers EJ, Kusters JG, Janssen HL. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* 2005; **41**: 771-778 [PMID: 15791617 DOI: 10.1002/hep.20649]

101 **Kim SJ**, Hsu C, Song YQ, Tay K, Hong XN, Cao J, Kim JS, Eom HS, Lee JH, Zhu J, Chang KM, Reksodiputro AH, Tan D, Goh YT, Lee J, Intragumtornchai T, Chng WJ, Cheng AL, Lim ST, Suh C, Kwong YL, Kim WS. Hepatitis B virus reactivation in B-cell lymphoma patients treated with rituximab: analysis from the Asia Lymphoma Study Group. *Eur J Cancer* 2013; **49**: 3486-3496 [PMID: 23910494 DOI: 10.1016/j.ejca.2013.07.006]

102 **Yeo W**, Chan TC, Leung NW, Lam WY, Mo FK, Chu MT, Chan HL, Hui EP, Lei KI, Mok TS, Chan PK. Hepatitis B virus reactivation in lymphoma patients with prior resolved hepatitis B undergoing anticancer therapy with or without rituximab. *J Clin Oncol* 2009; **27**: 605-611 [PMID: 19075267 DOI: 10.1200/JCO.2008.18.0182]

103 **Dervite I**, Hober D, Morel P. Acute hepatitis B in a patient with antibodies to hepatitis B surface antigen who was receiving rituximab. *N Engl J Med* 2001; **344**: 68-69 [PMID: 11187122 DOI: 10.1056/NEJM200101043440120]

104 **Yang SH**, Kuo SH. Reactivation of hepatitis B virus during rituximab treatment of a patient with follicular lymphoma. *Ann Hematol* 2008; **87**: 325-327 [PMID: 17932671 DOI: 10.1007/s00277-007-0396-1]

105 **Pei SN**, Chen CH, Lee CM, Wang MC, Ma MC, Hu TH, Kuo CY. Reactivation of hepatitis B virus following rituximab-based regimens: a serious complication in both HBsAg-positive and HBsAg-negative patients. *Ann Hematol* 2010; **89**: 255-262 [PMID: 19697028 DOI: 10.1007/s00277-009-0806-7]

106 **Hsu C**, Tsou HH, Lin SJ, Wang MC, Yao M, Hwang WL, Kao WY, Chiu CF, Lin SF, Lin J, Chang CS, Tien HF, Liu TW, Chen PJ, Cheng AL; Taiwan Cooperative Oncology Group. Chemotherapy-induced hepatitis B reactivation in lymphoma patients with resolved HBV infection: A prospective study. *Hepatology* 2013; Epub ahead of print [DOI: 10.1002/hep.26718]

107 **Kurokawa T**, Hase M, Tokuman N, Yoshida T. Immune reconstitution of B-cell lymphoma patients receiving CHOP-based chemotherapy containing rituximab. *Hematol Oncol* 2011; **29**: 5-9 [PMID: 20552573 DOI: 10.1002/hon.947]

108 **Peñalver FJ**, Alvarez-Larrán A, Díez-Martin JL, Gallur L, Jarque I, Caballero D, Díaz-Mediavilla J, Bustelos R, Fernández-Aceñero MJ, Cabrera JR. Rituximab is an effective and safe therapeutic alternative in adults with refractory and severe autoimmune hemolytic anemia. *Ann Hematol* 2010; **89**: 1073-1080 [PMID: 20526716 DOI: 10.1007/s00277-010-0997-y]

109 **Gudbrandsdottir S**, Birgens HS, Frederiksen H, Jensen BA, Jensen MK, Kjeldsen L, Klausen TW, Larsen H, Mourits-Andersen HT, Nielsen CH, Nielsen OJ, Plesner T, Pulczynski S, Rasmussen IH, Rønnov-Jessen D, Hasselbalch HC. Rituximab and dexamethasone vs dexamethasone monotherapy in newly diagnosed patients with primary immune thrombocytopenia. *Blood* 2013; **121**: 1976-1981 [PMID: 23293082 DOI: 10.1182/blood-2012-09-455691]

110 **Droz N**, Gilardin L, Cacoub P, Berenbaum F, Wendling D, Godeau B, Piette AM, Dernis E, Ebbo M, Fautrel B, Le Guenno G, Mekinian A, Bernard-Chabert B, Costedoat-Chalumeau N, Descloux E, Michot JM, Radenne S, Rigolet A, Rivière S, Yvin JL, Thibault V, Thabut D, Pol S, Guillevin L, Mouthon L, Terrier B. Kinetic profiles and management of hepatitis B virus reactivation in patients with immune-mediated inflammatory diseases. *Arthritis Care Res* (Hoboken) 2013; **65**: 1504-1514 [PMID: 23436730 DOI: 10.1002/acr.21990]

111 **Pasquet F**, Combarnous F, Macgregor B, Coppere B, Mausservey C, Ninet J, Hot A. Safety and efficacy of rituximab treatment for vasculitis in hepatitis B virus-associated type II cryoglobulinemia: a case report. *J Med Case Rep* 2012; **6**: 39 [PMID: 22284897 DOI: 10.1186/1752-1947-6-39]

112 **Cil T**, Altintas A, Tuzun Y, Pasa S, Isikdogan A. Hepatitis B virus reactivation induced by Yttrium-90-ibritumomab-tiuxetan. *Leuk Lymphoma* 2007; **48**: 1866-1868 [PMID: 17786727 DOI: 10.1080/10428190701509848)]

113 **Soong YL**, Lee KM, Lui HF, Chow WC, Tao M, Li Er Loong S. Hepatitis B reactivation in a patient receiving radiolabeled rituximab. *Ann Hematol* 2005; **84**: 61-62 [PMID: 15449029 DOI: 10.1007/s00277-004-0948-6]

114 **Mitka M**. FDA: Increased HBV reactivation risk with ofatumumab or rituximab. *JAMA* 2013; **310**: 1664 [PMID: 24150447 DOI: 10.1001/jama.2013.281115]

115 **Ogawa Y**, Ogura M, Suzuki T, Ando K, Uchida T, Shirasugi Y, Tobinai K, Lee JH, Kase M, Katsura K, Hotta T. A phase I/II study of ofatumumab (GSK1841157) in Japanese and Korean patients with relapsed or refractory B-cell chronic lymphocytic leukemia. *Int J Hematol* 2013; **98**: 164-170 [PMID: 23846385 DOI: 10.1007/s12185-013-1393-x]

116 **Cortelezzi A**, Sciumè M, Liberati AM, Vincenti D, Cuneo A, Reda G, Laurenti L, Zaja F, Marasca R, Chiarenza A, Gritti G, Orsucci L, Storti S, Angelucci E, Cascavilla N, Gobbi M, Mauro FR, Morabito F, Fabris S, Piciocchi A, Vignetti M, Neri A, Rossi D, Giannarelli D, Guarini A, Foà R. Bendamustine in combination with ofatumumab in relapsed or refractory chronic lymphocytic leukemia: a GIMEMA Multicenter Phase II Trial. *Leukemia* 2014; **28**: 642-648 [PMID: 24220274 DOI: 10.1038/leu.2013.334]

117 **Chen CJ**, Wang LY, Yu MW. Epidemiology of hepatitis B virus infection in the Asia-Pacific region. *J Gastroenterol Hepatol* 2000; **15** Suppl: E3-E6 [PMID: 10921373 DOI: 10.1046/j.1440-1746.2000.02124.x]

118 **Yeo W**, Johnson PJ. Diagnosis, prevention and management of hepatitis B virus reactivation during anticancer therapy. *Hepatology* 2006; **43**: 209-220 [PMID: 16440366 DOI: 10.1002/hep.21051]

119 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539 [PMID: 17256718 DOI: 10.1002/hep.21513]

120 **Liaw YF**, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, Guan R, Lau GK, Locarnini S. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008; **2**: 263-283 [PMID: 19669255 DOI: 10.1007/s12072-008-9080-3]

121 **European Association For The Study Of The Liver**. EASL Clinical Practice Guidelines: management of chronic hepatitis B. *J Hepatol* 2009; **50**: 227-242 [PMID: 19054588 DOI: 10.1016/j.jhep.2008.10.001]

122 **Weinbaum CM**, Williams I, Mast EE, Wang SA, Finelli L, Wasley A, Neitzel SM, Ward JW. Recommendations for identification and public health management of persons with chronic hepatitis B virus infection. *MMWR Recomm Rep* 2008; **57**: 1-20 [PMID: 18802412]

123 **Artz AS**, Somerfield MR, Feld JJ, Giusti AF, Kramer BS, Sabichi AL, Zon RT, Wong SL. American Society of Clinical Oncology provisional clinical opinion: chronic hepatitis B virus infection screening in patients receiving cytotoxic chemotherapy for treatment of malignant diseases. *J Clin Oncol* 2010; **28**: 3199-3202 [PMID: 20516452 DOI: 10.1200/JCO.2010.30.0673]

124 **Zurawska U**, Hicks LK, Woo G, Bell CM, Krahn M, Chan KK, Feld JJ. Hepatitis B virus screening before chemotherapy for lymphoma: a cost-effectiveness analysis. *J Clin Oncol* 2012; **30**: 3167-3173 [PMID: 22711851 DOI: 10.1200/JCO.2011.40.7510]

125 **Lok AS**, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 661-662 [PMID: 19714720 DOI: 10.1002/hep.23190]

126 **Liu CJ**, Chen PJ, Chen DS, Kao JH. Hepatitis B virus reactivation in patients receiving cancer chemotherapy: natural history, pathogenesis, and management. *Hepatol Int* 2011;

Epub ahead of print [PMID: 21670970 DOI: 10.1007/s12072-011-9279-6]
127 **Hsu C**, Hsiung CA, Su IJ, Hwang WS, Wang MC, Lin SF, Lin TH, Hsiao HH, Young JH, Chang MC, Liao YM, Li CC, Wu HB, Tien HF, Chao TY, Liu TW, Cheng AL, Chen PJ. A revisit of prophylactic lamivudine for chemotherapy-associated hepatitis B reactivation in non-Hodgkin's lymphoma: a randomized trial. *Hepatology* 2008; **47**: 844-853 [PMID: 18302293 DOI: 10.1002/hep.22106]
128 **Katz LH**, Fraser A, Gafter-Gvili A, Leibovici L, Tur-Kaspa R. Lamivudine prevents reactivation of hepatitis B and reduces mortality in immunosuppressed patients: systematic review and meta-analysis. *J Viral Hepat* 2008; **15**: 89-102 [PMID: 18184191 DOI: 10.1111/j.1365-2893.2007.00902.x]
129 **Lok AS**, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, Dienstag JL, Heathcote EJ, Little NR, Griffiths DA, Gardner SD, Castiglia M. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; **125**: 1714-1722 [PMID: 14724824 DOI: 10.1053/j.gastro.2003.09.033]
130 **Huang YH**, Hsiao LT, Hong YC, Chiou TJ, Yu YB, Gau JP, Liu CY, Yang MH, Tzeng CH, Lee PC, Lin HC, Lee SD. Randomized controlled trial of entecavir prophylaxis for rituximab-associated hepatitis B virus reactivation in patients with lymphoma and resolved hepatitis B. *J Clin Oncol* 2013; **31**: 2765-2772 [PMID: 23775967 DOI: 10.1200/JCO.2012.48.5938]
131 **Trembling PM**, Tanwar S, Rosenberg WM, Dusheiko GM. Treatment decisions and contemporary versus pending treatments for hepatitis C. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 713-728 [PMID: 24019151 DOI: 10.1038/nrgastro.2013.163]
132 **Lechner F**, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; **191**: 1499-1512 [PMID: 10790425 DOI: 10.1084/jem.191.9.1499]
133 **Nakamoto N**, Cho H, Shaked A, Olthoff K, Valiga ME, Kaminski M, Gostick E, Price DA, Freeman GJ, Wherry EJ, Chang KM. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog* 2009; **5**: e1000313 [PMID: 19247441 DOI: 10.1371/journal.ppat.1000313]
134 **Sugimoto K**, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 2003; **38**: 1437-1448 [PMID: 14647055 DOI: 10.1016/j.hep.2003.09.026]
135 **Martell M**, Esteban JI, Quer J, Genescà J, Weiner A, Esteban R, Guardia J, Gómez J. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* 1992; **66**: 3225-3229 [PMID: 1313927]
136 **Chang KM**, Rehermann B, McHutchison JG, Pasquinelli C, Southwood S, Sette A, Chisari FV. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. *J Clin Invest* 1997; **100**: 2376-2385 [PMID: 9410918 DOI: 10.1172/JCI119778]
137 **von Hahn T**, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P, McKeating JA. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 2007; **132**: 667-678 [PMID: 17258731 DOI: 10.1053/j.gastro.2006.12.008]
138 **Agnello V**, Chung RT, Kaplan LM. A role for hepatitis C virus infection in type II cryoglobulinemia. *N Engl J Med* 1992; **327**: 1490-1495 [PMID: 1383822 DOI: 10.1056/NEJM199211193272104]
139 **Dal Maso L**, Franceschi S. Hepatitis C virus and risk of lymphoma and other lymphoid neoplasms: a meta-analysis of epidemiologic studies. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 2078-2085 [PMID: 17119031 DOI: 10.1158/1055-9965.EPI-06-0308]
140 **Vento S**, Cainelli F, Mirandola F, Cosco L, Di Perri G, Solbiati M, Ferraro T, Concia E. Fulminant hepatitis on withdrawal of chemotherapy in carriers of hepatitis C virus. *Lancet* 1996; **347**: 92-93 [PMID: 8538348 DOI: 10.1016/S0140-6736(96)90212-3]
141 **Zuckerman E**, Zuckerman T, Douer D, Qian D, Levine AM. Liver dysfunction in patients infected with hepatitis C virus undergoing chemotherapy for hematologic malignancies. *Cancer* 1998; **83**: 1224-1230 [PMID: 9740089 DOI: 10.1002/(SICI)1097-0142(19980915)]
142 **Kawatani T**, Suou T, Tajima F, Ishiga K, Omura H, Endo A, Ohmura H, Ikuta Y, Idobe Y, Kawasaki H. Incidence of hepatitis virus infection and severe liver dysfunction in patients receiving chemotherapy for hematologic malignancies. *Eur J Haematol* 2001; **67**: 45-50 [PMID: 11553266 DOI: 10.1034/j.1600-0609.2001.067001045.x]
143 **Nosotti L**, D'Andrea M, Pitidis A, Pimpinelli F, Dessanti ML, Pisani F, Vignally P, Petti MC. Hepatitis C virus infection prevalence and liver dysfunction in a cohort of B-cell non-Hodgkin's lymphoma patients treated with immunochemotherapy. *Scand J Infect Dis* 2012; **44**: 70-73 [PMID: 21905952 DOI: 10.3109/00365548.2011.611819]
144 **Ennishi D**, Maeda Y, Niitsu N, Kojima M, Izutsu K, Takizawa J, Kusumoto S, Okamoto M, Yokoyama M, Takamatsu Y, Sunami K, Miyata A, Murayama K, Sakai A, Matsumoto M, Shinagawa K, Takaki A, Matsuo K, Kinoshita T, Tanimoto M. Hepatic toxicity and prognosis in hepatitis C virus-infected patients with diffuse large B-cell lymphoma treated with rituximab-containing chemotherapy regimens: a Japanese multicenter analysis. *Blood* 2010; **116**: 5119-5125 [PMID: 20823454 DOI: 10.1182/blood-2010-06-289231]
145 **Ennishi D**, Terui Y, Yokoyama M, Mishima Y, Takahashi S, Takeuchi K, Okamoto H, Tanimoto M, Hatake K. Monitoring serum hepatitis C virus (HCV) RNA in patients with HCV-infected CD20-positive B-cell lymphoma undergoing rituximab combination chemotherapy. *Am J Hematol* 2008; **83**: 59-62 [PMID: 17712791 DOI: 10.1002/ajh.21022]
146 **Coppola N**, Pisaturo M, Guastafierro S, Tonziello G, Sica A, Iodice V, Sagnelli C, Ferrara MG, Sagnelli E. Increased hepatitis C viral load and reactivation of liver disease in HCV RNA-positive patients with onco-haematological disease undergoing chemotherapy. *Dig Liver Dis* 2012; **44**: 49-54 [PMID: 21885355 DOI: 10.1016/j.dld.2011.07.016]
147 **Tsutsumi Y**, Ichiki K, Shiratori S, Kawamura T, Tanaka J, Asaka M, Imamura M, Masauzi N. Changes in hepatitis C virus antibody titer and viral RNA load in non-Hodgkin's lymphoma patients after rituximab chemotherapy. *Int J Lab Hematol* 2009; **31**: 468-470 [PMID: 18294236 DOI: 10.1111/j.1751-553X.2008.01034.x]
148 **Lake-Bakaar G**, Dustin L, McKeating J, Newton K, Freeman V, Frost SD. Hepatitis C virus and alanine aminotransferase kinetics following B-lymphocyte depletion with rituximab: evidence for a significant role of humoral immunity in the control of viremia in chronic HCV liver disease. *Blood* 2007; **109**: 845-846 [PMID: 17210867 DOI: 10.1182/blood-2006-08-041525]
149 **Halfon P**, Bourlière M, Halimi G, Khiri H, Bertezene P, Portal I, Botta-Fridlund D, Gauthier AP, Jullien M, Feryn JM, Gerolami V, Cartouzou G. Assessment of spontaneous fluctuations of viral load in untreated patients with chronic hepatitis C by two standardized quantitation methods: branched DNA and Amplicor Monitor. *J Clin Microbiol* 1998; **36**: 2073-2075 [PMID: 9650965]
150 **Mahale P**, Kontoyiannis DP, Chemaly RF, Jiang Y, Hwang JP, Davila M, Torres HA. Acute exacerbation and reactivation of chronic hepatitis C virus infection in cancer patients. *J Hepatol* 2012; **57**: 1177-1185 [PMID: 22871500 DOI: 10.1016/

j.jhep.2012.07.031]
151 **Torres HA**, Davila M. Reactivation of hepatitis B virus and hepatitis C virus in patients with cancer. *Nat Rev Clin Oncol* 2012; **9**: 156-166 [PMID: 22271089 DOI: 10.1038/nrclinonc.2012.1]
152 **D'Arena G**, Laurenti L, Capalbo S, D'Arco AM, De Filippi R, Marcacci G, Di Renzo N, Storti S, Califano C, Vigliotti ML, Tarnani M, Ferrara F, Pinto A. Rituximab therapy for chronic lymphocytic leukemia-associated autoimmune hemolytic anemia. *Am J Hematol* 2006; **81**: 598-602 [PMID: 16823816 DOI: 10.1002/ajh.20665]
153 **Dufour JF**, Pradat P, Ruivard M, Hot A, Dumontet C, Broussolle C, Trepo C, Sève P. Severe autoimmune cytopenias in treatment-naive hepatitis C virus infection: clinical description of 16 cases. *Eur J Gastroenterol Hepatol* 2009; **21**: 245-253 [PMID: 19279468 DOI: 10.1097/MEG.0b013e3283249908]
154 **Papaevangelou V**, Varsami M, Papadakis V, Zellos A, Parcharidou A, Papargyri S, Karentzou O, Manolaki N, Roma E, Polychronopoulou S. Hepatitis C treatment concomitant to chemotherapy as "salvage" therapy in children with hematologic malignancies. *Pediatr Infect Dis J* 2010; **29**: 277-280 [PMID: 19949358 DOI: 10.1097/INF.0b013e3181c2115a]
155 **Pellicelli AM**, Zoli V. Role of ribavirin in hepatitis flare in HCV-infected patients with B cell non Hodgkin's lymphoma treated with rituximab-containing regimens. *Dig Liver Dis* 2011; **43**: 501-502 [PMID: 21459059 DOI: 10.1016/j.dld.2010.12.018]
156 **Young NS**, Brown KE. Parvovirus B19. *N Engl J Med* 2004; **350**: 586-597 [PMID: 14762186 DOI: 10.1056/NEJMra030840]
157 **Sokal EM**, Melchior M, Cornu C, Vandenbroucke AT, Buts JP, Cohen BJ, Burtonboy G. Acute parvovirus B19 infection associated with fulminant hepatitis of favourable prognosis in young children. *Lancet* 1998; **352**: 1739-1741 [PMID: 9848349 DOI: 10.1016/S0140-6736(98)06165-0]
158 **Anderson MJ**, Higgins PG, Davis LR, Willman JS, Jones SE, Kidd IM, Pattison JR, Tyrrell DA. Experimental parvoviral infection in humans. *J Infect Dis* 1985; **152**: 257-265 [PMID: 2993431]
159 **Kurtzman GJ**, Ozawa K, Cohen B, Hanson G, Oseas R, Young NS. Chronic bone marrow failure due to persistent B19 parvovirus infection. *N Engl J Med* 1987; **317**: 287-294 [PMID: 3037373 DOI: 10.1056/NEJM198707303170506]
160 **Norbeck O**, Isa A, Pöhlmann C, Broliden K, Kasprowicz V, Bowness P, Klenerman P, Tolfvenstam T. Sustained CD8+ T-cell responses induced after acute parvovirus B19 infection in humans. *J Virol* 2005; **79**: 12117-12121 [PMID: 16140790 DOI: 10.1128/JVI.79.18.12117-12121.2005]
161 **Kasprowicz V**, Isa A, Tolfvenstam T, Jeffery K, Bowness P, Klenerman P. Tracking of peptide-specific CD4+ T-cell responses after an acute resolving viral infection: a study of parvovirus B19. *J Virol* 2006; **80**: 11209-11217 [PMID: 16943301 DOI: 10.1128/JVI.01173-06]
162 **Kuo SH**, Lin LI, Chang CJ, Liu YR, Lin KS, Cheng AL. Increased risk of parvovirus B19 infection in young adult cancer patients receiving multiple courses of chemotherapy. *J Clin Microbiol* 2002; **40**: 3909-3912 [PMID: 12409350]
163 **Heegaard ED**, Schmiegelow K. Serologic study on parvovirus b19 infection in childhood acute lymphoblastic leukemia during chemotherapy: clinical and hematologic implications. *J Pediatr Hematol Oncol* 2002; **24**: 368-373 [PMID: 12142785]
164 **Eid AJ**, Brown RA, Patel R, Razonable RR. Parvovirus B19 infection after transplantation: a review of 98 cases. *Clin Infect Dis* 2006; **43**: 40-48 [PMID: 16758416 DOI: 10.1086/504812]
165 **Li Y**, Dong Y, Jiang J, Yang Y, Liu K, Li Y. High prevelance of human parvovirus infection in patients with malignant tumors. *Oncol Lett* 2012; **3**: 635-640 [PMID: 22740966 DOI: 10.3892/ol.2012.548]
166 **Poplar S**, Allford S, Rooney N. Chronic parvovirus B19 infection leading to red cell aplasia following treatment of Hodgkin lymphoma. *Br J Haematol* 2010; **148**: 671 [PMID: 19681883 DOI: 10.1111/j.1365-2141.2009.07830.x]
167 **Sharma VR**, Fleming DR, Slone SP. Pure red cell aplasia due to parvovirus B19 in a patient treated with rituximab. *Blood* 2000; **96**: 1184-1186 [PMID: 10910942]
168 **Song KW**, Mollee P, Patterson B, Brien W, Crump M. Pure red cell aplasia due to parvovirus following treatment with CHOP and rituximab for B-cell lymphoma. *Br J Haematol* 2002; **119**: 125-127 [PMID: 12358915 DOI: 10.1046/j.1365-2141.2002.03778.x]
169 **Isobe Y**, Sugimoto K, Shiraki Y, Nishitani M, Koike K, Oshimi K. Successful high-titer immunoglobulin therapy for persistent parvovirus B19 infection in a lymphoma patient treated with rituximab-combined chemotherapy. *Am J Hematol* 2004; **77**: 370-373 [PMID: 15558805 DOI: 10.1002/ajh.20200]
170 **Klepfish A**, Rachmilevitch E, Schattner A. Parvovirus B19 reactivation presenting as neutropenia after rituximab treatment. *Eur J Intern Med* 2006; **17**: 505-507 [PMID: 17098597 DOI: 10.1016/j.ejim.2006.05.002]
171 **Hartmann JT**, Meisinger I, Kröber SM, Weisel K, Klingel K, Kanz L. Progressive bicytopenia due to persistent parvovirus B19 infection after immunochemotherapy with fludarabine/cyclophosphamide and rituximab for relapsed B cell lymphoma. *Haematologica* 2006; **91**: ECR49 [PMID: 17194655]
172 **Yang SH**, Lin LW, Fang YJ, Cheng AL, Kuo SH. Parvovirus B19 infection-related acute hepatitis after rituximab-containing regimen for treatment of diffuse large B-cell lymphoma. *Ann Hematol* 2012; **91**: 291-294 [PMID: 21538062 DOI: 10.1007/s00277-011-1238-8]
173 **Wong S**, Young NS, Brown KE. Prevalence of parvovirus B19 in liver tissue: no association with fulminant hepatitis or hepatitis-associated aplastic anemia. *J Infect Dis* 2003; **187**: 1581-1586 [PMID: 12721938 DOI: 10.1086/374781]
174 **Aksoy S**, Harputluoglu H, Kilickap S, Dede DS, Dizdar O, Altundag K, Barista I. Rituximab-related viral infections in lymphoma patients. *Leuk Lymphoma* 2007; **48**: 1307-1312 [PMID: 17613758 DOI: 10.1080/10428190701411441]
175 **Iyer A**, Mathur R, Deepak BV, Sinard J. Fatal adenoviral hepatitis after rituximab therapy. *Arch Pathol Lab Med* 2006; **130**: 1557-1560 [PMID: 17090202]
176 **Ronan BA**, Agrwal N, Carey EJ, De Petris G, Kusne S, Seville MT, Blair JE, Vikram HR. Fulminant hepatitis due to human adenovirus. *Infection* 2014; **42**: 105-111 [PMID: 23979854 DOI: 10.1007/s15010-013-0527-7]
177 **Ollier L**, Tieulie N, Sanderson F, Heudier P, Giordanengo V, Fuzibet JG, Nicand E. Chronic hepatitis after hepatitis E virus infection in a patient with non-Hodgkin lymphoma taking rituximab. *Ann Intern Med* 2009; **150**: 430-431 [PMID: 19293084 DOI: 10.7326/0003-4819-150-6-200903170-00026]
178 **Bermúdez A**, Marco F, Conde E, Mazo E, Recio M, Zubizarreta A. Fatal visceral varicella-zoster infection following rituximab and chemotherapy treatment in a patient with follicular lymphoma. *Haematologica* 2000; **85**: 894-895 [PMID: 10942955]
179 **Del Prete CJ**, Cohen NS. A case of rituximab-induced hepatitis. *Cancer Biother Radiopharm* 2010; **25**: 747-748 [PMID: 21204770 DOI: 10.1089/cbr.2010.0806]
180 **Mössner E**, Brünker P, Moser S, Püntener U, Schmidt C, Herter S, Grau R, Gerdes C, Nopora A, van Puijenbroek E, Ferrara C, Sondermann P, Jäger C, Strein P, Fertig G, Friess T, Schüll C, Bauer S, Dal Porto J, Del Nagro C, Dabbagh K, Dyer MJ, Poppema S, Klein C, Umaña P. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood* 2010; **115**: 4393-4402 [PMID: 20194898 DOI: 10.1182/blood-2009-06-225979]

181 **Dalle S**, Reslan L, Besseyre de Horts T, Herveau S, Herting F, Plesa A, Friess T, Umana P, Klein C, Dumontet C. Preclinical studies on the mechanism of action and the anti-lymphoma activity of the novel anti-CD20 antibody GA101. *Mol Cancer Ther* 2011; **10**: 178-185 [PMID: 21220500 DOI: 10.1158/1535-7163.MCT-10-0385]

182 **Goldenberg DM**, Rossi EA, Stein R, Cardillo TM, Czuczman MS, Hernandez-Ilizaliturri FJ, Hansen HJ, Chang CH. Properties and structure-function relationships of veltuzumab (hA20), a humanized anti-CD20 monoclonal antibody. *Blood* 2009; **113**: 1062-1070 [PMID: 18941114 DOI: 10.1182/blood-2008-07-168146]

P- Reviewers: Bensussan A, Lee YY, Shi ZJ **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Wu HL

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5315/wjh.v3.i2.44

World J Hematol 2014 May 6; 3(2): 44-48
ISSN 2218-6204 (online)

CASE REPORT

Chronic disseminated candidiasis complicated with a ruptured intracranial fungal aneurysm in ALL

Teppei Okawa, Toshiaki Ono, Akifumi Endo, Masatoshi Takagi, Masayuki Nagasawa

Teppei Okawa, Toshiaki Ono, Akifumi Endo, Masatoshi Takagi, Masayuki Nagasawa, Department of Developmental Biology, Post Graduate School, Tokyo Medical and Dental University, Tokyo 113-8519, Japan
Teppei Okawa, Masayuki Nagasawa, Department of Hematology Oncology and Immunology, Tokyo Bay Urayasu/Ichikawa Medical Center, Chiba 279-0001, Japan
Author contributions: Okawa T and Nagasawa M designed the report; Okawa T, Ono T, Endo A and Takagi M collected the patient's clinical data; Okawa T, and Nagasawa M analyzed the data and wrote the paper.
Supported by The Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture Japan, No. 24591541 to Masayuki Nagasawa
Correspondence to: Masayuki Nagasawa, MD, PhD, Department of Developmental Biology, Post Graduate School, Tokyo Medical and Dental University, 5-45, Yushima 1-chome, Bunkyo-ku, Tokyo 113-8519, Japan. mnagasawa.ped@tmd.ac.jp
Telephone: +81-3-58035250 Fax: +81-3-58035247
Received: September 30, 2013 Revised: December 23, 2013
Accepted: January 17, 2014
Published online: May 6, 2014

Key words: Disseminated candidiasis; β-D-glucan; Fungal cranial aneurysm; Acute lymphocytic leukemia; Bone marrow transplantation

Core tip: Chronic disseminated candidiasis and resulting fungal intracranial aneurysm is a life-threatening complication during the induction therapy of leukemia with a poor survival rate. However, intensive and patient anti-fungal treatment made the patient receive unrelated bone marrow transplantation successfully.

Okawa T, Ono T, Endo A, Takagi M, Nagasawa M. Chronic disseminated candidiasis complicated with a ruptured intracranial fungal aneurysm in ALL. *World J Hematol* 2014; 3(2): 44-48 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i2/44htm DOI: http://dx.doi.org/10.5315/wjh.v3.i2.44

Abstract

An 11-year-old boy with acute lymphocytic leukemia (ALL) contracted disseminated candidiasis during induction therapy, which was complicated with rupture of a fungal cranial aneurysm. Ventricular drainage and coil embolization of a residual aneurysm in combination with intensive antifungal therapy rescued the patient. Although clinical improvement was achieved, high fever and elevated levels of C-reactive protein and β-D-glucan continued for more than 10 mo. One year later, the ALL relapsed during maintenance therapy with methotrexate and 6-mercaptopurine. After salvage chemotherapy, the patient received unrelated bone marrow transplantation (BMT) in a non-complete remission condition and survived. During subsequent chemotherapy and BMT, no recurrence of the fungal infection was observed under the prophylactic anti-fungal therapy with micafungin.

INTRODUCTION

Disseminated fungal infection in acute leukemia patients is a serious complication, not only because it is difficult to manage and cure, but it also interferes with continuation and completion of the standard treatment regimen for leukemia, which consequently leads to an unfavorable outcome. We experienced an acute lymphocytic leukemia child who contracted a disseminated candidiasis with a resultant rupture of intracranial fungal aneurysm during the induction chemotherapy. Our clinical experience in this patient is very useful and informative for physicians in this field.

CASE REPORT

An 11-year-old boy with B-precursor acute lymphocytic

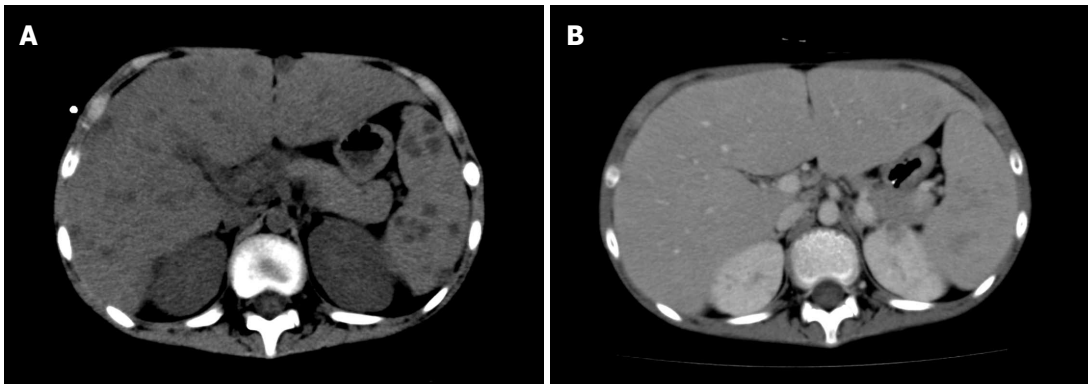


Figure 1 Computer tomography scan images of the liver. Computed tomography (CT) scans of the liver performed on the 51st d (A) and 239th d (B) are presented.

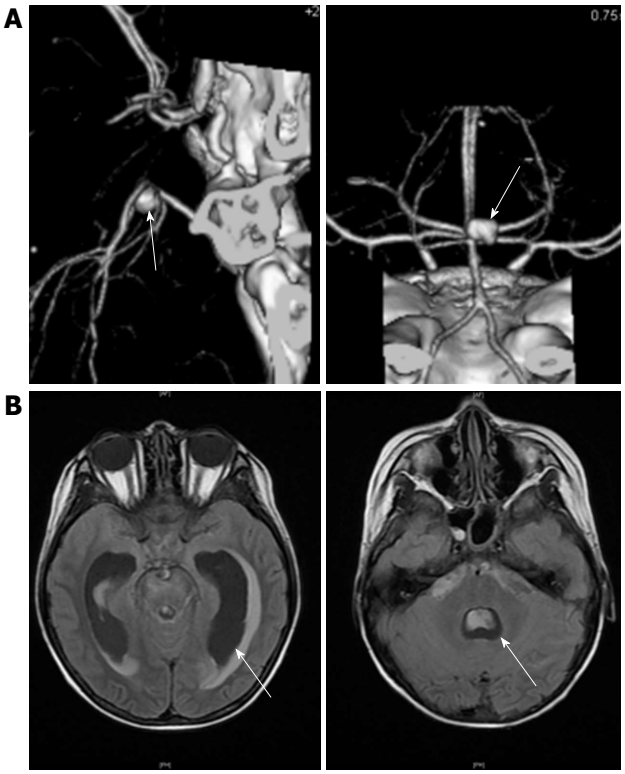


Figure 2 Three-dimensional reconstituted image of the intracranial aneurysm (A) and computer tomography scan (B). A: Arrows indicate intracranial aneurysm; B: Arrows indicate dilated ventricles and subdural hemorrhage.

leukemia (ALL) was admitted to our hospital. After confirming a good response to preceding prednisolone, TCCSG L09-16 (UMIN000003375) high risk (HR) induction chemotherapy was started. Two weeks later, the patient's white blood cell count (WBC) fell below 100/μL and fever was noted on day 19. Serum C-reactive protein (CRP) level was 2.38 mg/dL. Intravenous antibiotic [cefpirome sulfate (CPR)] was started on the same day. On the 22nd d, the patient's β-D-glucan level was below 6.0 pg/mL. Panipenem/betamipron (PAPM/BP) was administered instead of CPR, but changed to meropenem. Micafungin (MCFG; 6 mg/kg per day) was added and induction therapy was discontinued on the 29th d. The values of β-D-glucan and CRP increased to 27.4 pg/mL and 10.52 mg/dL, respectively, on the same day. Granulocyte colony-stimulating factor was started on the 37th day and liposomal amphotericin B (L-AMB; 6 mg/kg per day) was administered instead of MCFG on the 40th day. Whole-body computed tomography (CT) on the 40th day revealed no diagnostic findings. WBC increased to 3500/μL (neutrophils > 90%) on the 44th d. MCFG was re-started on the 48th d in addition to L-AMB. Although a repeated blood culture was negative, serum *Candida* antigen value was greater than 2 ng/mL on the 49th d. On the 51st d, the patient began to complain of an intermittent temporal headache. Whole-body CT on the 54th d revealed multiple lesions in the liver (Figure 1) and flucytosine (5-FC; 150 mg/kg per day) was added. On the 56th day, the patient's consciousness level suddenly dropped and he experienced vomiting and incontinence. CT and magnetic resonance imaging (MRI) (Figure 2) indicated subarachnoid hemorrhage and drainage from both lateral ventricles was performed. The cerebrospinal fluid (CSF) was bloody and negative for bacteria and fungi. On the next day, brain angiography revealed an aneurysm with a diameter of 7 mm on the right basilar artery and coil embolization was successfully performed on the same day.

The patient's condition was stabilized. His consciousness level occasionally fluctuated according to increased ventricular pressure, which rapidly improved on adjustment of ventricular drainage.

Because induction therapy for ALL was discontinued, maintenance therapy consisting of daily 6-mercaptopurine and weekly methotrexate was started on the 76th d. High-grade fever and elevated CRP levels were sustained, although the patient was clinically improving. Unexpectedly, the plasma β-D-glucan level gradually increased to more than 500 pg/mL. A CT scan on the 265th d revealed complete disappearance of multiple lesions in the liver. Five months later, CRP and β-D-glucan levels began to decrease slowly. 5-FC and L-AMB were discontinued on the 265th d and 335th d, respectively (Figure 3). On the 319th d, a ventricle-peritoneal shunt was performed. Ten months later, WBC gradually decreased with the progression of thrombocytopenia. A bone marrow examination on the 356th d confirmed the first relapse of ALL. Although the patient's β-D-glucan

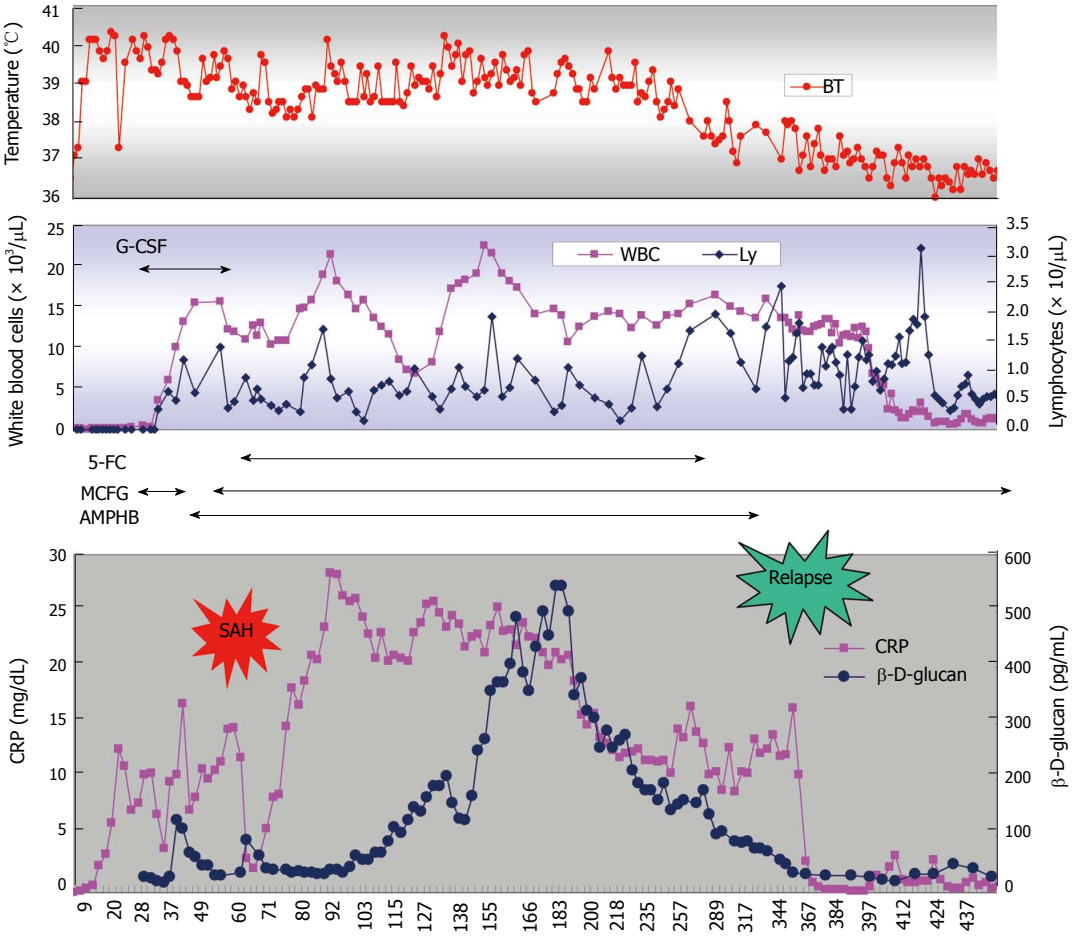


Figure 3 Clinical course of the patient. Initiation of prednisolone was designated as day 1. WBC: White blood cell; 5-FC: 5-flucytosine; MCFG: Micafungin; CRP: C-reactive protein.

level was still above 50 pg/mL, induction therapy based on the BFM95 protocol was started. After being in remission, he relapsed on the 680th d and on the 825th d. He received unrelated bone marrow transplantation (BMT) during non-remission status on the 839th d with a myeloablative conditioning regimen consisting of total body irradiation (TBI; 12 Gy), cyclophosphamide (60 mg/kg × 2) and etoposide (60 mg/kg). Graft versus host disease prophylaxis consisted of tacrolimus and short term methotrexate. Engraftment was on the 16th d and he became free of red cell and platelet cell transfusion on the 15th and 37th d after BMT respectively. He has been in remission for more than 3 mo after BMT. During the subsequent chemotherapy/BMT and thereafter, no recurrence of fungal infection was observed under the prophylactic use of MCFG.

DISCUSSION

Fungal infections account for 4% to 9% of neutropenic infections in patients with hematological malignancy[1]. Additionally, it has been reported that patients with ALL are at the highest risk for invasive candidiasis during the neutropenic period following induction chemotherapy[2]. Disseminated candidiasis is a rare, life-threatening extended form of *Candida* infection and its mortality exceeds 10%-50%[3]. Fungal aneurysms of the intracranial circulation are an extremely rare complication and clinically differ from the more common bacterial "mycotic" aneurysms, which are usually associated with infectious endocarditis[4-6]. Contrary to bacterial "mycotic" aneurysms, fungal aneurysms usually affect the circle of Willis and the proximal arterial tree, and surgical treatment is extremely difficult[6]. Mortality related to fungal aneurysms is extremely high and it exceeds 80%-90% when the aneurysms rupture[7,8]. According to the literature, cases of pediatric fungal aneurysm are very rare and most of them are complicated in patients with chronic mucocutaneous candidiasis, a rare primary immunodeficiency[9-11].

In our case, progression of disseminated candidiasis occurred within 3 wk after chemotherapy, which seems relatively fast. Once disseminated, a great deal of time is needed to treat the fungal infection even when the neutrophil count recovers[12]. It is known that liver lesions are not apparent under agranulocytic conditions because of the lack of inflammation[13]. It may be possible that an increase in neutrophils induces excessive inflammation, which sometimes results in tissue destruction. With intensive anti-fungal chemotherapy and surgical and endovascular treatment, our patient survived through the early critical episode. However, the subsequent clinical course of the patient was difficult and challenging. Once

CRP and β-D-glucan levels decreased after surgery, the CRP level increased again above 20 mg/dL and the β-D-glucan level increased gradually. Additionally, high fever continued for months.

In our case, clinical evidence of disseminated candidiasis was based on elevated β-D-glucan levels, the presence of serum *Candida* antigen and multiple liver lesions. Repeated blood or CSF cultures could not detect *Candida* spp. It has been reported that the positive predictive value of blood cultures for *Candida* is relatively low and the typical findings of a CT scan are clinically valuable for diagnosis with serological data[13]. Although histological and microbiological evidence could not be obtained, it is more likely that intracranial aneurysm was due to disseminated candidiasis from the clinical point of view. Guidelines for disseminated candidiasis recommend fluconazole, amphotericin B, voriconazole or caspofungin[14,15]. MCFG was recently reported to be as effective as caspofungin[16]. Because some strains of *Candida*, such as *C. glabrata* and *C. krusei*, are resistant to fluconazole, we selected a higher dose of MCFG for the first-line therapy. Considering the severity of infection, we added L-AMB to MCFG. Although elevated CRP and β-D-glucan levels with high fever continued as shown in Figure 3, the patient was clinically improving and we continued antifungal therapy. It is well known that humans do not produce enzymes that metabolize β-D-glucan. There may be 2 possible explanations for why β-D-glucan was increasing in the middle of the treatment course: one is that the destruction of *Candida* was accelerated at that time and another is that the *Candida* antigen was sequestered from circulation and drained into systemic circulation at that time. In light of the sustained high level of CRP and its later decrease in parallel with β-D-glucan, the former scenario seems to be more likely.

Fortunately, our patient survived and received BMT successfully with no recurrence of candidiasis or other fungal infections under the prophylactic use of MCFG thereafter. He has been in remission for more than 3 mo after BMT, although we have to be careful how long the patient remains in remission. With the recent advance of anti-fungal drugs, disseminated candidiasis is still a challenging complication during the treatment of hematological malignancy and is difficult to manage. However, intensive patient treatment has enabled us to accomplish chemotherapy and BMT successfully even in a high-risk patient with ALL[17].

COMMENTS

Case characteristics

An 11-year-old boy with acute lymphocytic leukemia who suffered from chronic disseminated candidiasis during remission induction chemotherapy.

Clinical diagnosis

Chronic disseminated candidiasis and fungal intracranial aneurysm and its rupture.

Differential diagnosis

Bacterial infection and intracranial hemorrhage due to granulocytopenia and thrombocytopenia

Laboratory diagnosis

Increased β-D-glucan and candida antigen in the serum

Imaging diagnosis

Whole computed tomography scan revealed multiple masses in the liver. Angiography disclosed intracranial aneurysm.

Treatment

Coiled embolization and long-term chemotherapy with combination of multiple anti-fungal drugs

Experiences and lessons

This case report emphasizes the difficulties of management of fungal infection during the chemotherapy of leukemia. Once it has occurred, not only control of fungal infection but also the control of leukemia is disturbed. However, bone marrow transplantation is not a contraindicated choice of therapy.

Peer review

This article is well described and some important messages emerge. This paper will therefore be very useful for hematologists and infectiologists.

REFERENCES

1 **Castagnola E**, Cesaro S, Giacchino M, Livadiotti S, Tucci F, Zanazzo G, Caselli D, Caviglia I, Parodi S, Rondelli R, Cornelli PE, Mura R, Santoro N, Russo G, De Santis R, Buffardi S, Viscoli C, Haupt R, Rossi MR. Fungal infections in children with cancer: a prospective, multicenter surveillance study. *Pediatr Infect Dis J* 2006; **25**: 634-639 [PMID: 16804435 DOI: 10.1097/01.inf.0000220256.69385.2e]

2 **Thaler M**, Pastakia B, Shawker TH, O'Leary T, Pizzo PA. Hepatic candidiasis in cancer patients: the evolving picture of the syndrome. *Ann Intern Med* 1988; **108**: 88-100 [PMID: 3276268 DOI: 10.7326/0003-4819-108-1-88]

3 **Hassan I**, Powell G, Sidhu M, Hart WM, Denning DW. Excess mortality, length of stay and cost attributable to candidaemia. *J Infect* 2009; **59**: 360-365 [PMID: 19744519 DOI: 10.1016/j.jinf.2009.08.020]

4 **Eishi K**, Kawazoe K, Kuriyama Y, Kitoh Y, Kawashima Y, Omae T. Surgical management of infective endocarditis associated with cerebral complications. Multicenter retrospective study in Japan. *J Thorac Cardiovasc Surg* 1995; **110**: 1745-1755 [PMID: 8523887 DOI: 10.1016/S0022-5223(95)70038-2]

5 **Oohara K**, Yamazaki T, Kanou H, Kobayashi A. Infective endocarditis complicated by mycotic cerebral aneurysm: two case reports of women in the peripartum period. *Eur J Cardiothorac Surg* 1998; **14**: 533-535 [PMID: 9860215 DOI: 10.1016/S1010-7940(98)00221-8]

6 **Watson JC**, Myseros JS, Bullock MR. True fungal mycotic aneurysm of the basilar artery: a clinical and surgical dilemma. *Cerebrovasc Dis* 1999; **9**: 50-53 [PMID: 9873163 DOI: 10.1159/000015896]

7 **Hurst RW**, Judkins A, Bolger W, Chu A, Loevner LA. Mycotic aneurysm and cerebral infarction resulting from fungal sinusitis: imaging and pathologic correlation. *AJNR Am J Neuroradiol* 2001; **22**: 858-863 [PMID: 11337328]

8 **Regelsberger J**, Elsayed A, Matschke J, Lindop G, Grzyska U, van den Boom L, Venne D. Diagnostic and therapeutic considerations for "mycotic" cerebral aneurysms: 2 case reports and review of the literature. *Cent Eur Neurosurg* 2011; **72**: 138-143 [PMID: 21830179]

9 **Marazzi MG**, Bondi E, Giannattasio A, Strozzi M, Savioli C. Intracranial aneurysm associated with chronic mucocutaneous candidiasis. *Eur J Pediatr* 2008; **167**: 461-463 [PMID: 17443345 DOI: 10.1007/s00431-007-0490-3]

10 **Leroy D**, Dompmartin A, Houtteville JP, Theron J. Aneurysm associated with chronic mucocutaneous candidiasis during long-term therapy with ketoconazole. *Dermatologica* 1989; **178**: 43-46 [PMID: 2492956 DOI: 10.1159/000248386]

11 **Loeys BL**, Van Coster RN, Defreyne LR, Leroy JG. Fungal intracranial aneurysm in a child with familial chronic mucocutaneous candidiasis. *Eur J Pediatr* 1999; **158**: 650-652 [PMID: 10445344 DOI: 10.1007/s004310051169]

12 **Kontny U**, Walsh TJ, Rossler J, Uhl M, Niemeyer CM. Successful treatment of refractory chronic disseminated candidiasis after prolonged administration of caspofungin in a child with acute myeloid leukemia. *Pediatr Blood Cancer* 2007; **49**: 360-362 [PMID: 16444677 DOI: 10.1002/pbc.20751]
13 **Semelka RC**, Kelekis NL, Sallah S, Worawattanakul S, Ascher SM. Hepatosplenic fungal disease: diagnostic accuracy and spectrum of appearances on MR imaging. *AJR Am J Roentgenol* 1997; **169**: 1311-1316 [PMID: 9353448 DOI: 10.2214/ajr.169.5.9353448]
14 **Ascioglu S**, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, Denning DW, Donnelly JP, Edwards JE, Erjavec Z, Fiere D, Lortholary O, Maertens J, Meis JF, Patterson TF, Ritter J, Selleslag D, Shah PM, Stevens DA, Walsh TJ. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis* 2002; **34**: 7-14 [PMID: 11731939 DOI: 10.1086/323335]
15 **Freifeld AG**, Bow EJ, Sepkowitz KA, Boeckh MJ, Ito JI, Mullen CA, Raad II, Rolston KV, Young JA, Wingard JR. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the infectious diseases society of america. *Clin Infect Dis* 2011; **52**: e56-e93 [PMID: 21258094]
16 **Spellberg BJ**, Filler SG, Edwards JE. Current treatment strategies for disseminated candidiasis. *Clin Infect Dis* 2006; **42**: 244-251 [PMID: 16355336 DOI: 10.1086/499057]
17 **Masood A**, Sallah S. Chronic disseminated candidiasis in patients with acute leukemia: emphasis on diagnostic definition and treatment. *Leuk Res* 2005; **29**: 493-501 [PMID: 15755501 DOI: 10.1016/j.leukres.2004.10.003]

P- Reviewers: Uderzo C, Zimmer J **S- Editor**: Zhai HH
L- Editor: Roemmele A **E- Edi**

ISSN 2218-6204 (online)

World Journal of *Hematology*

World J Hematol 2014 August 6; 3(3): 49-117

Published by Baishideng Publishing Group Inc

WJH

World Journal of Hematology

Contents

Quarterly Volume 3 Number 3 August 6, 2014

AIM AND SCOPE

World Journal of Hematology (*World J Hematol*, *WJH*, online ISSN 2218-6204, DOI: 10.5315) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning experimental, clinical, oncological and transplant hematology, transfusion science, hemostasis and thrombosis, evidence-based medicine, epidemiology and nursing. Priority publication will be given to articles concerning diagnosis and treatment of hematological diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

We encourage authors to submit their manuscripts to *WJH*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

INDEXING/ABSTRACTING

World Journal of Hematology is now indexed in Digital Object Identifier.

NAME OF JOURNAL
World Journal of Hematology

ISSN
ISSN 2218-6204 (online)

LAUNCH DATE
June 6, 2012

FREQUENCY
Quarterly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Hematology
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
August 6, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/2218-6204/g_info_20100722173604.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
doi:10.5315/wjh.v3.i3.49

World J Hematol 2014 August 6; 3(3): 49-70
ISSN 2218-6204 (online)

REVIEW

Current approach to relapsed acute lymphoblastic leukemia in children

Jose L Fuster

Jose L Fuster, Pediatric Hematology and Oncology Unit, Hospital Clínico Universitario Virgen de la Arrixaca, 30120 Murcia, Spain
Author contributions: Fuster JL solely contributed to this paper.
Supported by The Asociación Pablo Ugarte
Correspondence to: Jose L Fuster, MD, Pediatric Hematology and Oncology Unit, Hospital Clínico Universitario Virgen de la Arrixaca, Ctra, Madrid-Cartagena s/n, El Palmar, 30120 Murcia, Spain. josel.fuster@carm.es
Telephone: +34-96-8369082 Fax: +34-96-8369082
Received: January 29, 2014 Revised: May 31, 2014
Accepted: June 18, 2014
Published online: August 6, 2014

Abstract

Recurrent acute lymphoblastic leukaemia (ALL) is a common disease for pediatric oncologists and accounts for more deaths from cancer in children than any other malignancy. Although most patients achieve a second remission, about 50% of relapsed ALL patients do not respond to salvage therapy or suffer a second relapse and most children with relapse die. Treatment must be tailored after relapse of ALL, since outcome will be influenced by well-established prognostic features, including the timing and site of disease recurrence, the disease immunophenotype, and early response to retrieval therapy in terms of minimal residual disease (MRD). After reinduction chemotherapy, high risk (HR) patients are clear candidates for allogeneic stem cell transplantation (SCT) while standard risk patients do better with conventional chemotherapy and local therapy. Early MRD response assessment is currently applied to identify those patients within the more heterogeneous intermediate risk group who should undergo SCT as consolidation therapy. Recent evidence suggests distinct biological mechanisms for early *vs* late relapse and the recognition of the involvement of certain treatment resistance related genes as well cell cycle regulation and B-cell development genes at relapse, provides the opportunity to search for novel target therapies.

Key words: Children; Relapse; Acute lymphoblastic leukaemia

Core tip: Selected recent publications regarding the current management of childhood relapsed acute lymophoblastic leukemia have been reviewed. Controversies, current lines of investigation and possible future directions are discussed.

Fuster JL. Current approach to relapsed acute lymphoblastic leukemia in children. *World J Hematol* 2014; 3(3): 49-70 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i3/49.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i3.49

INTRODUCTION

Despite current cure rates above 75%[1], relapse is the most important obstacle in definite cure of children with acute lymphoblastic leukaemia (ALL)[2]. Depending on certain risk factors, such as age at diagnosis, sex, ethnicity, presenting white blood cell (WBC) count, hematopoietic lineage of the disease, cytogenetic abnormalities, and early response to primary therapy (Table 1)[3], approximately 20% of children diagnosed with ALL will experience relapse after current frontline therapy[4-17]. In a retrospective analysis of 9585 patients registered within 10 consecutive Children's Cancer Group (COG) studies, the relapsed cohort had a higher percentage of patients who fell in the age range < 1 or $\geqslant 10$ years, as well as a higher proportion of males, patients with initial WBC count > 100000/mL, and African American or Hispanic ethnicity. Slow early response was also associated with a higher risk of relapse. By contrast, there was no significant difference in the distribution

Table 1 Features at primary diagnosis of acute lymphoblastic leukaemia associated with an increased risk of relapse[3]

Clinical features		High-risk group stratification[1]
Age	Infants < 1 yr old	Yes
	⩾ 10 yr	Yes[2]
WBC	⩾ 50×10^9/L	Yes[2]
Sex	Male	No
Ethnicity	Blacks	No
	Native American	No
	Alaskan Native	No
	Hispanic	No
CNS status	CNS3	No
Response to therapy		
Morphological response	PPR	Yes
	Induction failure[3]	Yes
MRD ⩾ 0.01%	After induction (day 33)	Yes
	After consolidation (day 78)	
Biology		
Immunophenotype	T-cell	No
	Early T-cell precursor	Accepted by some study groups
Genetic alterations	*BCR-ABL1*	Yes
	MLL translocation	Yes if age < 1 yr
	Hypodiploidy (< 44 chromosomes)	Yes
	TCF3-PBX1 (E2A-PBX1)	No
	TCF3-HLF	Accepted by some study groups
	iAMP21	Accepted by some study groups
	BCR-ABL1-like ALL[4]	No
	IKZF1 mutation or deletion	No

[1]Some features have prognostic importance but are not commonly used in risk stratification; [2]The National Cancer Institute (NCI)/Rome risk criteria categorize as high risk patients all those patients with WBC ⩾ 50×10^9/L and/or age ⩾ 10 yr; [3]Induction failure: failure to achieve morphological remission after 4 to 6 wk of induction therapy; [4]The BCR-ABL1-like or Ph-like ALL with a gene expression profile similar to that of Ph + ALL. ALL: Acute lymphoblastic leukaemia; CNS: Central nervous system; CNS3: ⩾ 5 WBC/μL in cerebrospinal fluid with blasts or cranial nerve palsy; MRD: Minimal residual disease; PPR: Poor prednisone response defined as ⩾ 1×10^9/L leukemic blasts in the peripheral blood after 1 wk prednisone prophase; WBC: White blood cell count.

of the immunophenotype (B-precursor or T-cell) between the patients who relapsed *vs* those who did not[16]. Recurrent ALL is a relatively common disease for pediatric oncologists, and given the relatively high prevalence of newly diagnosed ALL, relapsed ALL still has a higher incidence than the new diagnoses of many of the most common pediatric malignancies and represents one of the most common childhood cancer. The number of children with ALL who experience treatment failure each year is similar to the number of children with newly diagnosed acute myeloid leukemia or rhabdomyosarcoma[2,18]. Moreover, relapsed ALL accounts for more deaths from cancer in children than any other malignancy and represents a major cause of death among children[17,19-23]. In the early 1980s, ALL relapse was regarded as an almost incurable disease[24]. Today, most patients achieve a second remission. However, about 50% of relapsed ALL patients do not respond to salvage therapy or suffer a second relapse[20-22,24-26]. For these patients, prognosis is extremely poor with survival rates below 10%[27]. Despite substantial second remission rates and a wide availability of haematopoietic stem cell transplantation (SCT), most children with relapse die[2].

After remission reinduction, recommendations for continuation therapy include ongoing intensive chemotherapy with or without radiation therapy or SCT. As in newly diagnosed patients, treatment must be tailored after relapse of ALL, since outcome will be influenced by several risk factors. Decisions regarding optimal postremission therapy in relapsed ALL are frequently based on well-established prognostic features, including the timing and site of disease recurrence, the disease immunophenotype, and, more recently, on evaluation of early response in terms of minimal residual disease (MRD) at the end of the reinduction phase[20,26,28-31]. With conventional approaches (intensive chemotherapy and/or SCT), disease free survival (DFS) rates range approximately from 20% to 50% depending on the study, time to end point, and the patient population. Though slightly different variables were measured, results from different study groups showed similar poor outcomes for patients in second complete remission (CR2)[16,20-22,24,25,32-34]. Thus, there is a relative lack of success in the induction of durable second remissions using conventional chemotherapy combinations and the benefits of SCT *vs* aggressive chemotherapy for different patient groups remain unclear. Although the best treatment approach for relapsed ALL remains uncertain, there is agreement that when relapse occurs early, leukemia-free survival remains dismal; most children still die despite aggressive chemoradiotherapy approaches, including transplantation, and novel salvage regimens are needed[18,21,24,35,36]. Relapsed ALL represents the focus of considerable pediatric re-

Table 2 Basic concepts of acute lymphoblastic leukemia relapse

Site of relapse				Ref.
BM	> 25% blasts in the BM (M3 marrow) and/or blasts cell in the PB	Isolated BM	No evidence of ALL in the CNS or any other site	[21,24,26,28, 30,36,38]
		Concurrent or combined	⩾ 5% blasts in the BM in combination with EM ALL	[22,24,26,28, 30,38,39]
Isolated CNS	⩾ 5 cells/mm^3 with leukemic blasts in a cytocentrifuge preparation of the cerebro-spinal fluid demonstrating leukemic blasts (cytomorphological) without major blood contamination (⩽ 20 erythrocytes/mm^3)[1] OR clinical signs of CNS disease OR a leukaemic mass found on cranial computed tomography or magnetic resonance imaging		< 5% blasts in the BM, no blasts in the PB and absence of leukemic infiltrations elsewhere	[24,25,28, 30,36,38,39]
Isolated testicular[2]	Leukemic infiltrations in the testis demonstrated by biopsy (both microscopically and immunologically)		< 5% blasts in the BM, no blasts in the PB and absence of leukemic infiltrations elsewhere	[24]
Other extramedullary	Leukemic infiltrations demonstrated by biopsy (both microscopically and immunologically)		< 5% blasts in the BM, no blasts in the peripheral blood and absence of leukemic infiltrations elsewhere	[24,38]
Length of first CR				
COG classification				[16,26,28, 29,36]
Early	Within 36 mo from initial diagnoses	Very early	< 18 mo from initial diagnoses	
		Intermediate	18-36 mo from initial diagnosis	
Late	⩾ 36 mo from initial diagnosis			
BFM classification				
Early	Occurring within 6 mo of the completion of frontline therapy	Very early	Within 18 mo from diagnosis	[42]
Late	More than 6 mo after the completion of frontline therapy			
Response evaluation after relapse				
CR[3]	M1 marrow	(< 5% blasts by bone marrow aspirate) in absence of clinical signs of disease with no evidence of circulating blasts or extramedullary disease and a recovered bone marrow[4]		[19,22,25, 28,30,38]
	M2 marrow	presence of 5% to 25% blasts in the BM aspirate by conventional morphology		[28]
	M3 marrow	presence of > 25% blasts in the BM aspirate by conventional morphology		[28]
CNS remission	< 5 WBC cells/mL regardless of cytologic evaluation			[36]
Remission of testicular relapse	Defined clinically by return to normal testicular size			[36]
Reinduction failure	Reinduction treatment not resulting in CR			[19]
Refractory patients	Surviving patients after reinduction failure			[19]
Relapse after a new remission	A pathologically confirmed M3 marrow (⩾ 25% leukemic blasts) or the presence of leukemia in any other site (*e.g.*, CNS, PB)			[19]
Treatment failure[5]	All cases of relapse and reinduction failure			[19]
MRD response	positive	Identification of ⩾ 0.01% blasts (1/10000) in the BM using flow cytometry–based assays		[28]
	negative	< 0.01% blasts in the BM using flow cytometry–based assays		[28]

[1]Some studies require the demonstration of the presence of leukemic cells in the CSF in two consecutive CSF samples taken with an interval of at least 24 h (van de Berg, PBC-2011; Barredo, JCO-2006); [2]In some studies, testicular relapse was diagnosed in case of uni- or bilateral painless enlargement of the testicles (Reismüller, BJH-2009; Saarinen-Pihkala, JCO-2006). In case of unilateral testicular relapse, excluding a subclinical involvement of the contralateral testis is recommended (Einsiedel, JCO-2005); [3]Second, third and subsequent remissions are designated as CR2, CR3 ... respectively; [4]Recovery of the BM is assumed in case that white blood cell (WBC) count is > 2.0 × 10^9/L and platelet count > 50 × 10^9/L (van de Berg, PBC-2011). Other studies considered the recovery of peripheral counts if absolute neutrophil count is ⩾ 750-1000/μL with platelet count ⩾ 75000-100000/μL) (Ko, JCO-2010, Raetz, JCO-2008, Raetz, 2012). Some studies consider patients without platelet recovery but fulfilling the remaining criteria for CR as "CR without platelets" (Ko, JCO-2010, Raetz, JCO-2008, Kolb, Leukemia-2003); [5]Treatment failures, development of a second malignant neoplasm, or death from any cause are generally considered events for disease-free survival (DFS) analysis (Ko, JCO-2010). ALL: Acute lymphoblastic leukaemia; BFM: Berlin-Frankfurt-Münster; BM: Bone marrow; CNS: Central nervous system; COG: Children Oncology Group; CR: Complete remission (complete response); EM: Extramedullary; MRD: Minimal residual disease; PB: Peripheral blood.

search and alternative treatment options exploring distinct mechanisms of action are being pursued[17,37]. New studies clearly need to address how to effectively treat relapsed patients and maintain durable remissions[16].

BASIC CONCEPTS OF ALL RELAPSE

Site of relapse and length of first remission are the major criteria for the classification of patients after first relapse. According to the site of relapse, patients are commonly classified as isolated marrow, concurrent marrow, isolated central nervous system (CNS), isolated testicular and other extramedullary relapses with or without CNS involvement (Table 2)[16].

Marrow relapse is generally defined as a bone marrow (BM) showing greater than 25% blasts (M3) by conventional morphology and/or blasts cells in the peripheral blood[21,24,26,28,30,36,38]. Isolated BM (medullary)

relapse is like marrow relapse but without evidence of ALL in the CNS, testicles or any other site. Combined BM relapse is defined as ⩾ 5% blasts in the BM in combination with extramedullary ALL[22,24,26,28,30,38,39]. Accordingly, isolated extramedullary relapses are those with a clinically-overt extramedullary manifestation of leukemia and less than 5% marrow infiltration[24]. Isolated CNS relapse is defined as ⩾ 5 cells/mm^3 in a cytocentrifuge preparation of the cerebro-spinal fluid demonstrating leukemic blasts (cytomorphological) without major blood contamination (⩽ 20 erythrocytes/mm^3) or clinical signs of CNS disease or a leukemic mass found on cranial computed tomography or magnetic resonance imaging, and < 5% blasts in the bone marrow, no blasts in the peripheral blood and the absence of leukemic infiltrations elsewhere (Table 2)[22,25,28,30,36,38,39]. Some studies require the demonstration of the presence of leukemic cells in the cerebrospinal fluid (CSF) in two consecutive CSF samples taken with an interval of at least 24 h[38,40]. Evidence suggests that submicroscopic involvement of the BM with leukemia is a frequent finding in patients with "apparently" isolated CNS relapse[41].

Isolated relapse elsewhere (testicle, skin, bone, orbita, mediastinum, lymph nodes, and tonsils) can be defined as leukemic infiltrations demonstrated by biopsy (both microscopically and immunologically), with < 5% blasts in the BM, no blasts in the peripheral blood and an absence of leukemic infiltrations elsewhere (Table 2)[38]. In some studies, testicular relapse was diagnosed in case of uni- or bilateral painless enlargement of the testicles[22,33]. In the case of unilateral testicular relapse, it is recommended to rule out a subclinical involvement of the contralateral testis[24].

Although a cut-off point between early and late relapses is often made at 3-6 mo after treatment cessation, the definitions for "early" *vs* "late" relapse differ slightly among different study groups. The COG categorizes relapses as "early" (recurrence occurring within 36 mo after initial diagnoses) or "late" (occurring ⩾ 36 mo after initial diagnosis). Early relapses are further classified as "very early", if they occur < 18 mo, or "intermediate", if they occur 18-36 mo after initial diagnosis[16,26,28,29,36]. For the Berlin-Frankfurt-Münster (BFM) group, the time-point of relapse is defined in relation to the date of primary diagnosis and the date of completion of primary therapy (*i.e.*, the end of the antileukemic therapy of the frontline protocol). Although completion of primary therapy often corresponds to the end of the maintenance therapy, in a few patients, it may correspond to the end of a short and intensive first line treatment, or to the end of an inadequately short primary treatment. For the BFM group, the end of frontline therapy is as much or even more important than the duration of the first remission. Therefore, those relapses occurring within 6 mo of the completion of frontline therapy are classified as "early", while "late" relapses are those occurring more than 6 mo after the completion of frontline therapy. The concept of "very early" relapse coincides with that of the COG classification (*i.e.*, relapses occurring within 18 mo after diagnosis)[42]. Thus, assuming that, for most patients, the duration of contemporary frontline treatment is 24 mo, there is a six months gap between the COG and the BFM criteria for the definition of late relapse so that relapses occurring between 30 and 36 mo after initial diagnosis should be considered as "late" within BFM trials, while COG trials should consider them as early relapses.

Patients are considered to have achieved a complete response (CR) if reinduction treatment results in an M1 marrow (< 5% blasts by BM aspirate) in the absence of clinical signs of disease with no evidence of circulating blasts or extramedullary disease and a recovered BM (Table 2)[19,22,25,28,30,38]. M2 and M3 marrow response are defined as the presence of 5% to 25% and > 25% blasts in the BM aspirate by conventional morphology, respectively[28]. Recovery of the BM is assumed in cases where the white blood cell (WBC) count is > 2.0 × 10^9/L and platelet count > 50 × 10^9/L[38]. Other studies considered the recovery of peripheral counts if absolute neutrophil count is ⩾ 750-1000/μL with platelet count ⩾ 75000-100000/μL)[19,28,29]. Some studies consider patients without platelet recovery but fulfilling the remaining criteria for CR as "CR without platelets"[19,28,43]. CNS remission is commonly defined as < 5 WBC cells/mL regardless of the cytologic evaluation and remission of testicular relapse is defined clinically by a return to normal testicular size[36]. Reinduction treatment not resulting in CR is generally termed reinduction failure and surviving patients are termed refractory[19]. Regarding MRD response, the identification of ⩾ 0.01% blasts (1/10000) in the BM using flow cytometry-based assays is generally assumed as MRD positive (negative < 0.01%)[28]. After a new remission is achieved, relapse is defined as a pathologically confirmed M3 marrow (⩾ 25% leukemic blasts) or the presence of leukemia in any other site (*e.g.*, CNS, peripheral blood)[19]. Relapses and reinduction failures are collectively termed treatment failures within most studies. Treatment failures, the development of a second malignant neoplasm, or death from any cause are generally considered events for DFS analysis[19].

CONTRIBUTING FACTORS FOR ALL RELAPSE

Clinical

Age and WBC at primary diagnosis of ALL are the most important clinical prognostic factors. Infants < 1 year old and children ⩾ 10 years have the worse prognosis (Table 1)[3]. Risk factors predicting CNS relapse after the first CR include T-cell immunophenotype, hyperleukocytosis, high-risk genetic abnormalities, and the presence of leukemic cells in the CSF at the time of diagnosis[44].

Biological

Understanding the biological factors contributing to relapse will probably contribute to identify new agents

Table 3 Clinical and biological data of early *vs* late relapses

	Clinical data	Biological explanation	Ref.	Biological evidence	Ref.
Early relapse	Patients failing to achieve CR2 with the same agents used at primary diagnosis usually do not respond to different drug combinations	Intrinsic drug resistance: the malignant cells responsible for relapse are present at diagnosis and are selected for during treatment	Yang *et al*[45], 2008	Genome-wide analysis of DNA CNAs and LOH on matched diagnostic and relapse BM samples revealed that the majority (94%) of relapse cases was related to the presenting diagnostic leukemic clone	Mullighan *et al*[46], 2008
	Equivalent post-relapse survival for patients undergoing different intensity regimens at primary diagnosis	The malignant cells responsible for relapse are present at diagnosis and mutate to a resistant phenotype through the acquisition of spontaneous mutations	Freyer *et al*[17], 2011	Primary diagnosis and relapse clones originates from a common ancestral clone and acquire distinct CNAs before emerging as the predominant clone at diagnosis or relapse	
	Decrease in CR rates after subsequent relapses and treatment attempts	Acquisition of resistance-conferring mutations induced by initial treatment	Ko *et al*[19], 2010	Adquisition of new genetic alterations at relapse, often involving cell proliferation and B-cell development pathway	Bhojwani *et al*[48], 2006 Yang *et al*[45], 2008 Mullighan *et al*[46], 2008 Hogan *et al*[49], 2011
Late relapse	The distribution of patients experiencing early and late relapses were highly skewed towards NCI HR in the former group and NCI standard risk in the latter	Late relapse represents de novo development of a second leukaemia from a common premalignant clone	Nguyen *et al*[16], 2008	Distinct patterns of gene expression in pairs of relapsed samples from patients who relapse early from those relapsing later	Bhojwani *et al*[48], 2006
				Pattern of *NOTCH1* mutations and genome-wide copy number showed a common clonal origin between diagnosis and early relapse but not for late relapses of T-cell ALL	Szczepanski *et al*[53], 2011
				Distinct pattern of deletions at the non-translocated *TEL* allele at primary diagnosis and relapse of *TEL-AML1*-positive ALL	Zuna *et al*[51], 2004

ALL: Acute lymphoblastic leukaemia; CAN: Copy number abnormalities; CR: Complete remission; CR2: Second complete remission; HR: High risk; LOH: Loss-of-heterozygosity; NCI: National Cancer Institute.

able to increase the chances of a sustained second remission and cure. Studying the biology of these diseases at diagnosis, in minimal residual disease states after selection by chemotherapy, and at relapse, provides a unique opportunity to dissect pathways and identify potential therapeutic strategies for relapsed childhood ALL and may improve our understanding of how to use current therapy as well as identifying new targets[16,37,45].

It has generally been assumed that relapse is the consequence of the emergence of a drug-resistant leukemia subclone which was already present at diagnosis and that was selected during frontline therapy. During initial therapy, this minor population would exhibit only moderate reduction relative to the bulk of the diagnostic leukemic cells but would rapidly expand before clinical relapse[45]. Although most relapsed patients achieve a second CR2 with drug combinations involving the same agents used at primary diagnosis, those patients who fail to enter in remission are not likely to be salvaged using different drug combinations, suggesting intrinsic drug resistance[45]. The equivalent post-relapse survival for patients undergoing different intensity regimens as first line therapy, suggests that the malignant cells responsible for relapse are present at diagnosis and mutate to a resistant phenotype through the acquisition of spontaneous mutations that are dependent on intrinsic genomic instability rather than treatment exposures[17]. Lesion specific backtracking studies revealed that in most cases the relapse clone existed as a minor sub-clone within the diagnostic sample prior to the initiation of therapy suggesting that the relapse clone was selected for during treatment. In only a minority (6%) of ALL cases did the relapse clone represent the emergence of a genetically distinct and thus unrelated second leukemia[46]. These findings indicate that the diagnosis and relapse clones originated from a common ancestral clone and acquired distinct copy number abnormalities (CNAs) before emerging as the predominant clones at diagnosis or relapse. In this model, relapse emerges from a drug-resistant subclone present at initial diagnosis that is selected during treatment regardless of the nature of the frontline therapy delivered[17]. This data support the hypothesis that many relapses may be the result of the selection of a relatively resistant clone already present at initial diagnosis rather than the generation of a novel clone by mutation[18,47]. Resistant leukemia subclones are probably present at primary diagnosis in those patients destined for early relapse. Early-relapse mechanisms appear to be more homogeneous and are suggestive of the selection of a resistant, more proliferative clone (Table 3)[48]. Alternatively, the acquisition of resistance-conferring mutations induced by initial treatment might be responsible

for the relative drug resistance noted at relapse[45]. For subsequent relapses and treatment attempts a significant decrease in CR rates is expected[19], which suggests the emergence of new mechanisms of resistance. According to this model, genomic studies carried out in samples from children at diagnosis and relapse demonstrated the acquisition of new genetic alterations at relapse, often involving cell proliferation and B-cell development pathways[45,46,48,49].

By contrast, late relapses may represent de novo development of a second leukemia from a common premalignant clone. Data regarding patients relapsing after being primarily treated within 10 Children Cancer Group (CCG) trials showed how the distribution of patients experiencing early, intermediate and late relapses were highly skewed toward National Cancer Institute (NCI) HR patients in the former group and NCI SR in the latter group. Although SR patients receive less intense therapy, these data suggest that intrinsic differences in the biology of the leukemic blasts are correlated with different mechanisms and the timing of relapse[16]. Distinct gene expression profiles were revealed for pediatric relapsed ALL patients at both early and late time points[49]. The analysis of microsatellite markers showed that some very late relapses of *TEL/AML1*+ positive leukemia most likely represent a new event that occurs in a quiescent precursor leukemia cell harboring an otherwise silent fusion gene that has escaped eradication during initial therapy[50]. Moreover, analysis of deletions at the non-translocated *TEL* allele study of relapsed *TEL-AML1*-positive ALL samples showed that the relapsed clone was related but distinct from the clone at initial diagnosis. This might explain the clinical responsiveness of many cases of late or off-treatment *TEL/AML1*+ ALL relapses[51]. Paired samples from patients experiencing early relapse are more similar in expression patterns than paired samples from those relapsing later[48]. Staal *et al*[52] using genome-wide expression array on purified leukemic cells, found that genes involved in a late or an early relapse identified clearly distinct pathways. Analyses of the TCR gene rearrangement status, pattern of *NOTCH1* mutations, and genome-wide copy number showed a common clonal origin between diagnosis and early relapses of T-cell ALL but not for the few cases of T-cell ALL late relapses, suggesting that these recurrences should be considered as a second T-ALL rather than a resurgence of the original clone[53]. These findings are suggestive of a model whereby late relapse is due to the acquisition of diverse secondary events that might occur in a distinct subpopulation such as a leukemic stem cell[48].

By comparing matched diagnosis and relapse samples, Bhojwani *et al*[48] found that certain genes involved in cell proliferation, protein biosynthesis, carbohydrate metabolism, and DNA replication/repair were among those highly expressed in relapsed *vs* newly diagnosed blasts. By contrast, some of the genes down-regulated at relapse compared with initial diagnosis included pro-apoptotic genes, antiproliferative genes and a putative tumor suppressor gene[48].

Treatment resistance related genes, such as *CDKN2A/B* and *MSH6*, *ETV6*, and cell cycle regulation and B-cell development (*PAX5*, *EBF1*, *IKZF1*) were shown to emerge at relapse, providing the opportunity to search for novel target therapies[37,45,48]. The discovery of these new genetic alterations associated with high rates of relapse (and shorter first remission), such as the rearrangement of *CRLF2*, *IKZF1* deletions/mutations and *JAK* family mutations, offers the potential for the identification of patients at diagnosis who should be treated more aggressively and with agents targeting those molecular lesions[45,54-56].

PROGNOSTIC FACTORS IN PATIENTS WITH RELAPSED ALL

During the last 2 decades several study groups such the Acute Lymphoblastic Leukemia-Relapse Study of the BFM Group (ALL-REZ BFM) have performed prospective controlled phase III trials to establish standardized treatment protocols with the primary goal of improving the prognosis of children with relapsed ALL and to evaluate risk factors, thereby allowing for risk-adapted treatment intensity[24].

Time to relapse (length of first remission), site of relapse and ALL-immunophenotype are well-established risk factors that can predict survival and constitute the most important prognostic determinants that can be used to stratify patients with a first relapse into different treatment groups[2,16,17,20,25-27,32,34,35,57].

Length of first complete remission

Before relapse, the median duration of the first complete remission (CR1) has been reported to be around 2.5 years[20,25,35]. Most ALL relapses occur during treatment or within the first 2 years after treatment completion, although relapses have been reported to occur even 10 years after diagnosis[2,18].

In a large series of 854 ALL relapses reported by the Nordic Society for Pediatric Hematology and Oncology (NOPHO), the median time from diagnosis to first relapse was 28 mo (range, 2-227 mo)[33]. According to Chessells *et al*[32] 74% of relapses occurred in the first 3 years after diagnosis, 4% after 6 years, and only 1% occurred more than 10 years after diagnosis. Reissmüller *et al*[22] reported a relative incidence of very early (within 18 mo from diagnosis), early (after 18 mo from diagnosis up to 6 mo after cessation of primary treatment) and late relapses (more than 6 mo after cessation of front-line therapy) of 41%, 22% and 37%, respectively. In a retrospective analysis of 1961 relapsed patients registered within 10 consecutive CCG studies, the duration of the CR1 for patients who relapsed varied according to NCI risk group at primary diagnosis, with shorter duration of remission coinciding largely with higher risk features at diagnosis[16]. The duration of the CR1 has been reported to vary with the site of relapse[34,35,41]. In the study re-

Table 4 Relative incidence of site of relapse

Isolated BM	Combined BM	Isolated EM	Isolated testis	Other isolated EM	Year	Ref.
42.90%	19.60%	37.50%			1996-2000	Malempati *et al*[34], 2007
47%	23%	30%			1995-2002	Roy *et al*[20], 2005
63%	16%	13%	7%		1981-1999	Reissmüller *et al*[22], 2009
57%	12%	15%	10%	2%	1972-1998	Chessells *et al*[32], 2003

BM: Bone marrow; EM: Extramedullary.

Table 5 Risk stratification after relapse

	BCP			T-cell		
	Isolated EM	Combined BM	Isolated BM	Isolated EM	Combined BM	Isolated BM
Risk stratification according to the BFM Group classification[42]						
Very early[1]	Intermediate	High	High	Intermediate	High	High
Early[1]	Intermediate	Intermediate	High	Intermediate	High	High
Late[1]	Standard	Intermediate	Intermediate	Standard	High	High
Risk stratification according to the United Kingdom ALLR3 Study classification[30]						
Very early[1]	High	High	High	High	High	High
Early[1]	Intermediate	Intermediate	High	Intermediate	High	High
Late[1]	Standard	Intermediate	Intermediate	Standard	High	High
Current approach to risk stratification according to I-BFM SG						
Very early[1]	High	High	High	High	High	High
Early[1]	Standard	Standard	High	Standard	High	High
Late[1]	Standard	Standard	Standard	Standard	High	High

[1]Very early, less than 18 mo from initial diagnosis; Early: More than 18 mo from initial diagnosis but < 6 mo from completion of primary treatment; Late: More than 6 mo after completion of primary treatment. BCP: B-cell precursor; BM: Bone marrow; EM: Extramedullary; I-BFM SG: International BFM Study Group.

ported by Malempati *et al*[34], the mean interval between day 28 of primary induction and relapse for all patients was 32.8 mo, CNS relapses tended to occur earlier (mean 23.1 mo), and testicular recurrences tended to occur later (40.5 mo) than BM relapses (mean 36.2 mo).

Timing of relapse has emerged as the most significant predictor of outcome and the most important factor for a second relapse is the duration of the first remission. Early relapse has worse prognoses than late relapse[16,17,20,22,25,32-35,38,57]. Some late relapses are thought to arise from a common precursor that retains the chemosensitivity of the original clone, which could explain the high cure rates achieved with chemotherapy alone in late relapses[30]. Ko *et al*[19] found CR rates of 83% for early first relapse and 93% for late first relapse. Breaking down early relapse into very early relapse (< 18 mo from diagnosis) and intermediate (18 to 36 mo from diagnosis), they found CR rates of 78% and 86%, respectively. EFS rates reported for early relapses ranged from 5% to 18% and 19% to 57% for late relapses[16,19,20,22,24,26,28]. Even when intensive salvage strategies including SCT are employed, longer-term EFS rates for early relapses are only 10% to 20%, compared with 40% to 50% for late relapses. These outcomes have been remarkably consistent over recent decades, irrespective of differences in the components of salvage regimens[21,24,28].

Site of relapse

The majority of relapses (60% to 80%) involve the bone marrow (BM) alone or together with extramedullary involvement, and more than 70% of relapses involving the BM are isolated BM relapses. Isolated CNS or testicular relapse or, much less frequently, relapse involving other extramedullary sites may also occur (Table 4)[20,22,32,34].

Bone marrow relapses are associated with a worse outcome than extramedullary relapses, with overall long-term survival rates of approximately 25%[16,17,19,22,33-34]. Survival at 3 to 6 years after relapse has been found to range from approximately 20% for isolated marrow relapse to 50%-80% for isolated extramedullary relapse, with combined-site (*i.e.*, marrow plus extramedullary) relapses having an intermediate outcome[16,20,22,25,32,34,40]. In extramedullary relapses, a clear distinction also has to be made for early relapses *vs* late relapses. Regarding early relapse, survival rates are higher for patients with isolated CNS relapse than for patients with either isolated or combined BM relapse, and this is also true for intermediate and late relapsing patients. Survival rates were also significantly higher for patients with concurrent marrow relapses compared to those with isolated marrow relapses[16,24].

Thus, involvement of an extramedullary site in patients with BM relapse has been identified as a favourable prognostic feature compared to patients without extramedullary involvement. To explain this fact, it has been hypothesized that combined BM relapses originate from the involved extramedullary compartment, in which the leukemic cells could survive the front-line chemotherapy

because they were protected by the blood-brain/testis barrier. Thus, relapses in extramedullary sites are often considered as relapses from malignant cells treated with suboptimal drug levels; due to their homing on these sanctuaries. Therefore, they may be more sensitive to chemotherapy than clones originating directly from the BM[24]. Five-year survival rates for isolated CNS range between 43.5% for early, and 78.2% for late relapses[16,40].

In the case of a testicular relapse, isolated relapse patients fare better, with an EFS of 58% *vs* 28% for combined relapses[20]. In the COG analysis reported by Nguyen *et al*[16], overall 5-year post-relapse survival rate after early isolated testicular relapse was lower (13%) than after intermediate (52%) or late (59.9%) relapses although this difference was not statistically significant.

Immunophenotype

The immunological lineage of the disease (B-cell precursor *vs* T-cell ALL) is another well recognized risk factor in childhood relapsed ALL. Late relapses of T-ALL are rare and make up approximately 10% of all recurrences[53]. The BFM group demonstrated that children with T-cell ALL BM relapses have a much worse prognosis than B-cell precursor ALL (BCP), irrespective of the time between diagnosis and recurrence[42]. In a report by investigators at St. Jude Children's Research Hospital, CR2 rates for this population were 60%, with a 5-year EFS of only 5% compared to 28.7% for B-cell lineage[25]. Other studies confirmed that the prognosis of patients with a first relapse of T-ALL is dismal, with only 15% to 25% of patients achieving durable remissions after second-line treatment[16,24]. Thus, apart from the fact that T-cell recurrences tend to occur early, T-cell immunophenotype itself is associated with a very poor outcome after relapse regardless of site and time to relapse[16,20-22,24-26,28,32].

Minimal residual disease

Minimal residual disease (MRD), measured either by flow cytometry or real-time polymerase chain reaction (PCR), may supplement morphologic response[29,58,59]. Rates of MRD positivity after reinduction for relapsed ALL are much higher than those observed in first-line ALL clinical trials[28]. The prognostic significance of MRD response at relapse has been assessed in several studies[28,31,60]. Persistence of MRD after re-induction/consolidation therapy (*i.e.*, after 5 and 12-13 wk from the beginning of treatment for relapse) influences prognosis in children with relapsed ALL. Children with MRD levels $< 1 \times 10^{-3}$ or 1×10^{-4} have been shown to carry a lower risk of recurrence than patients with higher levels of MRD[30,31,58,61,62].

Within the COG AALL01P2 study, five-year EFS probabilities differed in patients according to MRD response using flow cytometry-based assays at the end of the first block of chemotherapy (negative < 0.01%; positive ≥ 0.01%)[28]. The absence of MRD at the end of the first month of reinduction therapy portended better outcomes in all patients, and separately in early and late relapse patients. The combination of timing of relapse and MRD appeared to identify three groups of patients. Early relapse patients who were MRD positive had a dismal outcome, while late relapse patients who were MRD negative had an excellent outcome, approaching that seen in newly diagnosed patients. MRD-negative early relapse patients and MRD-positive late relapse patients appeared to form an intermediate group. MRD positivity was also correlated strongly with the duration of initial remission; those patients experiencing relapse at less than 18 mo from initial diagnosis had the highest proportion of MRD positivity[28,29]. In a prospective blinded study, Eckert *et al*[31] have recently reported that EFS and OS decreased and the cumulative incidence of relapse increased with increasing MRD level (quantified by PCR analysis of antigen receptors) after reinduction chemotherapy in intermediate-risk relapsed ALL patients treated by the ALL-REZ BFM P95/96 protocol. Patients of the lower MRD groups ($< 10^{-4}$ and $< 10^{-3}$ to $\geq 10^{-4}$) had an acceptable prognosis (EFS at 10 years 80% and 64%, respectively) compared to patients of the higher MRD groups ($< 10^{-3}$ to $\geq 10^{-2}$ and $\geq 10^{-2}$) who had EFS at 10 years of 36% and 4.8%, respectively. Multivariate analysis revealed that MRD after the second induction course was the only parameter independently predicting the occurrence of subsequent adverse events[31]. Conflicting results, however, were observed in the Medical Research Council (MRC) UKR3 trial, in which reinduction therapy with mitoxantrone was superior to that with idarubicin, yet no differences in the end of reinduction MRD were observed[30].

In a prospective and blinded study, the ALL-REZ BFM Study Group evaluated the impact of pre-transplantation MRD in children treated according to the ALL-REZ BFM 96 or 2002 protocol who received their transplantation in CR2 or third CR (CR3). MRD proved to be the most important determinant for subsequent relapse and survival after transplantation in univariate and multivariate analysis. The cut-off of less than 10^{-4} leukemic cells turned out to be a feasible discriminator between patients at high ($\geq 10^{-4}$ leukemic cells) or low risk ($< 10^{-4}$ leukemic cells) for subsequent relapse. According to these findings, patients classified as being intermediate risk with conventional clinical parameters could be further classified into a very HR subgroup if MRD proves to persist at a high level until transplantation[61]. In another study, classical risk factors such as immunophenotype, site of relapse, time to relapse, and others were only significant in patients who receive chemotherapy in CR2. These factors lost their relevance in patients undergoing SCT, and MRD remained the only independent prognostic variable in this setting[42]. Thus, MRD of leukemia both during second CR and before transplantation, has been reported to be a very strong prognostic factor for the ultimate outcome[61]. However, the Saint Jude group reported that, although MRD before transplantation was an independent predic-

tor of survival, patients with high levels of MRD (0.1% to < 5.0% leukemia cells) still had a reasonably good chance of survival (43%) after SCT, suggesting that the negative effect of MRD had been partially offset by recent improvements in the transplantation procedure[63].

Given its power as a prognostic factor, quantification of MRD at diagnosis of ALL relapse and regularly during therapy has become an essential tool to characterize the responsiveness of the disease and to allocate the patients to a risk adapted treatment. It is currently being incorporated for relapsed patients into a risk-classification algorithm for the management of relapsed ALL within the COG (Table 2)[28,29].

A similar stratification system was used in the UKALL R3 relapse trial[30], and is currently applied by the International BFM Study Group (I-BFM SG).

Although study designs are incorporating the use of MRD in order to quickly assess responses in patients with relapsed ALL who are treated with novel agents, at present MRD remains an unvalidated surrogate marker for this purpose[28,29]. To this regard, even when a clear superiority from one arm to the other was obtained regarding the primary outcome (*i.e.*, EFS), results from the UK ALLR3 trial failed to demonstrate a difference in MRD level at early assessment between both study arms[30].

Other prognostic factors

There is some debate in the literature on the prognostic factor of the white blood cell (WBC) count and the presence of blasts in the peripheral blood at the time of relapse[25,32,33]. There is some evidence that, among children with relapsed ALL, those who had WBC counts < 50000/μL at initial diagnosis are more likely to have favourable outcomes after relapse[16]. Age at primary diagnosis might influence outcome after relapse. Older age at diagnosis (≥ 10 years), as well as age younger than 1 year, has been associated with significantly inferior post-relapse outcome[16-17,33,35,57]. In a recent analysis of 1150 patients aged 0-18 years registered in four consecutive Austrian ALL-BFM trials, prognosis of relapsed leukaemia was significantly better for younger patients (patients aged 1-15 years at primary diagnosis) than for adolescent (*i.e.*, patients aged between 15 and 18 years at primary diagnosis) even when neither the time point or the site of relapse differed significantly between broth groups[64]. These results suggest that age at initial diagnosis is a prognostic factor in relapsed ALL, just as it is for newly diagnosed disease[29]. Certain unfavourable clonal cytogenetic abnormalities detected at primary diagnosis have been found to portend worse post-relapse survival[22,32]. Philadelphia chromosome-positive (Ph+) and 11q23 abnormalities were associated with early relapse and poorer prognosis[32]. The prognosis of children relapsing after first line treatment for Ph+ ALL, particularly for those relapsing after SCT, is poor[28,65]. The *ETV6/RUNX1* fusion gene has been associated with better outcome after relapse[22].

It has been debated whether the intensity of frontline treatment affects the outcome of patients after relapse[29]. Type of first treatment was reported to influence the outcome after relapse, with more recent regimens being associated with improved survival[32]. The Austrian BFM Study Group reported higher post-relapse EFS (but not survival) for 203 children with relapsed ALL who received treatment on the more recent of their frontline studies conducted during the 1980s and 1990s[22]. It might be expected that patients who relapse after receiving an inferior initial treatment regimen would have greater success in retrieval, and greater post-relapse survival than patients who relapse after receiving a superior initial treatment regimen, given that their leukemia clone at relapse should be "less resistant" after being exposed to less effective or intensive prior treatment[17]. However, data from 272 relapsed patients after primary therapy within the COG CCG-1961 Study, demonstrated that there was no difference in 3-year post-relapse survival between two groups of patients having primarily received augmented *vs* standard intensity post-induction intensification. For subjects initially treated with augmented (n = 109) *vs* standard-intensity (n = 163) post-induction intensification, the 3-year post-relapse survival was 36.4% *vs* 39.2%, respectively (P = 0.72). There was no difference in the median time-to-death post-relapse according to initial regimen, (10.5 mo for augmented *vs* 16.2 mo for standard-intensity, P = 0.27), and no difference in post-relapse survival was seen after adjusting for timing of relapse, site of relapse, age at diagnosis, and lineage of the leukemia[17]. Similarly, in a report of post-relapse survival rates in 1961 children previously enrolled on 10 consecutive CCG clinical trials, according to treatment era at initial diagnosis (trials conducted from 1988-1995 *vs* 1996-2002), with treatment intensity increasing over time, the post-relapse outcomes were nearly identical[16]. Thus, differing intensity of initial treatment, as reflected in either the cross-regimen setting of single studies (CCG-1952 and CCG-1961) or the trans-era context of sequential CCG/COG studies involving both standard- and high-risk patients, does not alter the generally poor outcome associated with relapsed childhood ALL of any initial risk category[17]. Prognosis seems to be particularly poor for those patients relapsing after SCT[22].

Finally, male sex, African American or Hispanic ethnicity, and central nervous system (CNS) disease at diagnosis were reported to be significant predictors of inferior post-relapse survival in children with newly diagnosed ALL who had been enrolled on COG clinical trials from 1988 to 2002[16].

Risk-stratification

The BFM cooperative Group developed a relapse score incorporating duration of first complete remission, site of relapse, and immunophenotype to classify patients as standard-, intermediate-, and high-risk, with 6-year post-relapse survival rate reaching 78%, 41% and 19%, re-

Table 6 Children oncology group approach to relapsed acute lymphoblastic leukaemia[29]

Relapse	Site	Time		MRD	
B-lineage	Marrow	Early	Chemotherapy *vs* chemotherapy plus novel agents	Negative	SCT
				Positive	Bridging study before HSCT
		Late	Chemotherapy *vs* chemotherapy plus novel agents	Negative	Continuation therapy
				Positive	SCT
	IEM	Early	Chemotherapy *vs* chemotherapy plus novel agents	Any	SCT
		Late	Chemotherapy *vs* chemotherapy plus novel agents	Negative	Continuation therapy
				Positive	SCT
T-lineage	Marrow	Early	Chemotherapy *vs* chemotherapy plus novel agents	Negative	SCT
		Late		Positive	Bridging study before HSCT
	IEM	Early	Chemotherapy *vs* chemotherapy plus novel agents		
		Late		Any	SCT

IEM: Isolated extramedullary; SCT: Hematopoietic stem cell transplantation.

spectively for patients receiving more modern treatment. According to this classification, all children with T-cell relapse involving the bone marrow at any time, and children with very early combined and very early or early isolated marrow non-T cell are classified as HR; very early or early isolated extramedullary relapse, irrespective of immunophenotype, as well as early or late combined BM and late isolated marrow BCP ALL relapse, are classified as intermediate risk (IR); while SR category correspond to late isolated extramedullary (both T and non-T cell immunophenotype) (Table 5)[32,42]. Among 1556 patients up to 18 years of age with first relapse of ALL enrolled in trials of ALL-REZ BFM between June 1983 and April 2001, the SR group comprised 5% of patients while 55% and 40% of all patients were allocated to the IR and HR, respectively[42].

In a retrospective review of 150 relapsed patients form four large pediatric oncology units in the United Kingdom, Roy *et al*[20] found that children with a very early isolated extramedullary relapse had a significantly poor outcome when compared with the rest of the IR group, and suggested modifying this risk stratification system. Accordingly, within the United Kingdom ALLR3 Study these patients were classified as high risk patients[30]. However, only two risk groups are currently considered by the I-BFM SG. The standard risk group includes patients with: (1) a late or early isolated extramedullary relapse of BCP or T-cell ALL; (2) a late or early combined BM/extramedullary relapse; and (3) a late isolated BM relapse of BCP ALL. The high risk group comprises those with a very early isolated extramedullary relapse of BCP or T-cell ALL; early isolated or very early isolated or combined BCP ALL, and any BM relapse of T-ALL (Table 5).

TREATMENT FOR RELAPSED ALL

Risk-adjusted selection of treatment

Salvage treatment after ALL relapse involves inducing a CR2 with conventional intensive chemotherapy and apply consolidation, re-intensification and maintenance therapy, or allogeneic stem-cell transplantation (SCT) as further intensification of treatment. As occurs with primary diagnosed ALL, successful treatment of relapse largely relies upon the risk-based treatment allocation of patients in order to maximize response to therapy while minimizing toxicity and adverse effects. Using the prognostic criteria such as first remission duration; site and immunophenotype of relapse; genetic alterations; and initial response to relapse therapy, distinct subgroups of relapsed ALL can be identified that may either be treated with chemoradiotherapy only or by additional allogeneic SCT (Table 6 and Figure 1)[27,29].

Reinduction

Current treatment approaches for relapsed ALL begin with reinduction therapy in an attempt to induce a CR2. Reinduction of patients with relapsed ALL commonly includes conventional agents largely identical to those used at initial diagnosis except with increased dose intensity or alternative schedules with reported rates of toxic deaths around 4%-5%[26,42,47].

Few randomized trials comparing different reinduction regimens in risk-stratified children with relapsed ALL have been conducted[30,39,66], and it remains unclear whether any reinduction combination in use today is significantly superior to any other[18,47]. The Pediatric Oncology Group compared every 2 wk and weekly pegylated asparaginase with vincristine, doxorubicin, and prednisone in a population including both early and late marrow relapse and obtained CR2 rates of 82% and 97%, respectively[66].

The BFM group randomized dose and duration of infusional methotrexate in reinduction, demonstrating similar outcomes between intermediate-dose (1 g/m^2 over 36 h) and high-dose (5 g/m^2 over 24 h) infusions[39]. In a trial conducted by the United Kingdom Children's Cancer Group (UKCCG) patients were allocated to receive either idarubicin or mitoxantrone during induction; after 3 blocks of therapy, HR and IR patients with MRD $\geqslant 10^{-4}$ received allogeneic SCT, whereas SR and IR patients with MRD $< 10^{-4}$ continued chemotherapy. EFS and OS were significantly higher in the mitoxantrone group. The 3-year OS was 69% in the mitoxantrone group (45% in the idarubicin), which overall represented

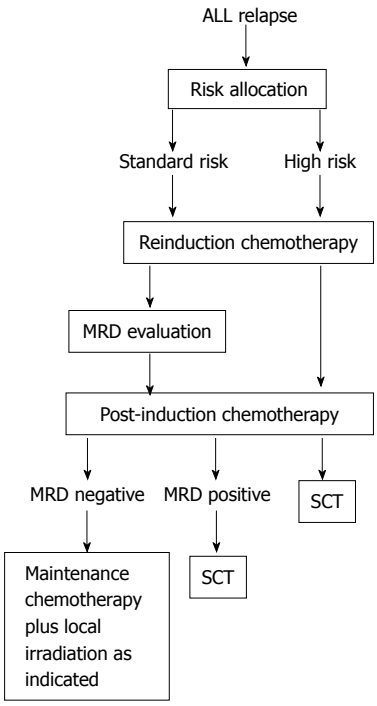


Figure 1 Algorithm for the management of relapsed acute lymphoblastic leukaemia in children. Risk allocation is based on immunophenotype, time and site of relapse (see Table 5). SCT: Allogeneic stem cell transplantation; MRD: Minimal residual disease.

a substantial improvement over preceding trials from the same investigators[30].

The conceptional backbone therapy of the ALL-REZ BFM Group is a series of short (*i.e.*, 5 to 7 d), intensive multiagent chemotherapy courses (block therapy), including most of the same traditional chemotherapy agents with known antileukemic efficacy, with an interval of 2 wk between the blocks to allow for regeneration of bone marrow aplasia, then followed by local irradiation therapy when indicated, and conventional maintenance therapy. This block therapy concept proved to be feasible, effective, and relatively well-tolerated and has been incorporated into many other treatment regimens for relapsed ALL and HR primary ALL worldwide[24,26]. A risk-adapted intensification of treatment by prolonging the intensive treatment phase in an ALL-REZ BFM Study could not prevent a high relapse rate in the high-risk group (early isolated or combined bone marrow relapse)[24]. Other treatment strategies with a more continuous therapy with repetitive application of comparably less intensive chemotherapy has been developed and used by the COG, and the UK ALL Relapse Study Group (MRC UKALLR), achieving comparable results[20,35]. It remains unclear whether short course intensive, or continuous less intensive chemotherapy constitute the most adequate and effective approach in treating childhood relapsed ALL or which subgroups of patients may benefit more from one approach or the other[24].

The COG conducted the AALL01P2 phase II pilot study with the primary objective of developing a safe and active reinduction regimen that could serve as a platform for evaluating the addition of promising new agents in future trials[28]. An objective of this study was to improve the depth of CR2 using three intensive non-overlapping blocks of chemotherapy derived from combinations that were previously shown to be effective in the management of recurrent ALL. With this regimen there was a 40% incidence of febrile neutropenia and a 19% incidence of documented infections[28]. Five toxic deaths occurred among 124 patients (4%) yielding a similar toxic death rate seen with other regimens[20,24,26,28,33,36,66].

The authors concluded that extending the duration of re-induction to three blocks appears to be beneficial for the group of patients with initial favourable morphologic responses and were MRD-negative at the end of the first month of treatment[28].

Reinduction remission rates for patients with a first relapse range from 71% to 95%, depending on the timing and site of relapse. CR2 rates for late bone marrow relapse typically approach 95%, whereas those for early relapse range from 70% to 85% and are frequently < 50% for very early relapses[16,19-22,24-26,28-30,32,34,35,42,66]. T-cell immunophenotype has been related with a lower remission rate[25]. Patients failing to achieve a CR2 after reinduction chemotherapy have a dismal prognosis[25,28,67]. Data from the BFM study group showed that only one third of children treated with curative intention after conventional reinduction failure obtained a CR2 (81% of them only after SCT) with a median survival of 255 d after the diagnosis of reinduction failure[67]. Given that further therapies with curative intent are associated with high treatment-related morbidity, mortality, and minimal survival, children with relapsed ALL and having no response to protocol-therapy should be eligible for innovative, ethically approved phase 1 or 2 trials[18,67].

For patients with HR relapsed ALL, the COG is currently exploring the role of adding novel agents to remission reinduction therapy (Table 6)[29]. The International Cooperative Group on Relapsed ALL conducts 2 randomized trials comparing the classic BFM reinduction therapy with that reported by the UKCCG in standard and intermediate risk patients, and with a novel regimen combining clofarabine, etoposide, and cyclophosphamide in high risk children, respectively[18,37].

Post-remission therapy: SCT vs continuation of chemotherapy

For patients with relapsed ALL who attain a second remission, no consensus on optimal therapy exists. All patients who reach a CR2 receive additional chemotherapy, even if SCT is planned. In general, a higher dose intensity is used than in first-line treatment but published data do not show one chemotherapy combination to be better than others[47].

For patients not allocated to SCT, a consolidation phase after induction chemotherapy followed by a prolonged continuation treatment is generally recommend-

ed[20,25,42]. According to the ALL-REZ BFM protocols, patients not allocated to SCT receive treatment consisting of alternating courses of polychemotherapy. At the end of intensive systemic chemotherapy, local radiotherapy is applied as indicated followed by conventional maintenance therapy up to 2 years[42].

Allogeneic SCT is a curative option for several hematologic malignancies and the current availability of several different stem cell sources has expanded this option for many children. High-dose myeloablative chemotherapy followed by SCT is an alternative to chemotherapy alone for relapsed ALL. Several published retrospective studies suggest some benefit for SCT, particularly for patients with early BM relapse[19,22,42,57]. With improved supportive care and better donor selection, the outcome after unrelated donor and matched sibling SCT for relapsed ALL has become more equal[68]. Therefore, the comparability of several different stem cell sources has expanded this option for many children and SCT has been widely used for patients with relapsed ALL. In the ALL-REZ BFM 87 study, SCT was associated with a superior EFS compared to chemotherapy/radiotherapy as postremission therapy alone, and the performance of SCT (included as time-dependent covariate) was an independent predictor of EFS. Results of autologous transplantation and chemotherapy were the same[24]. In the Austrian BFM Study Group report, patients who received SCT in second CR did significantly better than patients given chemotherapy only (10-year EFS 55% *vs* 33%) and this was even more obvious following an isolated BM relapse (10-year EFS 58% for SCT in second CR *vs* 22% for chemotherapy only)[22]. In a report by Eapen *et al*[57], children with an early BM first relapse of BCP ALL had lower rates of second relapse and higher rates of leukemia-free survival and OS if they received an HLA-matched sibling transplant with a TBI-containing regimen compared with a non-transplant approach. In contrast, for those with a late first relapse and second relapse, leukemia-free and OS rates were similar after chemotherapy alone and transplantation[57]. In a retrospective report from the NOPHO Study group, SCT led to increased long-term survival compared with chemotherapy irrespective of the length of first remission[33]. Matched-pair comparisons across BFM group ALL-REZ trials showed that unrelated donor transplantation achieved significantly better leukemia-free survival than chemotherapy in HR relapse but not in IR relapse. The EFS at 5 years was 17% for the chemotherapy group (0% for HR) and 42% for the SCT group (44% for HR) while rates of treatment related death were 4% and 30%, respectively[42].

Other studies suggest that the type of therapy after relapse does not affect outcome[21], and for a subgroup of patients with relapsed ALL, mainly represented by late BM and extramedullary relapses, combined chemotherapy and radiotherapy may yield durable second remissions[24]. From the BFM group, Borgmann *et al*[42] reported 39% EFS after transplantation for IR patients compared to 49% for non-transplanted patients. In line with such a statement are the data in a United Kingdom study including 256 patients, who were analyzed on the basis of HLA matched donor availability; no statistical benefit in outcome was seen[69]. Malempati *et al*[34] found no significant difference in EFS or OS between treatment with SCT or chemotherapy for any site of relapse or duration of the CR1, with a 2-year estimated EFS of 49.5% with SCT compared to 49.1% with chemotherapy for the entire group. For early BM relapse they also found no difference in treatment modality; the 2-year estimated EFS was 43.1% with SCT and 38.0% with chemotherapy; there was also no significant difference in EFS for late BM relapse according to treatment type: 2-year estimated EFS was 56.1% with SCT and 61.5% with chemotherapy. Similarly, 3-year estimated EFS after isolated CNS relapse was equivalent with either SCT or chemotherapy at 45% and 56.1%[34].

The analysis of the ALL REZ BFM 90 Study showed that SCT did not improve EFS for IR patients (represented by late isolated or combined BM and isolated extramedullary regardless of time point of relapse) or for those who received allogeneic HLA-compatible grafts; however, EFS was significantly higher after SCT in HR patients (early BM, very early isolated or combined BM and any relapse of T-lineage) than after the administration of chemo-radiotherapy alone. This group of patients when treated with conventional chemo-radiotherapy had a low chance of cure[26]. In the study by van de Berg *et al*[38], the benefits of the conditioning and the possible graft-*vs*-leukemia effect on patients undergoing SCT did not outweigh the benefit of prolonged, rotational chemotherapy for late relapses (including BM relapses); patients treated with chemotherapy only achieved a 65% survival rate[38].

The impact of type of donor (matched related *vs* unrelated or mismatched related) on outcome has not been demonstrated. Some studies claimed a clear advantage for matched related donor SCT or for matched unrelated donor SCT[42]. Long-term EFS rates from of above 40% have been reported with HLA-matched sibling donor SCT in CR2 after early relapse[57]. By contrast, others found no significant difference in outcome according to type of donor[2,32]. Results with umbilical cord transplantation are comparable to that obtained with unrelated donor SCT[70]. Unrelated donor registries and cord blood banks have increased the donor availability for the majority of patients lacking an HLA matched familial donor but the process of searching for an unrelated donor usually takes several months during which patients in CR2 are at risk of new relapse or even death from treatment related complications[33]. In this regard, reported time to transplant after relapse is commonly around 3 mo[19,26,28,34].

Haploidentical hematopoietic SCT (haplo-SCT) from a mismatched family member donor offers an alternative option for patients who lack a human leucocyte antigen (HLA)-matched donor[71]. The main obstacles are graft rejection, delayed immune reconstitution, graft-*vs*-host

disease (GvHD) and vulnerability to infections[71]. T-cell depletion can prevent overwhelming GvHD allowing the graft to contain large numbers of stem cells. This approach can reduce the risk of graft failure retaining CD34-negative stem cells and most other immune cells, thus allowing expedite immune reconstitution during the early post-transplant period. However, the absence of the T cell-mediated graft-*vs*-leukemia effect would render the recipients of a T cell-depleted allograft more susceptible to leukemia relapse. In this scenario, donor-*vs*-recipient NK alloreactivity has emerged as a crucial factor for the outcome of haplo-SCT. Ruggeri *et al*[72] reported a low relapse risk for patients with acute myeloid leukaemia transplanted from NK-alloreactive donors. This NK-mediated graft-*vs*-leukemia effect has also been documented in children with ALL[73]. Data from the Pediatric Diseases and the Acute Leukemia Working Parties of the European Blood and Marrow Transplant showed 34% and 22% EFS for children undergoing haplo-SCT in CR2 and CR3, respectively[74]. Therefore, a T cell-depleted haplo-SCT should be included in the treatment algorithm as a valuable option for patients with ALL in need of transplantation and lacking a matched donor, especially if an NK alloreactive relative exists. An unmanipulated HLA-haploidentical SCT has been proposed for those few patients who are unable to locate an HLA-compatible donor, a suitable umbilical cord blood unit, or an NK-alloreactive relative[18]. As stated by Locatelli *et al*[18], for those patients considered candidates for allo-SCT, the preferred source of stem cells should be a matched sibling donor; for those lacking an HLA-compatible family donor, an unrelated donor, umbilical cord blood or haploidentical family donor are suitable options.

Intensive chemo-radiotherapy has been administered before transplantation to reduce the burden of disease and induce immunosuppression in the host. Total body irradiation (TBI)-containing regimens before SCT from a matched sibling donor proved to be superior to chemotherapy alone and a non-TBI regimen in children with early relapse and BCP ALL who achieve a CR2. Transplantation with a TBI-containing regimen resulted in significantly lower risks of relapse, treatment failure, and overall mortality compared to non-TBI regimens and this was independent of the duration of the first remission[57]. Such conditioning regimens reduce graft rejection but they can cause considerable mortality due to severe toxicity, delayed immune restoration and severe infection, especially in heavily pre-treated patients. Moreover, DFS estimates are not appreciably improved by aggressive chemo-radiotherapy, as recurrent or refractory malignancies have usually become resistant to chemotherapy. These observations have encouraged the reassessment of conditioning strategies for transplantation. Newer strategies aim to minimise toxicity while allowing rapid engraftment and expediting immune reconstitution during the early post-transplant period, thereby protecting the host from infection and perhaps generating a graft-*vs*-tumour effect against disease relapse[71,75]. In contrast to traditional myeloablative conditioning regimens that use high doses of radiation or chemotherapy or both to suppress host immune responses and eradicate diseases, this approach relies almost exclusively on graft-*vs*-host effects for eradication of the underlying diseases. Reduced intensity or non-myeloablative conditioning regimens for haplo-SCT reduced mortality and have an acceptable rate of engraftment. However, delayed immune reconstitution, severe GvHD and infection continue to be impediments[71,75]. Elimination of TBI may reduce damage to organs that generate immune cells, while avoidance of anti-thymocyte globulin may prevent complications that include delayed immune reconstitution and Epstein-Barr virus associated lymphoproliferative disease[75]. Using a reduced intensity conditioning regimen (fludarabine, thiotepa, melfalan and OKT3) without TBI and without anti-thymocyte globulin in children with refractory haematological malignancy, a more rapid and robust immune reconstitution when compared to patients transplanted with a myeloablative conditioning regimen was reported. Studies with melphalan-based reduced-intensity conditioning regimens and T/B cell-depleted grafts show high engraftment rates. The risk of acute and chronic GvHD was significantly reduced by graft manipulation procedures (T/B cell depletion) and is comparable to that after matched unrelated donor transplantations[71,75]. Furthermore, the overall incidence of cytomegalovirus, Epstein-Barr virus and adenovirus viremia in the reduced intensity conditioning regimen group was less than that in the myeloablative conditioning regimen group[75]. Transplant related mortality could be effectively reduced by improved T cell recovery and close monitoring of viral loads followed by preemptive therapy[71].

Many factors complicate the analysis of published results comparing outcomes after SCT *vs* chemotherapy only, including intrinsic selection biases, different lengths of the interval between diagnosis of relapse and transplantation as well as disparate conditioning regimens, supportive strategies and stem cell sources[21,34]. Very often, reported trials assigned HR with matched family donors to allogeneic transplantation and those without donors to chemotherapy or autologous transplantation[47].

Another impediment in comparing reports is the more favourable outcomes of non-sibling donors SCT over the years. A matched-pair analysis of unrelated donor SCT *vs* chemotherapy revealed that only high-risk patients benefited from unrelated donor transplantations[42]. In a prospective randomized trial, Gaynon *et al*[21] found poor protocol adherence with small numbers of patients recruited to the chemotherapy arm. The authors speculated how this might be related to the known poor outcome of relapsed ALL and "a desire to do everything possible for children for whom aggressive chemotherapy had already failed once"[21,47].

Thus, much debate has centered on optimal pos-

tremission therapy including stem cell transplantation[18,33,42]. A recent meta-analysis demonstrated the variability of outcomes and conclusions among studies comparing SCT with chemotherapy for the treatment of ALL in second remission[76]. However, excluding patients with late relapse[38], in no comparison is outcome after transplantation worse than after chemotherapy alone[47]. To this regard, after the induction of the CR2, options for ongoing continuation therapy are frequently risk based in order to allocate patients to treatment regimens with adequate intensity and justifiable toxicity[18,29,31]. There are some patients with an acceptable EFS rate with chemotherapy alone, while other patients need to undergo allogeneic SCT after the 2CR or are even eligible for phase Ⅰ/Ⅱ trials with the chance of benefiting from new agents. Both the COG and the BFM as well as the I-BFM groups developed formal criteria for risk stratification for relapsed ALL with the main intention of identifying children for whom SCT might be better than continuation chemotherapy once a second remission is attained (Tables 5 and 6)[2,42].

Children with a very early (< 18 mo after diagnosis) or early (between 18 mo after diagnosis and 6 mo after cessation of frontline chemotherapy) isolated BM relapse, a very early BM/extramedullary combined relapse and all T-cell ALL with BM involvement at relapse diagnosis should be categorized as HR patients and should be allocated to SCT given that nearly all will suffer a subsequent relapse when being treated solely with conventional intensive post-induction chemotherapy. Allogeneic SCT with a matched donor is currently the preferred therapeutic option for these children after the CR2[20,26,29,33,38,42,69].

The outcomes for very early extramedullary recurrences without SCT have been inferior to those of early or late extramedullary relapse, with an EFS of < 50%, and SCT in CR2 has also been considered for these patients[20,40,41,77].

It remains unclear as to what will be the most effective approach for HR patients who continue to have high levels of disease before or after transplantation, as this is associated with a high incidence of relapse post-allogenic SCT[20]. In this scenario, further cytoreductive chemotherapy (clofarabine), immunomodulation, the application of new agents, and/or innovative transplant procedures might be considered.

Children with early or late (> 18 mo from initial diagnosis) isolated extramedullary relapse represent the SR group and, for these patients, outcomes have been very good with chemotherapy and site-directed radiation and there is no indication for SCT. However, intensive systemic therapy is essential for preventing later BM recurrences[20,26,29,31].

The largest group of patients (more than 50%) belongs to the IR group, in which treatment choices are the most difficult[32,42]. This group includes patients with BCP ALL with either late (> 6 mo after cessation of frontline chemotherapy) isolated BM relapse, or with a late or early combined BM/extramedullary relapse as well as early (including T-cell) isolated extramedullary relapses (Table 5)[31]. The optimal post-remission therapy for children with late B-cell precursor BM relapse (either isolated or combined) is controversial[20]. Intensive systemic therapy is essential for preventing later BM recurrences. However, the benefit of SCT for these patients has not been firmly established. SCT is associated with a 10% to 20% risk of peri-transplantation mortality, depending on donor type, and still has a substantial relapse rate[32,57]. While some studies report comparable results with both SCT and chemotherapy[26,57] others argue that the outcome of patients undergoing a transplant is poorer, and that SCT in late relapses is not beneficial[38]. Within the NOPHO study, patients with late BM relapse but with initial HR features and combined BM relapses did not do well on conventional chemotherapy. The authors recommended considering allo-SCT for these subgroups of patients[33]. Data from the DCOG Relapse ALL 98 protocol showed that patients with a late BM relapse undergoing a transplant had poorer outcomes than those undergoing CT only. Although the majority of these patients died from a relapse of leukaemia, the benefits of the conditioning and the possible graft-*vs*-leukemia effect after SCT did not outweigh the benefit of the prolonged chemotherapy[38]. The COG is currently investigating if outcome for patients with late (≥ 36 mo) B-cell precursor marrow relapse, can be improved by using the same AALL01P2 triple re-induction regimen followed by 2 years of intensive chemotherapy[28,78].

In the IR patients (with EFS rates greater than 40%) additional risk factors, such as the dynamics of treatment response assessed by MRD, would help to identify those patients at a high risk of subsequent relapse who are thus eligible for SCT[26,30]. MRD response is being integrated into risk classification schemes[18,29]. A cut-off point MRD after reinduction of 10^{-3} (quantified by PCR) was recently proposed by Eckert *et al*[31] to discriminate between patients with a good or a poor prognosis. Patients belonging to the group with MRD between 10^{-3} and 10^{-4} can be categorised as molecular good responders and allogeneic SCT would not be appropriate for these patients. In the subsequent trial ALL-REZ BFM 2002, this level of MRD after induction was applied to decide whether chemotherapy or SCT should be used as consolidation post-induction therapy[31]. A different cut-off MRD of 0.01% (10^{-4}) (measured by flow cytometry) was applied by the COG for prognostic assessment after the first, second and third treatment block of the AALL01P2 study in IR and HR relapses[28]. Within the UK ALLR3 study, patients with MRD ≥ 0.01% (10^{-4}) (quantified by PCR) at the end of induction were eligible for SCT[30]. A different preceding treatment and quantification method within each protocol might explain these differences in MRD cut-off levels as being predictive of outcome.

A major task of ongoing and future trials is to predict subsequent relapses more precisely, thus clarifying which

patients benefit from post-remission intensification by allogeneic SCT. In this context, not only the acute mortality and toxicity, but also the long-term sequelae of allogeneic SCT have to be taken into account[24].

Local therapy for extramedullary ALL

Therapeutic irradiation of manifest extramedullary leukemia in addition to systemic chemotherapy for patients experiencing CNS recurrence can be regarded as standard of care, since the disease is protected from chemotherapy by biological blood barriers in extramedullary sanctuary sites such as the CNS and the testes[18]. In accordance with other investigators, for patients with CNS involvement at relapse, we would encourage the use of an Ommaya reservoir during intensification. Intraventricular therapy has several theoretical advantages: a more uniform distribution of chemotherapy throughout the cerebrospinal fluid (CSF), higher ventricular levels than those achieved by lumbar administration, and prolonged concentration over time exposure to cell cycle active chemotherapy[36].

For patients with late isolated CNS relapse (not allocated to SCT), cranio-spinal irradiation is generally postponed until the end of intensive chemotherapy or even after the end of maintenance treatment, in order to avoid intolerability for chemotherapy[38]. The administration of 24 Gy and 15 Gy to cranium and spine, respectively are commonly recommended[38], although the adequate dose (18 *vs* 24 Gy) and mode of CNS irradiation (cranial *vs* craniospinal) remains controversial[44]. A 4-year EFS of 78% can be achieved after reduction of the radiation dose to 18 Gy in patients with B-cell precursor ALL whose initial remission lasted > 18 mo while in patients relapsing before 18 mo the EFS was 52%[40]. Further dose reduction (15 Gy) is recommended for patients with prior irradiation[18]. The addition of cranial irradiation, even in patients without obvious CNS-involvement (prophylactic cranial irradiation), was reported to significantly improve the outcome of patients with isolated BM-relapse by the ALL REZ BFM Study Group and was introduced from 1989 onwards[26]. If the CNS was involved at the time of relapse, patients received more intense intrathecal triple chemotherapy with methotrexate, cytarabine and prednisone[26]. In addition, cranial or cranio-spinal radiation was delivered in an age-dependent manner to all patients[22,24,26]. This strategy was adopted by other study groups[21,36]. However, whether protective CNS irradiation is necessary in patients with isolated BM relapse, remains controversial and, given the well documented radiation associated late effects, it is omitted by several groups in favour of intensified intrathecal chemotherapy[25,33].

Most study groups recommend local irradiation of both testes at 24 Gy regardless whether only one or both testes are involved at relapse. Within the BFM studies, orchidectomy has been the treatment of choice for the involved testicle in the case of testicular relapse. In unilateral testicular disease the clinically affected testis is removed and the remaining testis irradiated (15-18 Gy according to the results of biopsy)[22]. In the case of clinical unilateral or bilateral testicular involvement and no resection 24 Gy local irradiation is generally recommended[22,25,26,33,36]. Radiotherapy (24 Gy) for bilateral testicular recurrence is expected to induce infertility and significantly impair hormone production[18]. Within the DCOG Relapse ALL 98 Protocol, patients with late testicular relapses were treated without irradiation and without surgery[38].

A variety of other extramedullary sites may be involved in ALL relapse. Little data are available regarding the prognostic impact of these manifestations and on the necessity of local therapy. Since a blood barrier is not present in these sites, systemic chemotherapy is supposed to be effective. Thus, for an extramedullary relapse other than CNS and testis, no local therapy is generally considered apart from cases where local persistence of the disease occurs after induction/consolidation chemotherapy. In this situation, it is recommended to take a biopsy and to apply local irradiation therapy if vital leukemic cells are still present.

Treatment for second and subsequent relapses

Most treatment failures after the CR2 are related to subsequent relapses[21]. For 74 patients experiencing a second relapse and enrolled into ALL-REZ BFM trials before 2006, the median duration of the second CR was 7.5 mo (range, 18 d to 4.4 years)[27]. In this situation, a significant decrease in CR rates is expected[21]. A variety of multidrug regimens provide a 40% CR rate in the second and subsequent relapses[28]. Ko *et al*[19] reported CR rates of 44%, 27%, and 12% for third, fourth, and further therapeutic attempts, respectively. The subsequent CR rate was lower when CR was not achieved or was of short duration after the prior treatment attempt[19]. In contrast, the NOPHO study group reported a third complete remission (CR3) as high as 72% in 274 patients after the second relapse. In this study, those who never achieved 3CR had a shorter first remission, more BM relapses and shorter time intervals between the relapses, indicating a more aggressive disease. However, long-term survival was only 12%[33]. Few other data appear for DFS rates in the CR3 and beyond[19,20,27,33]. According to Ko *et al*[19], DFS among patients who achieve CR decreased with an increasing number of prior treatment attempts. Two and 5-year DFS for patients achieving CR after third therapeutic attempts was 31% and 15%, respectively. DFS increased with increasing duration of the prior remission[19]. In a report from the Austrian BFM Study Group, the median duration of CR after second relapse was 13 mo, with 10-year EFS rates of only 9% and 6% after the second and third relapses, respectively[22].

Concerning prognostic factors, the length of the CR2 and relapse site are relevant[27,32]. Reismüller *et al*[27] found that the duration of the second CR seemed to have an influence on EFS: 6% *vs* 21% for patients with a CR2 duration of less or more than 1.5 years, respec-

tively. In this report, the only other prognostic factors that proved to be statistically significant were site of first and second relapse with isolated extramedullary relapses faring better than isolated and combined BM relapses, and duration of the first CR[27]. Other reported factors associated with survival are NCI risk criteria at initial diagnosis, immunophenotype, presenting leucocyte count and length of first remission[19,32]. Additional extramedullary sites of disease were not significantly associated with DFS[18]. The prognosis for children with BM relapse after SCT, and children with a second relapse of T-cell ALL is dismal. In the latter group, this is mainly due to the lack of ability to achieve a CR3[27]. Patients who relapse after allogeneic SCT often have refractory disease and are particularly susceptible to chemotherapy-related toxicity[79].

Survival after second relapse was reported to vary according to treatment. The role of SCT for patients with a second or third relapse has been debated. Overall survival ranging from 20% to 36% was reported for those undergoing SCT compared with 10% to 15% for those with chemotherapy only[32,33]. Ko and coworkers[19] found increased survival for patients undergoing SCT, regardless of time to relapse or the number of prior relapses.

Given that only a very small group of patients with second ALL relapse has a realistic chance of cure, these patients are ideal candidates for phase Ⅰ/Ⅱ trials exploring new innovative drugs, with the consideration of SCT in those achieving a durable remission[18,27,29].

OUTCOME OF RELAPSED ALL

Outcomes for children with relapsed ALL have changed little over time despite efforts by many investigators to intensify therapy with approaches that often include SCT. Although clinical remission can be achieved in most (85%) relapses, the chance to experience a second relapse is still high and long-term survival rates do not exceed 40% to 50%[20-22,24-26,33,35,43]. Results from the CCG 1941 marrow relapse study showed that 50% of patients failed to enter remission, died from toxicity, or relapsed again after achieving a brief second remission[21]. The overall outcomes are dismal for patients who do not achieve a CR2 after an initial attempt[18,28]. In a recent large retrospective review within the Therapeutic Advances in Childhood Leukemia Consortium (TACL), Ko *et al*[19] found 27% 5-year DFS for patients in CR2. These results are similar to those generally reported by other study groups with DFS rates ranging from 16% to 39% depending on the study, time to end point, and the patient population[16,20-22,24,25,32-34].

Second malignancies such as primary brain tumors and acute myeloid leukemia are another matter of concern in relapsed ALL patients with an estimated actuarial incidence at 15 years from diagnosis of around 11%[32,57].

NEW PERSPECTIVES

Unfortunately, retrieval therapy is inadequate in most cases of relapsed ALL and most of these children succumb to their disease. Further intensification of chemotherapy is unlikely to cure additional patients. The failure of intensive chemotherapy to cure most children, as well as its related toxicity, makes it essential to search for new treatment approaches. Approximately one third of relapsed patients can be assigned to a "poor prognosis group" (early BM-relapse or any BM relapse of T-cell ALL), for whom no promising therapy regimen exist[24]. Moreover, the extremely poor survival after relapse underscores the need to focus on improving the outcome of the primary therapy for those patients who are unlikely to be salvaged if they relapse. Promising new therapies should be integrated into trials for subsets of higher risk patients at initial diagnosis[16]. Using analyses of DNA copy number abnormalities, gene expression, DNA methylation and sequencing in matched diagnosis/relapsed ALL BM samples, investigations are under way regarding the evolution of genetic lesions from diagnosis to relapse that lead to drug resistance and disease progression with the aim of identifying new potential biomarkers and therapeutic targets[45,46,48,49,52,53,78]. Further development and the use of targeted therapies or immune modulators may decrease residual disease and may improve the outcome in children with relapsed ALL treated with either intensive chemotherapy or SCT[34]. Offering uniform clinical trials for patients with relapsed ALL while gathering biological data in order to identify new agents not generally used in the treatment of ALL at primary treatment, are the focus of several current collaborative study groups[16].

Because responses to single-agent therapy have been poor, integrating new agents in combination with established chemotherapy platforms in a randomized manner has been adopted as a therapeutic approach by de COG with the aim of exploring improvements in CR2 and MRD rates as a measure to define new agent activity and, potentially, to more efficiently select candidate agents for future study[29,78]. Novel approaches include new formulations of existing chemotherapeutic agents, new antimetabolites and nucleoside analogs, monoclonal antibodies directed against leukemia-associated antigens, adoptive therapy approaches such as chimeric antigen receptor (CAR)-modified T cells, and molecularly targeted drugs such as the proteasome inhibitor bortezomib and JAK kinase, aurora kinase, and mammalian target of rapamycin (mTOR) inhibitors[18,29,37,80].

Intrathecal lyposomal cytarabine may have a role in relapsed ALL with CNS involvement and resistance to conventional therapy[18].

Clofarabine is a second-generation purine analog capable of inhibiting DNA synthesis/repair and inducing cell death[81]. Clofarabine has been granted accelerated approval both in Europe and in the United States for the treatment of pediatric patients with relapsed or refractory ALL who received at least 2 prior regimens of chemotherapy[18]. O'Connor *et al*[82] reported an overall response rate of 67% in 23 pediatric patients diagnosed with relapsed ALL. Clofarabine was safe and effective

when used in combination with cyclophosphamide and etoposide although a high risk of severe infection was noted, including fungal and viral infection. The response rate to treatment with a clofarabine-based regimen was inversely proportional to the number of prior treatment attempts. Durable remissions were achieved, allowing patients the option of hematopoietic stem cell transplantation with the potential of long term cure. Treatment was effective in 3 out of 5 infants with relapsed *MLL* rearranged ALL[82]. Thus, the use of clofarabine-based regimens should be considered in children with either resistant or second or subsequent BM relapse[18]. Nelarabine is an inhibitor of purine nucleoside phosphorylase. The FDA approved nelarabine in October 2005 for third-line treatment of patients with T-cell ALL/lymphoma[18]. In the COG AALL00P2 trial, patients with T-ALL with a poor early treatment response that predicted poor outcomes in previous trials attained a 5-year EFS rate of 69% with intensive chemotherapy plus nelarabine without increased toxicities. Non HR patients (< 1000/μL peripheral blood blasts on prednisone prephase day 8 and MRD < 1% at induction therapy day 36) who received nelarabine had a 5-year EFS rate of 74%[83].

Monoclonal antibodies directed to cell surface antigens expressed by leukemic blasts (epratuzumab, blinatumomab, inotuzumab ozogamicin, and moxetumomab pasudotox, among others), are ideal candidates. Combinations of monoclonal antibody and cytotoxic therapies may hold particular promise in relapsed ALL[2,29]. Epratuzumab is a humanized monoclonal antibody that binds to the third extracellular domain of CD22. CD22, a B-cell surface antigen, is highly expressed in more than 90% of cases of childhood B-precursor ALL. After binding, the receptor/antigen complex is rapidly internalized and appears to modulate B-cell activation and signaling. Given the high CD22 expression levels in B-precursor ALL, its mechanism of action distinct from cytotoxic agents, and a toxicity profile that could allow for combining it with dose-intensive chemotherapy, epratuzumab became an attractive agent to explore in relapsed ALL. Epratuzumab was the first agent tested by the COG in combination with an established reinduction platform in children and young adults with first, early BM relapses of $CD22^+$ ALL in an effort to improve CR2 rates[84]. Patients received four intravenous doses of epratuzumab, 360 mg/m^2 per dose, twice weekly during the 14-d reduction phase, followed by four weekly doses, 360 mg/m^2 per dose, administered with block 1 of the AALL01P2 chemotherapy regimen[28,84]. Epratuzumab administration was tolerated with acceptable toxicity, both as a single agent and when combined with chemotherapy. MRD responses in those who achieved remission were significantly more favourable in those who received epratuzumab (42% MRD-negative compared with 25% among historical controls) suggesting that the antibody may enhance response to cytotoxic chemotherapy[29,78,84]. However, the rates of CR2 did not differ compared with a historical control population treated with chemotherapy alone[84]. Based on these results, the COG will not pursue epratuzumab further[78,85].

T-cell engaging antibodies are bispecific antibodies designed to transiently engage primed cytotoxic effector memory T lymphocytes for the lysis of target cells. The T-cell engaging CD19/CD3-bispecific antibody blinatumomab can redirect T lymphocytes against CD19+ ALL blasts, which represents a new approach to the treatment of BCP ALL. Handgretinger *et al*[79] reported the first clinical experience in three pediatric patients with BCP ALL showing that blinatumomab, administered as a continuous 24 h intravenous infusion at 15 mg/m^2 per day for several weeks, was well tolerated and able to rapidly induce MRD-negative complete responses in refractory BCP ALL after multiple relapses and allogeneic HSCT. Blinatumumab is an attractive drug to be explored in the near future for children with second or greater relapsed or refractory ALL[78].

Bortezomib is a proteasome inhibitor, which renders leukemic cells more sensitive to the apoptotic effects of chemotherapy. A phase 1 study conducted by the TACL (TACL study, T2005-003) demonstrated that a standard dose of bortezomib (1.3 mg/m^2 given on days 1, 4, 8, and 11) can be safely combined with an intensive 4-drug reinduction regimen in children with relapsed ALL and showed promising activity in relapsed childhood ALL[86]. Within the phase 2 expansion of this combination (TACL study T2005-003) patients were eligible only after they failed 2 or 3 previous treatment regimens. The CR rate was 64% with an additional 9% of CR without platelet recovery for an overall response rate of 73% which was significantly better than in previous trials. BCP ALL patients had an 80% overall response rate while no T-cell ALL patients showed a response. The study reached its predefined early stopping rule for efficacy when 14 complete responses were observed among the first 22 patients enrolled. OS at 24 mo was estimated to be 41%. Lethal bacterial sepsis was the principal toxicity[87]. A study combining bortezomib with a 4-drug reinduction platform (the AALL01P2 triple reinduction regimen) is also in progress within the COG for patients with early BM relapse occurring within 36 mo of diagnosis[29,78]. This approach will also be explored by the I-BFM SG.

In relapse Ph+ ALL, second complete remissions can be obtained with the combination of imatinib and intensive chemotherapy[28,88]. For patients relapsing after treatment with imatinib, the use of scalating doses of imatinib or alternative tyrosine kinase inhibitors may overcome imatinib resistance and help to induce a new remission and a second SCT should be considered in this situation[88,89]. High *FLT3* expression identifies *MLL-AF4*+ ALL patients at very high risk of treatment failure and poor survival, emphasizing the value of ongoing/future clinical trials for FLT3 inhibitors[90]. The COG is conducting a phase I trial (ADVL1011) of the JAK inhibitor ruxolitinib, and plans to develop a trial of ruxolitinib combined with chemotherapy in relapsed ALL patients with *JAK* mutations. These mutations are

present in a proportion of cases of so-called Ph-like ALL overexpressing *CRLF2*. Similarly, patients with other fusion genes activating *ABL1*, *JAK2*, and *PDGFRB* might be treated with *ABL/PDGFRB* class tyrosine kinase inhibitors[78].

The occurrence of defective immune recovery after haploidentical SCT was associated with a high risk of severe infections, which heavily affected morbidity and mortality. Post-transplant CD8-depleted donor lymphocyte infusions are feasible and promote immune reconstitution[91]. Further attempts might be directed at increasing the alloreactive potential of the transplantation. Donor lymphocyte infusions have been advocated to convert stable mixed chimerism into full chimerism and have been used successfully in patients with persistent, relapsed, or progressive disease both after conventional and nonmyeloablative SCT to exert graft-*vs*-tumor effects, most notably in patients with chronic myeloid leukemia[92,93].

Adoptive immuno therapy was investigated mostly in children who have a functional thymus and lower incidence of GvHD compared with adults. Donor-*vs*-recipient NK alloreactivity has emerged as a crucial factor for the outcome of haplo-SCT[72,73]. Genetic engineering to endow T cells with receptors that bind leukaemia cell surface antigens such as CD19 or CD22, is another promising adoptive therapy approach. Immune cells are genetically modified to express chimeric antigen receptors (CAR) that contain a target recognition domain linked to an intracellular component that activate a signalling cascade[80]. Impressive antileukemic effects have been reported using CD19-CAR constructs in pediatric patients with relapsed/refractory BCP ALL[80,94].

CONCLUSION

Relapsed ALL remains a significant challenge for pediatric oncologists. According to recent reports regarding genetic and epigenetic signatures, two different biological mechanisms seem to distinguish early *vs* late ALL relapse. This might partially explain their distinct behaviour, therapy response and outcome. While SCT is generally accepted as the best option as post-induction consolidation therapy for HR patients after CR2, this seems to apply to only a subgroup of patient categorized as IR. Early response evaluation in terms of MRD after reinduction therapy seems to offer the best chance to stratify IR to SCT or conventional chemotherapy and it is currently been applied by several study groups. However, leukemia-free survival remains dismally low for many patients after relapse and, despite efforts by many investigators to intensify therapy with approaches that often include SCT, outcomes for these children have changed little over time. Relapsed ALL represents the focus of considerable pediatric research and alternative treatment options exploring distinct mechanisms of action are being pursued. Given the rarity of the disease, prospective clinical trials need to be coordinated within international cooperative groups.

ACKNOWLEDGEMENTS

The author wants to thank Professor Juan Jose Ortega Aramburu for his long and fruitful dedication to childhood leukemia and for encouraging me and many others to get involved in this field.

REFERENCES

1 **Pui CH**, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *J Clin Oncol* 2011; **29**: 551-565 [PMID: 21220611 DOI: 10.1200/JCO.2010.30.7405]
2 **Gaynon PS**. Childhood acute lymphoblastic leukaemia and relapse. *Br J Haematol* 2005; **131**: 579-587 [PMID: 16351633]
3 **Teachey DT**, Hunger SP. Predicting relapse risk in childhood acute lymphoblastic leukaemia. *Br J Haematol* 2013; **162**: 606-620 [PMID: 23808872 DOI: 10.1111/bjh.12442]
4 **Conter V**, Aricò M, Basso G, Biondi A, Barisone E, Messina C, Parasole R, De Rossi G, Locatelli F, Pession A, Santoro N, Micalizzi C, Citterio M, Rizzari C, Silvestri D, Rondelli R, Lo Nigro L, Ziino O, Testi AM, Masera G, Valsecchi MG. Long-term results of the Italian Association of Pediatric Hematology and Oncology (AIEOP) Studies 82, 87, 88, 91 and 95 for childhood acute lymphoblastic leukemia. *Leukemia* 2010; **24**: 255-264 [PMID: 20016536 DOI: 10.1038/leu.2009.250]
5 **Escherich G**, Horstmann MA, Zimmermann M, Janka-Schaub GE. Cooperative study group for childhood acute lymphoblastic leukaemia (COALL): long-term results of trials 82,85,89,92 and 97. *Leukemia* 2010; **24**: 298-308 [PMID: 20016530 DOI: 10.1038/leu.2009.249]
6 **Kamps WA**, van der Pal-de Bruin KM, Veerman AJ, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004. *Leukemia* 2010; **24**: 309-319 [PMID: 20016528 DOI: 10.1038/leu.2009.258]
7 **Liang DC**, Yang CP, Lin DT, Hung IJ, Lin KH, Chen JS, Hsiao CC, Chang TT, Peng CT, Lin MT, Chang TK, Jaing TH, Liu HC, Wang LY, Yeh TC, Jou ST, Lu MY, Cheng CN, Sheen JM, Chiou SS, Wu KH, Hung GY, Chen RL, Chen SH, Cheng SN, Chang YH, Chen BW, Ho WL, Wang JL, Lin ST, Hsieh YL, Wang SC, Chang HH, Yang YL, Huang FL, Chang CY, Chang WH, Lin KS. Long-term results of Taiwan Pediatric Oncology Group studies 1997 and 2002 for childhood acute lymphoblastic leukemia. *Leukemia* 2010; **24**: 397-405 [PMID: 20016538 DOI: 10.1038/leu.2009.248]
8 **Mitchell C**, Richards S, Harrison CJ, Eden T. Long-term follow-up of the United Kingdom medical research council protocols for childhood acute lymphoblastic leukaemia, 1980-2001. *Leukemia* 2010; **24**: 406-418 [PMID: 20010621 DOI: 10.1038/leu.2009.256]
9 **Möricke A**, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H, Ludwig WD, Ritter J, Harbott J, Mann G, Klingebiel T, Zintl F, Niemeyer C, Kremens B, Niggli F, Niethammer D, Welte K, Stanulla M, Odenwald E, Riehm H, Schrappe M. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. *Leukemia* 2010; **24**: 265-284 [PMID: 20010625 DOI: 10.1038/leu.2009.257]
10 **Pui CH**, Pei D, Sandlund JT, Ribeiro RC, Rubnitz JE, Raimondi SC, Onciu M, Campana D, Kun LE, Jeha S, Cheng C, Howard SC, Metzger ML, Bhojwani D, Downing JR, Evans WE, Relling MV. Long-term results of St Jude Total Therapy Studies 11, 12, 13A, 13B, and 14 for childhood acute lymphoblastic leukemia. *Leukemia* 2010; **24**: 371-382 [PMID:

20010620 DOI: 10.1038/leu.2009.252]
11 **Salzer WL**, Devidas M, Carroll WL, Winick N, Pullen J, Hunger SP, Camitta BA. Long-term results of the pediatric oncology group studies for childhood acute lymphoblastic leukemia 1984-2001: a report from the children's oncology group. *Leukemia* 2010; **24**: 355-370 [PMID: 20016527 DOI: 10.1038/leu.2009.261]
12 **Schmiegelow K**, Forestier E, Hellebostad M, Heyman M, Kristinsson J, Söderhäll S, Taskinen M. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. *Leukemia* 2010; **24**: 345-354 [PMID: 20010622 DOI: 10.1038/leu.2009.251]
13 **Silverman LB**, Stevenson KE, O'Brien JE, Asselin BL, Barr RD, Clavell L, Cole PD, Kelly KM, Laverdiere C, Michon B, Schorin MA, Schwartz CL, O'Holleran EW, Neuberg DS, Cohen HJ, Sallan SE. Long-term results of Dana-Farber Cancer Institute ALL Consortium protocols for children with newly diagnosed acute lymphoblastic leukemia (1985-2000). *Leukemia* 2010; **24**: 320-334 [PMID: 20016537 DOI: 10.1038/leu.2009.253]
14 **Tsuchida M**, Ohara A, Manabe A, Kumagai M, Shimada H, Kikuchi A, Mori T, Saito M, Akiyama M, Fukushima T, Koike K, Shiobara M, Ogawa C, Kanazawa T, Noguchi Y, Oota S, Okimoto Y, Yabe H, Kajiwara M, Tomizawa D, Ko K, Sugita K, Kaneko T, Maeda M, Inukai T, Goto H, Takahashi H, Isoyama K, Hayashi Y, Hosoya R, Hanada R. Long-term results of Tokyo Children's Cancer Study Group trials for childhood acute lymphoblastic leukemia, 1984-1999. *Leukemia* 2010; **24**: 383-396 [PMID: 20033052 DOI: 10.1038/leu.2009.260]
15 **Tsurusawa M**, Shimomura Y, Asami K, Kikuta A, Watanabe A, Horikoshi Y, Matsushita T, Kanegane H, Ohta S, Iwai A, Mugishima H, Koizumi S. Long-term results of the Japanese Childhood Cancer and Leukemia Study Group studies 811, 841, 874 and 911 on childhood acute lymphoblastic leukemia. *Leukemia* 2010; **24**: 335-344 [PMID: 20016539 DOI: 10.1038/leu.2009.259]
16 **Nguyen K**, Devidas M, Cheng SC, La M, Raetz EA, Carroll WL, Winick NJ, Hunger SP, Gaynon PS, Loh ML. Factors influencing survival after relapse from acute lymphoblastic leukemia: a Children's Oncology Group study. *Leukemia* 2008; **22**: 2142-2150 [PMID: 18818707 DOI: 10.1038/leu.2008.251]
17 **Freyer DR**, Devidas M, La M, Carroll WL, Gaynon PS, Hunger SP, Seibel NL. Postrelapse survival in childhood acute lymphoblastic leukemia is independent of initial treatment intensity: a report from the Children's Oncology Group. *Blood* 2011; **117**: 3010-3015 [PMID: 21193696 DOI: 10.1182/blood-2010-07-294678]
18 **Locatelli F**, Schrappe M, Bernardo ME, Rutella S. How I treat relapsed childhood acute lymphoblastic leukemia. *Blood* 2012; **120**: 2807-2816 [PMID: 22896001 DOI: 10.1182/blood-2012-02-265884]
19 **Ko RH**, Ji L, Barnette P, Bostrom B, Hutchinson R, Raetz E, Seibel NL, Twist CJ, Eckroth E, Sposto R, Gaynon PS, Loh ML. Outcome of patients treated for relapsed or refractory acute lymphoblastic leukemia: a Therapeutic Advances in Childhood Leukemia Consortium study. *J Clin Oncol* 2010; **28**: 648-654 [PMID: 19841326 DOI: 10.1200/JCO.2009.22.2950]
20 **Roy A**, Cargill A, Love S, Moorman AV, Stoneham S, Lim A, Darbyshire PJ, Lancaster D, Hann I, Eden T, Saha V. Outcome after first relapse in childhood acute lymphoblastic leukaemia - lessons from the United Kingdom R2 trial. *Br J Haematol* 2005; **130**: 67-75 [PMID: 15982346]
21 **Gaynon PS**, Harris RE, Altman AJ, Bostrom BC, Breneman JC, Hawks R, Steele D, Zipf T, Stram DO, Villaluna D, Trigg ME. Bone marrow transplantation versus prolonged intensive chemotherapy for children with acute lymphoblastic leukemia and an initial bone marrow relapse within 12 months of the completion of primary therapy: Children's Oncology Group study CCG-1941. *J Clin Oncol* 2006; **24**: 3150-3156 [PMID: 16717292]
22 **Reismüller B**, Attarbaschi A, Peters C, Dworzak MN, Pötschger U, Urban C, Fink FM, Meister B, Schmitt K, Dieckmann K, Henze G, Haas OA, Gadner H, Mann G. Long-term outcome of initially homogenously treated and relapsed childhood acute lymphoblastic leukaemia in Austria--a population-based report of the Austrian Berlin-Frankfurt-Münster (BFM) Study Group. *Br J Haematol* 2009; **144**: 559-570 [PMID: 19077160 DOI: 10.1111/j.1365-2141.2008.07499.x]
23 **Smith MA**, Seibel NL, Altekruse SF, Ries LA, Melbert DL, O'Leary M, Smith FO, Reaman GH. Outcomes for children and adolescents with cancer: challenges for the twenty-first century. *J Clin Oncol* 2010; **28**: 2625-2634 [PMID: 20404250 DOI: 10.1200/JCO.2009.27.0421]
24 **Einsiedel HG**, von Stackelberg A, Hartmann R, Fengler R, Schrappe M, Janka-Schaub G, Mann G, Hählen K, Göbel U, Klingebiel T, Ludwig WD, Henze G. Long-term outcome in children with relapsed ALL by risk-stratified salvage therapy: results of trial acute lymphoblastic leukemia-relapse study of the Berlin-Frankfurt-Münster Group 87. *J Clin Oncol* 2005; **23**: 7942-7950 [PMID: 16258094]
25 **Rivera GK**, Zhou Y, Hancock ML, Gajjar A, Rubnitz J, Ribeiro RC, Sandlund JT, Hudson M, Relling M, Evans WE, Pui CH. Bone marrow recurrence after initial intensive treatment for childhood acute lymphoblastic leukemia. *Cancer* 2005; **103**: 368-376 [PMID: 15599932]
26 **Tallen G**, Ratei R, Mann G, Kaspers G, Niggli F, Karachunsky A, Ebell W, Escherich G, Schrappe M, Klingebiel T, Fengler R, Henze G, von Stackelberg A. Long-term outcome in children with relapsed acute lymphoblastic leukemia after time-point and site-of-relapse stratification and intensified short-course multidrug chemotherapy: results of trial ALL-REZ BFM 90. *J Clin Oncol* 2010; **28**: 2339-2347 [PMID: 20385996 DOI: 10.1200/JCO.2009.25.1983]
27 **Reismüller B**, Peters C, Dworzak MN, Pötschger U, Urban C, Meister B, Schmitt K, Dieckmann K, Gadner H, Attarbaschi A, Mann G. Outcome of children and adolescents with a second or third relapse of acute lymphoblastic leukemia (ALL): a population-based analysis of the Austrian ALL-BFM (Berlin-Frankfurt-Münster) study group. *J Pediatr Hematol Oncol* 2013; **35**: e200-e204 [PMID: 23652878 DOI: 10.1097/MPH.0b013e318290c3d6]
28 **Raetz EA**, Borowitz MJ, Devidas M, Linda SB, Hunger SP, Winick NJ, Camitta BM, Gaynon PS, Carroll WL. Reinduction platform for children with first marrow relapse of acute lymphoblastic Leukemia: A Children's Oncology Group Study[corrected]. *J Clin Oncol* 2008; **26**: 3971-3978 [PMID: 18711187 DOI: 10.1200/JCO.2008.16.1414]
29 **Raetz EA**, Bhatla T. Where do we stand in the treatment of relapsed acute lymphoblastic leukemia? *Hematology Am Soc Hematol Educ Program* 2012; **2012**: 129-136 [PMID: 23233571 DOI: 10.1182/asheducation-2012.1.129]
30 **Parker C**, Waters R, Leighton C, Hancock J, Sutton R, Moorman AV, Ancliff P, Morgan M, Masurekar A, Goulden N, Green N, Révész T, Darbyshire P, Love S, Saha V. Effect of mitoxantrone on outcome of children with first relapse of acute lymphoblastic leukaemia (ALL R3): an open-label randomised trial. *Lancet* 2010; **376**: 2009-2017 [PMID: 21131038 DOI: 10.1016/S0140-6736(10)62002-8]
31 **Eckert C**, von Stackelberg A, Seeger K, Groeneveld TW, Peters C, Klingebiel T, Borkhardt A, Schrappe M, Escherich G, Henze G. Minimal residual disease after induction is the strongest predictor of prognosis in intermediate risk relapsed acute lymphoblastic leukaemia - long-term results of trial ALL-REZ BFM P95/96. *Eur J Cancer* 2013; **49**: 1346-1355 [PMID: 23265714 DOI: 10.1016/j.ejca.2012.11.010]
32 **Chessells JM**, Veys P, Kempski H, Henley P, Leiper A, Webb D, Hann IM. Long-term follow-up of relapsed

childhood acute lymphoblastic leukaemia. *Br J Haematol* 2003; **123**: 396-405 [PMID: 14616997]
33 **Saarinen-Pihkala UM**, Heilmann C, Winiarski J, Glomstein A, Abrahamsson J, Arvidson J, Békássy AN, Forestier E, Jonmundsson G, Schroeder H, Vettenranta K, Wesenberg F, Gustafsson G. Pathways through relapses and deaths of children with acute lymphoblastic leukemia: role of allogeneic stem-cell transplantation in Nordic data. *J Clin Oncol* 2006; **24**: 5750-5762 [PMID: 17179109]
34 **Malempati S**, Gaynon PS, Sather H, La MK, Stork LC. Outcome after relapse among children with standard-risk acute lymphoblastic leukemia: Children's Oncology Group study CCG-1952. *J Clin Oncol* 2007; **25**: 5800-5807 [PMID: 18089878]
35 **Lawson SE**, Harrison G, Richards S, Oakhill A, Stevens R, Eden OB, Darbyshire PJ. The UK experience in treating relapsed childhood acute lymphoblastic leukaemia: a report on the medical research council UKALLR1 study. *Br J Haematol* 2000; **108**: 531-543 [PMID: 10759711]
36 **Thomson B**, Park JR, Felgenhauer J, Meshinchi S, Holcenberg J, Geyer JR, Avramis V, Douglas JG, Loken MR, Hawkins DS. Toxicity and efficacy of intensive chemotherapy for children with acute lymphoblastic leukemia (ALL) after first bone marrow or extramedullary relapse. *Pediatr Blood Cancer* 2004; **43**: 571-579 [PMID: 15382275]
37 **Bhojwani D**, Pui CH. Relapsed childhood acute lymphoblastic leukaemia. *Lancet Oncol* 2013; **14**: e205-e217 [PMID: 23639321 DOI: 10.1016/S1470-2045(12)70580-6]
38 **van den Berg H**, de Groot-Kruseman HA, Damen-Korbijn CM, de Bont ES, Schouten-van Meeteren AY, Hoogerbrugge PM. Outcome after first relapse in children with acute lymphoblastic leukemia: a report based on the Dutch Childhood Oncology Group (DCOG) relapse all 98 protocol. *Pediatr Blood Cancer* 2011; **57**: 210-216 [PMID: 21337680 DOI: 10.1002/pbc.22946]
39 **von Stackelberg A**, Hartmann R, Bührer C, Fengler R, Janka-Schaub G, Reiter A, Mann G, Schmiegelow K, Ratei R, Klingebiel T, Ritter J, Henze G. High-dose compared with intermediate-dose methotrexate in children with a first relapse of acute lymphoblastic leukemia. *Blood* 2008; **111**: 2573-2580 [PMID: 18089849]
40 **Barredo JC**, Devidas M, Lauer SJ, Billett A, Marymont M, Pullen J, Camitta B, Winick N, Carroll W, Ritchey AK. Isolated CNS relapse of acute lymphoblastic leukemia treated with intensive systemic chemotherapy and delayed CNS radiation: a pediatric oncology group study. *J Clin Oncol* 2006; **24**: 3142-3149 [PMID: 16809737]
41 **Hagedorn N**, Acquaviva C, Fronkova E, von Stackelberg A, Barth A, zur Stadt U, Schrauder A, Trka J, Gaspar N, Seeger K, Henze G, Cavé H, Eckert C. Submicroscopic bone marrow involvement in isolated extramedullary relapses in childhood acute lymphoblastic leukemia: a more precise definition of "isolated" and its possible clinical implications, a collaborative study of the Resistant Disease Committee of the International BFM study group. *Blood* 2007; **110**: 4022-4029 [PMID: 17720883]
42 **Borgmann A**, von Stackelberg A, Hartmann R, Ebell W, Klingebiel T, Peters C, Henze G. Unrelated donor stem cell transplantation compared with chemotherapy for children with acute lymphoblastic leukemia in a second remission: a matched-pair analysis. *Blood* 2003; **101**: 3835-3839 [PMID: 12732501]
43 **Kolb EA**, Steinherz PG. A new multidrug reinduction protocol with topotecan, vinorelbine, thiotepa, dexamethasone, and gemcitabine for relapsed or refractory acute leukemia. *Leukemia* 2003; **17**: 1967-1972 [PMID: 14513046]
44 **Pui CH**, Howard SC. Current management and challenges of malignant disease in the CNS in paediatric leukaemia. *Lancet Oncol* 2008; **9**: 257-268 [PMID: 18308251]
45 **Yang JJ**, Bhojwani D, Yang W, Cai X, Stocco G, Crews K, Wang J, Morrison D, Devidas M, Hunger SP, Willman CL, Raetz EA, Pui CH, Evans WE, Relling MV, Carroll WL. Genome-wide copy number profiling reveals molecular evolution from diagnosis to relapse in childhood acute lymphoblastic leukemia. *Blood* 2008; **112**: 4178-4183 [PMID: 18768390 DOI: 10.1182/blood-2008-06-165027]
46 **Mullighan CG**, Phillips LA, Su X, Ma J, Miller CB, Shurtleff SA, Downing JR. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science* 2008; **322**: 1377-1380 [PMID: 19039135 DOI: 10.1126/science.1164266]
47 **Bailey LC**, Lange BJ, Rheingold SR, Bunin NJ. Bone-marrow relapse in paediatric acute lymphoblastic leukaemia. *Lancet Oncol* 2008; **9**: 873-883 [PMID: 18760243 DOI: 10.1016/S1470-2045(08)70229-8]
48 **Bhojwani D**, Kang H, Moskowitz NP, Min DJ, Lee H, Potter JW, Davidson G, Willman CL, Borowitz MJ, Belitskaya-Levy I, Hunger SP, Raetz EA, Carroll WL. Biologic pathways associated with relapse in childhood acute lymphoblastic leukemia: a Children's Oncology Group study. *Blood* 2006; **108**: 711-717 [PMID: 16822902]
49 **Hogan LE**, Meyer JA, Yang J, Wang J, Wong N, Yang W, Condos G, Hunger SP, Raetz E, Saffery R, Relling MV, Bhojwani D, Morrison DJ, Carroll WL. Integrated genomic analysis of relapsed childhood acute lymphoblastic leukemia reveals therapeutic strategies. *Blood* 2011; **118**: 5218-5226 [PMID: 21921043 DOI: 10.1182/blood-2011-04-345595]
50 **Ford AM**, Fasching K, Panzer-Grümayer ER, Koenig M, Haas OA, Greaves MF. Origins of "late" relapse in childhood acute lymphoblastic leukemia with TEL-AML1 fusion genes. *Blood* 2001; **98**: 558-564 [PMID: 11468150]
51 **Zuna J**, Ford AM, Peham M, Patel N, Saha V, Eckert C, Köchling J, Panzer-Grümayer R, Trka J, Greaves M. TEL deletion analysis supports a novel view of relapse in childhood acute lymphoblastic leukemia. *Clin Cancer Res* 2004; **10**: 5355-5360 [PMID: 15328172]
52 **Staal FJ**, de Ridder D, Szczepanski T, Schonewille T, van der Linden EC, van Wering ER, van der Velden VH, van Dongen JJ. Genome-wide expression analysis of paired diagnosis-relapse samples in ALL indicates involvement of pathways related to DNA replication, cell cycle and DNA repair, independent of immune phenotype. *Leukemia* 2010; **24**: 491-499 [PMID: 20072147 DOI: 10.1038/leu.2009.286]
53 **Szczepanski T**, van der Velden VH, Waanders E, Kuiper RP, Van Vlierberghe P, Gruhn B, Eckert C, Panzer-Grümayer R, Basso G, Cavé H, Stadt UZ, Campana D, Schrauder A, Sutton R, van Wering E, Meijerink JP, van Dongen JJ. Late recurrence of childhood T-cell acute lymphoblastic leukemia frequently represents a second leukemia rather than a relapse: first evidence for genetic predisposition. *J Clin Oncol* 2011; **29**: 1643-1649 [PMID: 21357790 DOI: 10.1200/JCO.2010.30.2877]
54 **Mullighan CG**, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, Ma J, Liu W, Cheng C, Schulman BA, Harvey RC, Chen IM, Clifford RJ, Carroll WL, Reaman G, Bowman WP, Devidas M, Gerhard DS, Yang W, Relling MV, Shurtleff SA, Campana D, Borowitz MJ, Pui CH, Smith M, Hunger SP, Willman CL, Downing JR. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med* 2009; **360**: 470-480 [PMID: 19129520 DOI: 10.1056/NEJMoa0808253]
55 **Mullighan CG**, Zhang J, Harvey RC, Collins-Underwood JR, Schulman BA, Phillips LA, Tasian SK, Loh ML, Su X, Liu W, Devidas M, Atlas SR, Chen IM, Clifford RJ, Gerhard DS, Carroll WL, Reaman GH, Smith M, Downing JR, Hunger SP, Willman CL. JAK mutations in high-risk childhood acute lymphoblastic leukemia. *Proc Natl Acad Sci USA* 2009; **106**: 9414-9418 [PMID: 19470474 DOI: 10.1073/pnas.0811761106]
56 **Harvey RC**, Mullighan CG, Chen IM, Wharton W, Mikhail FM, Carroll AJ, Kang H, Liu W, Dobbin KK, Smith MA, Carroll WL, Devidas M, Bowman WP, Camitta BM, Reaman GH, Hunger SP, Downing JR, Willman CL. Rearrangement of CRLF2 is associated with mutation of JAK kinases,

alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. *Blood* 2010; **115**: 5312-5321 [PMID: 20139093 DOI: 10.1182/blood-2009-09-245944]

57 **Eapen M**, Raetz E, Zhang MJ, Muehlenbein C, Devidas M, Abshire T, Billett A, Homans A, Camitta B, Carroll WL, Davies SM. Outcomes after HLA-matched sibling transplantation or chemotherapy in children with B-precursor acute lymphoblastic leukemia in a second remission: a collaborative study of the Children's Oncology Group and the Center for International Blood and Marrow Transplant Research. *Blood* 2006; **107**: 4961-4967 [PMID: 16493003]

58 **Bader P**, Hancock J, Kreyenberg H, Goulden NJ, Niethammer D, Oakhill A, Steward CG, Handgretinger R, Beck JF, Klingebiel T. Minimal residual disease (MRD) status prior to allogeneic stem cell transplantation is a powerful predictor for post-transplant outcome in children with ALL. *Leukemia* 2002; **16**: 1668-1672 [PMID: 12200679]

59 **Knechtli CJ**, Goulden NJ, Hancock JP, Grandage VL, Harris EL, Garland RJ, Jones CG, Rowbottom AW, Hunt LP, Green AF, Clarke E, Lankester AW, Cornish JM, Pamphilon DH, Steward CG, Oakhill A. Minimal residual disease status before allogeneic bone marrow transplantation is an important determinant of successful outcome for children and adolescents with acute lymphoblastic leukemia. *Blood* 1998; **92**: 4072-4079 [PMID: 9834212]

60 **Coustan-Smith E**, Gajjar A, Hijiya N, Razzouk BI, Ribeiro RC, Rivera GK, Rubnitz JE, Sandlund JT, Andreansky M, Hancock ML, Pui CH, Campana D. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia after first relapse. *Leukemia* 2004; **18**: 499-504 [PMID: 14981525]

61 **Bader P**, Kreyenberg H, Henze GH, Eckert C, Reising M, Willasch A, Barth A, Borkhardt A, Peters C, Handgretinger R, Sykora KW, Holter W, Kabisch H, Klingebiel T, von Stackelberg A. Prognostic value of minimal residual disease quantification before allogeneic stem-cell transplantation in relapsed childhood acute lymphoblastic leukemia: the ALL-REZ BFM Study Group. *J Clin Oncol* 2009; **27**: 377-384 [PMID: 19064980 DOI: 10.1200/JCO.2008.17.6065]

62 **Paganin M**, Zecca M, Fabbri G, Polato K, Biondi A, Rizzari C, Locatelli F, Basso G. Minimal residual disease is an important predictive factor of outcome in children with relapsed 'high-risk' acute lymphoblastic leukemia. *Leukemia* 2008; **22**: 2193-2200 [PMID: 18754029 DOI: 10.1038/leu.2008.227]

63 **Leung W**, Pui CH, Coustan-Smith E, Yang J, Pei D, Gan K, Srinivasan A, Hartford C, Triplett BM, Dallas M, Pillai A, Shook D, Rubnitz JE, Sandlund JT, Jeha S, Inaba H, Ribeiro RC, Handgretinger R, Laver JH, Campana D. Detectable minimal residual disease before hematopoietic cell transplantation is prognostic but does not preclude cure for children with very-high-risk leukemia. *Blood* 2012; **120**: 468-472 [PMID: 22517895 DOI: 10.1182/blood-2012-02-409813]

64 **Pichler H**, Reismüller B, Steiner M, Dworzak MN, Pötschger U, Urban C, Meister B, Schmitt K, Panzer-Grümayer R, Haas OA, Attarbaschi A, Mann G. The inferior prognosis of adolescents with acute lymphoblastic leukaemia (ALL) is caused by a higher rate of treatment-related mortality and not an increased relapse rate--a population-based analysis of 25 years of the Austrian ALL-BFM (Berlin-Frankfurt-Münster) Study Group. *Br J Haematol* 2013; **161**: 556-565 [PMID: 23480776 DOI: 10.1111/bjh.12292]

65 **Aricò M**, Schrappe M, Hunger SP, Carroll WL, Conter V, Galimberti S, Manabe A, Saha V, Baruchel A, Vettenranta K, Horibe K, Benoit Y, Pieters R, Escherich G, Silverman LB, Pui CH, Valsecchi MG. Clinical outcome of children with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukemia treated between 1995 and 2005. *J Clin Oncol* 2010; **28**: 4755-4761 [PMID: 20876426 DOI: 10.1200/JCO.2010.30.1325]

66 **Abshire TC**, Pollock BH, Billett AL, Bradley P, Buchanan GR. Weekly polyethylene glycol conjugated L-asparaginase compared with biweekly dosing produces superior induction remission rates in childhood relapsed acute lymphoblastic leukemia: a Pediatric Oncology Group Study. *Blood* 2000; **96**: 1709-1715 [PMID: 10961868]

67 **von Stackelberg A**, Völzke E, Kühl JS, Seeger K, Schrauder A, Escherich G, Henze G, Tallen G. Outcome of children and adolescents with relapsed acute lymphoblastic leukaemia and non-response to salvage protocol therapy: a retrospective analysis of the ALL-REZ BFM Study Group. *Eur J Cancer* 2011; **47**: 90-97 [PMID: 20970323 DOI: 10.1016/j.ejca.2010.09.020]

68 **Dini G**, Zecca M, Balduzzi A, Messina C, Masetti R, Fagioli F, Favre C, Rabusin M, Porta F, Biral E, Ripaldi M, Iori AP, Rognoni C, Prete A, Locatelli F. No difference in outcome between children and adolescents transplanted for acute lymphoblastic leukemia in second remission. *Blood* 2011; **118**: 6683-6690 [PMID: 22010101]

69 **Harrison G**, Richards S, Lawson S, Darbyshire P, Pinkerton R, Stevens R, Oakhill A, Eden OB. Comparison of allogeneic transplant versus chemotherapy for relapsed childhood acute lymphoblastic leukaemia in the MRC UKALL R1 trial. MRC Childhood Leukaemia Working Party. *Ann Oncol* 2000; **11**: 999-1006 [PMID: 11038037]

70 **Smith AR**, Baker KS, Defor TE, Verneris MR, Wagner JE, Macmillan ML. Hematopoietic cell transplantation for children with acute lymphoblastic leukemia in second complete remission: similar outcomes in recipients of unrelated marrow and umbilical cord blood versus marrow from HLA matched sibling donors. *Biol Blood Marrow Transplant* 2009; **15**: 1086-1093 [PMID: 19660721 DOI: 10.1016/j.bbmt.2009.05.005]

71 **Chen X**, Hale GA, Barfield R, Benaim E, Leung WH, Knowles J, Horwitz EM, Woodard P, Kasow K, Yusuf U, Behm FG, Hayden RT, Shurtleff SA, Turner V, Srivastava DK, Handgretinger R. Rapid immune reconstitution after a reduced-intensity conditioning regimen and a CD3-depleted haploidentical stem cell graft for paediatric refractory haematological malignancies. *Br J Haematol* 2006; **135**: 524-532 [PMID: 17010105]

72 **Ruggeri L**, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, Stern M, Pende D, Perruccio K, Burchielli E, Topini F, Bianchi E, Aversa F, Martelli MF, Velardi A. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood* 2007; **110**: 433-440 [PMID: 17371948]

73 **Pende D**, Marcenaro S, Falco M, Martini S, Bernardo ME, Montagna D, Romeo E, Cognet C, Martinetti M, Maccario R, Mingari MC, Vivier E, Moretta L, Locatelli F, Moretta A. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* 2009; **113**: 3119-3129 [PMID: 18945967 DOI: 10.1182/blood-2008-06-164103]

74 **Klingebiel T**, Cornish J, Labopin M, Darbyshire P, Handgretinger R, Balduzzi A, Owoc-Lempach J, Fagioli F, Or R, Peters C, Aversa F, Polge E, Dini G, Rocha V; Pediatric Diseases and Acute Leukemia Working Parties of the European Group for Blood and Marrow Transplantation (EBMT). Results and factors influencing outcome after fully haploidentical hematopoietic stem cell transplantation in children with very high-risk acute lymphoblastic leukemia: impact of center size: an analysis on behalf of the Acute Leukemia and Pediatric Disease Working Parties of the European Blood and Marrow Transplant group. *Blood* 2010; **115:** 3437-3446 [DOI: 10.1182/blood-2009-03-207001]

75 **Lang P**, Handgretinger R. Haploidentical SCT in children:

an update and future perspectives. *Bone Marrow Transplant* 2008; **42** Suppl 2: S54-S59 [PMID: 18978746 DOI: 10.1038/bmt.2008.285]

76 **Hahn T**, Wall D, Camitta B, Davies S, Dillon H, Gaynon P, Larson RA, Parsons S, Seidenfeld J, Weisdorf D, McCarthy PL. The role of cytotoxic therapy with hematopoietic stem cell transplantation in the therapy of acute lymphoblastic leukemia in children: an evidence-based review. *Biol Blood Marrow Transplant* 2005; **11**: 823-861 [PMID: 16275588]

77 **Harker-Murray PD**, Thomas AJ, Wagner JE, Weisdorf D, Luo X, DeFor TE, Verneris MR, Dusenbery KE, MacMillan ML, Tolar J, Baker KS, Orchard PJ. Allogeneic hematopoietic cell transplantation in children with relapsed acute lymphoblastic leukemia isolated to the central nervous system. *Biol Blood Marrow Transplant* 2008; **14**: 685-692 [PMID: 18489994 DOI: 10.1016/j.bbmt.2008.03.011]

78 **Hunger SP**, Loh ML, Whitlock JA, Winick NJ, Carroll WL, Devidas M, Raetz EA. Children's Oncology Group's 2013 blueprint for research: acute lymphoblastic leukemia. *Pediatr Blood Cancer* 2013; **60**: 957-963 [PMID: 23255467 DOI: 10.1002/pbc.24420]

79 **Handgretinger R**, Zugmaier G, Henze G, Kreyenberg H, Lang P, von Stackelberg A. Complete remission after blinatumomab-induced donor T-cell activation in three pediatric patients with post-transplant relapsed acute lymphoblastic leukemia. *Leukemia* 2011; **25**: 181-184 [PMID: 20944674 DOI: 10.1038/leu.2010.239]

80 **Fry TJ**, Mackall CL. T-cell adoptive immunotherapy for acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program* 2013; **2013**: 348-353 [PMID: 24319203 DOI: 10.1182/asheducation-2013.1.348]

81 **Jeha S**. Recent progress in the treatment of acute lymphoblastic leukemia: clofarabine. *Hematol Oncol Clin North Am* 2009; **23**: 1137-1144, viii [PMID: 19825457 DOI: 10.1016/j.hoc.2009.07.011]

82 **O'Connor D**, Sibson K, Caswell M, Connor P, Cummins M, Mitchell C, Motwani J, Taj M, Vora A, Wynn R, Kearns PR. Early UK experience in the use of clofarabine in the treatment of relapsed and refractory paediatric acute lymphoblastic leukaemia. *Br J Haematol* 2011; **154**: 482-485 [PMID: 21689087 DOI: 10.1111/j.1365-2141.2011.08752.x]

83 **Dunsmore KP**, Devidas M, Linda SB, Borowitz MJ, Winick N, Hunger SP, Carroll WL, Camitta BM. Pilot study of nelarabine in combination with intensive chemotherapy in high-risk T-cell acute lymphoblastic leukemia: a report from the Children's Oncology Group. *J Clin Oncol* 2012; **30**: 2753-2759 [PMID: 22734022 DOI: 10.1200/JCO.2011.40.8724]

84 **Raetz EA**, Cairo MS, Borowitz MJ, Blaney SM, Krailo MD, Leil TA, Reid JM, Goldenberg DM, Wegener WA, Carroll WL, Adamson PC. Chemoimmunotherapy reinduction with epratuzumab in children with acute lymphoblastic leukemia in marrow relapse: a Children's Oncology Group Pilot Study. *J Clin Oncol* 2008; **26**: 3756-3762 [PMID: 18669463 DOI: 10.1200/JCO.2007.15.3528]

85 **Raetz EA**, Cairo MS, Borowitz MJ, Lu XM, Devidas M, Reid JM, Goldenberg DM, Wegener WA, Whitlock JA, Adamson PC, Hunger SP, Carroll WL Reinduction chemoimmunotherapy with epratuzumab in relapsed acute lymphoblastic leukemia (ALL) in children, adolescents, and young adults: Results from Children's Oncology Group (COG) study ADVL04P2. *Blood* (ASH Annual Meeting Abstracts) 2011; **118**: 573a

86 **Messinger Y**, Gaynon P, Raetz E, Hutchinson R, Dubois S, Glade-Bender J, Sposto R, van der Giessen J, Eckroth E, Bostrom BC. Phase I study of bortezomib combined with chemotherapy in children with relapsed childhood acute lymphoblastic leukemia (ALL): a report from the therapeutic advances in childhood leukemia (TACL) consortium. *Pediatr Blood Cancer* 2010; **55**: 254-259 [PMID: 20582937 DOI: 10.1002/pbc.22456]

87 **Messinger YH**, Gaynon PS, Sposto R, van der Giessen J, Eckroth E, Malvar J, Bostrom BC. Bortezomib with chemotherapy is highly active in advanced B-precursor acute lymphoblastic leukemia: Therapeutic Advances in Childhood Leukemia & amp; Lymphoma (TACL) Study. *Blood* 2012; **120**: 285-290 [PMID: 22653976 DOI: 10.1182/blood-2012-04-418640]

88 **Fuster JL**, Bermúdez M, Galera A, Llinares ME, Calle D, Ortuño FJ. Imatinib mesylate in combination with chemotherapy in four children with de novo and advanced stage Philadelphia chromosome-positive acute lymphoblastic leukemia. *Haematologica* 2007; **92**: 1723-1724 [PMID: 18056006]

89 **Millot F**, Cividin M, Brizard F, Chomel JC, Méchinaud F, Guilhot F. Successful second allogeneic stem cell transplantation in second remission induced by dasatinib in a child with Philadelphia chromosome positive acute lymphoblastic leukemia. *Pediatr Blood Cancer* 2009; **52**: 891-892 [PMID: 19202569 DOI: 10.1002/pbc.21938]

90 **Chillón MC**, Gómez-Casares MT, López-Jorge CE, Rodriguez-Medina C, Molines A, Sarasquete ME, Alcoceba M, Miguel JD, Bueno C, Montes R, Ramos F, Rodríguez JN, Giraldo P, Ramírez M, García-Delgado R, Fuster JL, González-Díaz M, Menendez P. Prognostic significance of FLT3 mutational status and expression levels in MLL-AF4+ and MLL-germline acute lymphoblastic leukemia. *Leukemia* 2012; **26**: 2360-2366 [PMID: 22705992]

91 **Dodero A**, Carniti C, Raganato A, Vendramin A, Farina L, Spina F, Carlo-Stella C, Di Terlizzi S, Milanesi M, Longoni P, Gandola L, Lombardo C, Corradini P. Haploidentical stem cell transplantation after a reduced-intensity conditioning regimen for the treatment of advanced hematologic malignancies: posttransplantation CD8-depleted donor lymphocyte infusions contribute to improve T-cell recovery. *Blood* 2009; **113**: 4771-4779 [PMID: 19211934 DOI: 10.1182/blood-2008-10-183723]

92 **Bethge WA**, Hegenbart U, Stuart MJ, Storer BE, Maris MB, Flowers ME, Maloney DG, Chauncey T, Bruno B, Agura E, Forman SJ, Blume KG, Niederwieser D, Storb R, Sandmaier BM. Adoptive immunotherapy with donor lymphocyte infusions after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. *Blood* 2004; **103**: 790-795 [PMID: 14525766]

93 **Peggs KS**, Mackinnon S. Cellular therapy: donor lymphocyte infusion. *Curr Opin Hematol* 2001; **8**: 349-354 [PMID: 11604574]

94 **Grupp SA**, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* 2013; **368**: 1509-1518 [PMID: 23527958 DOI: 10.1056/NEJMoa1215134]

P- Reviewer: Behfar M, Imashuku S, Ramirez M, Shimoni A
S- Editor: Ji FF **L- Editor**: A **E- Editor:** Lu YJ

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
doi:10.5315/wjh.v3.i3.71

World J Hematol 2014 August 6; 3(3): 71-84
ISSN 2218-6204 (online)

REVIEW

Hemophagocytic lymphohistiocytosis: Recent progress in the pathogenesis, diagnosis and treatment

Shinsaku Imashuku

Shinsaku Imashuku, the Department of Laboratory Medicine, Uji-Tokushukai Medical Center, Kyoto 611-0042, Japan
Author contributions: Imashuku S contributed to this paper.
Correspondence to: Shinsaku Imashuku, MD, PhD, Consultant to the Department of Laboratory Medicine, Uji-Tokushukai Medical Center, 86 Kasugamori, Ogura-cho, Uji, Kyoto 611-0042, Japan. shinim95@mbox.kyoto-inet.or.jp
Telephone: +81-774-201111 Fax: +81-774-202336
Received: March 9, 2014 Revised: May 9, 2014
Accepted: June 18, 2014
Published online: August 6, 2014

Abstract

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome that develops as a primary (familial/hereditary) or secondary (non-familial/hereditary) disease characterized in the majority of the cases by hereditary or acquired impaired cytotoxic T-cell (CTL) and natural killer responses. The molecular mechanisms underlying impaired immune homeostasis have been clarified, particularly for primary diseases. Familial HLH (familial hemophagocytic lymphohistiocytosis type 2-5, Chediak-Higashi syndrome, Griscelli syndrome type 2, Hermansky-Pudlak syndrome type 2) develops due to a defect in lytic granule exocytosis, impairment of (signaling lymphocytic activation molecule)-associated protein, which plays a key role in CTL activity [*e.g.*, X-linked lymphoproliferative syndrome (XLP) 1], or impairment of X-linked inhibitor of apoptosis, a potent regulator of lymphocyte homeostasis (*e.g.*, XLP2). The development of primary HLH is often triggered by infections, but not in all. Secondary HLH develops in association with infection, autoimmune diseases/rheumatological conditions and malignancy. The molecular mechanisms involved in secondary HLH cases remain unknown and the pathophysiology is not the same as primary HLH. For either primary or secondary HLH cases, immunosuppressive therapy should be given to control the hypercytokinemia with steroids, cyclosporine A, or intravenous immune globulin, and if primary HLH is diagnosed, immunochemotherapy with a regimen containing etoposide or anti-thymocyte globulin should be started. Thereafter, allogeneic hematopoietic stem-cell transplantation is recommended for primary HLH or secondary refractory disease (especially EBV-HLH).

Key words: Alemtuzumab; Anti-thymocyte globulin; Cyclosporine A; Epstein-Barr virus; Etoposide; Hematopoietic stem-cell transplantation; Hemophagocytic lymphohistiocytosis; Hereditary diseases; Immunochemotherapy; Intravenous immunoglobulin; Molecular diagnosis; Rituximab; Steroids

Core tip: This review discusses the diagnostic criteria for hemophagocytic lymphohistiocytosis (HLH), the algorithms used to identify the underlying immune defects at the molecular level, and the optimal therapeutic approaches. For any HLH cases, a screening for primary HLH should be made following the diagnostic algorithm. During the process, immunosuppressive therapy should be started to control the hypercytokinemia with steroids, cyclosporine A, or intravenous immune globulin, and if primary HLH is confirmed, immunochemotherapy with a regimen containing etoposide or anti-thymocyte globulin should be given. Supportive measures to control hemorrhage/organ dysfunction are also required. In cases of primary HLH or secondary/refractory HLH, timely allogeneic hematopoietic stem cell transplantation is recommended.

Imashuku S. Hemophagocytic lymphohistiocytosis: Recent progress in the pathogenesis, diagnosis and treatment. *World J Hematol* 2014; 3(3): 71-84 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i3/71.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i3.71

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a primary (familial/hereditary) or secondary (non-familial/hereditary) hyperinflammatory and hypercytokinemic syndrome[1,2]. Immune homeostasis is maintained by regulating the proliferation and apoptosis of activated lymphocytes and their associated granule-dependent cytotoxic activity, which plays a critical role in defense against tumor cells and cells infected with viruses[3]. The hypercytokinemia conditions associated with HLH are caused by cytokine releases from activated T cells and macrophages as a result of impaired activation-induced cell death due to uncontrolled immune responses resulting from the impaired ability of cytotoxic T-cell (CTL) and natural killer (NK) cells to kill their target cells[4-6]. Thus, marked activation of macrophages occurs, which results in hypercytokinemia. Previous reviews of primary HLH identified a hereditary impairment in the molecules involved in the multistep processes of cytolysis (from cell activation to the release of perforin and granzymes)[7,8]. Indeed, primary HLH is caused by loss-of-function mutations in the genes encoding perforin and various molecules involved in the transport, fusion and exocytosis of secretory vesicles[4-8]. In other forms of HLH, particularly Epstein-Barr virus (EBV)-driven primary HLH, deficiency in SAP (which is important for CTL function) is responsible for the marked reduction in CTL and NK cell activity; however, in XIAP (which is important for apoptosis), CTL and NK cell activity are not altered[9-11]. Secondary HLH develops in apparently immunocompetent subjects; however, some of these subjects show acquired functional reductions in CTL and NK cell activity, which are associated with viral, bacterial or parasite infections, metabolic diseases, autoimmune diseases/rheumatological conditions (termed as macrophage activation syndrome; MAS), or malignancy[12-19]. Initial diagnostic work-up in the diagnosis of HLH includes the detection of the expansion of $CD8^+$ T cell subset in the peripheral blood[20,21] and the identification of the factors that trigger the development of HLH, particularly infectious agents[22,23]. In addition, for all HLH cases, molecular screening must be performed to determine whether the disease is primary or secondary[6,24,25]. The assay of NK or CTL activity[26,27] and flow cytometric analysis of molecules, such as perforin, Munc 13-4, SAP/XIAP, is essential for rapid diagnosis[6,24,25,28] (Figure 1). Age-related factors were emphasized in the past because primary HLH usually develops during the first 3 years of life; however, more recently, late-onset cases have been identified[29-33]. The diversity of clinical features associated with primary HLH has been examined using detailed genotype-phenotype analysis methods. Among infectious agents, EBV plays a major role in cases of infection-associated primary or secondary HLH. Thus, quantification of cell-free or peripheral mononuclear cell EBV-DNA levels is extremely useful for the diagnosis of EBV-HLH and chronic active EBV infection (CAEBV)-related HLH[34-36]. The HLH-94 or HLH-2004 regimens employed in many centers has led to a significant improvement in the therapeutic results due to the efficacy of a combination of immunochemotherapy and allogeneic HSCT for treating primary and secondary HLH, especially for refractory EBV-HLH[37-39]; however, the diversity of the clinical features associated with primary HLH raises questions regarding the appropriate timing of HSCT[40-48].

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Clinical features

The initial symptoms of HLH include persistent fever, hepatic and/or renal dysfunction, splenomegaly, hemorrhagic diathesis, neurological symptoms, and other features, caused by hyperinflammatory conditions[1,2,38,49-52]. The clinical features of primary and secondary cases are not significantly different; however, some types of primary HLH are associated with hypogammaglobulinemia-related symptoms (*e.g.*, FHL5 and XLP1)[45,53], enteropathy and renal tubular dysfunction due to the epithelial abnormalities in FHL5[54] and oculocutaneous albinism in patients with Griscelli syndrome type 2 (GS-2), Chediak-Higashi syndrome (CHS), and Hermansky-Pudlak syndrome type 2 (HPS-II)[55-59], although occurrence of HLH in HPS-II deficiency is limited to a single case[58]. The most ominous findings in cases of HLH are central nervous system (CNS) disease[60-62] or an association with primary or therapy-related hematological malignancies[63-65]. The HLH conditions do not show the same severity, which is determined by the type of NK deficiency[26,27] in association with the type of genetic mutations in the primary HLH[42-48], or the degree of lymphoproliferation as represented by serum soluble IL-2R levels in the secondary HLH[66]. HLH occurs in all age groups, from premature infants and neonates to the elderly, but the majority of primary HLH cases occur in early infancy. For cases occurring during the fetal and neonatal periods[67,68], pre- or post-natal molecular diagnosis is essential[69]. Primary HLH can also develop in adolescents and adults[29-33]. Thus, especially in this older age group, a molecular diagnosis is recommended to enable a definite diagnosis of primary or secondary HLH.

Triggers and underlying diseases

The most common "trigger" for HLH is infectious disease. Viral and other types of infection cause secondary HLH[13,14,22,23,49,50]. Among them, EBV-HLH and CAEBV-related HLH, which are defined by the specific diagnostic criteria[34-36], are the most common form of secondary HLH; however, infection-induced HLH also occurs in individuals with primary HLH, MAS, and malignancy. Post-organ transplant-HLH, or post-HSCT-HLH, is a distinct subtype of secondary HLH that was described recently[70-72]. Among the various malignancies, lymphoma-associated HLH (LAHS) is the most common[73-75]. Progress in molecular diagnostic techniques

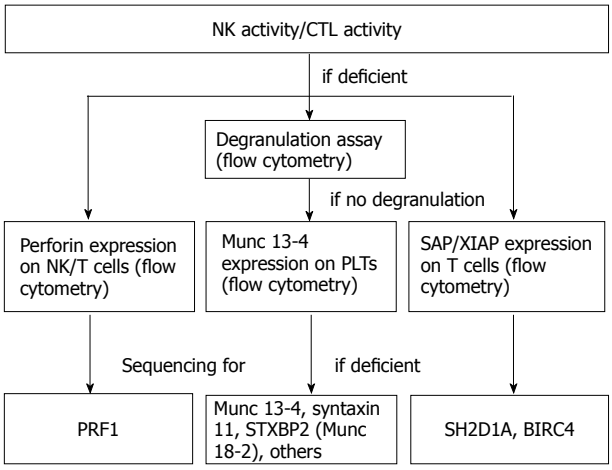


Figure 1 Diagnostic work up algorithm for a patient with primary hemophagocytic lymphohistiocytosis (familial hemophagocytic lymphohistiocytosis and X-linked lymphoproliferative syndrome)[6,22,23]. Degranulation assay is useful for familial hemophagocytic lymphohistiocytosis (FHL) type 3-5. It is advised to perform flow cytometry of peripheral blood mononuclear cells to detect the expansion of CD8+ T cells as first step of diagnosing hemophagocytic lymphohistiocytosis (HLH) prior to the work up algorism.

has led to the identification of molecular abnormality of primary HLH in cases of secondary HLH[76,77] as well as in hematological malignancies[63,64,78-80]. In addition, genotype-phenotype correlations have been identified in patients with primary HLH, particularly those associated with FHL2, FHL3, and FHL5[42-46] and with XLP[53,81-83]. It is these types of studies that identified the existence of atypical late-developing primary HLH cases in adolescents and adults[29-33], and the identification of which raises questions about how promptly HSCT should be introduced.

Laboratory findings and immunopathological features

The cardinal laboratory features associated with HLH include bicytopenia, high levels of serum ferritin, triglyceride, transaminases, lactate dehydrogenase and soluble IL-2R. Serum creatinine and BUN levels are often elevated, while plasma fibrinogen is decreased. Deficient NK activity has generally been noted[12,14-17,26,27]. Hemophagocytosis is observed on bone marrow smears or in lymph node or liver biopsies; however, the detection of hemophagocytes is not mandatory for the diagnosis. Although detection of abnormal karyotypes in bone marrow cells is rare in patients with HLH, they are occasionally detectable in cases of EBV-HLH, correlated with CAEBV[84]. Immunopathological features in HLH are characterized by uncontrolled activation of T cells, especially a significant increase in the subpopulation of CD8+ T cells with clonal expansion[20,21] and macrophages in association with overproduction of various cytokines[85,86].

Molecular genetics

The molecular defects associated with primary HLH are listed in Table 1. Molecular abnormalities have been identified in the perforin-granzyme cytotoxic molecule pathway (in FHL type 2-5, GS-2, CHS, HPS-Ⅱ), T-cell activation pathway (in XLP1), the apoptotic pathway (in XLP2), and the inducible T-cell kinase pathway (in ITK deficiency)[11,87-100]. More recently, CD27 deficiency[101,102] and magnesium transporter 1 (MAGT1) deficiency, also termed as XMEN (X-linked immunodeficiency with Mg^{2+} defect, EBV infection and neoplasm)[103], were identified. These EBV-driven ITK, or CD27 deficiency and XMEN give rise to EBV- associated lymphoproliferative disease (LPD), but does not primarily predispose to HLH; although hemophagocytosis was described in some of the cases[100,102]. These novel discoveries are expected to help elucidate the molecular mechanisms causing the inherited forms of EBV-LPD and HLH.

Table 1 List of primary hemophagocytic lymphohistiocytosis

Disease	Molecular abnormalities (chromosome location)
CTL molecule dysfunction	
Pore formation	
FHL2	Perforin (10q21-2)
Vesicle priming fusion	
FHL3	Munc13-4/Unc 13D (17q25)
FHL4	Syntaxin 11 (6q24)
FHL5	STXBP2/Munc18-2 (19p13)
Vesicle docking/trafficking	
Chediak-Higashi syndrome	LYST (1q42.1-42.2)
Griscelli syndrome, type 2	Rab27a (15q21)
Hermansky-Pudlak syndrome Ⅱ	AP-3 (3q24)
EBV-driven	
XLP1	SAP/SH2D1A (Xq25)
XLP2 (XIAP)	BIRC4 (Xq24-25)
ITK deficiency[1]	ITK (5q34)
CD27 deficiency[1]	CD27 (12p13)
XMEN[1]	MAGT1 (Xq21.1)

[1]Major clinical features in these diseases are not hemophagocytic lymphohistiocytosis (HLH) but Epstein-Barr virus (EBV)-associated lymphoproliferative disease. FHL: Familial hemophagocytic lymphohistiocytosis; XLP: X-linked lymphoproliferative disease; ITK: IL-2-inducible T cell kinase; XMEN: X-linked immunodeficiency with Mg^{2+} defect, EBV infection and neoplasia; MAGT1: Magnesium transporter 1, LYST is also called CHS1 gene. Association of hemophagocytosis was described in some cases.

Diagnosis and differential diagnosis

Achieving the definitive diagnosis of HLH is often challenging[104,105]. Currently, HLH is diagnosed according to globally accepted diagnostic criteria shown in Table 2[38]. Differential diagnoses include fulminant hepatitis or acute hepatic failure[106], severe sepsis, systemic inflammatory response syndrome, and other hyperinflammatory conditions[107]. In the differentiation of primary and secondary HLH, screening measures are employed, which include NK and CTL activity determination, degranulation assays as well as flow cytometric assay of the expression of perforin and other molecules (Figure 1)[6,24-27]. More recently, Western blot analysis was found to be useful to screen for primary HLH by detecting FHL-related proteins in platelets[28]. An accurate diagnosis is made by performing mutation analysis of the genes

Table 2 Diagnostic guidelines for hemophagocytic lymphohistiocytosis[38]

The diagnosis of HLH can be established if one of either (1) or (2) below is fulfilled	
(1) A molecular diagnosis consistent with HLH	
(2) Clinical diagnostic criteria fulfilled for 5 out of the 8 criteria below	
Clinical criteria	1 fever
	2 splenomegaly
Routine laboratory criteria	3 bicytopenia (Hb < 90 g/L, platelets < 100×10^9/L, neutrophils < 1.0×10^9/L)
	4 Hypertriglyceridemia (> 3.0 mmol/L) and/or hypofibrinogenemia (< 1.5 g/L)
Specific histopathological/marker criteria	5 hemophagocytosis
	6 low or absent NK cell activity
	7 hyperferritinemia (> 500 μg/L)
	8 hyper-sIL-2R-nemia (> 2400 U/mL)

HLH: Hemophagocytic lymphohistiocytosis; NK: Natural killer.

responsible for these hereditary diseases. EBV-HLH is diagnosed using a combination of HLH diagnostic criteria and EBV-specific data (*i.e.*, the number of EBV-DNA copies and antibody expression patterns in the serum)[34-36]. Although the majority of EBV-HLH cases in Asia are thought to be secondary HLH, molecular and genetic analyses need to be performed to determine whether they are in fact primary HLH, particularly in patients with refractory EBV-HLH[6,44,81-83,99-103]. In Europe, some patients with FHL3 or FHL5 presented with clinical features suggestive of CAEBV-related HLH[44]. Also, since the risk of malignancy is high in the condition of CTL dysfunction, patients presenting with hematological malignancies could be searched for primary HLH-related gene mutations[78-80].

Prognostic factors and clinical outcome

The ultimate treatment goal of HLH is to have disease-free survival without CNS sequelae and treatment-related acute myeloid leukemia (t-AML). The outcome of HLH depends on the severity of clinical features at the onset and types of HLH (primary or secondary). In particular, primary HLH, refractory EBV-HLH and LAHS without treatment have a poor outcome. In principle, primary HLH cases are fatal if HSCT is not performed[108]. In refractory secondary HLH, immunotherapy may not be curative, when the patients require both salvage chemotherapy and HSCT (Figure 2). Preferably, it is essential to perform HSCT before the development of CNS disease or of t-AML. In the prognostic analysis of HLH, it was found that after initial treatment, death during the acute phase occurs in 10%-15% of patients, usually due to life-threatening infections, hemorrhage, and/or irreversible organ dysfunction[37,109,110]. Death at the later stages of treatment is often due to reactivation of the disease and adverse effects associated with HSCT[109,110]. These data indicate the requirement of improved outcome of HSCT in the treatment of HLH. Although late onset cases of primary HLH are believed to carry a better prognosis, there is a report that adolescents and young adults with HLH who undergo allogeneic HSCT are at increased risk of mortality compared to younger patients[111]. The factors suggestive of a poor prognosis for those with EBV-HLH are summarized in Table 3.

Table 3 Poor prognostic factors in Epstein-Barr virus-hemophagocytic lymphohistiocytosis[34]

Persistent increase of cell-free EBV genome copies
Chromosome abnormality
Correlation with chronic active EBV infection (CAEBV)[1]
In association with primary HLH
Severe organ dysfunction, such as renal failure, CNS hemorrhage
Choice of treatment, such as timing of etoposide use, HSCT

[1]CAEBV is often associated with 1 and 2 of the above. CNS: Central nervous system; HSCT: Hematopoietic stem cell transplantation; EBV: Epstein-Barr virus; HLH: Hemophagocytic lymphohistiocytosis.

TREATMENT OF HLH

General considerations and supportive therapy

Any patients with HLH can be treated first with immunosuppressive regimens designed to control the hypercytokinemia and hyperinflammation. Such treatments include steroids (prednisolone or dexamethasone), cyclosporine A (CSA), or intravenous immune globulin (IVIG). During the initial period of therapy, finding out the triggering factors and underlying diseases as well as molecular diagnostic analyses are recommended to determine familial or non-familial diseases (Figure 1). If confirmed, primary HLH is similarly treatable with HLH-directed immunochemotherapy[6,37-41,112-116]. On the other hand, if apparent infection-triggered HLH is confirmed, rigorous treatment of any identified infectious agents is important. For any secondary HLH, application of treatment should aim to target the underlying diseases. Patients with very severe cases of HLH requiring hemodynamic and respiratory support are treated in the intensive care unit. Inotropic agents are life-saving for those that are hemodynamically unstable[117,118]. Antibacterial or antifungal agents are also required to treat opportunistic infections due to HLH-related neutropenia. Because severe thrombocytopenia and coagulopathy are both life-threatening conditions, the patient may require infusions of concentrated platelets, fresh frozen plasma, fibrinogen, and recombinant thrombomodulin[49,51,119]. Although there is no definite consensus on its benefit, plasma exchange or exchange transfusion may be used to treat the hypercytokinemia and reduce the hemorrhagic tendency during the initial treatment phase[120,121]. The addition of acyclovir to the therapeutic regimen for those with EBV-HLH is not thought to be beneficial because there is no objective evidence showing a clinical improvement using this drug[122]. However, acyclovir is useful for treating neonatal herpes simplex virus (HSV)-HLH in infancy[49,123,124]. Indeed, a combination of high-dose acyclovir, steroid pulse therapy, IVIG, and blood

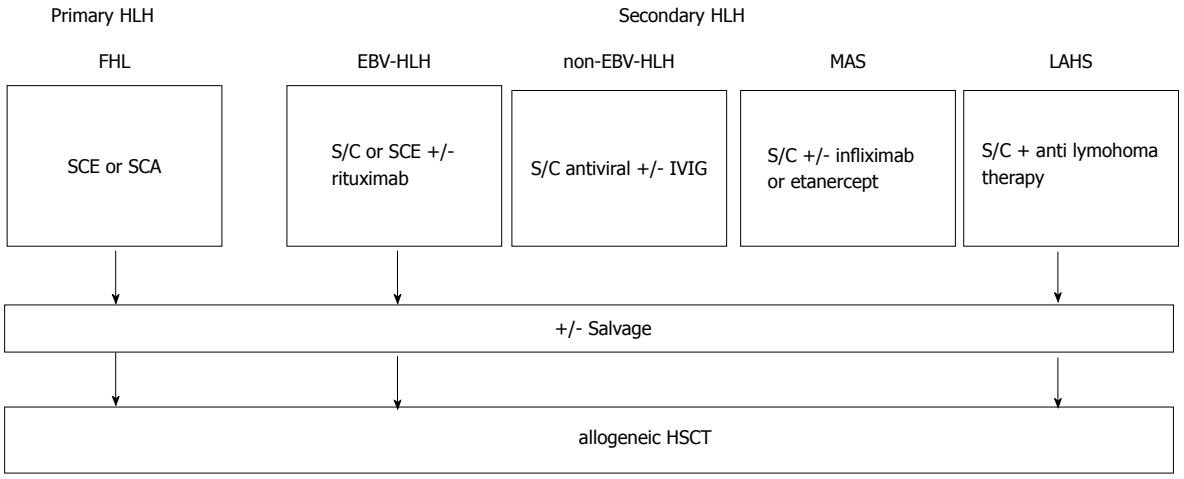


Figure 2 Flow chart illustrates the treatment pathways in hemophagocytic lymphohistiocytosis. Hematopoietic stem cell transplantation (HSCT) is required for the majority of primary hemophagocytic lymphohistiocytosis (HLH) and some of LAHS and EBV-HLH. FHL: Familial hemophagocytic lymphohistiocytosis; MAS: Macrophage activation syndrome; LAHS: Lymphoma-associated HLH; SCE: Steroids + cyclosporine A + etoposide; SCA: Steroids + cyclosporine A + ATG; IVIG: Intravenous immune globulin; S/C: Steroids alone or cyclosporine A alone or two drug combination.

transfusion has proved successful for treating neonatal HSV-HLH[125]. In patients with mycobacterium tuberculosis-associated HLH, early diagnostic confirmation and the timely administration of antituberculous medication is crucial for an improved outcome[126]. For patients with leishmania-related HLH, amphotericin B was shown to be effective[127]; however, for those with human immunodeficiency virus-related HLH, outcome remains poor even in the era of highly active antiretroviral therapy[128].

The efficacy of intrathecal chemotherapy for treating CNS disease in HLH patients has not been sufficiently evaluated. At present, the outcome for HLH patients with CNS disease is poor, even when treated with a combination of systemic immunochemotherapy and intrathecal chemotherapy[60-62]. The HLH-94 study tested the ability of a high systemic dose of dexamethasone to prevent the development of CNS disease. In addition, the study examined the use of intrathecal methotrexate in patients showing neurological symptoms at the time of disease onset; however, neither treatment appeared to prevent the exacerbation of CNS disease[37,62]. Data show that at the time of HLH diagnosis, neurological symptoms were already present in 37% of patients, and abnormal findings regarding the CSF were made in 52%; in all, 63% of patients had either neurological symptoms or abnormal CSF findings. CNS sequelae were more common in the latter group and, consistent with this, a substantial proportion of HLH survivors suffer neurological sequelae. Thus, early diagnosis of HLH and an evaluation of the CNS status including the CSF, coupled with early systemic HLH therapy, is crucial; in addition, the timely use of HSCT should be considered if reactivation of HLH with or without CNS disease is suspected to develop or to be exacerbated during the treatment[37,62,129]. However, since childhood survivors of HLH even after HSCT are shown to be at risk of long-term cognitive and psychosocial difficulties[130], prospective and systematic long-term follow-up of neurological function in these post-HSCT patients is essential.

Suppression of inflammation

In the past, IVIG was used to treat various types of HLH[49,131,132]; however, this form of treatment seems best suited to enterovirus-, hepatitis-, cytomegalovirus-, or bacteria-associated HLH[133-137]. Combined treatment with antibiotics and IVIG resulted in the full recovery of a patient with Group G streptococcal endocarditis-associated HLH[138]. Treatment with steroids alone can be effective for some HLH cases[139]. CSA quickly and efficiently suppresses the cytokines secreted by dysregulated T-cells and activated macrophages; indeed, CSA is able to control various cytokine-related pathological conditions[140,141]. Currently, the majority of HLH cases are treated first with a combination of steroids and CSA[49,140-142]. The prompt and continuous infusion of CSA (1-3 mg/kg per day over several days) is required to alleviate the cytokine "storm" as quickly as possible in patients with severe HLH, but without renal failure[49]. In addition, CSA treatment effectively supports neutrophil recovery especially in severely neutropenic Asian EBV-HLH patients during the acute phase[143].

Combined immunotherapy or immunochemotherapy

The etoposide/steroid/CSA triple combination was used in the HLH-94 or HLH-2004 regimen, consisting of 8 wk of initial therapy followed by continuation therapy and allogeneic HSCT if required. This regimen is now used in many centers to treat HLH, which comprises of dexamethasone (starting dose, 10 mg/d, Ⅳ or PO, followed by tapering), CSA (dose adjusted to obtain trough levels of 200 μg/L, PO, daily), and etoposide (150 mg/m^2, Ⅳ; a total of 10 doses during the initial 8 wk). This regimen has considerably improved the outcome for HLH patients: in the initial analysis comprising 113 patients, the 3-year survival rate was 45% (± 10%)[37]. The CNS outcomes in the patients treated with this regimen

were published in 2008[62]. Long-term follow-up results for 227 patients were published in 2011, where the estimated 5-year survival rate was 54% ± 6%, and the 5-year survival rate for 124 patients who received HSCT was 66% ± 8%[109]. The same group also suggested that this regimen should be revised as HLH-2004[58]; however, the therapeutic results of HLH-2004 have not yet been published. The HLH-94 regimen was also found to be effective when used to treat secondary EBV-HLH[144-146]. On the other hand, along the usage of HLH-94-type HLH treatment, several cases of t-AML were reported[65]. A French group used an ATG/steroid/CSA combination to treat patients with primary HLH[116,147,148], and similar to the etoposide/steroid/CSA regimen, this combination was also followed by HSCT. The effectiveness of this treatment was first described in 1993[147], where the regimen comprised steroids (2-5 mg/kg per day methylprednisolone, Ⅳ, followed by tapering), rabbit ATG (5-10 mg/kg per day for 5 d), and CSA (4-6 mg/kg per day, PO, daily). The study results were published in 2007[148]. In 38 consecutive patients with use of 45 courses of ATG, this regimen resulted in a rapid and complete response in 73%, a partial response in 24%, and no response in only one patient. Subsequent HSCT, when performed early after a complete or partial response, led to a high cure rate of 16 out of 19 cases. Overall, 21 of the 38 patients survived and there were four toxicity-related deaths. The same group also published the HSCT results for 48 patients in 2006[116]. Unfortunately, no direct comparison is possible between the therapeutic results performed after the ATG-regimen or after the HLH-94-type regimen. A regimen comprising HIT (hybrid immunotherapy)-HLH, which uses a combination of ATG/etoposide in the initial treatment phase, is currently being tested (unpublished; Jordan M, Histiocyte Society Clinical Studies 2013).

Other treatments

Rituximab is an effective treatment for some cases of EBV-HLH and has been used as a form of pre-emptive B-cell-directed therapy in patients with XLP1-related EBV-HLH or other severe forms of EBV-HLH in which EBV resides within B cells[149-152]. More recently, Chellapandian *et al*[153] examined 42 EBV-HLH cases and found that a combination of rituximab and conventional immunochemotherapy improved patient symptoms and reduced both the viral load and the level of inflammation. In the past, rituximab was thought to be unsuitable as a treatment for Asian patients with EBV-HLH in which EBV resides in T-cells or NK cells; however, the inclusion of rituximab in the initial treatment regimen may be useful in such cases[154]. R-CHOP (a combination of rituximab, doxorubicin, vincristine, cyclophosphamide and prednisolone) as well as R-etoposide are an effective combination for treating EBV-LPD-associated HLH[152]. The combination of rituximab and CSA induced remission in one patient with EBV-HLH occurring in association with CHS[57]. In addition, intrathecal rituximab is an effective treatment for post-transplant EBV-positive CNS lesions[155,156]. Alemtuzumab is effective as a bridge to allogeneic HSCT in primary HLH patients undergoing salvage treatment[157]. Marsh *et al*[158] reported that of 22 patients who received alemtuzumab (median dose, 1 mg/kg; range, 0.1-8.9 mg/kg) over a median of 4 d (range, 2-10), 64% experienced a partial response within 2 weeks. Indeed, 77% survived and underwent allogeneic HSCT, where the adverse events, including cytomegalovirus and adenovirus viremia, were reported to be "acceptable". Alemtuzumab has also been used to treat refractory MAS[159]. As other biological and experimental agents, the anti-CD25 antibody (daclizumab) was successfully used in a single adult patient with HLH[160] and the anti-TNF-α antibody (infliximab/etanercept) is an effective treatment for MAS[161-165]. Because IFN-γ plays a major role in the pathogenesis of HLH, a humanized anti-IFN-γ antibody, NI-0501(NovImmune), is currently being tested as a future treatment for the disease (unpublished; Arico M, Histiocyte Society Clinical Studies 2013), based on the murine model studies[166,167]. A study in XMEN patients showed that magnesium supplementation is an effective treatment because magnesium restores decreased intracellular free Mg^{2+} levels and corrects defective expression of NK activating receptor (NKG2D), while concurrently reducing the number of EBV-infected cells *in vivo*[103].

Allogeneic hematopoietic stem cell transplantation

Patients with primary HLH and those with refractory secondary HLH are candidates for allogeneic HSCT[37-41,112-116,168]. Primary HLH cases with nonsense (disruptive) gene mutations such as premature stop codon, or sequence frameshift generally develop symptoms in early infancy, thus require early introduction of HSCT. In these cases, delayed HSCT may have a risk of reactivation of HLH, development of CNS disease or hematological malignancies. Those with missense (hypomorphic) mutations may often wait for transplantation until adolescence or young adulthood. In secondary HLH, HSCT is planned whenever the disease becomes refractory to immunochemotherapy. For HSCT, reduced intensity conditioning (RIC) rather than myeloablative conditioning (MAC) is the preferred regimen because it results in better patient survival; however, the RIC regimen may result in mixed donor chimerism during the post-transplant period[41]. Landman-Parker *et al*[169] showed that partial engraftment of donor bone marrow cells after HSCT is sufficient to obtain long-term remission in patients with primary HLH. Experimental transplantation of perforin-deficient mice showed that 10%-20% perforin-expressing cells, with either mixed hematopoietic or CD8 (+) T-cell chimerism, are sufficient to re-establish immune regulation[170]. These data suggest that stable levels of donor chimerism (> 10%) could maintain remission in the HLH patients after HSCT. Of the 40 HLH patients who underwent allogeneic HSCT between 2003 and 2009 in Cincinnati, 14 received MAC comprising busulfan, cyclophosphamide,

and ATG plus or minus etoposide, while 26 patients received RIC comprising fludarabine, melphalan, and alemtuzumab. All patients engrafted successfully, and the overall estimated 3-year survival after HSCT was 43% for those receiving MAC and 92% for those receiving RIC ($P = 0.0001$)[41]. In Japan, 57 patients (43 with primary HLH and 14 with EBV-HLH) underwent HSCT between 1995 and 2005. Data show that EBV-HLH patients had a better prognosis after HSCT than primary HLH patients, also demonstrating that the RIC-conditioning regimen significantly improves the outcome of patients undergoing allogeneic HSCT[40].

CONCLUSION

Recent progress has been reviewed on how to understand the pathogenesis, how to diagnose and how to make treatment decisions in patients with HLH. Although the outcomes have significantly improved over the past decade, further refinement of treatment is required at the initial phase of the disease as well as pre- and post HSCT periods with special care for CNS disease in order to promise a cure with excellent quality of life in these patients with HLH.

REFERENCES

1 **Janka GE**. Familial and acquired hemophagocytic lymphohistiocytosis. *Annu Rev Med* 2012; **63**: 233-246 [PMID: 22248322 DOI: 10.1146/annurev-med-041610-134208]
2 **Chandrakasan S**, Filipovich AH. Hemophagocytic lymphohistiocytosis: advances in pathophysiology, diagnosis, and treatment. *J Pediatr* 2013; **163**: 1253-1259 [PMID: 23953723 DOI: 10.1016/j.jpeds.2013.06.053]
3 **Pachlopnik Schmid J**, Côte M, Ménager MM, Burgess A, Nehme N, Ménasché G, Fischer A, de Saint Basile G. Inherited defects in lymphocyte cytotoxic activity. *Immunol Rev* 2010; **235**: 10-23 [PMID: 20536552]
4 **Cetica V**, Pende D, Griffiths GM, Aricò M. Molecular basis of familial hemophagocytic lymphohistiocytosis. *Haematologica* 2010; **95**: 538-541 [PMID: 20378576 DOI: 10.3324/haematol.2009.019562]
5 **Gholam C**, Grigoriadou S, Gilmour KC, Gaspar HB. Familial haemophagocytic lymphohistiocytosis: advances in the genetic basis, diagnosis and management. *Clin Exp Immunol* 2011; **163**: 271-283 [PMID: 21303357 DOI: 10.1111/j.1365-2249.2010.04302.x]
6 **Jordan MB**, Allen CE, Weitzman S, Filipovich AH, McClain KL. How I treat hemophagocytic lymphohistiocytosis. *Blood* 2011; **118**: 4041-4052 [PMID: 21828139 DOI: 10.1182/blood-2011-03-278127]
7 **de Saint Basile G**, Ménasché G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol* 2010; **10**: 568-579 [PMID: 20634814 DOI: 10.1038/nri2803]
8 **de Saint Basile G**, Ménasché G, Latour S. Inherited defects causing hemophagocytic lymphohistiocytic syndrome. *Ann N Y Acad Sci* 2011; **1246**: 64-76 [PMID: 22236431 DOI: 10.1111/j.1749-6632.2011.06307.x]
9 **Dupré L**, Andolfi G, Tangye SG, Clementi R, Locatelli F, Aricò M, Aiuti A, Roncarolo MG. SAP controls the cytolytic activity of CD8+ T cells against EBV-infected cells. *Blood* 2005; **105**: 4383-4389 [PMID: 15677558 DOI: 10.1182/blood-2004-08-3269]
10 **Snow AL**, Marsh RA, Krummey SM, Roehrs P, Young LR, Zhang K, van Hoff J, Dhar D, Nichols KE, Filipovich AH, Su HC, Bleesing JJ, Lenardo MJ. Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. *J Clin Invest* 2009; **119**: 2976-2989 [PMID: 19759517]
11 **Rigaud S**, Fondanèche MC, Lambert N, Pasquier B, Mateo V, Soulas P, Galicier L, Le Deist F, Rieux-Laucat F, Revy P, Fischer A, de Saint Basile G, Latour S. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature* 2006; **444**: 110-114 [PMID: 17080092 DOI: 10.1038/nature05257]
12 **Green MR**, Kennell AS, Larche MJ, Seifert MH, Isenberg DA, Salaman MR. Natural killer cell activity in families of patients with systemic lupus erythematosus: demonstration of a killing defect in patients. *Clin Exp Immunol* 2005; **141**: 165-173 [PMID: 15958083 DOI: 10.1016/j.jinf.2008.02.013]
13 **Rajagopala S**, Dutta U, Chandra KS, Bhatia P, Varma N, Kochhar R. Visceral leishmaniasis associated hemophagocytic lymphohistiocytosis--case report and systematic review. *J Infect* 2008; **56**: 381-388 [PMID: 18405976]
14 **Bogdan C**. Natural killer cells in experimental and human leishmaniasis. *Front Cell Infect Microbiol* 2012; **2**: 69 [PMID: 22919660 DOI: 10.3389/fcimb.2012.00069]
15 **Lee SJ**, Cho YN, Kim TJ, Park SC, Park DJ, Jin HM, Lee SS, Kee SJ, Kim N, Yoo DH, Park YW. Natural killer T cell deficiency in active adult-onset Still's Disease: correlation of deficiency of natural killer T cells with dysfunction of natural killer cells. *Arthritis Rheum* 2012; **64**: 2868-2877 [PMID: 22605480 DOI: 10.1002/art.34514]
16 **Villanueva J**, Lee S, Giannini EH, Graham TB, Passo MH, Filipovich A, Grom AA. Natural killer cell dysfunction is a distinguishing feature of systemic onset juvenile rheumatoid arthritis and macrophage activation syndrome. *Arthritis Res Ther* 2005; **7**: R30-R37 [PMID: 15642140 DOI: 10.1186/ar1453]
17 **Yoshida Y**, Machigashira K, Suehara M, Arimura H, Moritoyo T, Nagamatsu K, Osame M. Immunological abnormality in patients with lysinuric protein intolerance. *J Neurol Sci* 1995; **134**: 178-182 [PMID: 8747863 DOI: 10.1016/0022-510X(95)00237-1]
18 **Georgeson GD**, Szony BJ, Streitman K, Kovács A, Kovács L, László A. Natural killer cell cytotoxicity is deficient in newborns with sepsis and recurrent infections. *Eur J Pediatr* 2001; **160**: 478-482 [PMID: 11548185 DOI: 10.1007/s004310100773]
19 **Mazodier K**, Marin V, Novick D, Farnarier C, Robitail S, Schleinitz N, Veit V, Paul P, Rubinstein M, Dinarello CA, Harlé JR, Kaplanski G. Severe imbalance of IL-18/IL-18BP in patients with secondary hemophagocytic syndrome. *Blood* 2005; **106**: 3483-3489 [PMID: 16020503 DOI: 10.1182/blood-2005-05-1980]
20 **Kasahara Y**, Yachie A. Cell type specific infection of Epstein-Barr virus (EBV) in EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. *Crit Rev Oncol Hematol* 2002; **44**: 283-294 [PMID: 12467968 DOI: 10.1016/S1040-8428(02)00119-1]
21 **Wada T**, Sakakibara Y, Nishimura R, Toma T, Ueno Y, Horita S, Tanaka T, Nishi M, Kato K, Yasumi T, Ohara O, Yachie A. Down-regulation of CD5 expression on activated CD8+ T cells in familial hemophagocytic lymphohistiocytosis with perforin gene mutations. *Hum Immunol* 2013; **74**: 1579-1585 [PMID: 24051121 DOI: 10.1016/j.humimm.2013.09.001]
22 **Imashuku S**. Differential diagnosis of hemophagocytic syndrome: underlying disorders and selection of the most effective treatment. *Int J Hematol* 1997; **66**: 135-151 [PMID: 9277044 DOI: 10.1016/S0925-5710(97)00584-7]
23 **Ansuini V**, Rigante D, Esposito S. Debate around infection-dependent hemophagocytic syndrome in paediatrics. *BMC Infect Dis* 2013; **13**: 15 [PMID: 23324497 DOI: 10.1186/1471-2334-13-15]

24 **Marcenaro S**, Gallo F, Martini S, Santoro A, Griffiths GM, Aricó M, Moretta L, Pende D. Analysis of natural killer-cell function in familial hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. *Blood* 2006; **108**: 2316-2323 [PMID: 16778144 DOI: 10.1182/blood-2006-04-015693]

25 **Bryceson YT**, Pende D, Maul-Pavicic A, Gilmour KC, Ufheil H, Vraetz T, Chiang SC, Marcenaro S, Meazza R, Bondzio I, Walshe D, Janka G, Lehmberg K, Beutel K, zur Stadt U, Binder N, Arico M, Moretta L, Henter JI, Ehl S. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes. *B lood* 2012; **119**: 2754-2763 [PMID: 22294731 DOI: 10.1182/blood-2011-08-374199]

26 **Schneider EM**, Lorenz I, Müller-Rosenberger M, Steinbach G, Kron M, Janka-Schaub GE. Hemophagocytic lymphohistiocytosis is associated with deficiencies of cellular cytolysis but normal expression of transcripts relevant to killer-cell-induced apoptosis. *Blood* 2002; **100**: 2891-2898 [PMID: 12351400 DOI: 10.1182/blood-2001-12-0260]

27 **Horne A**, Zheng C, Lorenz I, Löfstedt M, Montgomery SM, Janka G, Henter JI, Marion Schneider E. Subtyping of natural killer cell cytotoxicity deficiencies in haemophagocytic lymphohistocytosis provides therapeutic guidance. *Br J Haematol* 2005; **129**: 658-666 [PMID: 15916689 DOI: 10.1111/j.1365-2141.2005.05502.x]

28 **Murata Y**, Yasumi T, Shirakawa R, Izawa K, Sakai H, Abe J, Tanaka N, Kawai T, Oshima K, Saito M, Nishikomori R, Ohara O, Ishii E, Nakahata T, Horiuchi H, Heike T. Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4 protein. *Blood* 2011; **118**: 1225-1230 [PMID: 21653941 DOI: 10.1182/blood-2011-01-329540]

29 **Ueda I**, Kurokawa Y, Koike K, Ito S, Sakata A, Matsumora T, Fukushima T, Morimoto A, Ishii E, Imashuku S. Late-onset cases of familial hemophagocytic lymphohistiocytosis with missense perforin gene mutations. *Am J Hematol* 2007; **82**: 427-432 [PMID: 17266056 DOI: 10.1002/ajh.20878]

30 **Nagafuji K**, Nonami A, Kumano T, Kikushige Y, Yoshimoto G, Takenaka K, Shimoda K, Ohga S, Yasukawa M, Horiuchi H, Ishii E, Harada M. Perforin gene mutations in adult-onset hemophagocytic lymphohistiocytosis. *Haematologica* 2007; **92**: 978-981 [PMID: 17606450 DOI: 10.3324/haematol.11233]

31 **Beaty AD**, Weller C, Levy B, Vogler C, Ferguson WS, Bicknese A, Knutsen AP. A teenage boy with late onset hemophagocytic lymphohistiocytosis with predominant neurologic disease and perforin deficiency. *Pediatr Blood Cancer* 2008; **50**: 1070-1072 [PMID: 18074390 DOI: 10.1002/pbc.21438]

32 **Sieni E**, Cetica V, Piccin A, Gherlinzoni F, Sasso FC, Rabusin M, Attard L, Bosi A, Pende D, Moretta L, Aricò M. Familial hemophagocytic lymphohistiocytosis may present during adulthood: clinical and genetic features of a small series. *PLoS One* 2012; **7**: e44649 [PMID: 22970278 DOI: 10.1371/journal.pone.0044649]

33 **Weisfeld-Adams JD**, Mehta L, Rucker JC, Dembitzer FR, Szporn A, Lublin FD, Introne WJ, Bhambhani V, Chicka MC, Cho C. Atypical Chédiak-Higashi syndrome with attenuated phenotype: three adult siblings homozygous for a novel LYST deletion and with neurodegenerative disease. *Orphanet J Rare Dis* 2013; **8**: 46 [PMID: 23521865 DOI: 10.1186/1750-1172-8-46]

34 **Imashuku S**. Clinical features and treatment strategies of Epstein-Barr virus-associated hemophagocytic lymphohis tiocytosis. *Crit Rev Oncol Hematol* 2002; **44**: 259-272 [PMID: 12467966 DOI: 10.1016/S1040-8428(02)00117-8]

35 **Kimura H**, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, Matsuyama T, Morishima T. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J Clin Microbiol* 1999; **37**: 132-136 [PMID: 9854077]

36 **Okano M**, Kawa K, Kimura H, Yachie A, Wakiguchi H, Maeda A, Imai S, Ohga S, Kanegane H, Tsuchiya S, Morio T, Mori M, Yokota S, Imashuku S. Proposed guidelines for diagnosing chronic active Epstein-Barr virus infection. *Am J Hematol* 2005; **80**: 64-69 [PMID: 16138335 DOI: 10.1002/ajh.20398]

37 **Henter JI**, Samuelsson-Horne A, Aricò M, Egeler RM, Elinder G, Filipovich AH, Gadner H, Imashuku S, Komp D, Ladisch S, Webb D, Janka G. Treatment of hemophagocytic lymphohistiocytosis with HLH-94 immunochemotherapy and bone marrow transplantation. *Blood* 2002; **100**: 2367-2373 [PMID: 12239144 DOI: 10.1182/blood-2002-01-0172]

38 **Henter JI**, Horne A, Aricó M, Egeler RM, Filipovich AH, Imashuku S, Ladisch S, McClain K, Webb D, Winiarski J, Janka G. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 2007; **48**: 124-131 [PMID: 16937360 DOI: 10.1002/pbc.21039]

39 **Horne A**, Janka G, Maarten Egeler R, Gadner H, Imashuku S, Ladisch S, Locatelli F, Montgomery SM, Webb D, Winiarski J, Filipovich AH, Henter JI. Haematopoietic stem cell transplantation in haemophagocytic lymphohistiocytosis. *Br J Haematol* 2005; **129**: 622-630 [PMID: 15916685]

40 **Ohga S**, Kudo K, Ishii E, Honjo S, Morimoto A, Osugi Y, Sawada A, Inoue M, Tabuchi K, Suzuki N, Ishida Y, Imashuku S, Kato S, Hara T. Hematopoietic stem cell transplantation for familial hemophagocytic lymphohistiocytosis and Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in Japan. *Pediatr Blood Cancer* 2010; **54**: 299-306 [PMID: 19827139]

41 **Marsh RA**, Vaughn G, Kim MO, Li D, Jodele S, Joshi S, Mehta PA, Davies SM, Jordan MB, Bleesing JJ, Filipovich AH. Reduced-intensity conditioning significantly improves survival of patients with hemophagocytic lymphohistiocytosis undergoing allogeneic hematopoietic cell transpla ntation. *Blood* 2010; **116**: 5824-5831 [PMID: 20855862 DOI: 10.1182/blood-2010-04-282392]

42 **Trizzino A**, zur Stadt U, Ueda I, Risma K, Janka G, Ishii E, Beutel K, Sumegi J, Cannella S, Pende D, Mian A, Henter JI, Griffiths G, Santoro A, Filipovich A, Aricò M. Genotype-phenotype study of familial haemophagocytic lymphohistiocytosis due to perforin mutations. *J Med Genet* 2008; **45**: 15-21 [PMID: 17873118 DOI: 10.1136/jmg.2007.052670]

43 **Sieni E**, Cetica V, Santoro A, Beutel K, Mastrodicasa E, Meeths M, Ciambotti B, Brugnolo F, zur Stadt U, Pende D, Moretta L, Griffiths GM, Henter JI, Janka G, Aricò M. Genotype-phenotype study of familial haemophagocytic lymphohistiocytosis type 3. *J Med Genet* 2011; **48**: 343-352 [PMID: 21248318 DOI: 10.1136/jmg.2010.085456]

44 **Rohr J**, Beutel K, Maul-Pavicic A, Vraetz T, Thiel J, Warnatz K, Bondzio I, Gross-Wieltsch U, Schündeln M, Schütz B, Woessmann W, Groll AH, Strahm B, Pagel J, Speckmann C, Janka G, Griffiths G, Schwarz K, zur Stadt U, Ehl S. Atypical familial hemophagocytic lymphohistiocytosis due to mutations in UNC13D and STXBP2 overlaps with primary immunodeficiency diseases. *Haematologica* 2010; **95**: 2080-2087 [PMID: 20823128 DOI: 10.3324/haematol.2010.029389]

45 **Pagel J**, Beutel K, Lehmberg K, Koch F, Maul-Pavicic A, Rohlfs AK, Al-Jefri A, Beier R, Bomme Ousager L, Ehlert K, Gross-Wieltsch U, Jorch N, Kremens B, Pekrun A, Sparber-Sauer M, Mejstrikova E, Wawer A, Ehl S, zur Stadt U, Janka G. Distinct mutations in STXBP2 are associated with variable clinical presentations in patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL5). *Blood* 2012; **119**: 6016-6024 [PMID: 22451424 DOI: 10.1182/blood-2011-12-398958]

46 **Meeths M**, Entesarian M, Al-Herz W, Chiang SC, Wood SM, Al-Ateeqi W, Almazan F, Boelens JJ, Hasle H, Ifversen M, Lund B, van den Berg JM, Gustafsson B, Hjelmqvist H, Nordenskjöld M, Bryceson YT, Henter JI. Spectrum

of clinical presentations in familial hemophagocytic lymphohistiocytosis type 5 patients with mutations in STXBP2. *Blood* 2010; **116**: 2635-2643 [PMID: 20558610 DOI: 10.1182/blood-2010-05-282541]

47 **Zhang K**, Jordan MB, Marsh RA, Johnson JA, Kissell D, Meller J, Villanueva J, Risma KA, Wei Q, Klein PS, Filipovich AH. Hypomorphic mutations in PRF1, MUNC13-4, and STXBP2 are associated with adult-onset familial HLH. *Blood* 2011; **118**: 5794-5798 [PMID: 21881043 DOI: 10.1182/blood-2011-07-370148]

48 **Sepulveda FE**, Debeurme F, Ménasché G, Kurowska M, Côte M, Pachlopnik Schmid J, Fischer A, de Saint Basile G. Distinct severity of HLH in both human and murine mutants with complete loss of cytotoxic effector PRF1, RAB27A, and STX11. *Blood* 2013; **121**: 595-603 [PMID: 23160464 DOI: 10.1182/blood-2012-07-440339]

49 **Imashuku S**. Advances in the management of hemophagocytic lymphohistiocytosis. *Int J Hematol* 2000; **72**: 1-11 [PMID: 10979202]

50 **Ishii E**, Ohga S, Imashuku S, Yasukawa M, Tsuda H, Miura I, Yamamoto K, Horiuchi H, Takada K, Ohshima K, Nakamura S, Kinukawa N, Oshimi K, Kawa K. Nationwide survey of hemophagocytic lymphohistiocytosis in Japan. *Int J Hematol* 2007; **86**: 58-65 [PMID: 17675268 DOI: 10.1532/IJH97.07012]

51 **Baars JW**, de Boer JP, Wagstaff J, Roem D, Eerenberg-Belmer AJ, Nauta J, Pinedo HM, Hack CE. Interleukin-2 induces activation of coagulation and fibrinolysis: resemblance to the changes seen during experimental endotoxaemia. *Br J Haematol* 1992; **82**: 295-301 [PMID: 1419810 DOI: 10.1111/j.1365-2141.1992.tb06421.x]

52 **Nawathe PA**, Ravindranath TM, Satwani P, Baird JS. Severe hemorrhagic coagulopathy with hemophagocytic lymphohistiocytosis secondary to Epstein-Barr virus-associated T-cell lymphoproliferative disorder. *Pediatr Crit Care Med* 2013; **14**: e176-e181 [PMID: 23439459 DOI: 10.1097/PCC.0b013e3182720f94]

53 **Pachlopnik Schmid J**, Canioni D, Moshous D, Touzot F, Mahlaoui N, Hauck F, Kanegane H, Lopez-Granados E, Mejstrikova E, Pellier I, Galicier L, Galambrun C, Barlogis V, Bordigoni P, Fourmaintraux A, Hamidou M, Dabadie A, Le Deist F, Haerynck F, Ouachée-Chardin M, Rohrlich P, Stephan JL, Lenoir C, Rigaud S, Lambert N, Milili M, Schiff C, Chapel H, Picard C, de Saint Basile G, Blanche S, Fischer A, Latour S. Clinical similarities and differences of patients with X-linked lymphoproliferative syndrome type 1 (XLP-1/SAP deficiency) versus type 2 (XLP-2/XIAP deficiency). *Blood* 2011; **117**: 1522-1529 [PMID: 21119115 DOI: 10.1182/blood-2010-07-298372]

54 **Stepensky P**, Bartram J, Barth TF, Lehmberg K, Walther P, Amann K, Philips AD, Beringer O, Zur Stadt U, Schulz A, Amrolia P, Weintraub M, Debatin KM, Hoenig M, Posovszky C. Persistent defective membrane trafficking in epithelial cells of patients with familial hemophagocytic lymphohistiocytosis type 5 due to STXBP2/MUNC18-2 mutations. *Pediatr Blood Cancer* 2013; **60**: 1215-1222 [PMID: 23382066 DOI: 10.1002/pbc.24475]

55 **Meeths M**, Bryceson YT, Rudd E, Zheng C, Wood SM, Ramme K, Beutel K, Hasle H, Heilmann C, Hultenby K, Ljunggren HG, Fadeel B, Nordenskjöld M, Henter JI. Clinical presentation of Griscelli syndrome type 2 and spectrum of RAB27A mutations. *Pediatr Blood Cancer* 2010; **54**: 563-572 [PMID: 19953648]

56 **Trottestam H**, Beutel K, Meeths M, Carlsen N, Heilmann C, Pasić S, Webb D, Hasle H, Henter JI. Treatment of the X-linked lymphoproliferative, Griscelli and Chédiak-Higashi syndromes by HLH directed therapy. *Pediatr Blood Cancer* 2009; **52**: 268-272 [PMID: 18937330 DOI: 10.1002/pbc.21790]

57 **Ogimi C**, Tanaka R, Arai T, Kikuchi A, Hanada R, Oh-Ishi T. Rituximab and cyclosporine therapy for accelerated phase Chediak-Higashi syndrome. *Pediatr Blood Cancer* 2011; **57**: 677-680 [PMID: 21681939 DOI: 10.1002/pbc.23231]

58 **Enders A**, Zieger B, Schwarz K, Yoshimi A, Speckmann C, Knoepfle EM, Kontny U, Müller C, Nurden A, Rohr J, Henschen M, Pannicke U, Niemeyer C, Nurden P, Ehl S. Lethal hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type II. *Blood* 2006; **108**: 81-87 [PMID: 16551969 DOI: 10.1182/blood-2005-11-4413]

59 **Jessen B**, Bode SF, Ammann S, Chakravorty S, Davies G, Diestelhorst J, Frei-Jones M, Gahl WA, Gochuico BR, Griese M, Griffiths G, Janka G, Klein C, Kögl T, Kurnik K, Lehmberg K, Maul-Pavicic A, Mumford AD, Pace D, Parvaneh N, Rezaei N, de Saint Basile G, Schmitt-Graeff A, Schwarz K, Karasu GT, Zieger B, Zur Stadt U, Aichele P, Ehl S. The risk of hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type 2. *Blood* 2013; **121**: 2943-2951 [PMID: 23403622 DOI: 10.1182/blood-2012-10-463166]

60 **Haddad E**, Sulis ML, Jabado N, Blanche S, Fischer A, Tardieu M. Frequency and severity of central nervous system lesions in hemophagocytic lymphohistiocytosis. *Blood* 1997; **89**: 794-800 [PMID: 9028310]

61 **Imashuku S**, Hyakuna N, Funabiki T, Ikuta K, Sako M, Iwai A, Fukushima T, Kataoka S, Yabe M, Muramatsu K, Kohdera U, Nakadate H, Kitazawa K, Toyoda Y, Ishii E. Low natural killer activity and central nervous system disease as a high-risk prognostic indicator in young patients with hemophagocytic lymphohistiocytosis. *Cancer* 2002; **94**: 3023-3031 [PMID: 12115393 DOI: 10.1002/cncr.10515]

62 **Horne A**, Trottestam H, Aricò M, Egeler RM, Filipovich AH, Gadner H, Imashuku S, Ladisch S, Webb D, Janka G, Henter JI. Frequency and spectrum of central nervous system involvement in 193 children with haemophagocytic lymphohistiocytosis. *Br J Haematol* 2008; **140**: 327-335 [PMID: 18076710 DOI: 10.1111/j.1365-2141.2007.06922.x]

63 **Clementi R**, Locatelli F, Dupré L, Garaventa A, Emmi L, Bregni M, Cefalo G, Moretta A, Danesino C, Comis M, Pession A, Ramenghi U, Maccario R, Aricò M, Roncarolo MG. A proportion of patients with lymphoma may harbor mutations of the perforin gene. *Blood* 2005; **105**: 4424-4428 [PMID: 15728124 DOI: 10.1182/blood-2004-04-1477]

64 **Trapani JA**, Thia KY, Andrews M, Davis ID, Gedye C, Parente P, Svobodova S, Chia J, Browne K, Campbell IG, Phillips WA, Voskoboinik I, Cebon JS. Human perforin mutations and susceptibility to multiple primary cancers. *Oncoimmunology* 2013; **2**: e24185 [PMID: 23734337 DOI: 10.4161/onci.24185]

65 **Imashuku S**. Etoposide-related secondary acute myeloid leukemia (t-AML) in hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 2007; **48**: 121-123 [PMID: 17066467 DOI: 10.1002/pbc.21082]

66 **Imashuku S**, Hibi S, Sako M, Ishida Y, Mugishima H, Chen J, Tsunematsu Y. Soluble interleukin-2 receptor: a useful prognostic factor for patients with hemophagocytic lympho histiocytosis. *Blood* 1995; **86**: 4706-4707 [PMID: 8541568]

67 **Woods CW**, Bradshaw WT, Woods AG. Hemophagocytic lymphohistiocytosis in the premature neonate. *Adv Neonatal Care* 2009; **9**: 265-273 [PMID: 20010142 DOI: 10.1097/ANC.0b013e3181c1fff7]

68 **Bechara E**, Dijoud F, de Saint Basile G, Bertrand Y, Pondarré C. Hemophagocytic lymphohistiocytosis with Munc13-4 mutation: a cause of recurrent fatal hydrops fetalis. *Pediatrics* 2011; **128**: e251-e254 [PMID: 21646258 DOI: 10.1542/peds.2010-0764]

69 **zur Stadt U**, Pruggmayer M, Jung H, Henter JI, Schneider M, Kabisch H, Janka G. Prenatal diagnosis of perforin gene mutations in familial hemophagocytic lymphohistiocytosis (FHLH). *Prenat Diagn* 2002; **22**: 80-81 [PMID: 11810660 DOI: 10.1002/pd.231]

70 **Soyama A**, Eguchi S, Takatsuki M, Hidaka M, Tomonaga

T, Yamanouchi K, Miyazaki K, Inokuma T, Tajima Y, Kanematsu T. Hemophagocytic syndrome after liver transplantation: report of two cases. *Surg Today* 2011; **41**: 1524-1530 [PMID: 21969156 DOI: 10.1007/s00595-010-4512-9]

71 **Takagi S**, Masuoka K, Uchida N, Ishiwata K, Araoka H, Tsuji M, Yamamoto H, Kato D, Matsuhashi Y, Kusumi E, Ota Y, Seo S, Matsumura T, Matsuno N, Wake A, Miyakoshi S, Makino S, Ohashi K, Yoneyama A, Taniguchi S. High incidence of haemophagocytic syndrome following umbilical cord blood transplantation for adults. *Br J Haematol* 2009; **147**: 543-553 [PMID: 19709082 DOI: 10.1111/j.1365-2141.2009.07863.x]

72 **Koyama M**, Sawada A, Yasui M, Inoue M, Kawa K. Encouraging results of low-dose etoposide in the treatment of early-onset hemophagocytic syndrome following allogeneic hematopoietic stem cell transplantation. *Int J Hematol* 2007; **86**: 466-467 [PMID: 18192120 DOI: 10.1007/BF02984009]

73 **Shimazaki C**, Inaba T, Okano A, Hatsuse M, Takahashi R, Hirai H, Sudo Y, Ashihara E, Adachi Y, Murakami S, Saigo K, Tsuda H, Fujita N, Nakagawa M. Clinical characteristics of B-cell lymphoma-associated hemophagocytic syndrome (B-LAHS): comparison of CD5+ with CD5- B-LAHS. *Intern Med* 2001; **40**: 878-882 [PMID: 11579948 DOI: 10.2169/internalmedicine.40.878]

74 **Takahashi N**, Miura I, Chubachi A, Miura AB, Nakamura S. A clinicopathological study of 20 patients with T/natural killer (NK)-cell lymphoma-associated hemophagocytic syndrome with special reference to nasal and nasal-type NK/T-cell lymphoma. *Int J Hematol* 2001; **74**: 303-308 [PMID: 11721967 DOI: 10.1007/BF02982065]

75 **Fung KM**, Chakrabarty JH, Kern WF, Magharyous H, Gehrs BC, Li S. Intravascular large B-cell lymphoma with hemophagocytic syndrome (Asian variant) in a Caucasian patient. *Int J Clin Exp Pathol* 2012; **5**: 448-454 [PMID: 22808298]

76 **Balta G**, Azik FM, Gurgey A. Defective UNC13D gene-associated familial hemophagocytic lymphohistiocytosis triggered by visceral leishmaniasis: a diagnostic challenge. *J Pediatr Hematol Oncol* 2014; **36**: e42-e45 [PMID: 23774160 DOI: 10.1097/MPH.0b013e31829b7f22]

77 **Hazen MM**, Woodward AL, Hofmann I, Degar BA, Grom A, Filipovich AH, Binstadt BA. Mutations of the hemophagocytic lymphohistiocytosis-associated gene UNC13D in a patient with systemic juvenile idiopathic arthritis. *Arthritis Rheum* 2008; **58**: 567-570 [PMID: 18240215 DOI: 10.1002/art.23199]

78 **Clementi R**, Dagna L, Dianzani U, Dupré L, Dianzani I, Ponzoni M, Cometa A, Chiocchetti A, Sabbadini MG, Rugarli C, Ciceri F, Maccario R, Locatelli F, Danesino C, Ferrarini M, Bregni M. Inherited perforin and Fas mutations in a patient with autoimmune lymphoproliferative syndrome and lymphoma. *N Engl J Med* 2004; **351**: 1419-1424 [PMID: 15459303 DOI: 10.1056/NEJMoa041432]

79 **Chang TY**, Jaffray J, Woda B, Newburger PE, Usmani GN. Hemophagocytic lymphohistiocytosis with MUNC13-4 gene mutation or reduced natural killer cell function prior to onset of childhood leukemia. *Pediatr Blood Cancer* 2011; **56**: 856-858 [PMID: 21370424 DOI: 10.1002/pbc.22846]

80 **Machaczka M**, Klimkowska M, Chiang SC, Meeths M, Müller ML, Gustafsson B, Henter JI, Bryceson YT. Development of classical Hodgkin's lymphoma in an adult with biallelic STXBP2 mutations. *Haematologica* 2013; **98**: 760-764 [PMID: 23100279 DOI: 10.3324/haematol.2012.073098]

81 **Booth C**, Gilmour KC, Veys P, Gennery AR, Slatter MA, Chapel H, Heath PT, Steward CG, Smith O, O'Meara A, Kerrigan H, Mahlaoui N, Cavazzana-Calvo M, Fischer A, Moshous D, Blanche S, Pachlopnik Schmid J, Latour S, de Saint-Basile G, Albert M, Notheis G, Rieber N, Strahm B, Ritterbusch H, Lankester A, Hartwig NG, Meyts I, Plebani A, Soresina A, Finocchi A, Pignata C, Cirillo E, Bonanomi S, Peters C, Kalwak K, Pasic S, Sedlacek P, Jazbec J, Kanegane H, Nichols KE, Hanson IC, Kapoor N, Haddad E, Cowan M, Choo S, Smart J, Arkwright PD, Gaspar HB. X-linked lymphoproliferative disease due to SAP/SH2D1A deficiency: a multicenter study on the manifestations, management and outcome of the disease. *Blood* 2011; **117**: 53-62 [PMID: 20926771 DOI: 10.1182/blood-2010-06-284935]

82 **Marsh RA**, Madden L, Kitchen BJ, Mody R, McClimon B, Jordan MB, Bleesing JJ, Zhang K, Filipovich AH. XIAP deficiency: a unique primary immunodeficiency best classified as X-linked familial hemophagocytic lymphohistiocytosis and not as X-linked lymphoproliferative disease. *Blood* 2010; **116**: 1079-1082 [PMID: 20489057 DOI: 10.1182/blood-2010-01-256099]

83 **Filipovich AH**, Zhang K, Snow AL, Marsh RA. X-linked lymphoproliferative syndromes: brothers or distant cousins ? *Blood* 2010; **116**: 3398-3408 [PMID: 20660790 DOI: 10.1182/blood-2010-03-275909]

84 **Imashuku S**, Hibi S, Tabata Y, Itoh E, Hashida T, Tsunamoto K, Ishimoto K, Kawano F. Outcome of clonal hemophagocytic lymphohistiocytosis: analysis of 32 cases. *Leuk Lymphoma* 2000; **37**: 577-584 [PMID: 11042518 DOI: 10.3109/10428190009058510]

85 **Osugi Y**, Hara J, Tagawa S, Takai K, Hosoi G, Matsuda Y, Ohta H, Fujisaki H, Kobayashi M, Sakata N, Kawa-Ha K, Okada S, Tawa A. Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. *Blood* 1997; **89**: 4100-4103 [PMID: 9166851]

86 **Takada H**, Ohga S, Mizuno Y, Suminoe A, Matsuzaki A, Ihara K, Kinukawa N, Ohshima K, Kohno K, Kurimoto M, Hara T. Oversecretion of IL-18 in haemophagocytic lymphohistiocytosis: a novel marker of disease activity. *Br J Haematol* 1999; **106**: 182-189 [PMID: 10444185 DOI: 10.1046/j.1365-2141.1999.01504.x]

87 **Stepp SE**, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 1999; **286**: 1957-1959 [PMID: 10583959 DOI: 10.1126/science.286.5446.1957]

88 **Feldmann J**, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, Lambert N, Ouachée-Chardin M, Chedeville G, Tamary H, Minard-Colin V, Vilmer E, Blanche S, Le Deist F, Fischer A, de Saint Basile G. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell* 2003; **115**: 461-473 [PMID: 14622600 DOI: 10.1016/S0092-8674(03)00855-9]

89 **zur Stadt U**, Schmidt S, Kasper B, Beutel K, Diler AS, Henter JI, Kabisch H, Schneppenheim R, Nürnberg P, Janka G, Hennies HC. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum Mol Genet* 2005; **14**: 827-834 [PMID: 15703195 DOI: 10.1093/hmg/ddi076]

90 **zur Stadt U**, Rohr J, Seifert W, Koch F, Grieve S, Pagel J, Strauss J, Kasper B, Nürnberg G, Becker C, Maul-Pavicic A, Beutel K, Janka G, Griffiths G, Ehl S, Hennies HC. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. *Am J Hum Genet* 2009; **85**: 482-492 [PMID: 19804848 DOI: 10.1016/j.ajhg.2009.09.005]

91 **Côte M**, Ménager MM, Burgess A, Mahlaoui N, Picard C, Schaffner C, Al-Manjomi F, Al-Harbi M, Alangari A, Le Deist F, Gennery AR, Prince N, Cariou A, Nitschke P, Blank U, El-Ghazali G, Ménasché G, Latour S, Fischer A, de Saint Basile G. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J Clin Invest* 2009; **119**: 3765-3773 [PMID: 19884660 DOI: 10.1172/JCI40732]

92 **Sayos J**, Wu C, Morra M, Wang N, Zhang X, Allen D, van Schaik S, Notarangelo L, Geha R, Roncarolo MG, Oettgen

H, De Vries JE, Aversa G, Terhorst C. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* 1998; **395**: 462-469 [PMID: 9774102 DOI: 10.1038/26683]

93 **Coffey AJ**, Brooksbank RA, Brandau O, Oohashi T, Howell GR, Bye JM, Cahn AP, Durham J, Heath P, Wray P, Pavitt R, Wilkinson J, Leversha M, Huckle E, Shaw-Smith CJ, Dunham A, Rhodes S, Schuster V, Porta G, Yin L, Serafini P, Sylla B, Zollo M, Franco B, Bolino A, Seri M, Lanyi A, Davis JR, Webster D, Harris A, Lenoir G, de St Basile G, Jones A, Behloradsky BH, Achatz H, Murken J, Fassler R, Sumegi J, Romeo G, Vaudin M, Ross MT, Meindl A, Bentley DR. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet* 1998; **20**: 129-135 [PMID: 9771704 DOI: 10.1038/2424]

94 **Nichols KE**, Harkin DP, Levitz S, Krainer M, Kolquist KA, Genovese C, Bernard A, Ferguson M, Zuo L, Snyder E, Buckler AJ, Wise C, Ashley J, Lovett M, Valentine MB, Look AT, Gerald W, Housman DE, Haber DA. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc Natl Acad Sci USA* 1998; **95**: 13765-13770 [PMID: 9811875 DOI: 10.1073/pnas.95.23.13765]

95 **Karim MA**, Nagle DL, Kandil HH, Bürger J, Moore KJ, Spritz RA. Mutations in the Chediak-Higashi syndrome gene (CHS1) indicate requirement for the complete 3801 amino acid CHS protein. *Hum Mol Genet* 1997; **6**: 1087-1089 [PMID: 9215679 DOI: 10.1093/hmg/6.7.1087]

96 **Ménasché G**, Pastural E, Feldmann J, Certain S, Ersoy F, Dupuis S, Wulffraat N, Bianchi D, Fischer A, Le Deist F, de Saint Basile G. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genet* 2000; **25**: 173-176 [PMID: 10835631 DOI: 10.1038/76024]

97 **Huizing M**, Scher CD, Strovel E, Fitzpatrick DL, Hartnell LM, Anikster Y, Gahl WA. Nonsense mutations in ADTB3A cause complete deficiency of the beta3A subunit of adaptor complex-3 and severe Hermansky-Pudlak syndrome type 2. *Pediatr Res* 2002; **51**: 150-158 [PMID: 11809908 DOI: 10.1203/00006450-200202000-00006]

98 **Jones ML**, Murden SL, Brooks C, Maloney V, Manning RA, Gilmour KC, Bharadwaj V, de la Fuente J, Chakravorty S, Mumford AD. Disruption of AP3B1 by a chromosome 5 inversion: a new disease mechanism in Hermansky-Pudlak syndrome type 2. *BMC Med Genet* 2013; **14**: 42 [PMID: 23557002 DOI: 10.1186/1471-2350-14-42]

99 **Huck K**, Feyen O, Niehues T, Rüschendorf F, Hübner N, Laws HJ, Telieps T, Knapp S, Wacker HH, Meindl A, Jumaa H, Borkhardt A. Girls homozygous for an IL-2-inducible T cell kinase mutation that leads to protein deficiency develop fatal EBV-associated lymphoproliferation. *J Clin Invest* 2009; **119**: 1350-1358 [PMID: 19425169 DOI: 10.1172/JCI37901]

100 **Stepensky P**, Weintraub M, Yanir A, Revel-Vilk S, Krux F, Huck K, Linka RM, Shaag A, Elpeleg O, Borkhardt A, Resnick IB. IL-2-inducible T-cell kinase deficiency: clinical presentation and therapeutic approach. *Haematologica* 2011; **96**: 472-476 [PMID: 21109689 DOI: 10.3324/haematol.2010.033910]

101 **van Montfrans JM**, Hoepelman AI, Otto S, van Gijn M, van de Corput L, de Weger RA, Monaco-Shawver L, Banerjee PP, Sanders EA, Jol-van der Zijde CM, Betts MR, Orange JS, Bloem AC, Tesselaar K. CD27 deficiency is associated with combined immunodeficiency and persistent symptomatic EBV viremia. *J Allergy Clin Immunol* 2012; **129**: 787-793.e6 [PMID: 22197273 DOI: 10.1016/j.jaci.2011.11.013]

102 **Salzer E**, Daschkey S, Choo S, Gombert M, Santos-Valente E, Ginzel S, Schwendinger M, Haas OA, Fritsch G, Pickl WF, Förster-Waldl E, Borkhardt A, Boztug K, Bienemann K, Seidel MG. Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27. *Haematologica* 2013; **98**: 473-478 [PMID: 22801960 DOI: 10.3324/haematol.2012.068791]

103 **Chaigne-Delalande B**, Li FY, O'Connor GM, Lukacs MJ, Jiang P, Zheng L, Shatzer A, Biancalana M, Pittaluga S, Matthews HF, Jancel TJ, Bleesing JJ, Marsh RA, Kuijpers TW, Nichols KE, Lucas CL, Nagpal S, Mehmet H, Su HC, Cohen JI, Uzel G, Lenardo MJ. Mg2+ regulates cytotoxic functions of NK and CD8 T cells in chronic EBV infection through NKG2D. *Science* 2013; **341**: 186-191 [PMID: 23846901 DOI: 10.1126/science.1240094]

104 **Rosado FG**, Kim AS. Hemophagocytic lymphohistiocytosis: an update on diagnosis and pathogenesis. *Am J Clin Pathol* 2013; **139**: 713-727 [PMID: 23690113 DOI: 10.1309/AJCP4ZDKJ4ICOUAT]

105 **Lehmberg K**, Ehl S. Diagnostic evaluation of patients with suspected haemophagocytic lymphohistiocytosis. *Br J Haematol* 2013; **160**: 275-287 [PMID: 23206255 DOI: 10.1111/bjh.12138]

106 **Parizhskaya M**, Reyes J, Jaffe R. Hemophagocytic syndrome presenting as acute hepatic failure in two infants: clinical overlap with neonatal hemochromatosis. *Pediatr Dev Pathol* 1999; **2**: 360-366 [PMID: 10347280 DOI: 10.1007/s100249900135]

107 **Castillo L**, Carcillo J. Secondary hemophagocytic lymphohistiocytosis and severe sepsis/ systemic inflammatory response syndrome/multiorgan dysfunction syndrome/macrophage activation syndrome share common intermediate phenotypes on a spectrum of inflammation. *Pediatr Crit Care Med* 2009; **10**: 387-392 [PMID: 19325510 DOI: 10.1097/PCC.0b013e3181a1ae08]

108 **Aricò M**, Janka G, Fischer A, Henter JI, Blanche S, Elinder G, Martinetti M, Rusca MP. Hemophagocytic lymphohistiocytosis. Report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. *Leukemia* 1996; **10**: 197-203 [PMID: 8637226]

109 **Trottestam H**, Horne A, Aricò M, Egeler RM, Filipovich AH, Gadner H, Imashuku S, Ladisch S, Webb D, Janka G, Henter JI. Chemoimmunotherapy for hemophagocytic lymphohistiocytosis: long-term results of the HLH-94 treatment protocol. *Blood* 2011; **118**: 4577-4584 [PMID: 21900192 DOI: 10.1182/blood-2011-06-356261]

110 **Imashuku S**, Teramura T, Tauchi H, Ishida Y, Otoh Y, Sawada M, Tanaka H, Watanabe A, Tabata Y, Morimoto A, Hibi S, Henter JI. Longitudinal follow-up of patients with Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. *Haematologica* 2004; **89**: 183-188 [PMID: 15003893]

111 **Chandrakasan S**, Marsh RA, Bellman D. Adolescents and young adults with HLH who undergo allogeneic HCT are at increased risk of mortality compared to younger patients. Program of the Histiocyte Society Annual Meeting, 2013: 27

112 **Gross TG**, Filipovich AH, Conley ME, Pracher E, Schmiegelow K, Verdirame JD, Vowels M, Williams LL, Seemayer TA. Cure of X-linked lymphoproliferative disease (XLP) with allogeneic hematopoietic stem cell transplantation (HSCT): report from the XLP registry. *Bone Marrow Transplant* 1996; **17**: 741-744 [PMID: 8733691]

113 **Marsh RA**, Rao K, Satwani P, Lehmberg K, Müller I, Li D, Kim MO, Fischer A, Latour S, Sedlacek P, Barlogis V, Hamamoto K, Kanegane H, Milanovich S, Margolis DA, Dimmock D, Casper J, Douglas DN, Amrolia PJ, Veys P, Kumar AR, Jordan MB, Bleesing JJ, Filipovich AH. Allogeneic hematopoietic cell transplantation for XIAP deficiency: an international survey reveals poor outcomes. *Blood* 2013; **121**: 877-883 [PMID: 23131490 DOI: 10.1182/blood-2012-06-432500]

114 **Hamidieh AA**, Pourpak Z, Yari K, Fazlollahi MR, Hashemi S, Behfar M, Moin M, Ghavamzadeh A. Hematopoietic stem cell transplantation with a reduced-intensity conditioning regimen in pediatric patients with Griscelli syndrome type 2. *Pediatr Transplant* 2013; **17**: 487-491 [PMID: 23714271 DOI:

10.1111/petr.12092]
115 **Rihani R**, Barbar M, Faqih N, Halalsheh H, Hussein AA, Al-Zaben AH, Rahman FA, Sarhan M. Unrelated cord blood transplantation can restore hematologic and immunologic functions in patients with Chediak-Higashi syndrome. *Pediatr Transplant* 2012; **16**: E99-E105 [PMID: 21450011 DOI: 10.1111/j.1399-3046.2010.01461.x]
116 **Ouachée-Chardin M**, Elie C, de Saint Basile G, Le Deist F, Mahlaoui N, Picard C, Neven B, Casanova JL, Tardieu M, Cavazzana-Calvo M, Blanche S, Fischer A. Hematopoietic stem cell transplantation in hemophagocytic lymphohistiocytosis: a single-center report of 48 patients. *Pediatrics* 2006; **117**: e743-e750 [PMID: 16549504 DOI: 10.1542/peds.2005-1789]
117 **Mischler M**, Fleming GM, Shanley TP, Madden L, Levine J, Castle V, Filipovich AH, Cornell TT. Epstein-Barr virus-induced hemophagocytic lymphohistiocytosis and X-linked lymphoproliferative disease: a mimicker of sepsis in the pediatric intensive care unit. *Pediatrics* 2007; **119**: e1212-e1218 [PMID: 17403820 DOI: 10.1542/peds.2006-1534]
118 **Letsas KP**, Filippatos GS, Delimpasi S, Spanakis N, Kounas SP, Efremidis M, Tsakris A, Kardaras F. Enterovirus-induced fulminant myocarditis and hemophagocytic syndrome. *J Infect* 2007; **54**: e75-e77 [PMID: 16733067 DOI: 10.1016/j.jinf.2006.04.006]
119 **Uni M**, Yoshimi A, Maki H, Maeda D, Nakazaki K, Nakamura F, Fukayama M, Kurokawa M. Successful treatment with recombinant thrombomodulin for B-cell lymphoma-associated hemophagocytic syndrome complicated by disseminated intravascular coagulation. *Int J Clin Exp Pathol* 2013; **6**: 1190-1194 [PMID: 23696942]
120 **Kizaki Z**, Fukumochi H, Sawai T, Ishimura K, Esumi N, Nagai T, Todo S, Imashuku S. [Exchange transfusion and combination chemotherapy in the treatment of 4 cases of childhood malignant histiocytosis]. *Rinsho Ketsueki* 1987; **28**: 845-851 [PMID: 3669324]
121 **Matsumoto Y**, Naniwa D, Banno S, Sugiura Y. The efficacy of therapeutic plasmapheresis for the treatment of fatal hemophagocytic syndrome: two case reports. *Ther Apher* 1998; **2**: 300-304 [PMID: 10227760 DOI: 10.1111/j.1744-9987.1998.tb00127.x]
122 **Sullivan JL**, Byron KS, Brewster FE, Baker SM, Ochs HD. X-linked lymphoproliferative syndrome. Natural history of the immunodeficiency. *J Clin Invest* 1983; **71**: 1765-1778 [PMID: 6306053 DOI: 10.1016/0002-9343(82)90102-4]
123 **Suzuki N**, Morimoto A, Ohga S, Kudo K, Ishida Y, Ishii E. Characteristics of hemophagocytic lymphohistiocytosis in neonates: a nationwide survey in Japan. *J Pediatr* 2009; **155**: 235-238.e1 [PMID: 19446847 DOI: 10.1016/j.jpeds.2009.02.050]
124 **Imashuku S**, Tanaka T, Togari H. Detection of hemophagocytes in blood smears in fatal disseminated neonatal herpes simplex virus infection. *Int J Pediatr Hematol/Oncol* 2000; **6**: 425-428
125 **Yamada K**, Yamamoto Y, Uchiyama A, Ito R, Aoki Y, Uchida Y, Nagasawa H, Kimura H, Ichiyama T, Fukao T, Kohno Y. Successful treatment of neonatal herpes simplex-type 1 infection complicated by hemophagocytic lymphohistiocytosis and acute liver failure. *Tohoku J Exp Med* 2008; **214**: 1-5 [PMID: 18212481 DOI: 10.1620/tjem.214.1]
126 **Brastianos PK**, Swanson JW, Torbenson M, Sperati J, Karakousis PC. Tuberculosis-associated haemophagocytic syndrome. *Lancet Infect Dis* 2006; **6**: 447-454 [PMID: 16790385 DOI: 10.1016/S1473-3099(06)70524-2]
127 **Cançado GG**, Freitas GG, Faria FH, de Macedo AV, Nobre V. Hemophagocytic lymphohistiocytosis associated with visceral leishmaniasis in late adulthood. *Am J Trop Med Hyg* 2013; **88**: 575-577 [PMID: 23324220 DOI: 10.4269/ajtmh.12-0563]
128 **Fardet L**, Lambotte O, Meynard JL, Kamouh W, Galicier L, Marzac C, de Labarthe A, Cabane J, Lebbe C, Coppo P, Molina JM, Martinez V. Reactive haemophagocytic syndrome in 58 HIV-1-infected patients: clinical features, underlying diseases and prognosis. *AIDS* 2010; **24**: 1299-1306 [PMID: 20559036 DOI: 10.1097/QAD.0b013e328339e55b]
129 **Shuper A**, Attias D, Kornreich L, Zaizov R, Yaniv I. Familial hemophagocytic lymphohistiocytosis: improved neurodevelopmental outcome after bone marrow transplantation. *J Pediatr* 1998; **133**: 126-128 [PMID: 9672524 DOI: 10.1016/S0022-3476(98)70190-8]
130 **Jackson J**, Titman P, Butler S, Bond K, Rao A, Veys P, Chiesa R, Leiper A, Riley L, Gilmour K, Amrolia P, Rao K. Cognitive and psychosocial function post hematopoietic stem cell transplantation in children with hemophagocytic lymphohistiocytosis. *J Allergy Clin Immunol* 2013; **132**: 889-95.e1-3 [PMID: 23987797]
131 **Larroche C**, Bruneel F, André MH, Bader-Meunier B, Baruchel A, Tribout B, Genereau T, Zunic P. [Intravenously administered gamma-globulins in reactive hemaphagocytic syndrome. Multicenter study to assess their importance, by the immunoglobulins group of experts of CEDIT of the AP-HP]. *Ann Med Interne* (Paris) 2000; **151**: 533-539 [PMID: 11139652]
132 **Emmenegger U**, Spaeth PJ, Neftel KA. Intravenous immunoglobulin for hemophagocytic lymphohistiocytosis? *J Clin Oncol* 2002; **20**: 599-601 [PMID: 11786591]
133 **Lindamood KE**, Fleck P, Narla A, Vergilio JA, Degar BA, Baldwin M, Wintermark P. Neonatal enteroviral sepsis/meningoencephalitis and hemophagocytic lymphohistiocytosis: diagnostic challenges. *Am J Perinatol* 2011; **28**: 337-346 [PMID: 21089006 DOI: 10.1055/s-0030-1268710]
134 **Fukazawa M**, Hoshina T, Nanishi E, Nishio H, Doi T, Ohga S, Hara T. Neonatal hemophagocytic lymphohistiocytosis associated with a vertical transmission of coxsackievirus B1. *J Infect Chemother* 2013; **19**: 1210-1213 [PMID: 23757031 DOI: 10.1007/s10156-013-0629-2]
135 **Katsibardi K**, Moschovi MA, Theodoridou M, Spanakis N, Kalabalikis P, Tsakris A, Tzortzatou-Stathopoulou F. Enterovirus-associated hemophagocytic syndrome in children with malignancy: report of three cases and review of the literature. *Eur J Pediatr* 2008; **167**: 97-102 [PMID: 17318619 DOI: 10.1007/s00431-007-0436-9]
136 **Hot A**, Madoux MH, Viard JP, Coppéré B, Ninet J. Successful treatment of cytomegalovirus-associated hemophagocytic syndrome by intravenous immunoglobulins. *Am J Hematol* 2008; **83**: 159-162 [PMID: 17849465 DOI: 10.1002/ajh.21008]
137 **Tai CM**, Liu CJ, Yao M. Successful treatment of acute hepatitis A-associated hemophagocytic syndrome by intravenous immunoglobulin. *J Formos Med Assoc* 2005; **104**: 507-510 [PMID: 16091828]
138 **Naffaa M**, Awad J, Oren I, Braun E, Lavi N. Group G streptococcal endocarditis-associated hemophagocytic syndrome. *Int J Infect Dis* 2013; **17**: e1237-e1239 [PMID: 23816411 DOI: 10.1016/j.ijid.2013.05.004]
139 **Shiraishi A**, Ohga S, Doi T, Ishimura M, Takimoto T, Takada H, Miyamoto T, Abe Y, Hara T. Treatment choice of immunotherapy or further chemotherapy for Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 2012; **59**: 265-270 [PMID: 22183955 DOI: 10.1002/pbc.24039]
140 **Abella EM**, Artrip J, Schultz K, Ravindranath Y. Treatment of familial erythrophagocytic lymphohistiocytosis with cyclosporine A. *J Pediatr* 1997; **130**: 467-470 [PMID: 9063426 DOI: 10.1016/S0022-3476(97)70212-9]
141 **Mouy R**, Stephan JL, Pillet P, Haddad E, Hubert P, Prieur AM. Efficacy of cyclosporine A in the treatment of macrophage activation syndrome in juvenile arthritis: report of five cases. *J Pediatr* 1996; **129**: 750-754 [PMID: 8917244 DOI: 10.1016/S0022-3476(96)70160-9]
142 **Tsuda H**. The use of cyclosporin-A in the treatment of

virus-associated hemophagocytic syndrome in adults. *Leuk Lymphoma* 1997; **28**: 73-82 [PMID: 9498706]

143 **Imashuku S**, Hibi S, Kuriyama K, Tabata Y, Hashida T, Iwai A, Kato M, Yamashita N, Oda MUchida M, Kinugawa N, Sawada M, Konno M. Management of severe neutropenia with cyclosporin during initial treatment of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis. *Leuk Lymphoma* 2000; **36**: 339-346 [PMID: 10674906 DOI: 10.3109/10428190009148855]

144 **Imashuku S**, Hibi S, Ohara T, Iwai A, Sako M, Kato M, Arakawa H, Sotomatsu M, Kataoka S, Asami K, Hasegawa D, Kosaka Y, Sano K, Igarashi N, Maruhashi K, Ichimi R, Kawasaki H, Maeda N, Tanizawa A, Arai K, Abe T, Hisakawa H, Miyashita H, Henter JI. Effective control of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis with immunochemotherapy. Histiocyte Society. *Blood* 1999; **93**: 1869-1874 [PMID: 10068659]

145 **Imashuku S**, Kuriyama K, Teramura T, Ishii E, Kinugawa N, Kato M, Sako M, Hibi S. Requirement for etoposide in the treatment of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. *J Clin Oncol* 2001; **19**: 2665-2673 [PMID: 11352958]

146 **Lee JS**, Kang JH, Lee GK, Park HJ. Successful treatment of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis with HLH-94 protocol. *J Korean Med Sci* 2005; **20**: 209-214 [PMID: 15831988 DOI: 10.3346/jkms.2005.20.2.209]

147 **Stéphan JL**, Donadieu J, Ledeist F, Blanche S, Griscelli C, Fischer A. Treatment of familial hemophagocytic lymphohistiocytosis with antithymocyte globulins, steroids, and cyclosporin A. *Blood* 1993; **82**: 2319-2323 [PMID: 8400285]

148 **Mahlaoui N**, Ouachée-Chardin M, de Saint Basile G, Neven B, Picard C, Blanche S, Fischer A. Immunotherapy of familial hemophagocytic lymphohistiocytosis with antithymocyte globulins: a single-center retrospective report of 38 patients. *Pediatrics* 2007; **120**: e622-e628 [PMID: 17698967 DOI: 10.1542/peds.2006-3164]

149 **Milone MC**, Tsai DE, Hodinka RL, Silverman LB, Malbran A, Wasik MA, Nichols KE. Treatment of primary Epstein-Barr virus infection in patients with X-linked lymphoproliferative disease using B-cell-directed therapy. *Blood* 2005; **105**: 994-996 [PMID: 15494422 DOI: 10.1182/blood-2004-07-2965]

150 **Bond J**, Shahdadpuri R, Mc Mahon C, O'marcaigh A, Cotter M, Smith O. Successful treatment of acute Epstein-Barr virus infection associated with X-linked lymphoproliferative disorder with rituximab. *Pediatr Blood Cancer* 2007; **49**: 761-762 [PMID: 17066466 DOI: 10.1002/pbc.21081]

151 **Balamuth NJ**, Nichols KE, Paessler M, Teachey DT. Use of rituximab in conjunction with immunosuppressive chemotherapy as a novel therapy for Epstein Barr virus-associated hemophagocytic lymphohistiocytosis. *J Pediatr Hematol Oncol* 2007; **29**: 569-573 [PMID: 17762500 DOI: 10.1097/MPH.0b013e3180f61be3]

152 **Bosman G**, Langemeijer SM, Hebeda KM, Raemaekers JM, Pickkers P, van der Velden WJ. The role of rituximab in a case of EBV-related lymphoproliferative disease presenting with haemophagocytosis. *Neth J Med* 2009; **67**: 364-365 [PMID: 19767670]

153 **Chellapandian D**, Das R, Zelley K, Wiener SJ, Zhao H, Teachey DT, Nichols KE. Treatment of Epstein Barr virus-induced haemophagocytic lymphohistiocytosis with rituximab-containing chemo-immunotherapeutic regimens. *Br J Haematol* 2013; **162**: 376-382 [PMID: 23692048 DOI: 10.1111/bjh.12386]

154 **Imashuku S**, Kudo N, Kubo K, Yachie A. Are regimens containing rituximab effective in the initial treatment of Epstein-Barr virus-positive natural killer cell lymphoproliferative disease-associated hemophagocytic lymphohistiocytosis? *Int J Hematol* 2013; **98**: 375-377 [PMID: 23975519 DOI: 10.1007/s12185-013-1419-4]

155 **Bonney DK**, Htwe EE, Turner A, Kelsey A, Shabani A, Hughes S, Hughes I, Wynn RF. Sustained response to intrathecal rituximab in EBV associated Post-transplant lymphoproliferative disease confined to the central nervous system following haematopoietic stem cell transplant. *Pediatr Blood Cancer* 2012; **58**: 459-461 [PMID: 21584931 DOI: 10.1002/pbc.23134]

156 **Czyzewski K**, Styczynski J, Krenska A, Debski R, Zajac-Spychala O, Wachowiak J, Wysocki M. Intrathecal therapy with rituximab in central nervous system involvement of post-transplant lymphoproliferative disorder. *Leuk Lymphoma* 2013; **54**: 503-506 [PMID: 22873830 DOI: 10.3109/10428194.2012.718342]

157 **Strout MP**, Seropian S, Berliner N. Alemtuzumab as a bridge to allogeneic SCT in atypical hemophagocytic lymphohistiocytosis. *Nat Rev Clin Oncol* 2010; **7**: 415-420 [PMID: 20404855 DOI: 10.1038/nrclinonc.2010.40]

158 **Marsh RA**, Allen CE, McClain KL, Weinstein JL, Kanter J, Skiles J, Lee ND, Khan SP, Lawrence J, Mo JQ, Bleesing JJ, Filipovich AH, Jordan MB. Salvage therapy of refractory hemophagocytic lymphohistiocytosis with alemtuzumab. *Pediatr Blood Cancer* 2013; **60**: 101-109 [PMID: 22522603 DOI: 10.1002/pbc.24188]

159 **Keith MP**, Pitchford C, Bernstein WB. Treatment of hemophagocytic lymphohistiocytosis with alemtuzumab in systemic lupus erythematosus. *J Clin Rheumatol* 2012; **18**: 134-137 [PMID: 22426581 DOI: 10.1097/RHU.0b013e31824e8d9b]

160 **Olin RL**, Nichols KE, Naghashpour M, Wasik M, Shelly B, Stadtmauer EA, Vogl DT. Successful use of the anti-CD25 antibody daclizumab in an adult patient with hemophagocytic lymphohistiocytosis. *Am J Hematol* 2008; **83**: 747-749 [PMID: 18615554 DOI: 10.1002/ajh.21236]

161 **Henzan T**, Nagafuji K, Tsukamoto H, Miyamoto T, Gondo H, Imashuku S, Harada M. Success with infliximab in treating refractory hemophagocytic lymphohistiocytosis. *Am J Hematol* 2006; **81**: 59-61 [PMID: 16369976 DOI: 10.1002/ajh.20462]

162 **Oda Y**, Urushidani Y, Ooi S, Endoh A, Nakamura R, Adachi K, Fukushima H. Hemophagocytic lymphohistiocytosis in a rheumatoid arthritis patient treated with infliximab. *Intern Med* 2012; **51**: 655-657 [PMID: 22449679 DOI: 10.2169/internalmedicine.51.5687]

163 **Ideguchi H**, Ohno S, Takase K, Hattori H, Kirino Y, Takeno M, Ishigatsubo Y. Successful treatment of refractory lupus-associated haemophagocytic lymphohistiocytosis with infliximab. *Rheumatology* (Oxford) 2007; **46**: 1621-1622 [PMID: 17726035 DOI: 10.1093/rheumatology/kem205]

164 **Takahashi N**, Naniwa T, Banno S. Successful use of etanercept in the treatment of acute lupus hemophagocytic syndrome. *Mod Rheumatol* 2008; **18**: 72-75 [PMID: 18161002 DOI: 10.3109/s10165-007-0006-z]

165 **Kikuchi H**, Yamamoto T, Asako K, Takayama M, Shirasaki R, Ono Y. Etanercept for the treatment of intractable hemophagocytic syndrome with systemic lupus erythematosus. *Mod Rheumatol* 2012; **22**: 308-311 [PMID: 21773824 DOI: 10.1007/s10165-011-0500-1]

166 **Jordan MB**, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood* 2004; **104**: 735-743 [PMID: 15069016 DOI: 10.1182/blood-2003-10-3413]

167 **Pachlopnik Schmid J**, Ho CH, Chrétien F, Lefebvre JM, Pivert G, Kosco-Vilbois M, Ferlin W, Geissmann F, Fischer A, de Saint Basile G. Neutralization of IFNgamma defeats haemophagocytosis in LCMV-infected perforin- and Rab27a-deficient mice. *EMBO Mol Med* 2009; **1**: 112-124 [PMID: 20049711 DOI: 10.1002/emmm.200900009]

168 **Sato E**, Ohga S, Kuroda H, Yoshiba F, Nishimura M, Nagasawa M, Inoue M, Kawa K. Allogeneic hematopoietic stem cell transplantation for Epstein-Barr virus-associated T/natural killer-cell lymphoproliferative disease in Japan. *Am J Hematol* 2008; **83**: 721-727 [PMID: 18626884 DOI: 10.1002/

ajh.21247]
169 **Landman-Parker J**, Le Deist F, Blaise A, Brison O, Fischer A. Partial engraftment of donor bone marrow cells associated with long-term remission of haemophagocytic lymphohistiocytosis. *Br J Haematol* 1993; **85**: 37-41 [PMID: 8251408 DOI: 10.1111/j.1365-2141.1993.tb08642.x]
170 **Terrell CE**, Jordan MB. Mixed hematopoietic or T-cell chimerism above a minimal threshold restores perforin-dependent immune regulation in perforin-deficient mice. *Blood* 2013; **122**: 2618-2621 [PMID: 23974195 DOI: 10.1182/blood-2013-06-508143]

P- Reviewer: Lozano ML **S- Editor**: Ji FF
L- Editor: Roemmele A **E- Editor:** Lu YJ

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
doi:10.5315/wjh.v3.i3.85

World J Hematol 2014 August 6; 3(3): 85-92
ISSN 2218-6204 (online)

MINIREVIEWS

Myelofibrosis: Prognostication and cytoreductive treatment

Margherita Maffioli, Domenica Caramazza, Barbara Mora, Michele Merli, Francesco Passamonti

Margherita Maffioli, Domenica Caramazza, Barbara Mora, Michele Merli, Francesco Passamonti, Division of Hematology, Department of Medicine, University Hospital Ospedale di Circolo e Fondazione Macchi, 21100 Varese, Italy
Author contributions: Maffioli M and Passamonti F wrote the paper; Maffioli M, Caramazza D, Mora B, Merli M and Passamonti F discussed the mail topics of the paper and all gave the final approval of the manuscript.
Correspondence to: Francesco Passamonti, MD, Division of Hematology, Department of Medicine, University Hospital Ospedale di Circolo e Fondazione Macchi, Viale L. Borri 57, 21100 Varese, Italy. francesco.passamonti@ospedale.varese.it
Telephone: +39-332-393648 Fax: +39-332-393648
Received: November 2, 2013 Revised: February 8, 2014
Accepted: June 10, 2014
Published online: August 6, 2014

Abstract

Myeloproliferative neoplasms include three diseases: polycythemia vera, essential thrombocythemia and primary myelofibrosis (PMF), currently diagnosed according to the 2008 World Health Organization criteria. Patients with PMF may encounter many complications, and, among these, disease progression is the most severe. Concerning prognostication of Myelofibrosis (MF), the International Prognostic scoring system (IPSS) (International Prognostic Scoring System) model at diagnosis and the Dynamic IPSS (DIPSS) anytime during the course of the disease may be useful to define survival of MF patients. The IPSS and the DIPSS are based on age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25 × 10^9/L, and circulating blast cells 1% or greater. Cytogenetic profile and mutational analysis seem to be the next step to implement MF prognostication. Concerning treatments, hydroxyurea has been considered until now the drug of choice when an anti-myeloproliferative effect is needed, but recent data on JAK inhibitors demonstrated a significant effect of these drugs on splenomegaly and symptoms.

Key words: Myelofibrosis; Prognosis; JAK2; Ruxolitinib; Fedratinib; Momelotinib

Core tip: Myelofibrosis (MF) is a mutational/clinical-complex disease. Prognostication of MF is based on the International Prognostic scoring system (IPSS) model at diagnosis and on the Dynamic IPSS thereafter. Factors included in both models are: age > 65 years, constitutional symptoms, hemoglobin < 10 g/dL, leukocytes > 25 × 10^9/L, and circulating blast cells 1% or greater. Cytogenetic profile and mutational status help to better discriminate within each IPSS category. JAK inhibitors are new promising therapies with a molecular target, translating into a clinical benefit: spleen reduction MF-symptoms refief. Among JAK inhibitor, ruxolitinib has been approved for MF.

Maffioli M, Caramazza D, Mora B, Merli M, Passamonti F. Myelofibrosis: Prognostication and cytoreductive treatment. *World J Hematol* 2014; 3(3): 85-92 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i3/85.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i3.85

PROGNOSTICATION IN PMF

Among myeloproliferative neoplasms (MPN), primary myelofibrosis (PMF) has the most heterogeneous clinical presentation, including anemia, splenomegaly, leukocytosis or leukopenia, thrombocytosis or thrombocytopenia, and constitutional symptoms (fever, weight loss, night sweats). Available estimate of survival in MF fixes the median value at 6 years ranging from few months to many years[1]. Causes of death may be summarized into bone marrow failure (severe anemia, bleeding due to thrombocytopenia, and infections due to leukopenia) in 25%-30% of patients, leukemic transformation, named blast phase (BP), in 10%-20% of patients, cardiovascular

complications in 15%-20%, and portal hypertension in 10%.

Many factors affect survival in PMF such as advanced age[1,2], anemia[1], red blood cell transfusion need[1], leukopenia[3], leukocytosis[1], thrombocytopenia[1], peripheral blast count[1], systemic symptoms[1], hepatic myeloid metaplasia[4-22], decreased marrow cellularity with higher degree of fibrosis[23], higher degree of microvessel density[1], high number of circulating CD34-positive cells[1], cytogenetic abnormalities[1], the *JAK2* (V617F) mutation[1], some new mutation[1] and high level of some cytokines[24-30].

PROGNOSTIC FACTORS IN PMF

Cytogenetic abnormalities

Cytogenetic analysis has a role to identify an abnormal profile that provides evidence of clonality. Although most PMF patients' bone marrow aspirate results in a "dry tap", karyotype analysis can be performed on peripheral blood[29]. Among MPN, PMF shows the highest aberration rate with approximately 30% of patients carrying an abnormal karyotype at diagnosis[9]. The most frequent isolated abnormalities in PMF involve chromosome 1, 8, 9, 13 and 20[1]. Sole abnormalities of chromosome 7 were reported in 7% of PMF in a dedicated analysis and 7q- was the most frequent[1]. Concerning prognostic relevance of cytogenetic changes in PMF, recent studies have been consistently claiming an impact on survival[1]. Three studies, each comprising 202, 200 and 131 patients showed a favorable prognostic value for sole 20q- or sole 13q-[1]. A further study of 433 PMF patients refined a two-tired cytogenetic-risk stratification: unfavorable and favorable karyotype[1]. In detail, this study identified a high risk profile for cytogenetics when patients carry sole abnormalities of i(17q), -5/5q-, 12p-, 11q23 rearrangement, inv(3), sole +8 or sole -7/7q-, complex karyotype (three or more abnormalities), and a low-risk profile when patients carry normal diploid, or sole abnormalities not included in the high-risk profile. The respective 5-year survival rates were 8% and 51%. The presence of monosomal karyotype, which is defined as two or more autosomal monosomies or a single autosomal monosomy associated with at least one structural abnormality, identified a subset of patients with unfavorable karyotype associated with extremely poor overall and leukemia-free survival, as demonstrated in a study of 793 PMF patients[31].

Mutational profile

In PMF the prognostic role of the oncogenic mutations involving *MPL* and *JAK2* has been assessed, overall, not showing a significant effect on survival[1]. Concerning *JAK2* (V617F) allele burden, there is evidence that having a lower allele load implies a worse survival. In one study[7], survival was significantly reduced in the lower quartile compared with upper quartiles and *JAK2*^{wt} patients, mostly because of infections. In the second paper[27], Kaplan-Meier plots revealed significantly shortened overall and leukemia-free survival for the lower quartile allele burden group, mostly related to BP transformation. Intriguing results were obtained in PMF patients receiving allogeneic hematopoietic stem cell transplantation (HSCT)[32]. In 139 out of 162 patients with known *JAK2* (V617F) mutation status who received HSCT after reduced-intensity conditioning, overall survival was significantly reduced in patients harboring *JAK2*^{wt} compared with *JAK2* mutated patients. In addition, patients who cleared *JAK2* in the peripheral blood six months post-HSCT had a significant lower risk of relapse: this highlights the importance of complete molecular response in MF.

Mutations in the *EZH2* gene, acting through modifying chromatin structure and rendering genes involved in apoptosis inaccessible for transcription, have been found in roughly 6% of PMF[1]. Recently, in 370 PMF and 148 post-PV/ET MF genotyped for mutations of *EZH2* a total of 25 different mutations were detected. *EZH2*-mutated PMF patients had significantly higher leukocyte counts, blast cell counts, and larger spleens at diagnosis, and most of them (53%) were in the high-risk International Prognostic scoring system (IPSS) category. Leukemia-free survival (LFS) and overall survival (OS) were significantly reduced in *EZH2*-mutated PMF patients[1].

Mutations in *IDH* were detected in 4% of patients: 7 patients with mutations of *IDH2* (5 R140Q, 1 R140W and 1 R172G) and 5 of *IDH1* (3 R132S and 2 R132C)[24]. The estimate of survival disclosed that *IDH* mutations are associated with inferior OS and LFS. In addition, a more pronounced effect for the mutant *IDH* on OS and LFS was demonstrated in the context of the *JAK2* mutation.

After the identification of recurrent somatic mutations that involved different components of the RNA splicing machinery and other spliceosome-related genes in myelodysplastic syndromes, these mutations have been investigated in MPNs. A study on 155 MF identified *SF3B1* mutations in 6.5% of the patients: 4 (40%) K700E, 4 (40%) K666T/N/M, 1 (10%) H662D and 1 (10%) N626S and failed to demonstrate any prognostic relevance[11]. In a subsequent study on 187 patients, 17% harbored *SRSF2* monoallelic mutations affecting residue P95[1]. Significant associations were demonstrated between *SRSF2* mutations and advanced age, *IDH* mutations, and higher DIPSS-plus risk category. Finally, *SRSF2* mutations were associated with shortened OS and LFS.

Very recently, a total of 879 MF patients were studied to determine the individual and combinatorial prognostic relevance of somatic mutations in *ASXL1, SRSF2, EZH2, TET2, DNMT3A, CBL, IDH1, IDH2, MPL* and *JAK2* in patients with MF[1]. Analysis was performed in 483 European patients and the seminal observations were validated in 396 patients from Mayo Clinic, Rochester, United States. Of these, *ASXL1, SRSF2*, and *EZH2* mutations inter-independently predicted shortened survival. However, only *ASXL1* mutations remained

significant in the context of the International Prognostic Scoring System (IPSS) with a hazard ratio (HR) of 2.02. These observations were validated in the Mayo Clinic cohort, where investigators found that *ASXL1, SRSF2* and *EZH2* mutations were independently associated with poor survival, but only *ASXL1* mutations held prognostic relevance independently from the Dynamic IPSS (DIPSS)-plus model, with a HR of 1.4. In the European cohort, LFS was negatively affected by *IDH1/2, SRSF2* and *ASXL1* mutations and in the Mayo cohort by *IDH1* and *SRSF2* mutations. In conclusion, the study identified *ASXL1* mutations as the most relevant to be included in the patient's evaluation besides the IPSS models. *ASXL1* mutations are however present at low frequency (less than 20%) in lower risk IPSS categories.

However, the most recent discovery in MF is the occurrence of *CALR* (calreticulin) mutation in patients with MPN without *JAK2* or *MPL* mutation[10,16] and in familial MPN[12]. In MF a clear association with survival has been documented[26].

Proinflammatory cytokines

The abnormal cytokine profile in PMF means that PMF is, at least in part, an inflammatory disease. Cytokines contribute to clinical phenotype, bone marrow fibrosis, angiogenesis, extramedullary hematopoiesis and constitutional symptoms. The interest on cytokines in PMF has recently arisen as JAK inhibitors may quickly reduce several proinflammatory cytokines[1]. Mayo Clinic investigators found that among 30 cytokines tested in a cohort of 90 treatment-naive patients with PMF, high levels of IL-8, IL-2R, IL-12, IL-15 correlate with inferior survival independently from conventional risk stratification[31]. In detail, the presence of 3-fold increased levels of one or both IL-8 and IL-2R may predict worse survival. C-reactive protein (CRP) is a simple marker of systemic inflammation mediated by cytokines, mainly IL-6, and it has been found in MF as well as in ET and PV at higher level than in healthy controls. In PV and ET, high-sensitivity CRP (hsCRP) correlated with a higher risk of vascular complications. In MF hsCRP seems to be associated with a higher incidence of BP and this seems independent from models used to predict survival in MF. To explain this association investigators even speculated on a mutagenic role of chronic oxidative stress on the stem cell, but this seems premature as, for example, new JAK inhibitors, which are able to reduce CRP expression, don't affect leukemic evolution.

Plasma immunoglobulin free light chain

Plasma immunoglobulin FLC might be considered as a surrogate marker of host immune response. free light chain (FLC) (κ or λ) values above the upper limit of normal have been documented in 33% of 240 patients with PMF[18]. Increases in FLC were significantly associated with increased creatinine and advanced age in PMF. In multivariable analysis, increased FLC predicted shortened survival independently from age, creatinine, and other conventional risk factors. No correlations were seen with LFS, karyotype, or *JAK2, MPL*, or *IDH* mutations. In patients with PMF who were studied by cytokine profiling, the prognostic value of an increased FLC level was independent of that from circulating IL-2R or IL-8 levels.

Red blood cell transfusion dependency

Criteria to define red blood cell (RBC) transfusion dependency in PMF have been published[5], and recently updated[6]. Experts considered a volume of 2 units of RBC/month over three months to be the most appropriate observational interval and RBC-transfusion frequency to define a person as RBC-transfusion-dependent. In general, the cutoff level of hemoglobin to define the need of RBC transfusion is 8.5 g/dL. The prognostic impact of RBC transfusion need was examined in 254 consecutive patients, of whom 24% required RBC transfusions at diagnosis and 9% became RBC transfusion dependent during the first year after diagnosis[1]. RBC transfusion need clearly separated two groups with different survivals: 35 mo (transfused from diagnosis), 25 mo (transfused within 1 year), and 117 months (not transfused). RBC transfusion need had an IPSS-independent prognostic power downgrading or upgrading prognosis within specific IPSS categories. This result was confirmed by a study on 288 consecutive patients with PMF[4].

Only 5% of transfusion-independent patients have iron overload as compared to 72% of transfusion-dependent patients[1]. Iron homeostasis is potentially an intriguing pathway in MF. Prognostic interdependence among serum hepcidin (key regulator of iron homeostasis), serum ferritin, hemoglobin of < 10 g/dL, and RBC transfusion requirement has been described although only increased hepcidin and ferritin levels had independent prognostic value for survival in MF. Homeostatic control of hepcidin by iron is preserved in MF, as demonstrated by the strong positive correlation between hepcidin and ferritin levels. In addition, the absence of correlation between hepcidin and circulating inflammatory cytokine levels indicates that hepcidin levels are mainly controlled by iron loading or advanced disease and not by inflammatory signal. The role of iron chelation in MF has not been yet investigated, but some reports showed improvement in term of iron deposits and hemoglobin level[15].

Prognostic models for survival at diagnosis of PMF

In the last years many prognostic models have been developed in PMF. The most used in the past was the Lille score[3], recently replaced by the IPSS[1].

IPSS score

The IPSS was defined through the collaboration of seven centers under the auspices of the IWG-MRT in 2009[1]. After a systematic individual case review, the database included 1054 patients with PMF defined accord-

Table 1 Score values for international prognostic scoring system and dynamic international prognostic scoring system

Parameter	Scores	
	IPSS	DIPSS
Age > 65 yr	1	1
Hemoglobin < 10 g/dL	1	2
Leukocyte count > 25 × 10^9/L	1	1
Blast cells ⩾ 1%	1	1
Constitutional symptoms	1	1

IPSS: Score 0 for low risk, score 1 for intermediate risk-1, score 2 for intermediate risk-2, score ⩾ 3 for high risk; DIPSS: Score 0 for low risk, score 1-2 for intermediate risk-1, score 3-4 for intermediate risk-2, score 5-6 for high risk.

Table 2 Score values dynamic international prognostic scoring system-plus

Parameter	Score value
DIPSS intermediate-1	1
DIPSS intermediate-2	2
DIPSS high risk	3
Unfavorable cytogenetics	1
Red blood cell need	1
Platelet < 100 ×10^9/L	1

DIPSS-plus: Score 0 for low risk, score 1 for intermediate risk-1, score 2-3 for intermediate risk-2, score 4-6 for high risk; DIPSS: Dynamic International Prognostic scoring system-plus.

ing to the WHO classification system, excluding post-PV and post-ET MF and prefibrotic PMF. This is the largest prognostic study ever performed in PMF. Median survival was 69 mo. Multivariate analysis of parameters obtained at disease diagnosis identified age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25 × 10^9/L, and circulating blast cells 1% or greater as predictors of shortened survival. Based on the presence of 0 (low risk), 1 (intermediate risk-1), 2 (intermediate risk-2) or greater than or equal to 3 (high risk) of these variables, four risk groups with no overlapping in their survival curves were generated (Table 1). The four risk categories were well balanced: 22% of patients fell into the low risk category, 29% in the intermediate risk-1, 28% in the intermediate risk-2 and 21% in the high risk. Median survivals were 135 mo for low risk patients, 95 mo for intermediate-1 patients, 48 mo for intermediate-2 patients, and 27 mo for high risk patients.

Among these patients, 409 patients had available cytogenetic analysis at diagnosis: an abnormal karyotype implied a shorter survival primarily restricted to patients in the intermediate-1 and -2 risk categories. Concerning the *JAK2* (V617F) mutation, no association was observed between *JAK2* status and prognostic score or survival.

Dynamic models for survival in PMF

The progressive nature of PMF generated interest in defining new so-called dynamic models, such as the dynamic-IPSS (DIPSS) and the most recent DIPSS-Plus. In a non time-dependent analysis (models at diagnosis), patients are assigned to a risk group on the basis of the assessment of risk factors at diagnosis, and are followed in the same category irrespective of the acquisition of other risk factors during disease course. According to a dynamic model, patients contribute to the estimate of survival in a category only as long as they do not acquire further risk factors, then they shift to a higher category according to their new score.

DIPSS model

The DIPSS was developed in 525 PMF patients regularly followed[1]. DIPSS risk factors are age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25 × 10^9/L, and circulating blast cells 1% or greater. The scoring system of DIPSS is different from IPSS (Table 1). The resulting DIPSS risk categories are low (score 0), intermediate-1 (score 1 or 2), intermediate-2 (score 3 or 4) and high (score 5 or 6). Median survival was not reached in low risk patients; it was 14.2 years in intermediate-1, 4 years in intermediate-2, and 1.5 years in high risk. From a practical point of view, anytime a decision has to be made on the basis of an updated prognostic status, the parameters of the DIPSS models will be checked and corresponding values will be assigned. The sum of the values will allow allocating the patient into a risk category (low, intermediate-1, intermediate-2, high) and cumulative survival can be estimated. It is obvious that the corresponding cumulative probability of survival at each time point of the follow-up should be read considering the time elapsed since diagnosis. This estimate remains applicable thereafter until the patient changes risk category. The DIPPS model was also able to predict also the evolution to BP[20].

Very recently Scott *et al*[23] found that DIPSS categories at the time of HSCT predict post-transplant outcome in 170 patients with PMF (related donor, 86; unrelated donor, 84). After a median follow-up of 5.9 years, the median survivals have not been reached for DIPSS low and intermediate-1 risk groups, and were 7 and 2.5 years for intermediate-2 and high-risk patients, respectively.

DIPSS-plus model

This model was produced in 793 patients with PMF of which 428 were referred within and 365 after their first year of diagnosis[1]. This composite model included as worse prognostic factors the unfavorable cytogenetics as previously grouped (complex, sole or two including +8, -7/7q-, i(17q), inv (3), -5/5q-, 12p-, 11q23 rearrangements), RBC transfusion need, platelet count lower than 100 × 10^9/L, and DIPSS categories. According to the model, 1 point each was assigned to DIPSS intermediate-1 risk, unfavorable karyotype, platelets lower than 100 × 10^9/L, and RBC transfusion need, while DIPSS intermediate-2 and high risk were assigned 2 and 3

points, respectively (Table 2). On the basis of this scoring system, four categories were generated: low risk (0 adverse points; median survival, 185 mo), intermediate-1 risk (1 adverse point; median survival, 78 mo), intermediate-2 risk (2-3 adverse points; median survival, 35 mo), and high risk (4-6 adverse points; median survival, 16 mo). It's interesting to note that DIPSS-plus investigators found a proportion of patients in each DIPSS risk category with RBC transfusion need, unfavorable karyotype, and thrombocytopenia, namely 0% (RBC transfusion need), 7% (unfavorable karyotype), and 7% (thrombocytopenia) in low risk patients, 13%, 12%, and 18% in intermediate-1 risk patients, 56%, 17%, and 32% in intermediate-2 risk patients; and 69%, 23%, and 47% in high risk patients, respectively. This sheds light into the possibility of better stratifying patients with lower risk categories.

ANTI-MYELOPROLIFERATIVE AGENTS

The ELN guidelines recommended to use hydroxyurea (HU) as drug of choice when an anti-myeloproliferative effect is needed in MPNs[1]. However, data available on HU are scant. The most complete study on HU in MF evaluated retrospectively 40 patients[1]. Reasons for treatment were constitutional symptoms (55%), symptomatic splenomegaly (45%), thrombocytosis (40%), leukocytosis (28%), pruritus (10%), and bone pain (8%). Responses on different symptoms/clinical findings were as follows: bone pain in 100%, constitutional symptoms in 82%, pruritus in 50%, splenomegaly in 40%, and anemia in 12.5%. According to the IWG-MRT criteria[1], clinical improvement was achieved in 16 patients (40%). Despite the high rate, the median duration of response was 13.2 mo. Worsening of anemia or appearance of pancytopenia were observed in half of the patients.

JAK inhibitors

In the last few years several medicines with anti JAK properties, named JAK inhibitors (JAKi) have been studied. Among these, ruxolitinib is the only approved in many States and available for clinical practice. Other compounds are nowadays under phase 3 investigation (fedratinib, momelotinib, pacritinib), while others are being tested in phase 1-2 studies (www.clinicaltrials.gov). For the practical purpose of this review only ruxolitinib, fedratinib and momelotinib will be discussed in detail as only data published as a full paper will be taken into account.

Ruxolitinib

A phase Ⅰ/Ⅱ trial with ruxolitinib (oral drug) was conducted in 152 patients with PMF or post-PV/post-ET MF. Eligible subjects were therapy-requiring patients, refractory, relapsed, intolerant to previous therapy, or patients with intermediate or high-risk Lille score, if at diagnosis. Main exclusion criteria were thrombocytopenia (platelets < 100×10^9/L) and neutropenia. Applying IWG-MRT criteria[1], 44% of patients obtained a clinical improvement of spleen size (≥ 50% reduction from baseline, measured by palpation) at 3 mo and responses were maintained at 12 mo in more than 70% of patients. The majority of patients had more than 50% improvement in constitutional symptoms mostly due to the activity against pro-inflammatory cytokines[1]. The reduction of the *JAK2*(V617F) allele burden was modest. This study was mainly conducted at MD Anderson Cancer Center (MDACC), Houston, and at Mayo Clinic, Rochester. Two comparisons of outcomes from this phase Ⅰ/Ⅱ trial with historical controls have been performed separately in the two centers to test the effect of ruxolitinib on survival. Mayo Clinic investigators compared 51 patients who received ruxolitinib at Mayo Clinic with 410 patients from the Institutional database not showing any difference in term of survival[28]. The second study compared 107 patients treated with ruxolitinib at MDACC with 310 patients (from three different centers) matched for the phase 1-2 study entry criteria, as controls[1]. A survival benefit for patients treated with ruxolitinib was demonstrated. In addition, the study demonstrated that patients treated with ruxolitinib who obtained a reduction of spleen size greater than 50% have a significantly better survival than those who did not[1].

Two prospective randomized trials with ruxolitinib have been published: COMFORT-1 (155 ruxolitinib *vs* 151 placebo)[1] and COMFORT-2 (146 ruxolitinib vs. 73 best available therapy, BAT)[1]. In COMFORT-1, the primary endpoint (reduction of spleen volume by MRI equal to or greater than 35%) at week 24 was reached in 42% of patients in the ruxolitinib arm and in 1% of those in the placebo arm. At week 24, 46% of patients receiving ruxolitinib and 5% of those receiving placebo experienced symptom alleviation by at least 50%, as measured by the modified Myelofibrosis Symptom Assessment Form (MF-SAF)[14]. Patients treated with ruxolitinib experienced relief of abdominal discomfort, early satiety, night sweats, itching, musculoskeletal pain[1]. In the COMFORT-2 trial the primary endpoint (the same as the COMFORT-1 study but evaluated at week 48) was reached in 28% of patients treated with ruxolitinib and in 0% of those receiving BAT; at week 24 the figures were 32% and 0%, respectively. Mean improvements from baseline in FACT-LymS (Functional Assessment of Cancer Therapy-Lymphoma System) were greater in the ruxolitinib arm.

Recently, the long-term (median time, 2 years) data from the COMFORT-1 trial has been published: 100 of 155 patients randomized to ruxolitinib were still receiving treatment[33]. Mean spleen volume reductions in the ruxolitinib group were 32% at week 24 and 35% at week 96; improvements in quality of life measures were also maintained. Improved survival was observed for ruxolitinib (n = 27 deaths) *vs* placebo (n = 41 deaths) with a hazard ratio of 0.58 (95%CI: 0.36-0.95). Dose-dependent anemia and thrombocytopenia were the most common adverse events in the ruxolitinib group, but these

events rarely led to discontinuation. The incidence of new-onset grade 3 or 4 anemia (29% and 11%, respectively) and thrombocytopenia (9% and 3%, respectively) reported in the first 6 mo of therapy decreased over time to less than 5% for anemia and less than 2% for thrombocytopenia. Mean hemoglobin values reached a nadir of 10%-12% below baseline between weeks 8 and 12 and stabilized over time to a new steady-state slightly below baseline by week 24, and then remained stable throughout the remaining follow-up. In the first 6 mo of treatment, the most common non-hematologic adverse events that occurred more frequently in the ruxolitinib group compared with the placebo group were ecchymosis, headache and dizziness. Under ruxolitinib the rate of non-hematologic adverse events reduced over time. Two patients originally randomized to receive ruxolitinib developed BP at the time of the primary analysis[1] and no further cases were reported in this group.

COMFORT-2 trialists updated the 3 year-follow with 45% (66 of 146) of those originally randomized to ruxolitinib remaining on treatment. The 3-year probability to maintain spleen response (greater than 35%, by MRI) was 50% among patients achieving such degree of response. Ruxolitinib continues to be well tolerated. Anemia and thrombocytopenia were the main toxicities, but they were generally manageable, improved over time, and rarely led to treatment discontinuation (1% and 3.6% of patients, respectively). Other adverse events of special interest included leukopenia, bleeding, infections, thromboembolic events, elevated transaminase levels, increased systolic blood pressure, weight gain. The rate of these events generally decreased with longer exposure to ruxolitinib treatment, with the highest rates occurring within the first 6 mo of treatment. Among these events, infections occurred in 50% of patients between weeks 0-24 and included bronchitis, gastroenteritis, nasopharyngitis, urinary tract infections. The rate of infections becomes 25% in weeks 144-168. Over the entire course of the study, 2 patients (1.4%) in the ruxolitinib arm had tuberculosis. No single non-hematologic adverse event led to definitive ruxolitinib discontinuation in more than one patient. Finally, patients randomized to ruxolitinib showed longer overall survival than those randomized to BAT (HR = 0.48, 95%CI: 0.28-0.85).

Both COMFORTs trial included patients with placebo or BAT who crossed to ruxolitinib: this makes impossible the evaluation of the net effect of ruxolitinib over comparators in the long term. Very recently, a comparison of survival from diagnosis of the DIPSS cohort (350 PMF, selection criteria, patients who become intermediate-2 and high risk IPSS, blast cell count lower than 10%) and the COMFORT-2 cohort (100 patients, intermediate-2 and high risk IPSS, blast cell count lower than 10%, selection criteria, PMF) has been published. This demonstrated an advantage in term of survival using ruxolitinib (COMFORT-2) *vs* standard therapy (DIPSS)[21].

Taken together the COMFORT trials showed that ruxolitinib, a drug with a good safety profile, improves two clinical needs of patients: splenomegaly and MF-related symptoms. However, reactivation of infections such as tuberculosis or viral hepatitis has been reported in very few case reports[1] and this underlines the need for a careful observation of patients during follow-up. *In vitro* data[8] demonstrated that ruxolitinib significantly affects dendritic cell differentiation and function leading to impaired T-cell activation[13], potentially resulting in increased infection rates in ruxolitinib-treated patients. Though requiring adequante monitoring for these potential side effects, data on survival advantage are really interesting and place this drug as a new potential first line therapy in MF patients at higher risk.

Fedratinib, SAR302503

In a phase Ⅰ-Ⅱ trial, fedratinib was administered orally once a day to 59 patients with intermediate and high-risk MF[1]. By six and 12 cycles of treatment, 39% and 47% of patients, respectively, had achieved a spleen response per IWG-MRT criteria. The majority of patients with leukocytosis or thrombocytosis at baseline achieved normalization of blood counts after six (57% and 90%, respectively) and 12 (56% and 88%, respectively) cycles. Beside the effect on splenomegaly, the majority of patients with constitutional symptoms, fatigue, pruritus had a durable resolution. Grade 3 to 4 hematologic adverse events included anemia (occurring in 35% of 37 patients who were not RBC transfusion dependent at baseline), thrombocytopenia (24%) and neutropenia (10%). At doses ranging between 240 mg and 520 mg, two of five RBC transfusion-independent patients became RBC transfusion-dependent and two of nine had grade 3/4 thrombocytopenia. The main non-hematologic adverse events included all grades nausea (69%), diarrhea (64%) vomiting (58%), all self-limited and controlled by symptomatic treatments. Asymptomatic increase of lipase, AST, ALT, and creatinine have been reported in roughly one quarter of patients. A randomized, blinded, placebo-controlled study of fedratinib (dose 400 mg or 500 mg daily), named JAKARTA, in patients with intermediate-2 or high risk MF is ongoing with the objective to evaluate the reduction of spleen volume by MRI equal to or greater than 35%. Unfortunately, despite this pivotal study met the primary endpoint in both dose groups, cases consistent with Wernicke's encephalopathy have been reported in patients participating in fedratinib trials. Following a thorough risk-benefit analysis, the risk to patient safety was considered to outweigh the benefit that fedratinib would bring to patients. All clinical trials involving fedratinib have been halted, and fedratinib treatment discontinued in patients enrolled in ongoing trials.

Momelotinib, CYT387

Momelotinib was studied in a phase 1/2 trial in patients with high or intermediate risk MF[17]. Pre-planned safety and efficacy analysis has been completed for the initial

60 patients. In the dose-escalation phase, the maximum-tolerated dose was 300 mg/d based on reversible grade 3 headache and asymptomatic hyperlipasemia. Twenty-one and 18 additional patients were accrued at two biologically effective doses, 300 mg/d and 150 mg/d, respectively. Anemia and spleen responses, per IWG-MRT criteria, were 59% and 48%, respectively. Among 33 patients who were RBC-transfused in the month prior to study entry, 70% achieved a minimum 12-wk period without transfusions. Most patients experienced constitutional symptoms improvement. Grade 3/4 adverse reactions included thrombocytopenia (32%), hyperlipasemia (5%), elevated liver transaminases (3%) and headache (3%). New-onset treatment-related peripheral neuropathy was observed in 22% of patients (sensory symptoms, grade 1). A phase 3 study to determine the efficacy of momelotinib *vs* ruxolitinib in MF patients naive of JAKi is ongoing.

CONCLUSION

Concerning prognostication of MF, the IPSS model at diagnosis and the DIPSS anytime during the course of the disease may be useful to define survival of MF patients. The IPSS and the DIPSS are based on age greater than 65 years, presence of constitutional symptoms, hemoglobin level less than 10 g/dL, leukocyte count greater than 25×10^9/L, and circulating blast cells 1% or greater. Cytogenetic profile and mutational analysis seem to be the next step to implement MF prognostication. Taking together all available clinical data on MF, one may conclude that JAKi give a benefit to patients with MF, by reducing spleen size of about 50% in approximately 30%-40% of patients and by abolishing symptoms in the vast majority of patients. However, effect on these disease manifestations should be balanced with the safety profile[19]. Anemia and thrombocytopenia are on-target toxicities expected with all JAKi. Infections should be monitored with ruxolitinib, drug with the longest time of observation, but might be expected with all JAKi. Other toxicities may involve non-JAK2 targets, as in case of gastrointestinal events during therapy with fedratinib or in the case of neurological toxicity for momelotinib.

REFERENCES

1 **Passamonti F,** Cervantes F, Vannucchi AM, Morra E, Rumi E, Pereira A, Guglielmelli P, Pungolino E, Caramella M, Maffioli M, Pascutto C, Lazzarino M, Cazzola M, Tefferi A. A dynamic prognostic model to predict survival in primary myelofibrosis: a study by the IWG-MRT (International Working Group for Myeloproliferative Neoplasms Research and Treatment). *Blood* 2010; **115**: 1703-1708 [DOI: 10.1182/blood-2009-09-245837]

2 **Cervantes** FB, Dupriez A, Pereira F, Passamonti JT, Reilly E, Morra AM, Vannucchi RA, Mesa JL, Demory G, Barosi E, Rumi A. New Prognostic Scoring System for Primary Myelofibrosis Based on a Study of the International Working Group for Myelofibrosis Research and Treatment. *Blood* 2009; **113**: 2895-901 [DOI: 10.1182/blood-2008-07-170449]

3 **Le Bousse-Kerdilès MC**, Chevillard S, Charpentier A, Romquin N, Clay D, Smadja-Joffe F, Praloran V, Dupriez B, Demory JL, Jasmin C, Martyré MC. Differential expression of transforming growth factor-beta, basic fibroblast growth factor, and their receptors in CD34+ hematopoietic progenitor cells from patients with myelofibrosis and myeloid metaplasia. *Blood* 1996; **88**: 4534-4546 [PMID: 8977245]

4 **Elena C**, Passamonti F, Rumi E, Malcovati L, Arcaini L, Boveri E, Merli M, Pietra D, Pascutto C, Lazzarino M. Red blood cell transfusion-dependency implies a poor survival in primary myelofibrosis irrespective of IPSS and DIPSS. *Haematologica* 2011; **96**: 167-170 [PMID: 20884708 DOI: 10.3324/haematol.2010.031831]

5 **Gale RP,** Barosi G, Barbui T, Cervantes F, Dohner K, Dupriez B, Gupta V, Harrison C, Hoffman R, Kiladjian JJ, Mesa RMF, Mullin M, Passamonti F, Ribrag V, Roboz G, Saglio G, Vannucchi A, Verstovsek S. What Are Rbc-Transfusion-Dependence and -Independence? *Leuk Res* 2011; **35**: 8-11 [DOI: 10.1016/j.leukres.2010.07.015]

6 **Gale RP,** Barosi G, Barbui T, Cervantes F, Dohner K, Dupriez B, Gupta V, Harrison C, Hoffman R, Kiladjian JJ, Mesa RMF, Mullin M, Passamonti F, Ribrag V, Roboz G, Saglio G, Vannucchi A, Verstovsek S. Rbc-Transfusion Guidelines Update. *Leuk Res* 2012; **36**: 659-60 [DOI: 10.1016/j.leukres.2012.01.023]

7 **Guglielmelli P**, Barosi G, Specchia G, Rambaldi A, Lo Coco F, Antonioli E, Pieri L, Pancrazzi A, Ponziani V, Delaini F, Longo G, Ammatuna E, Liso V, Bosi A, Barbui T, Vannucchi AM. Identification of patients with poorer survival in primary myelofibrosis based on the burden of JAK2V617F mutated allele. *Blood* 2009; **114**: 1477-1483 [PMID: 19549988 DOI: 10.1182/blood-2009-04-216044]

8 **Heine A**, Held SA, Daecke SN, Wallner S, Yajnanarayana SP, Kurts C, Wolf D, Brossart P. The JAK-inhibitor ruxolitinib impairs dendritic cell function in vitro and in vivo. *Blood* 2013; **122**: 1192-1202 [PMID: 23770777 DOI: 10.1182/blood-2013-03-484642]

9 **Hussein K**, Huang J, Lasho T, Pardanani A, Mesa RA, Williamson CM, Ketterling RP, Hanson CA, Van Dyke DL, Tefferi A. Karyotype complements the International Prognostic Scoring System for primary myelofibrosis. *Eur J Haematol* 2009; **82**: 255-259 [PMID: 19215287 DOI: 10.1111/j.1600-0609.2009.01216]

10 **Klampfl T**, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, Them NC, Berg T, Gisslinger B, Pietra D, Chen D, Vladimer GI, Bagienski K, Milanesi C, Casetti IC, Sant'Antonio E, Ferretti V, Elena C, Schischlik F, Cleary C, Six M, Schalling M, Schönegger A, Bock C, Malcovati L, Pascutto C, Superti-Furga G, Cazzola M, Kralovics R. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 2013; **369**: 2379-2390 [PMID: 24325356 DOI: 10.1056/NEJMoa1311347]

11 **Lasho TL**, Finke CM, Hanson CA, Jimma T, Knudson RA, Ketterling RP, Pardanani A, Tefferi A. SF3B1 mutations in primary myelofibrosis: clinical, histopathology and genetic correlates among 155 patients. *Leukemia* 2012; **26**: 1135-1137 [PMID: 22064353 DOI: 10.1038/leu.2011.320]

12 **Maffioli M,** Genoni A, Caramazza D, Mora B, Bussini A, Merli M, Giorgino T, Casalone R, Passamonti F. Looking for CALR mutations in familial myeloproliferative neoplasms. *Leukemia* 2014; **28**: 1357-1360 [PMID: 24441291 DOI: 10.1038/leu.2014.33]

13 **Massa M**, Rosti V, Campanelli R, Fois G, Barosi G. Rapid and long-lasting decrease of T-regulatory cells in patients with myelofibrosis treated with ruxolitinib. *Leukemia* 2014; **28**: 449-451 [PMID: 24145312 DOI: 10.1038/leu.2013.296]

14 **Mesa R,** Schwager AS, Radia D, Cheville A, Hussein K, Niblack J, Pardanani AD, Steensma DP, Litzow MR, Rivera CE, Camoriano J, Verstovsek S, Sloan J, Harrison C, Kantarjian H, Tefferi A. The Myelofibrosis Symptom As-

sessment Form (Mfsaf): An Evidence-Based Brief Inventory to Measure Quality of Life and Symptomatic Response to Treatment in Myelofibrosis. *Leuk Res* 2009; **33**: 1199-203 [DOI: 10.1016/j.leukres.2009.01.035]

15 **Messa E,** Cilloni D, Messa F, Arruga F, Roetto A, Saglio G. Deferasirox Treatment Improved the Hemoglobin Level and Decreased Transfusion Requirements in Four Patients with the Myelodysplastic Syndrome and Primary Myelofibrosis. *Acta Haematol* 2008; **120**: 70-74 [DOI: 10.1155/2010/756289]

16 **Nangalia J,** Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, Avezov E, Li J, Kollmann K, Kent DG, Aziz A, Godfrey AL, Hinton J, Martincorena I, Van Loo P, Jones AV, Guglielmelli P, Tarpey P, Harding HP, Fitzpatrick JD, Goudie CT, Ortmann CA, Loughran SJ, Raine K, Jones DR, Butler AP, Teague JW, O'Meara S, McLaren S, Bianchi M, Silber Y, Dimitropoulou V, Bloxham D, Mudie L, Maddison M, Robinson B, Keohane C, Maclean C, Hill K, Orchard K, Tauro S, Du MQ, Greaves M, Bowen D, Huntly BJ, Harrison CN, Cross NC, Ron D, Vannucchi AM, Papaemmanuil E, Campbell PJ, Green AR. Somatic Calr Mutations in Myeloproliferative Neoplasms with Nonmutated Jak2. *N Engl J Med* 2013; **369**: 2391-405 [DOI: 10.1056/NEJMoa1312542]

17 **Pardanani A**, Laborde RR, Lasho TL, Finke C, Begna K, Al-Kali A, Hogan WJ, Litzow MR, Leontovich A, Kowalski M, Tefferi A. Safety and efficacy of CYT387, a JAK1 and JAK2 inhibitor, in myelofibrosis. *Leukemia* 2013; **27**: 1322-1327 [PMID: 23459451]

18 **Pardanani A,** Lasho TL, Finke CM, Rajkumar SV, Singh PP, Ketterling RP, Hanson CA, Katzmann JA, Tefferi A. Polyclonal Immunoglobulin Free Light Chain Levels Predict Survival in Myeloid Neoplasms. *J Clin Oncol* 2012; **30**: 1087-1094 [DOI. 10.1200/JCO.2011.39.0310]

19 **Passamonti F**. Balancing efficacy and safety of JAK inhibitors in myelofibrosis. *Leuk Res* 2014; **38**: 290-291 [PMID: 24444869]

20 **Passamonti F,** Cervantes F, Vannucchi AM, Morra E, Rumi E, Cazzola M, Tefferi A. Dynamic International Prognostic Scoring System (Dipss) Predicts Progression to Acute Myeloid Leukemia in Primary Myelofibrosis. *Blood* 2010; **116**: 2857-2858 [DOI. 10.1182/blood-2010-06-293415]

21 **Passamonti F**, Maffioli M, Cervantes F, Vannucchi AM, Morra E, Barbui T, Caramazza D, Pieri L, Rumi E, Gisslinger H, Knoops L, Kiladjian JJ, Mora B, Hollaender N, Pascutto C, Harrison C, Cazzola M. Impact of ruxolitinib on the natural history of primary myelofibrosis: a comparison of the DIPSS and the COMFORT-2 cohorts. *Blood* 2014; **123**: 1833-1835 [PMID: 24443442 DOI: 10.1182/blood-2013-12-544411]

22 **Pereira A**, Bruguera M, Cervantes F, Rozman C. Liver involvement at diagnosis of primary myelofibrosis: a clinicopathological study of twenty-two cases. *Eur J Haematol* 1988; **40**: 355-361 [PMID: 3366226]

23 **Chelloul N**, Briere J, Laval-Jeantet M, Najean Y, Vorhauer W, Jacquillat C. Prognosis of myeloid metaplasia with myelofibrosis. *Biomedicine* 1976; **24**: 272-280 [PMID: 990377]

24 **Scott BL,** Gooley TA, Sorror ML, Rezvani AR, Linenberger ML, Grim J, Sandmaier BM, Myerson D, Chauncey TR, Storb R, Buxhofer-Ausch V, Radich JP, Appelbaum FR, Deeg HJ. The Dynamic International Prognostic Scoring System for Myelofibrosis Predicts Outcomes after Hematopoietic Cell *Transplantation Blood* 2012; **119**: 2657-2664 [DOI: 10.1182/blood-2011-08-372904]

25 **Tefferi A,** Jimma T, Sulai NH, Lasho TL, Finke CM, Knudson RA, McClure RF, Pardanani A. Idh Mutations in Primary Myelofibrosis Predict Leukemic Transformation and Shortened Survival: Clinical Evidence for Leukemogenic Collaboration with Jak2v617f. *Leukemia* 2011; **26**: 475-480 [doi: 10.1038/leu.2011.253]

26 **Tefferi A,** Lasho TL, Finke CM, Knudson RA, Ketterling R, Hanson CH, Maffioli M, Caramazza D, Passamonti F, Pardanani A.CALR *vs* JAK2 *vs* MPL-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. *Leukemia* 2014; **28**: 1472-1477 [PMID: 24402162 DOI: 10.1038/leu.2014.3]

27 **Tefferi A**, Lasho TL, Huang J, Finke C, Mesa RA, Li CY, Wu W, Hanson CA, Pardanani A. Low JAK2V617F allele burden in primary myelofibrosis, compared to either a higher allele burden or unmutated status, is associated with inferior overall and leukemia-free survival. *Leukemia* 2008; **22**: 756-761 [PMID: 18216871 DOI: 10.1038/sj.leu.2405097]

28 **Tefferi A**, Litzow MR, Pardanani A. Long-term outcome of treatment with ruxolitinib in myelofibrosis. *N Engl J Med* 2011; **365**: 1455-1457 [PMID: 21995409 DOI: 10.1056/NEJMc1109555]

29 **Tefferi A**, Meyer RG, Wyatt WA, Dewald GW. Comparison of peripheral blood interphase cytogenetics with bone marrow karyotype analysis in myelofibrosis with myeloid metaplasia. *Br J Haematol* 2001; **115**: 316-319 [PMID: 11703327]

30 **Tefferi A**, Vaidya R, Caramazza D, Finke C, Lasho T, Pardanani A. Circulating interleukin (IL)-8, IL-2R, IL-12, and IL-15 levels are independently prognostic in primary myelofibrosis: a comprehensive cytokine profiling study. *J Clin Oncol* 2011; **29**: 1356-1363 [PMID: 21300928 DOI: 10.1200/JCO.2010.32.9490]

31 **Vaidya R**, Caramazza D, Begna KH, Gangat N, Van Dyke DL, Hanson CA, Pardanani A, Tefferi A. Monosomal karyotype in primary myelofibrosis is detrimental to both overall and leukemia-free survival. *Blood* 2011; **117**: 5612-5615 [PMID: 21450904 DOI: 10.1182/blood-2010-11-320002]

32 **Alchalby H**, Badbaran A, Zabelina T, Kobbe G, Hahn J, Wolff D, Bornhäuser M, Thiede C, Baurmann H, Bethge W, Hildebrandt Y, Bacher U, Fehse B, Zander AR, Kröger N. Impact of JAK2V617F mutation status, allele burden, and clearance after allogeneic stem cell transplantation for myelofibrosis. *Blood* 2010; **116**: 3572-3581 [PMID: 20489052 DOI: 10.1182/blood-2009-12-260588]

33 **Verstovsek S,** Mesa RA, Gotlib J, Levy RS, Gupta V, DiPersio JF, Catalano JV, Deininger MW, Miller CB, Silver RT, Talpaz M, Winton EF, Harvey JH, Arcasoy MO, Hexner EO, Lyons RM, Paquette R, Raza A, Vaddi K, Erickson-Viitanen S, Sun W, Sandor V, Kantarjian HM. Efficacy, Safety and Survival with Ruxolitinib Treatment in Patients with Myelofibrosis: Results of a Median 2-Year Follow-up of Comfort-I. *Haematologica* 2013; **99**: 292-298 [DOI: 10.3324/haematol.2013.087650]

P- Reviewer: Ren G, Ribera JM **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Lu YJ

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
doi:10.5315/wjh.v3.i3.93

World J Hematol 2014 August 6; 3(3): 93-104
ISSN 2218-6204 (online)

ORIGINAL ARTICLE

Tbl3 encodes a WD40 nucleolar protein with regulatory roles in ribosome biogenesis

Jindong Wang, Schickwann Tsai

Jindong Wang, Schickwann Tsai, Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT 84132, United States
Author contributions: Wang J designed and constructed various expression vectors, performed localization and ribosome profiling, analyzed the data, prepared the figures and co-wrote the manuscript; Tsai S designed the project, performed cell culture experiments, analyzed the data, prepared the figures and co-wrote the manuscript.
Supported by In part by a grant from the St. Perres Fund, No. 11-02011
Correspondence to: Schickwann Tsai, MD, PhD, Department of Medicine, University of Utah School of Medicine, 5C402, 30 North 1900 East, Salt Lake City, UT 84132, United States. schickwann.tsai@hsc.utah.edu
Telephone: +1-801-5850495 Fax: +1-801-5850496
Received: October 27, 2013 Revised: February 15, 2014
Accepted: June 18, 2014
Published online: August 6, 2014

Abstract

AIM: To investigate the subcellular localization and the function of mouse transducin β-like 3 (Tbl3).

METHODS: The coding sequence of mouse Tbl3 was cloned from the cDNAs of a promyelocyte cell line by reverse transcription-polymerase chain reaction. Fusion constructs of Tbl3 and enhanced green fluorescent protein (EGFP) were transfected into fibroblasts and examined by fluorescence microscopy to reveal the subcellular localization of tbl3. To search for nucleolar targeting sequences, scanning deletions of Tbl3-EGFP were constructed and transfected into fibroblasts. To explore the possible function of Tbl3, small hairpin RNAs (shRNAs) were used to knock down endogenous Tbl3 in mouse promyelocytes and fibroblasts. The effects of Tbl3 knockdown on ribosomal RNA (rRNAs) synthesis or processing were studied by labeling cells with 5,6-^{3}H-uridine followed by a chase with fresh medium for various periods. Total RNAs were purified from treated cells and subjected to gel electrophoresis and Northern analysis. Ribosome profiling by sucrose gradient centrifugation was used to compare the amounts of 40S and 60S ribosome subunits as well as the 80S monosome. The impact of Tbl3 knockdown on cell growth and proliferation was examined by growth curves and colony assays.

RESULTS: The largest open reading frame of mouse Tbl3 encodes a protein of 801 amino acids (AA) with an apparent molecular weight of 89-90 kilodalton. It contains thirteen WD40 repeats (an ancient protein-protein interaction motif) and a carboxyl terminus that is highly homologous to the corresponding region of the yeast nucleolar protein, utp13. Virtually nothing is known about the biological function of Tbl3. All cell lines surveyed expressed Tbl3 and the level of expression correlated roughly with cell proliferation and/or biosynthetic activity. Using Tbl3-EGFP fusion constructs we obtained the first direct evidence that Tbl3 is targeted to the nucleoli in mammalian cells. However, no previously described nucleolar targeting sequences were found in Tbl3, suggesting that the WD40 motif and/or other topological features are responsible for nucleolar targeting. Partial knockdown (by 50%-70%) of mouse Tbl3 by shRNA had no discernable effects on the processing of the 47S pre-ribosomal RNA (pre-rRNA) or the steady-state levels of the mature 28S, 18S and 5.8S rRNAs but consistently increased the expression level of the 47S pre-rRNA by two to four folds. The results of the current study corroborated the previous finding that there was no detectable rRNA processing defects in zebra fish embryos with homozygous deletions of zebra fish Tbl3. As ribosome production consumes the bulk of cellular energy and biosynthetic precursors, dysregulation of pre-rRNA synthesis can have negative effects on cell growth, proliferation and differentiation. Indeed, partial knockdown of Tbl3 in promyelocytes severely impaired their proliferation. The inhibitory effect of Tbl3 knockdown was also observed in fibroblasts, resulting in an 80% reduction in colony formation. Taken

together, these results indicate that Tbl3 is a newly recognized nucleolar protein with regulatory roles at very early stages of ribosome biogenesis, perhaps at the level of rRNA gene transcription.

CONCLUSION: Tbl3 is a newly recognized nucleolar protein with important regulatory roles in ribosome biogenesis.

Key words: Nucleolus; Nucleolar protein; Ribosome biogenesis; Ribosomal RNA; Pre-ribosomal RNA

Core tip: The mouse gene transducin β-like 3 (Tbl3) encodes a protein with thirteen WD40 protein-protein interaction motifs and is the mammalian homologue of yeast utp13. Virtually nothing is known about the function of tbl3. In this report, we provide the first direct evidence that Tbl3 is targeted to the nucleoli and plays an important role in regulating the synthesis of the 47S pre-ribosomal RNA, *i.e.*, at very early stages of ribosome biogenesis. This activity has never been described before and sets Tbl3 apart from all other known nucleolar proteins. TBL3 may provide an attractive target for anti-neoplastic therapy.

Wang J, Tsai S. *Tbl3* encodes a WD40 nucleolar protein with regulatory roles in ribosome biogenesis. *World J Hematol* 2014; 3(3): 93-104 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i3/93.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i3.93

INTRODUCTION

Ribosomes are essential for protein synthesis. Defects in ribosome biogenesis or "ribosomopathy" underlie several devastating hematological disorders such as the Diamond-Blackfan anemia, Shwachman-Bodian-Diamond syndrome, dyskeratosis congenita and the 5q- myelodysplastic syndrome[1,2].

The production of ribosomes is a highly coordinated, multi-step process that starts in the nucleoli, the organelles that form around the "nucleolar organizers" on the long arms of acrocentric chromosomes (human chromosomes 13, 14, 15, 21 and 22)[3]. The nucleolar organizers consist of 400 or so rRNA genes (rDNAs). The production of ribosomes begins with the transcription of the rDNAs by RNA polymerase I (RNA Pol I), which synthesizes a long primary transcript, the 47S pre-rRNA, that is then covalently modified (*e.g.*, 2'-O-ribose methylation and pseudouridylation) and processed into mature 28S, 18S and 5.8S rRNAs. The rRNAs are assembled into pre-ribosomes with about eighty ribosomal proteins plus the 5S rRNA, which is separately transcribed by RNA polymerase Ⅲ outside the nucleolus. A large number (> 150) of small nucleolar RNAs and nonribosomal proteins participate in the post-transcriptional modification, processing and assembly of rRNAs to produce pre-ribosomes, which undergo further maturation to become nascent 40S and 60S ribosomal subunits before being exported into the cytoplasm. Most steps of rRNA processing occur co-transcriptionally with pre-rRNA synthesis[4-7]. As the production of ribosomes consumes the bulk of cellular energy[8], defective or dysregulated ribosome production can affect many aspects of cellular physiology especially cell proliferation.

Due to the relative simplicity and the ease of genetic manipulations in the yeasts, considerably more has been learned about the functions of non-ribosomal nucleolar proteins in yeasts than in mammalian cells. Still, the functions of many yeast nonribosomal nucleolar proteins remain unknown. In mammals, the total number of non-ribosomal proteins involved in ribosome biogenesis is even larger, reflecting the greater complexities of eukaryotic rRNA modification, processing, ribosome assembly, transport and other nucleolar events. Recent proteomic analyses of purified human nucleoli listed over 200 to 700 nonribosomal nucleolar proteins[3,9-11]. The exact functions of many of these proteins are unknown.

In this report, we describe the initial functional characterization of murine transducin-β-like 3 (Tbl3). At least four proteins have been described as "transducin-β-like" based on DNA sequence analysis, including transducin-β-like 1 (Tbl1), transducin-β-like related 1 (Tblr1), transducin-β-like 2 (Tbl2) and tbl3. tbl1 and tblr1 have been shown to be transcription co-regulators (co-repressors) in the signal transduction pathway of retinoic acid receptor-α[12-15]. Tbl2 is a putative protein associated with the Williams-Beuren syndrome based on gene mapping but its function is entirely unknown[16]. Very little is known about tbl3[17]. A literature search yielded 8 publications that mentioned *Tbl3*. Of these, one is a gene mapping study and two are disease-gene association studies[18-20]. Three are proteomic studies[9-11]. The seventh describes the composition of the co-repressor complex associated with the retinal photoreceptor-specific nuclear receptor (PNR)[21]. The most recent report is from the authors and collaborators and focuses on the phenotypic characterization of a zebra fish mutant, *ceylon* (*cey*) with homozygous deletions of *Tbl3* (in addition to four other uncharacterized genes)[22]. The *cey* mutant embryos have normal tissue specification but the sizes of some organs or cell populations such eye, pancreas, T cells and erythrocytes are markedly diminished due to reduced cellular proliferation, which in turn is attributed to cell cycle slowing as a result of *Tbl3* deficiency. The proliferative defect of the *cey* mutant becomes apparent 3-4 d post fertilization (dpf). All mutant embryos die by 10-14 dpf due to depletion of maternally derived *Tbl3* mRNA in the embryos[22]. The embryonic lethality of *Tbl3* deletion highlights its importance in tissue/organ development. However neither the site of

Tbl3 expression nor the mechanism of cell cycle slowing was elucidated. Here, we provide direct experimental evidence that mouse Tbl3 is targeted to the nucleoli where it plays an important role(s) in regulating the production of the 47S pre-rRNA.

MATERIALS AND METHODS

Expression vectors

The full-length coding sequence of mouse Tbl3 was amplified from oligo d(T)-primed cDNAs of MPRO (Mouse Promyelocyte) cells[23], cloned in frame into the *Hin*dⅢ/*Sal* I sites of the pEGFP C3 (for N-terminal fusion with EGFP) and pEGFP N1 vector (for C-terminal fusion)(Clontech). The truncation mutants of Tbl3 (del 1-632, del 1-400, del 632-801, del 401-632) were generated by polymerase chain reaction (PCR) amplification of the desired coding sequences and cloned into the *Hind*Ⅲ/*Sal* I sites of pEGFP N1 or C3 vector. The N-terminal deletion mutants (del 1-10, del 1-12, del 1-16 del 1-20) and C-terminal deletion mutants (del 777-801, del 753-776, del 733-752, del 713-732, del 693-712, del 673-692, del 653-672, del 633-652) were also generated using PCR-based deletion methods. All plasmids were sequenced to ensure accuracy. DNAs were purified using a plasmid DNA purification kit (Qiagen) and further purified by phenol-chloroform extraction and ethanol precipitation.

Short hairpin RNA knockdown vectors

The short hairpin RNA or shRNA sequences for mouse *Tbl3* were designed using the shRNA Sequence Designer (Clontech). The sequences of the two shRNAs we used in the current study are: 5'ccggTGCCAAGGATCAGAGCATAttcaagaga-TATGCTCTGATCCTTGGCAttttttg and 5'ccggTGGCCATTACCTCTTCTGTttcaagagaACAGAAGAGGTAATGGCCAttttttg (uppercases denote *Tbl3*-derived sequences). Synthetic oligonucleotides were annealed and cloned into the *Age* I/*Eco*R I sites of the pMKO 1p vector (referred to as pMKO hereafter)[24]. pMKO contains a puromycin resistance gene expression cassette and confers puromycin resistance in transfected mammalian cells. Both constructs provided specific knockdown but the first shRNA exhibited a stronger knockdown effect and was used in most experiments. Either pMKO or pMKO-Luc shRNA (Luc stands for luciferase) was used as the negative control in all knockdown experiments with similar results.

Cell cultures

NIH 3T3 cells were transfected with the pEGFP N1 or C3 fusion constructs by calcium phosphate method. MPRO cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL) and the conditioned medium of BHK/HM-5 cell line as a source of murine granulocyte-macrophage colony stimulating factor (10% vol/vol)[23]. MPRO cells were transfected with 10 μg of pMKO (or pMKO-Luc shRNA) *vs* pMKO-Tbl3 shRNA DNAs per 2.5×10^6 cells by electroporation using Gene Pulser Xcell (Bio-Rad). Transfected cells were selected with puromycin (0.375 μg/mL)(Sigma) for 8-10 d to establish stable transfectants.

Fibroblast colony assay

LAP-3 fibroblasts[25] were transfected in triplicates with 5 μg of pMKO (or pMKO-Luc shRNA) *vs* pMKO-Tbl3 shRNA by the Lipofectamine (Life Technologies) method in parallel to minimize variation in toxicity and transfection efficiency. After 24-48 h, transfected cells were detached by trypsin/EDTA and subcultured at 1:10-20 ratios in new 60-mm tissue culture dishes and selected with puromycin (1.5 μg/mL) for 5-10 d. Colonies were fixed and stained *in situ* with Coomassie Stain (BioRad).

Northern analyses

Total cellular RNAs were extracted from the same starting numbers of MPRO cells stably transfected with pMKO (or pMKO-Luc shRNA) or pMKO-Tbl3 shRNA in parallel using an RNeasy kit (Qiagen). Total RNAs extracted from equal numbers of starting cells were electrophoresed in 1% formaldehyde-agarose gels, blotted onto Hybond-N (Amersham) and hybridized with a randomly primed, ^{32}P-labled *Tbl3* probe in RapidHyb buffer (Amersham) at 70 ℃ for 3 h, followed by two washings with 2 × standard sodium citrate buffer (SSC) plus 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min. The final wash was done with 0.1 × SSC plus 0.1% SDS at 56 ℃ for 15 min.

^{3}H-uridine labeling and fluorography

To study the synthesis and processing of pre-rRNA, equal numbers of stably transfected MPRO/pMKO (or pMKO-Luc shRNA) and MPRO/pMKO-Tbl3 shRNA were labeled with 2.5 μCi/mL of 5,6-^{3}H-uridine (Perkin Elmer) for 30 min at 37 ℃, washed with phosphate buffered saline (PBS) and chased for 0-180 min at 37 ℃ in nonradioactive media. Total RNAs were purified using an RNeasy kit. In some experiments, Trizol agent was used to recover the 5.8S rRNA quantitatively. Total RNAs from equal numbers of starting cells were electropheresed in 1% formaldehyde-agarose gels and blotted onto Hybond-N. For fluorography, Northern blots were spray-coated with En^{3}Hance Spray (Perkin Elmer) and exposed to X-ray films directly without covering at -80 ℃ for 1-14 d. The fluor graphs were analyzed using a BioRad Gel Doc XR+.

Ribosome profiling by sucrose gradient centrifugation

For ribosome profiling, equal numbers of MPRO/pMKO (or MPRO/pMKO-Luc shRNA) and MPRO/pMKO-Tbl3 shRNA were washed with PBS and pelleted at 4 ℃ and re-suspended in polysome lysis buffer containing 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 20 mmol/L HEPES (pH 7.4), 1% NP-40, 1 mmol/L dithiothreitol, heparin sodium (200 μg/mL), phenylmethylsulfonyl

fluoride (1.0 mmol/L) (Sigma) and RNasin (100 unit/mL)(Promega). Cells were homogenized using a 1-ml syringe with a 25-gauge needle for eight times and centrifuged at 8000 *g* for 10 min at 4 ℃. The supernatant was layered on a 10%-45% (wt/vol) sucrose density gradient made in the polysome gradient buffer (100 mmol/L KCl, 5 mmol/L $MgCl_2$, 20 mmol/L HEPES, pH 7.4) at 38000 *g* for 3 h at 4 ℃ in a Beckman SW55Ti rotor and analyzed using a UA-6 Absorbance Detector (Isco). Of note, no cycloheximide was added to the lysates or sucrose gradients to allow the polysomes to dissociate into a single peak of 80S monosomes (*i.e.,* 40S plus 60S subunits) to facilitate the comparison of the total amounts of ribosomes.

Western blots

NIH3T3 cells expressing Tbl3-EGFP fusion protein were lysed in RIPA buffer. Lysates were denatured and electrophoresed in a 4%-12% denaturing SDS PAGE (NuPage; InVitrogen) along with Magic Mark XP Western Protein Standard (Life Technologies), blotted onto Immobilon-P (Millipore), probed with a rat monoclonal anti-GFP antibody (Pierce), followed by biotinylated goat anti-rat Igs (Pierce) and streptavidin-conjugated horseradish peroxidase (Amersham) and visualized by enhanced chemiluminescence.

RESULTS

Tbl3 contains thirteen WD40 repeats

The cDNA of the largest open reading frame of mouse *Tbl3* was cloned from the MPRO[23] cell line by RT-PCR. It contains 2,406 nucleotides (nt) and encodes a protein of 801 aa. Sequence alignments show that it is highly homologous to human TBL3, *Schizosaccharomyces pombe* utp13, and *Saccharomyces cerevisiae* utp13 (Figure 1). Thirteen WD40 repeats (also known as beta-transducin repeats) are present in mouse tbl3. The WD40 repeat consists of the consensus aa sequence [$X_{6\text{-}94}$-(GH-$X_{23\text{-}41}$-WD)] and is found in many proteins involved in signal transduction, rRNA processing, gene regulation, vesicular trafficking and cytoskeletal assembly[26,27]. Crystallography studies reveal that the WD40 repeats cluster together to form a β-propeller structure, which serves as a rigid platform for multiple protein-protein interactions[27]. A second region of homology is found in the C-terminus of mouse Tbl3 and yeast utp13. The conserved sequence in the C-terminus has not been found in any other protein. It is likely that this region contains a functional domain(s) unique to mouse Tbl3 and yeast utp13.

Tbl3 expression correlates with cell proliferation and/or biosynthetic activity

Tbl3 is expressed in all cell lines and tissues that we have examined. In all cases, there is only one band of *Tbl3* mRNA measuring approximately 3 kb in length in each cell type (Figure 2). The level of *Tbl3* mRNA varies significantly from one cell type to another and roughly correlates with the proliferative or biosynthetic activity of the cells. Among the cell lines surveyed, erythroleukemia (HCD57)[28], macrophage (J774), B-cell lymphoma (BaF3), T-cell lymphoma (EL4) and NIH3T3 fibroblasts express the highest levels of *Tbl3*. In normal tissues, hepatocytes, myocardium, testes, hematopoietic progenitors, $CD8^+$ T cells and $CD14^+$ monocytes express 3-5 times more *Tbl3* than the average tissue (not shown).

Tbl3 is targeted to the nucleoli

To study the subcellular localization of mouse tbl3, we constructed an expression vector expressing mouse Tbl3 as a fusion protein with EGFP in its C-terminus. The vector was transfected into NIH3T3 cells (or CV-1 or 293 cells). Fluorescence microscopy revealed that the overwhelming majority of the fusion protein was targeted to the nucleoli with a very small fraction appearing in the nucleoplasm (Figure 3A-E). The same result was obtained when EGFP was fused to the N-terminus of Tbl3 (not shown). The small nucleoplasmic pool (in a very fine punctate pattern at high magnifications) may represent genuine extra-nucleolar distribution of Tbl3 or an overexpression artefact. Western blot analysis using a monoclonal anti-GFP antibody yielded a single band of tbl3-EGFP fusion protein of approximately 116 kD (Figure 3F). Thus, the deduced apparent molecular weight of mouse Tbl3 is 89-90 kDa.

Sequence requirement for nucleolar targeting by tbl3

No previously reported nucleolar targeting motifs have been identified in Tbl3. To determine the aa sequence required for nucleolar localization of tbl3, we constructed a series of scanning deletion mutants of Tbl3 as EGFP fusion proteins (Figure 4). These constructs were transfected into NIH3T3 fibroblasts and examined by fluorescence microscopy. Among the constructs examined, only the full-length Tbl3 and those with very short (10-16 aa) N-terminal deletions could localize to the nucleoli. The remaining constructs yielded a diffuse pattern throughout the cytoplasm and nucleoplasm (but largely excluded from the nucleoli), a pattern that is indistinguishable from that of EGFP *per se*. This result suggests that the overall topology and/or the WD40 repeats rather than a unique localization signal are responsible for the nucleolar localization of tbl3.

shRNA-mediated knockdown of *Tbl3* increases the level of newly synthesized 47S pre-rRNA

To study the function of *Tbl3*, we used the MPRO murine promyelocyte cell line as a model system. MPRO was established from a normal mouse marrow[23]. The differentiation of MPRO is reversibly blocked at the promyelocyte stage. DNA array and proteomic studies have shown that MPRO, unlike most transformed leukemia cell lines, closely reflects the physiology of normal promyelocytes[29]. To investigate the function of *Tbl3* in MPRO, we trasnsfected the MRPO cells with either

```
S.pombe utp13     -----MAPIGEKKRFELEKSIEPIYTG--GPVAFDSNEKILVTALTDRIIGTRSETGER- 52
S.cerevisiae      ---------MDLKTSYKGISLNPIYAGSSAVATVSENGKILATPVLDEININIDLTPGSRK 51
Mouse tbl3        MAETAAGLCRFKANYAVERKIEPFYKG--GKAQLDQTGHYLFCVCGTKVNILDVASGAL- 57
Human TBL3        MAETAAGVGRFKTNYAVERKIEPFYKG--GKAQLDQTGQHLFCVCGTRVNILEVASGAV- 57
                                .:::*:* *  . . .... : *      .:      .*

S.pombe           -LFSIKKDEDDYVTALAITSDSKKLIAAFRSRLLTIYEIPSGRRIKSMK-AHETPVITMT 110
S.cerevisiae      ILHKISNEDEQEITALKLTPDGQYLTYVSQAQLLKIFHLKTGKVVRSMK-ISS-PSYILD 109
Mouse             -LRSLEQEDQEDITSFDLSPDDEVLVTASRALLLAQWAWREGTVTRLWKAIHTAPVASMA 116
Human             -LRSLEQEDQEDITAFDLSPDNEVLVTASRALLLAQWAWQEGSVTRLWKAIHTAPVATMA 116
                   * .:.::::: :*:: ::.*.: *  . :: **  :    *   :  *     *   :

S.pombe           IDPTNTLLATGGAEGLVKVWDIAGAYVTHSFRGHGGVISALCFGKHQN--TWVLASGADD 168
S.cerevisiae      ADSTSTLLAVGGTDGSIIVVDIENGYITHSFKGHGGTISSLKFYGQLNSKIWLLASGDTN 169
Mouse             FDATSTLLATGGCDGAVRVWDIVQHYGTHHFRGSPGVVHLVAFHPDPTR--LLLFSSAVD 174
Human             FDPTSTLLATGGCDGAVRVWDIVRHYGTHHFRGSPGVVHLVAFHPDPTR--LLLFSSATD 174
                   *.*.****.** :* : * **   * ** *:*  *.:  : *  . .    :* *.  :

S.pombe           SRVRLWDLNSSRSMAVFEGHSSVIRGLTFEPTGS------FLLSGSRDKTVQVWNI---K 219
S.cerevisiae      GMVKVWDLVKRKCLHTLQEHTSAVRGLDIIEVPDNDEPSLNLLSGGRDDIINLWDFNMKK 229
Mouse             TSIRVWSLQDRSCLAVLTAHYSAVTSLSFSEGGH------TMLSSGRDKICIVWDL---Q 225
Human             AAIRVWSLQDRSCLAVLTAHYSAVTSLAFSADGH------TMLSSGRDKICIIWDL---Q 225
                    ::*.* .  .: .:  * *.: .* :            :**..**.   :*::   :

S.pombe           KRSAVRTIPVFHSVEAIGWVNGQPE--------EKILYTAGEGNLILAWDWKSGSRLDPG 271
S.cerevisiae      KCKLLKTLPVNQQVESCGFLKDGDG--------KRIIYTAGGDAIFQLIDSESGSVLKR- 280
Mouse             SYQTTRTVPVFESVEASVLLPEQPAPALGVKSSGLHFLTAGDQGILRVWEAASGQCVYTQ 285
Human             SCQATRTVPVFESVEAAVLLPEEPVSQLGVKSPGLYFLTAGDQGTLRVWEAASGQCVYTQ 285
                  . .  :*:** ..**:   :                : ***    :   :  **. :

S.pombe           VDTTHSETNAIIQVVPFSENTLLSVHSDLSLLLR-----KRVPGEGFITIKKLNGSFDEV 326
S.cerevisiae      TNKPIEELFIIGVLPILSNSQMFLVLSDQTLQLINVEEDLKNDEDTIQVTSSIAGNHGII 340
Mouse             PQMPGLRQELTHCTLARAADLLLTVTADHNLLLY--------EAHSLQLQKQFAGYSEEV 337
Human             AQPPGPGQELTHCTLAHTAGVVLTATADHNLLLY--------EARSLRLQKQFAGYSEEV 337
                   : .             : . :: . :* .* *          :   ..: *    :

S.pombe           IDCAWIG--DDHLAVCSNTEFIDVISTDGT--------QVFGVLEGHTDIVLTLDSSEDG 376
S.cerevisiae      ADMRYVGPELNKLALATNSPSLRIIPVPDLSGPEASLPLDVEIYEGHEDLLNSLDATEDG 400
Mouse             LDVRFLGPSDSHIVVASNSPCLKVFELQTL---------ACQILHGHTDIVLALDVFRKG 388
Human             LDVRFLGPEDSHVVVASNSPCLKVFELQTS---------ACQILHGHTDIVLALDVFRKG 388
                   *  ::*   .::.:.:*:  : ::                : .** *:: :**  ..*

S.pombe           VWLATGAKDNTVRLWNLNIEDNVYKCIHVFTGHTASVTAVALGPLDVNGYPTFLASSSQD 436
S.cerevisiae      LWIATASKDNTAIVWRYNENSCKFDIYAKYIGHSAAVTAVGLPNIVSKGYPEFLLTASND 460
Mouse             WLFASCAKDQSIRIWKMN-KAGQVACVAQGSGHTHSVGTICCSRLKES----FLVTGSQD 443
Human             WLFASCAKDQSVRIWRMN-KAGQVMCVAQGSGHTHSVGTVCCSRLKES----FLVTGSQD 443
                    :*: :**::  :*. * :           **: :* ::    :  .    ** :.*:*

S.pombe           RTLKRFNLGSQLN-------KSDFSNRAVWTIKAHDRDVNAIQVSKDGRIIASASQDKTI 489
S.cerevisiae      LTIKKWIIPKPTASM-----DVQIIKVSEYTRHAHEKDINALSVSPNDSIFATASYDKTC 515
Mouse             CTVKLWPLPEALLAKSTAADSGPVLLQAQTTRRCHDKDINSLAVSPNDKLLATGSQDRTA 503
Human             CTVKLWPLPKALLSKNTAPDNGPILLQAQTTQRCHDKDINSVAIAPNDKLLATGSQDRTA 503
                   *:* : : .          .  .   :  * :.*::*:*:: :: :. ::*:.* *:*

S.pombe           KLWDSSTGEVVGVLRGHRRGVWACSFNPFSRQLASGSGDRTIRIWNVDTQQCVQTLEGHT 549
S.cerevisiae      KIWNLENGELEATLANHKRGLWDVSFCQYDKLLATSSGDKTVKIWSLDTFSVMKTLEGHT 575
Mouse             KLWALPQCQLLGVFTGHRRGLWNVQFSPTDQVLATASADGTIKLWALQDFSCLKTFEGHD 563
Human             KLWALPQCQLLGVFSGHRRGLWCVQFSPMDQVLATASADGTIKLWALQDFSCLKTFEGHD 563
                  *:*     :: ..: .*:**:*  .*   .: **:.*.* *:::* ::  . ::*:***

S.pombe           GAILKLIYISQGTQVVSAAADGLVKVWSLSSGECVATLDNHEDRVWALASRFDGSLLVSG 609
S.cerevisiae      NAVQRCSFINKQKQLISCGADGLIKIWDCSSGECLKTLDGHNNRLWALSTMNDGDMIVSA 635
Mouse             ASVLKVAFVSRGSQLLSSGSDGLLKLWTIKSNECVRTLDAHEDKVWGLHCSQLDDHAITG 623
Human             ASVLKVAFVSRGTQLLSSGSDGLVKLWTIKNNECVRTLDAHEDKVWGLHCSRLDDHALTG 623
                   :: :  ::.: .*::*..:***:*:*  ...**: *** *::::*.*     ..  ::.

S.pombe           GADAVVSVWKDVTEEYIAKQAEELERRVEAEQLLSNFEQTEDWQQAIALALSLDRPHGLL 669
S.cerevisiae      DADGVFQFWKDCTEQEIEEEQEKAKLQVEQEQSLQNYMSKGDWTNAFLLAMTLDHPMRLF 695
Mouse             GSDSRIILWKDVTEAEQAEEQAKREEQVIKQQELDNLLHEKRYLRALGLAISLDRPHTVL 683
Human             ASDSRVILWKDVTEAEQAEEQARQEEQVVRQQELDNLLHEKRYLRALGLAISLDRPHTVL 683
                   :*. . .*** **    ::  . : :*  :* *.*      : .*: **::**:*  ::

S.pombe           RLFERVMTAPHQPNSITG-------NKDVDNVLVQLPDHQLIILFQRIRDWNTNSKTSMV 722
S.cerevisiae      NVLKRALGESRSRQDTEEGKIEVIFNEELDQAISILNDEQLILLMKRCRDWNTNAKTHTI 755
Mouse             TVIQAIRRDPE-------------ACEKLEATVLRLRRDQKEALLRFCVTWNTNSRHCHE 730
Human             TVIQAIRRDPE-------------ACEKLEATMLRLRRDQKEALLRFCVTWNTNSRHCHE 730
                   :::     ..               :.:: .:  *  .*   *::    ****::

S.pombe           AQRLLRLLLHSYSPEHLLKLSGIKDILDSMIPYTDRHLARVNDLIEDSYIVDYVI----- 777
S.cerevisiae      AQRTIRCILMHHNIAKLSEIPGMVKIVDAIIPYTQRHFTRVDNLVEQSYILDYALVEMDK 815
Mouse             AQAVLGVLLRHEAPEELLAYDGVRGSLEALLPYTERHFQRLSRTLQAATFLDFLWHNMKL 790
Human             AQAVLGVLLRREAPEELLAYEGVRAALEALLPYTERHFQRLSRTLQAAAFLDFLWHNMKL 790
                  **  :  :*      .*    *:   :::::***:**: *:.  :: : ::*:

S.pombe           -------------------
S.cerevisiae      LF----------------- 817
Mouse             SPCPAAAPPAL-------- 801
Human             -PVPAAAPTPWETHKGALP 808
```

Figure 1 Sequence analyses of mouse transducin β-like 3, human transducin β-like 3 and yeast upt13. Amino acid sequence alignment of mouse transducin β-like 3 (Tbl3), human TBL3, *Schizosaccharomyces pombe* (*S. pombe*) utp13 and *Saccharomyces cerevisiae* (*S. cerevisiae*) utp13 using the Clustal program. "*" indicates identical aa; ":" indicates conserved substitution; "." indicates semiconserved substitution.

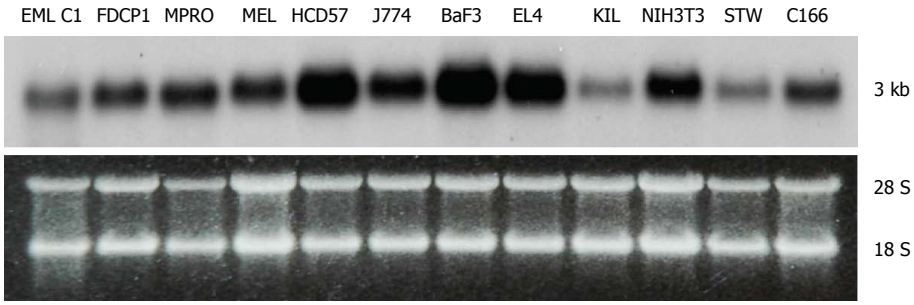


Figure 2 Tissue expression of transducin β-like 3. Northern blot analysis of the transducin β-like 3 (Tbl3) message (approximately 3 kb) in select mouse cell lines. The bottom panel is the corresponding ethidium bromide-stained gel showing similar loading. Each lane contained 10 μg of total RNA. The 28S and 18S rRNAs are indicated. EML C1: A multipotent hematopoietic progenitor line[38]; FDCP1: A bipotent granulocyte-monocyte progenitor line; MPRO: A promyelocytic line[23]; MEL: A murine erythroleukemia line; HCD57: An erythroblast line[28]; J774: A macrophage line; BaF3: A pre-B lymphoma line; EL4: A T cell-like lymphoma line; KIL: A natural killer line[39]; NIH3T3: An embryonic fibroblast-like line; STW: A bone marrow stromal cell line; C166: A yolk sac-derived endothelial line.

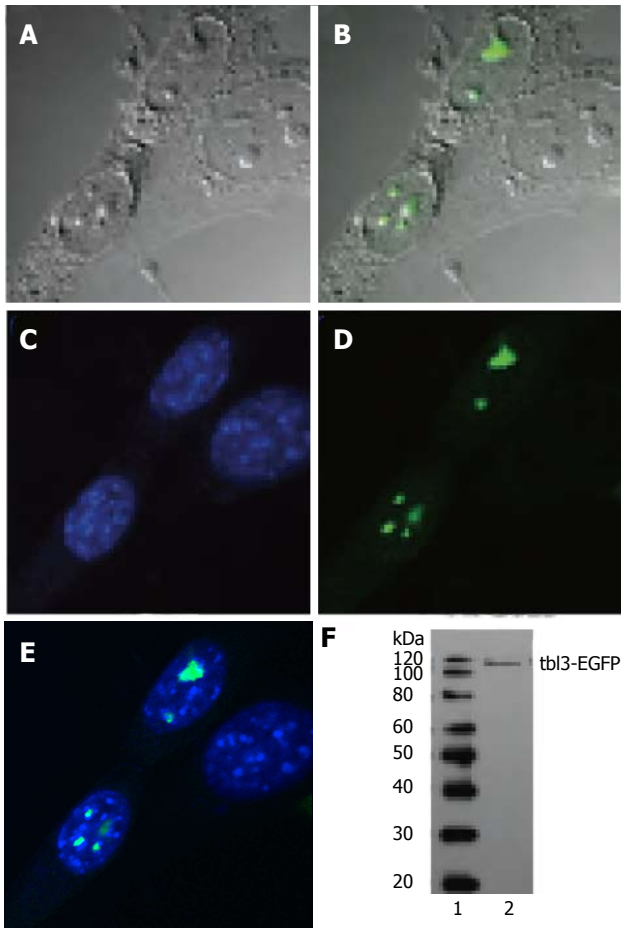


Figure 3 Nucleolar localization of Tbl3-enhanced green fluorescent protein. NIH 3T3 cells were transfected with the p-enhanced green fluorescent protein (EGFP) N1-transducin β-like 3 (Tbl3) construct. Cells were examined by fluorescence microscopy 20 h. after transfection. A: Differential interference contrast (DIC) microscopy of three fibroblasts revealing the nucleoli; B: Merged tbl3-EGFP green fluorescence and DIC of the cells shown in panel A. The nucleolar localization of tbl3-EGFP was verified by confocal microscopy; C: DAPI staining of DNA in the nuclei of the same field; D: Green fluorescence alone of the same field; E: DAPI plus tbl3-EGFP; F: Western detection of the tbl3-EGFP fusion protein. NIH 3T3 cells expressing tbl3-EGFP were lysed in RIPA buffer. The lysate (5 μg protein) was run on a 4%-12% NuPAGE gel (lane 2) along with Magic Mark size markers (lane 1), blotted and probed with an anti-GFP monoclonal antibody and visualized by enhanced chemiluminescence.

pMKO (or pMKO-Luc shRNA; negative controls) or pMKO-Tbl3 shRNA (1 or 2) vector and selected with puromycin to obtain stable transfectants. To avoid natural selection of variants with growth advantages, only low-passage (< 6) stable transfectants were used. Northern blot analyses demonstrated that the levels of *Tbl3* mRNA were knocked down by approximately 50%-70% in the pMKO-Tbl3 shRNA transfectants while the levels of *Tbl3* mRNA remained unchanged in the pMKO transfectants (Figure 5A, upper panel). The levels of housekeeping genes such as β-actin were unaffected by pMKO-Tbl3 shRNA (Figure 5A, middle panel).

To investigate the effects of *Tbl3* knockdown on newly transcribed pre-rRNA and its subsequent processing, we performed ^{3}H-uridine pulse-chase experiments in the MPRO transfectants. ^{3}H-uridine is phosphorylated after entering the cells and incorporated into newly transcribed RNAs, most of which are rRNAs. There was no detectable difference in the amounts of steady-state 28S and 18S rRNAs as revealed by ethidium bromide staining (Figure 5B and C, lower panels) and spectrophotometry. There was also no difference in the steady-state levels of the 5.8S rRNA on prolonged exposure (not shown). Intriguingly the levels of ^{3}H-uridine-labeled, newly transcribed 47S pre-rRNA were consistently increased by 2 to 4 fold in pMKO-Tbl3 shRNA transfectants (Figure 5B and C, upper panels). Furthermore, there was no obvious delay or defect in the processing of the 47S pre-rRNA as evidenced by similar rates of disappearance in the negative control and shRNA groups (Figure 5D) and by the proportional and contemporaneous appearance of the ^{3}H-uridine-labeled 28S and 18S rRNAs (and 5.8S rRNA; not shown) and the 41S, 36S and 32S processing intermediates (Figure 5B and C, upper panels). The key bands in lane 10 (Neg-1) and lane 12 (shRNA) of Figure 5B were further analyzed and the signal volume of the 28S rRNA was assigned a value of 1.00 to facilitate comparison. The calculated ratios of ^{3}H-labeled 32S:28S:18S rRNAs are 0.47:1.00:0.66 for the negative control group and 0.28:1.00:0.65 for the shRNA group. This result is consistent with the interpretation that there is no obvious delay in processing. Ribosome profiling by sucrose gradient centrifugation revealed that there was also no significant difference in the amount or ratio of the 40S and 60S ribosomal subunits and the 80S monosomes in

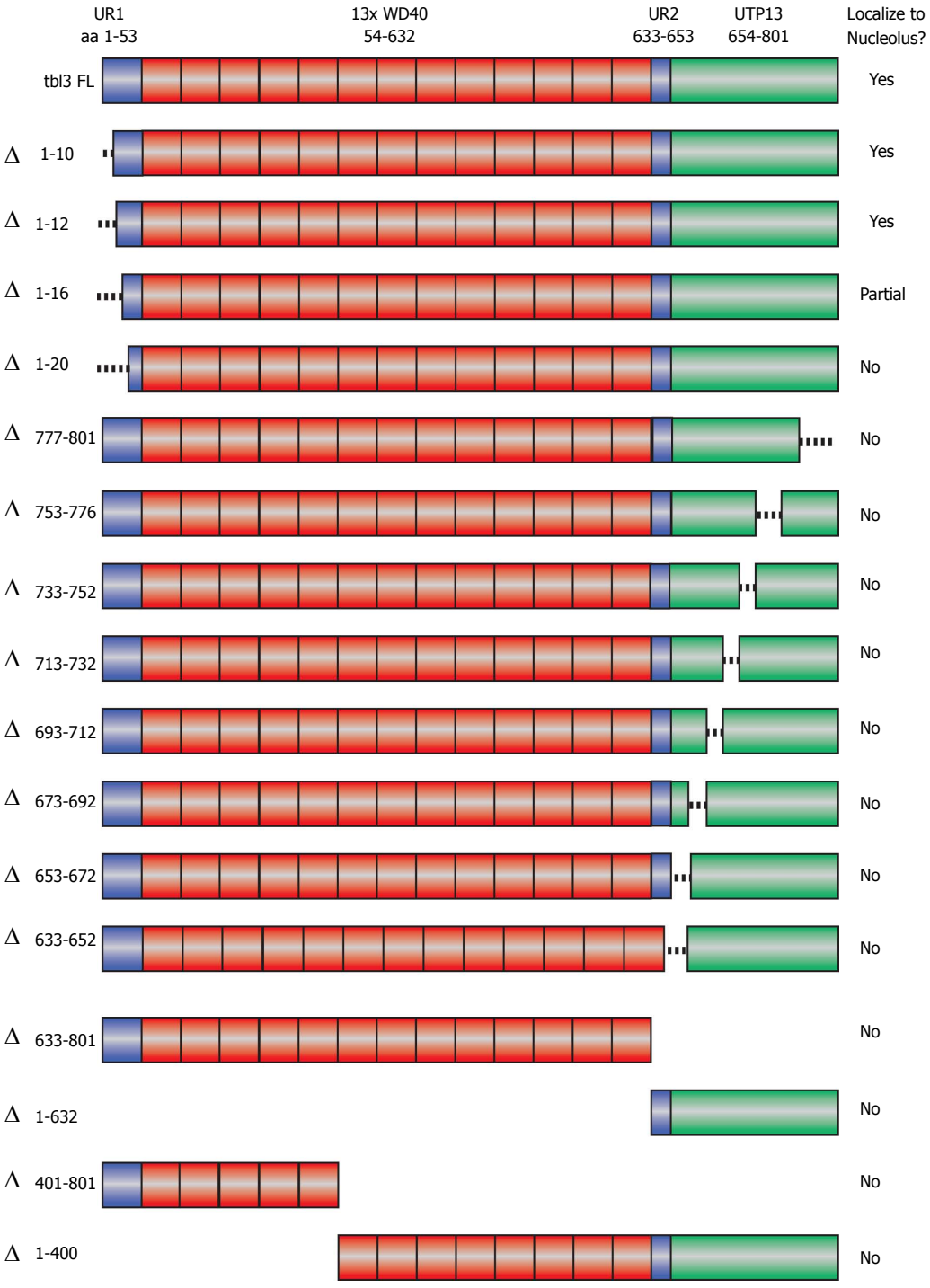


Figure 4 Scanning deletions of tbl3 and their effects on nucleolar targeting. A schematic summary of the deletions of transducin β-like 3 (tbl3) tested in the localization study. Shown at the very top is the modular structure of tbl3. "Δ" indicates deleted aa. Deletion mutants were expressed as C-terminal enhanced green fluorescent protein (EGFP) fusion proteins in NIH3T3 and examined by fluorescence microscopy. The results of localization are summarized in the right column. UR1: Unique region 1 (aa 1-53); WD40: Region containing thirteen WD40 repeats (aa 54-632); UR2: Unique region 2 (aa 633-653); UTP13: Conserved C-terminal domain (aa 654-801).

MPRO/pMKO (or MPRO/pMKO-Luc shRNA; negative controls) *vs* MPRO/pMKO-Tbl3 shRNA cells (Figure 6). This is consistent with the previous finding that the steady-state levels of 28S, 18S and 5.8S rRNA were not affected by Tbl3 knockdown (Figure 5B and C, lower panels). Together, the results of ^{3}H-uridine pulse-chase and ribosome sucrose gradient centrifugation indicate that *Tbl3* knockdown leads to increased levels of the 47S pre-rRNA but has no detectable effects on the processing of pre-rRNAs or the amount of steady-state 28S, 18S, 5.8S rRNAs or the amounts of the 40S and 60S subunits or the 80S monosomes.

Tbl3 knockdown impairs the proliferation of promyelocytes

During the course of the study we noticed that the

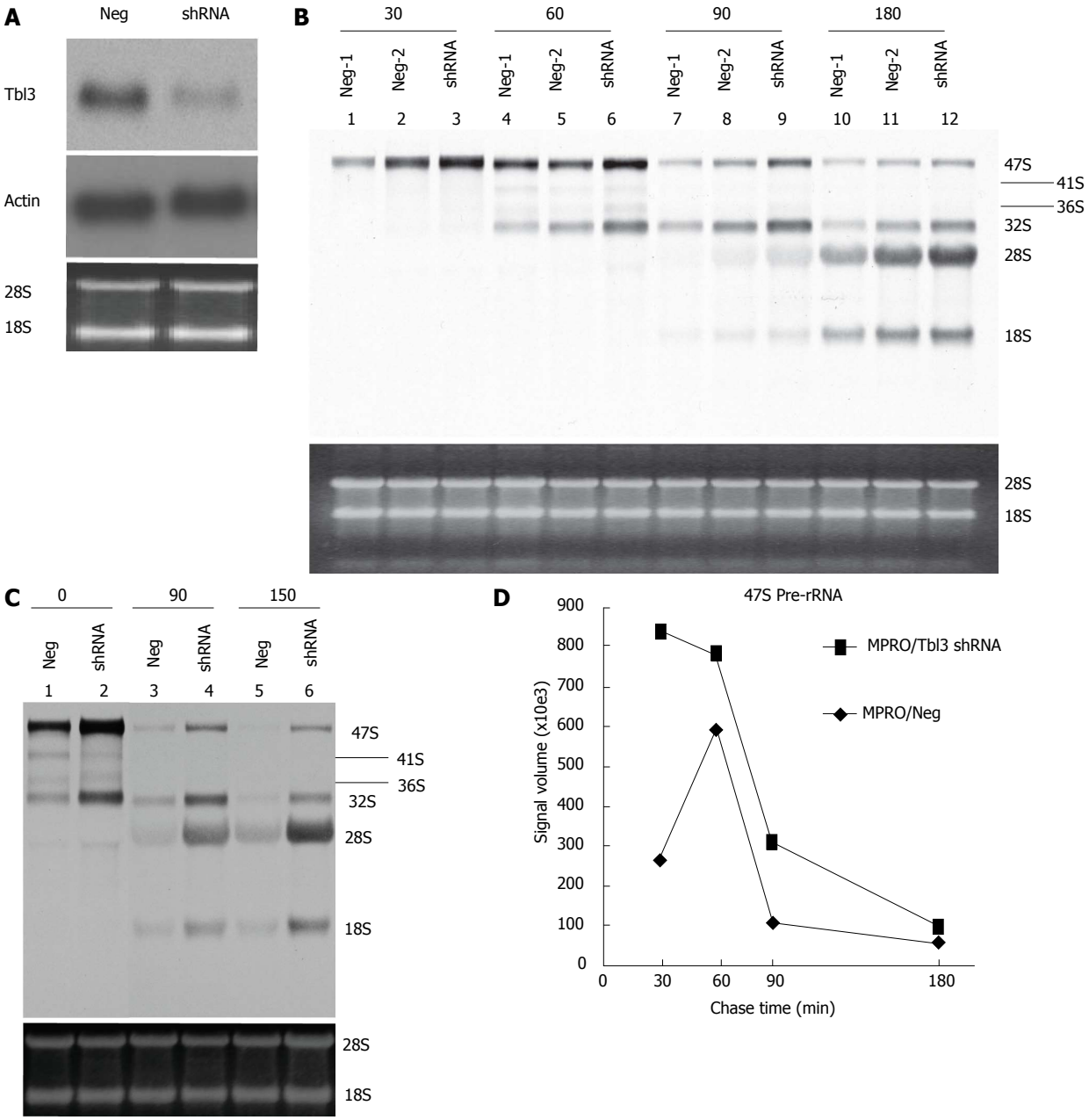


Figure 5 Effects of transducin β-like 3 knockdown on rRNA levels. A: Specific knockdown of transducin β-like 3 (Tbl3) by small hairpin RNAs (shRNAs). MRPO cells were transfected with pMKO (negative control) *vs* pMKO-Tbl3 shRNA and selected with puromycin to establish stable transfectants. Ten μg of total RNAs of stable transfectants were subjected to Northern analysis using a ^{32}P-labeled Tbl3 probe. The knockdown effect is 50%-70%. Middle panel: The same blot probed with a β-actin probe. Bottom panel: ethidium bromide-stained gel showing equal loading. The positions of the 28S and 18S rRNA are indicated; B: Tbl3 knockdown increases the level of newly synthesized 47S pre-rRNA but has no discernible effect on rRNA processing. MPRO stably transfected with pMKO (negative control) or pMKO-Tbl3 shRNA were pulse labeled with ^{3}H-uridine for 30 min, washed and chased for 0, 30, 60, 90 and 180 min with fresh medium without ^{3}H-uridine. Total RNAs were purified, electrophoresed and Northern blotted. ^{3}H-uridine-labeled rRNAs were visualized by fluorography. Tbl3 knockdown consistently increases the level of the 47S pre-rRNA by about 2-4 fold. Two negative controls (Neg-1 and Neg-2) are included to show the range of variation in signal strength in the negative control group. Bottom panel: Ethidium bromide-stained gel showing similar steady-state levels of 28S and 18S rRNAs (and the 5.8S rRNA; not shown). Each lane contained total RNAs purified from equal numbers of starting cells. No adjustment was made on the basis of RNA concentration or yield; C: An independent Tbl3 knockdown experiments similar to that described in B but the analysis was performed after 0, 90 and 150 minutes of chase. The 41S and 36S are better visualized in this blot. Bottom panel: Ethidium bromide-stained gel showing similar steady-state levels of 28S and 18S rRNAs (and the 5.8S rRNA; not shown). Each lane contained total RNAs purified from equal numbers of starting cells. No adjustment was made on the basis of RNA concentration or yield; D: Time course of disappearance of ^{3}H-uridine-labeled 47S pre-rRNAs. The fluorograph in B was analyzed and the measured signal volume was plotted against time. Note that ^{3}H-uridine incorporation peaked earlier (30 min *vs* 60 min) in the shRNA group, consistent with the notion that tbl3 knockdown increased the rate of pre-rRNA synthesis. There is no evidence of delay of processing of the 47S pre-rRNA in the shRNA group during the most relevant (60-90 min) or later (90-180 min) period judging from the slopes of decline.

growth of newly established MPRO/pMKO-Tbl3 shRNA transfectants was very slow compared with MPRO/pMKO (or MPRO/pMKO-Luc shRNA) or the parental MPRO. Calculation based on the time it took for the same number of newly established MPRO/pMKO-Tbl3 shRNA stable transfectants to reach the same population

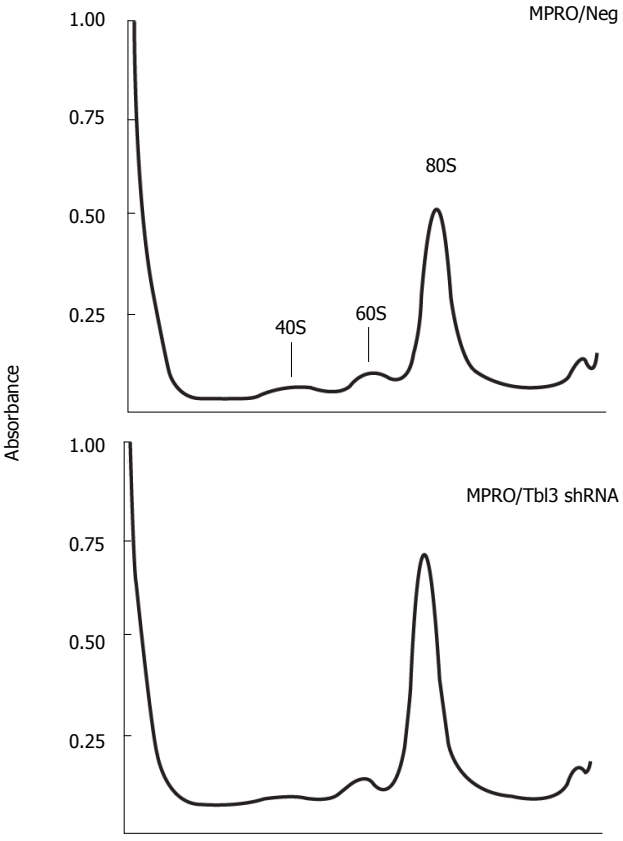


Figure 6 Knockdown of transducin β-like 3 has no discernable effect on ribosome profiles. Cell lysates prepared from equal numbers of MPRO/pMKO *vs* pMKO-transducin β-like 3 (Tbl3) small hairpin RNAs (shRNAs) stable transfectants were analyzed by sucrose gradient centrifugation in the absence of cycloheximide, followed by absorbance measurement at 254 nmol/L. (The omission of cycloheximide allowed polysomes to dissociate into 80S monosomes to facilitate the comparison of the total amount of 80S monosomes.) The peaks corresponding to the 40S and 60S ribosomal subunits and the 80S monosomes are indicated. The slight difference in the 80S monosome peaks is due to unequal loading and well within experimental variations.

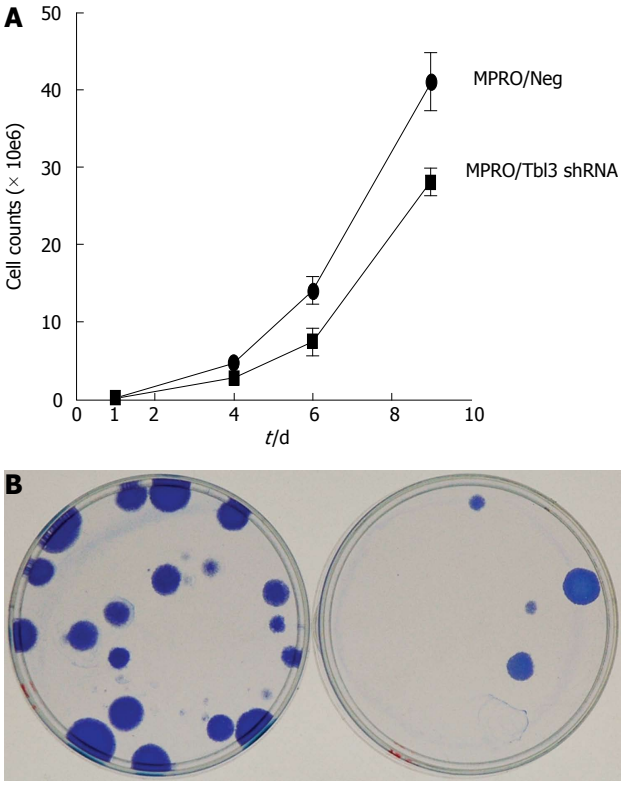


Figure 7 Knockdown of transducin β-like 3 inhibits cellular proliferation. A: Growth curves of stable MPRO/pMKO (negative control) vs MPRO/pMKO-transducin β-like 3 (Tbl3) small hairpin RNAs (shRNAs) transfectants. Each culture was started with 105 low-passage stable transfectants. Each data symbol represents the mean of triplicates and the standard deviation; B: Tbl3 knockdown markedly inhibits the proliferation of fibroblasts, left: LAP3/Neg; right: LAP3/Tbl3 shRNA. LAP-3 fibroblasts were transfected with pMKO or pMKO-Tbl3 shRNA and selected with puromycin (1.5 μg/mL) for 5-10 d to allow colony formation of stable transfectants. Colonies were fixed and stained with Coomassie blue *in situ* and photographed. Plates shown are representative of three independent experiments.

size as MPRO/pMKO indicates that the doubling time of MPRO/pMKO-Tbl3 shRNA was 3-4 times longer than that of MPRO/pMKO in newly established transfectants. As the population expanded from a few transfectants to approximately 10^6 cells, the doubling time of MPRO/pMKO-Tbl3 shRNA became progressively shorter, apparently due to the selection or outgrowth of variants that were less affected by the knockdown effects and hence enjoyed a higher proliferative rate. Nevertheless, the growth rate of MPRO/pMKO-Tbl3 shRNA was still slower compared with MPRO/pMKO in the 2-3 wk after the initial expansion following transfection and puromycin election (Figure 7A).

Tbl3 knockdown markedly impairs the proliferation of fibroblasts

To further examine the effects of *Tbl3* knockdown on cellular proliferation, we used the LAP3 fibroblast cell line as the model system. The LAP3 fibroblast cell line is derived from NIH3T3 (transformed embryonic cells) but is substantially "weakened" compared with the parental line[25]. Theoretical considerations and empiric experience indicate that it is particularly suitable for uncovering defects in ribosome biogenesis[25]. LAP3 cells were transfected with pMKO (or pMKO-Luc shRNA; negative controls) *vs* pMKO-Tbl3 shRNA (1 or 2) in triplicates in parallel and allowed to form macroscopic colonies in the presence of puromycin to select for stable integrants. The colonies were stained with Coomassie blue *in situ* to allow direct comparison. As shown in Figure 7B, the pMKO-Tbl3 shRNA-transfected group showed a great reduction (by about 80%) in colony numbers, indicating that *Tbl3* knockdown also has deleterious effects on fibroblast cell growth and proliferation.

DISCUSSION

In this report we provide direct evidence that mouse Tbl3 is targeted to the nucleoli (Figure 3A-D). This finding implicates Tbl3 in the ribosome biogenesis pathway and/or other nucleolar events. The results of targeting studies using a series of scanning deletion mutants of Tbl3 suggest that no particular nucleolar targeting

sequence is involved (Figure 4). Thus, Tbl3 likely associates with other proteins in the nucleoli *via* its WD40 protein-protein interaction motifs and/or other topological features and this forms the basis for its nucleolar localization.

To explore the function of mammalian *Tbl3*, we used the shRNA approach to achieve a partial knockdown. In retrospect, a partial rather than complete knockdown is desirable since a complete knockdown is likely incompatible with cellular survival based on what we have learned from our cell line studies and from the *cey* zebra fish embryos[22]. First, we looked at the processing of the 28S, 18S and 5.8S rRNAs. Then, we focused on the newly synthesized 47S pre-rRNA by pulse-labeling cells with ^{3}H-uridine. The latter approach allowed us to focus on newly synthesized pre-rRNA and minimized the contribution of post-transcriptional processing. As shown in Figure 5B and C (bottom panels), *Tbl3* knockdown had no discernable effect on the steady-state levels of the mature 28S, 18S and 5.8S rRNAs. Instead, it consistently increased the level of newly synthesized 47S pre-rRNA by two to four folds (Figure 5B and C, upper panels and Figure 5D). The higher levels of newly synthesized 47S pre-rRNA could result, *a priori*, from increased synthesis or decreased processing of the 47S pre-rRNA or both. If the elevated level of 47S pre-rRNA resulted from decreased processing, then we expect to see a slower rate of disappearance of the ^{3}H-labeled 47S pre-rRNA or a higher 47S to 28S (or 47S to 18S or 47S to 58S) ratio or the ratio of 28S to any processing intermediate in cells with *Tbl3* knockdown. However, our results showed that rates of disappearance of 47S pre-rRNA were very similar (Figure 5D) and the various ratios remained very similar in MPRO/pMKO-Tbl3 shRNA and in MPRO/pMKO (negative control) at all time points examined (Figure 5B and C, upper panels). This finding argues against a processing defect that would alter the size or quantity of the 28S, 18S and 5.8S rRNAs and raises the possibility that *Tbl3* knockdown primarily increased the synthesis of the 47S pre-rRNA. This interpretation is consistent with the previous conclusion that higher levels of steady-state 47S pre-rRNA reflect higher rates of rDNA transcription in a given cell type[30].

What could be the potential mechanisms by which Tbl3 regulates the synthesis of the 47S pre-rRNA? As Tbl3 is the mammalian homologue of yeast utp13, a review of the data on yeast utp13 may shed some light. Both mouse Tbl3 and yeast utp13 contain thirteen WD40 protein-protein interaction repeats and a conserved region in the C-terminus that is unique to both Tbl3 and utp13. The presence of many WD40 repeats in Tbl3 and utp13 indicates that they very likely form a complex or complexes with other proteins. Indeed, proteomic studies indicate that yeast utp13 forms a primary subcomplex, the so-called "utp-B" complex, with five other nucleolar proteins, namely utp 6, utp 18, utp 21, dip2 and pwp2[31]. This primary subcomplex in turn associates with other subcomplexes such as "UTP-A" and "UTP-C" complexes as well as additional nucleolar proteins to form a 90S megacomplex, which is known as the "small subunit (SSU) processome". The SSU is assembled co-transcriptionally at the 5' end of the newly transcribed pre-rRNA[31]. The SSU megacomplex is visible on electron microscopy as a terminal knob on the leading end of the elongating pre-rRNA still attached to the rDNA chromatin in yeast nucleoli[32]. By analogy, mammalian Tbl3 may similarly form a megacomplex *via* its multiple WD40 motifs with other proteins involved in the synthesis and/or processing of pre-rRNA and associates co-transcriptionally with the 5' end of the pre-rRNA. Given the observation that Tbl3 knockdown increases the production of 47S pre-rRNA, we hypothesize that Tbl3 is an important component of a putative pathway that normally provides feedback inhibition to the rDNA transcription machinery to coordinate the synthesis of the 47S pre-rRNA with the subsequent processing of rRNAs and ribosome assembly. Without coordination or feedback regulation, the rate of pre-RNA synthesis may become out of sync with that of processing and assembly and this mismatch may cause errors or waste in ribosome biogenesis[33,34].

Since the production of rRNAs and ribosomes consumes the lion share of cellular energy and biosynthetic precursors, over production of rRNAs inevitably will deprive cells of the energy and biosynthetic precursors needed for other physiologic processes and result in decreased cellular proliferation. Thus the consumption or siphoning away of cellular energy and biosynthetic precursors in overproduction of pre-rRNA may explain at least in part the inhibitory effect of *Tbl3* knockdown on cellular proliferation in mammalian cells (Figure 7A and B). As Tbl3 contains thirteen WD40 repeats it is possible that Tbl3 interacts with other nuclear or nucleolar proteins directly or indirectly involved in cell cycle regulation or DNA synthesis in addition to its interaction with components of SSU. The previously described detection of Tbl3 in the co-repressor complex of PNR in retina may well represent such an extra-nucleolar role[21]. However, we believe that the main role of Tbl3 is in the nucleoli as most (approximately 99%) if not all Tbl3 protein is found in that organelle (Figure 3A-D).

As we mentioned to earlier, complete depletion of yeast utp13 results in severe 18S rRNA processing defects and lethality in yeasts[32]. Considering the fact that Tbl3 is the mammalian homologue of yeast utp13, it is surprising that no rRNA processing defect was detected in the current study of mouse cells with partial *Tbl3* knockdown (Figure 5). Similarly, there were no detectable defects in rRNA processing in the *Tbl3*-/*Tbl3*- (*cey*) zebra fish embryos (supplemental Figure 6 of ref. 22). How can one reconcile these differences? The simplest explanation is that yeast utp13 and mammalian Tbl3 (or zebra fish tbl3) have evolved divergently in functionality. It is also quite possible that additional or redundant factors or mechanisms have evolved in mammals (or zebra fish) for rRNA processing such that a deficiency of Tbl3

alone has no discernible impact on rRNA processing. Still another possibility is that Tbl3 has more than one function and while a total deficiency is required to completely block the rRNA processing function, a partial deficiency is sufficient to interfere with the regulation of pre-rRNA synthesis.

The clinical importance of ribosomes is underscored by the fact that defects in ribosomes or ribosome biogenesis are the causes of several bone marrow failure syndromes such as the Diamond-Blackfan anemia (defective erythropoiesis due to rRNA processing defects caused by mutations in ribosomal protein genes *RPS17* or *RPS19* or *RPS24*)[35] and Shwachman-Bodian-Diamond syndrome (bone marrow failure, exocrine pancreas insufficiency, skeletal abnormalities and disposition to myelodysplastic syndrome and acute myeloid leukemia; caused by uncoupling of GTP hydrolysis from eIF6 release on the 60S ribosome subunit)[36]. More recently, a subtype of myelodysplastic syndrome, the so-called "5q- syndrome", has been experimentally phenocopied by RNAi-mediated haploinsufficiency of *RPS14*[37]. The propensity of ribosomopathy disorders to afflict hematopoiesis probably reflects the sensitivity of the highly proliferative hematopoietic progenitors to any disturbances in DNA or protein synthesis. Given the pronounced inhibitory effect of partial *Tbl3* knockdown on cellular proliferation in the current study, it is tempting to speculate that a loss of function mutation or deletion of human *TBL3* may also lead to a bone marrow failure-like syndrome. Furthermore, TBL3 may provide an attractive target for anti-tumor therapy.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Lester Lau and Dmitri Pestov for providing the LAP-3 fibroblasts.

COMMENTS

Background

Transducin β-like 3 (tbl3) encodes a protein with thirteen WD40 protein-protein interaction motifs. Virtually nothing is known about the function of Tbl3 including its subcellular localization and the general nature of its function. This report describes the first direct evidence that Tbl3 is targeted to the nucleoli and plays an important role in regulating the synthesis of the 47S pre-ribosomal RNA (pre-rRNA), *i.e.*, the first step in the production of ribosomes, which are indispensable for protein synthesis and hence the survival and normal functions of all cells.

Research frontiers

This article addresses a sizable gap in the understanding of the very early stage of the production of ribosomes. Understanding the function(s) of Tbl3 may lead to the development of new antibiotics and anti-tumor drugs.

Innovations and breakthroughs

Tbl3 is a newly identified nucleolar protein with a previously unrecognized regulatory function in the very early stage of ribosome production.

Applications

Given the pronounced inhibitory effect of *Tbl3* knockdown on cellular proliferation as demonstrated in the current study, the authors suspect that a loss of function mutation or deletion of human *TBL3* may also lead to a bone marrow failure-like syndrome or other developmental defects. Furthermore, TBL3 may provide a good target for anti-cancer therapy by interfering with the production of ribosomes at a very early stage.

Terminology

Ribosomopathy: defects in ribosome production and/or function including the synthesis, modification and processing of ribosomal RNA and assembly of ribosomes. Bone marrow failure syndromes: diseases caused by proliferative and/or differentiation defects in the production of blood cells by bone marrow stem cells.

Peer review

Authors provided the direct evidence that murine Tbl3 protein is targeted to compartment of nucleoli and plays an important role in synthesis of 47S pre-rRNA. Thus, this protein is important for regulation of ribosome biosynthesis. Manuscript is well written and new information about localization of Tbl3 protein in nucleolus is very interesting from the complex view on proteome of nucleolus.

REFERENCES

1 **Narla A**, Ebert BL. Ribosomopathies: human disorders of ribosome dysfunction. *Blood* 2010; **115**: 3196-3205 [PMID: 20194897 DOI: 10.1182/blood-2009-10-178129]
2 **Liu JM**, Ellis SR. Ribosomes and marrow failure: coincidental association or molecular paradigm? *Blood* 2006; **107**: 4583-4588 [PMID: 16507776 DOI: 10.1182/blood-2005-12-4831]
3 **Boisvert FM**, van Koningsbruggen S, Navascués J, Lamond AI. The multifunctional nucleolus. *Nat Rev Mol Cell Biol* 2007; **8**: 574-585 [PMID: 17519961 DOI: 10.1038/nrm2184]
4 **Kressler D**, Hurt E, Bassler J. Driving ribosome assembly. *Biochim Biophys Acta* 2010; **1803**: 673-683 [PMID: 19879902]
5 **Tschochner H**, Hurt E. Pre-ribosomes on the road from the nucleolus to the cytoplasm. *Trends Cell Biol* 2003; **13**: 255-263 [PMID: 12742169 DOI: 10.1016/S0962-8924(03)00054-0]
6 **Lapeyre B**. Conserved ribosomal RNA modification and their putative roles in ribosome biogenesis and translation. *Topics Curr Genetics* 2005; **12**: 263-284 [DOI: 10.1007/b105433]
7 **Raska I**, Koberna K, Malínský J, Fidlerová H, Masata M. The nucleolus and transcription of ribosomal genes. *Biol Cell* 2004; **96**: 579-594 [PMID: 15519693 DOI: 10.1016/j.biolcel.2004.04.015]
8 **Warner JR**. The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci* 1999; **24**: 437-440 [PMID: 10542411 DOI: 10.1016/S0968-0004(99)01460-7]
9 **Scherl A**, Couté Y, Déon C, Callé A, Kindbeiter K, Sanchez JC, Greco A, Hochstrasser D, Diaz JJ. Functional proteomic analysis of human nucleolus. *Mol Biol Cell* 2002; **13**: 4100-4109 [PMID: 12429849 DOI: 10.1091/mbc.E02-05-0271]
10 **Andersen JS**, Lyon CE, Fox AH, Leung AK, Lam YW, Steen H, Mann M, Lamond AI. Directed proteomic analysis of the human nucleolus. *Curr Biol* 2002; **12**: 1-11 [PMID: 11790298 DOI: 10.1016/S0960-9822(01)00650-9]
11 **Andersen JS**, Lam YW, Leung AK, Ong SE, Lyon CE, Lamond AI, Mann M. Nucleolar proteome dynamics. *Nature* 2005; **433**: 77-83 [PMID: 15635413 DOI: 10.1038/nature03207]
12 **Bassi MT**, Ramesar RS, Caciotti B, Winship IM, De Grandi A, Riboni M, Townes PL, Beighton P, Ballabio A, Borsani G. X-linked late-onset sensorineural deafness caused by a deletion involving OA1 and a novel gene containing WD-40 repeats. *Am J Hum Genet* 1999; **64**: 1604-1616 [PMID: 10330347 DOI: 10.1086/302408]
13 **Zhang J**, Kalkum M, Chait BT, Roeder RG. The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2. *Mol Cell* 2002; **9**: 611-623 [PMID: 11931768 DOI: 10.1016/S1097-2765(02)00468-9]
14 **Yoon HG**, Chan DW, Huang ZQ, Li J, Fondell JD, Qin J, Wong J. Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. *EMBO J* 2003; **22**: 1336-1346 [PMID: 12628926 DOI: 10.1093/emboj/cdg120]
15 **Perissi V**, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG.

A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* 2004; **116**: 511-526 [PMID: 14980219 DOI: 10.1016/S0092-8674(04)00133-3]

16 **Pérez Jurado LA**, Wang YK, Francke U, Cruces J. TBL2, a novel transducin family member in the WBS deletion: characterization of the complete sequence, genomic structure, transcriptional variants and the mouse ortholog. *Cytogenet Cell Genet* 1999; **86**: 277-284 [PMID: 10575226 DOI: 10.1159/000015319]

17 **Kawai J**, Shinagawa A, Shibata K, Yoshino M, Itoh M, Ishii Y, Arakawa T, Hara A, Fukunishi Y, Konno H, Adachi J, Fukuda S, Aizawa K, Izawa M, Nishi K, Kiyosawa H, Kondo S, Yamanaka I, Saito T, Okazaki Y, Gojobori T, Bono H, Kasukawa T, Saito R, Kadota K, Matsuda H, Ashburner M, Batalov S, Casavant T, Fleischmann W, Gaasterland T, Gissi C, King B, Kochiwa H, Kuehl P, Lewis S, Matsuo Y, Nikaido I, Pesole G, Quackenbush J, Schriml LM, Staubli F, Suzuki R, Tomita M, Wagner L, Washio T, Sakai K, Okido T, Furuno M, Aono H, Baldarelli R, Barsh G, Blake J, Boffelli D, Bojunga N, Carninci P, de Bonaldo MF, Brownstein MJ, Bult C, Fletcher C, Fujita M, Gariboldi M, Gustincich S, Hill D, Hofmann M, Hume DA, Kamiya M, Lee NH, Lyons P, Marchionni L, Mashima J, Mazzarelli J, Mombaerts P, Nordone P, Ring B, Ringwald M, Rodriguez I, Sakamoto N, Sasaki H, Sato K, Schönbach C, Seya T, Shibata Y, Storch KF, Suzuki H, Toyo-oka K, Wang KH, Weitz C, Whittaker C, Wilming L, Wynshaw-Boris A, Yoshida K, Hasegawa Y, Kawaji H, Kohtsuki S, Hayashizaki Y. Functional annotation of a full-length mouse cDNA collection. *Nature* 2001; **409**: 685-690 [PMID: 11217851 DOI: 10.1038/35055500]

18 **Weinstat-Saslow DL**, Germino GG, Somlo S, Reeders ST. A transducin-like gene maps to the autosomal dominant polycystic kidney disease gene region. *Genomics* 1993; **18**: 709-711 [PMID: 8307582 DOI: 10.1016/S0888-7543(05)80380-5]

19 **Yoshida T**, Kato K, Yokoi K, Oguri M, Watanabe S, Metoki N, Yoshida H, Satoh K, Aoyagi Y, Nozawa Y, Yamada Y. Association of genetic variants with hemorrhagic stroke in Japanese individuals. *Int J Mol Med* 2010; **25**: 649-656 [PMID: 20198315]

20 **Yoshida T**, Kato K, Yokoi K, Oguri M, Watanabe S, Metoki N, Yoshida H, Satoh K, Aoyagi Y, Nozawa Y, Yamada Y. Association of gene polymorphisms with chronic kidney disease in Japanese individuals. *Int J Mol Med* 2009; **24**: 539-547 [PMID: 19724895]

21 **Takezawa S**, Yokoyama A, Okada M, Fujiki R, Iriyama A, Yanagi Y, Ito H, Takada I, Kishimoto M, Miyajima A, Takeyama K, Umesono K, Kitagawa H, Kato S. A cell cycle-dependent co-repressor mediates photoreceptor cell-specific nuclear receptor function. *EMBO J* 2007; **26**: 764-774 [PMID: 17255935 DOI: 10.1038/sj.emboj.7601548]

22 **Hutchinson SA**, Tooke-Locke E, Wang J, Tsai S, Katz T, Trede NS. Tbl3 regulates cell cycle length during zebrafish development. *Dev Biol* 2012; **368**: 261-272 [PMID: 22659140 DOI: 10.1016/j.ydbio.2012.05.024]

23 **Tsai S**, Collins SJ. A dominant negative retinoic acid receptor blocks neutrophil differentiation at the promyelocyte stage. *Proc Natl Acad Sci USA* 1993; **90**: 7153-7157 [PMID: 8394011 DOI: 10.1073/pnas.90.15.7153]

24 **Stewart SA**, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY, An DS, Sabatini DM, Chen IS, Hahn WC, Sharp PA, Weinberg RA, Novina CD. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 2003; **9**: 493-501 [PMID: 12649500 DOI: 10.1261/rna.2192803]

25 **Strezoska Z**, Pestov DG, Lau LF. Bop1 is a mouse WD40 repeat nucleolar protein involved in 28S and 5. 8S RRNA processing and 60S ribosome biogenesis. *Mol Cell Biol* 2000; **20**: 5516-5528 [PMID: 10891491 DOI: 10.1128/MCB.20.15.5516-5528.2000]

26 **Neer EJ**, Schmidt CJ, Nambudripad R, Smith TF. The ancient regulatory-protein family of WD-repeat proteins. *Nature* 1994; **371**: 297-300 [PMID: 8090199 DOI: 10.1038/371297a0]

27 **Smith TF**, Gaitatzes C, Saxena K, Neer EJ. The WD repeat: a common architecture for diverse functions. *Trends Biochem Sci* 1999; **24**: 181-185 [PMID: 10322433 DOI: 10.1016/S0968-0004(99)01384-5]

28 **Ruscetti SK**, Janesch NJ, Chakraborti A, Sawyer ST, Hankins WD. Friend spleen focus-forming virus induces factor independence in an erythropoietin-dependent erythroleukemia cell line. *J Virol* 1990; **64**: 1057-1062 [PMID: 2154592]

29 **Lian Z**, Wang L, Yamaga S, Bonds W, Beazer-Barclay Y, Kluger Y, Gerstein M, Newburger PE, Berliner N, Weissman SM. Genomic and proteomic analysis of the myeloid differentiation program. *Blood* 2001; **98**: 513-524 [PMID: 11468144 DOI: 10.1182/blood.V98.3.513]

30 **Cui C**, Tseng H. Estimation of ribosomal RNA transcription rate in situ. *Biotechniques* 2004; **36**: 134-138 [PMID: 14740495]

31 **Pérez-Fernández J**, Román A, De Las Rivas J, Bustelo XR, Dosil M. The 90S preribosome is a multimodular structure that is assembled through a hierarchical mechanism. *Mol Cell Biol* 2007; **27**: 5414-5429 [PMID: 17515605 DOI: 10.1128/MCB.00380-07]

32 **Dragon F**, Gallagher JE, Compagnone-Post PA, Mitchell BM, Porwancher KA, Wehner KA, Wormsley S, Settlage RE, Shabanowitz J, Osheim Y, Beyer AL, Hunt DF, Baserga SJ. A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature* 2002; **417**: 967-970 [PMID: 12068309 DOI: 10.1038/nature00769]

33 **Grummt I**. Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes Dev* 2003; **17**: 1691-1702 [PMID: 12865296 DOI: 10.1101/gad.1098503R]

34 **Gallagher JE**, Dunbar DA, Granneman S, Mitchell BM, Osheim Y, Beyer AL, Baserga SJ. RNA polymerase I transcription and pre-rRNA processing are linked by specific SSU processome components. *Genes Dev* 2004; **18**: 2506-2517 [PMID: 15489292 DOI: 10.1101/gad.1226604]

35 **Choesmel V**, Fribourg S, Aguissa-Touré AH, Pinaud N, Legrand P, Gazda HT, Gleizes PE. Mutation of ribosomal protein RPS24 in Diamond-Blackfan anemia results in a ribosome biogenesis disorder. *Hum Mol Genet* 2008; **17**: 1253-1263 [PMID: 18230666 DOI: 10.1093/hmg/ddn015]

36 **Finch AJ**, Hilcenko C, Basse N, Drynan LF, Goyenechea B, Menne TF, González Fernández A, Simpson P, D'Santos CS, Arends MJ, Donadieu J, Bellanné-Chantelot C, Costanzo M, Boone C, McKenzie AN, Freund SM, Warren AJ. Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes Shwachman-Diamond syndrome. *Genes Dev* 2011; **25**: 917-929 [PMID: 21536732 DOI: 10.1101/gad.623011]

37 **Ebert BL**, Pretz J, Bosco J, Chang CY, Tamayo P, Galili N, Raza A, Root DE, Attar E, Ellis SR, Golub TR. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. *Nature* 2008; **451**: 335-339 [PMID: 18202658 DOI: 10.1038/nature06494]

38 **Tsai S**, Bartelmez S, Sitnicka E, Collins S. Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. *Genes Dev* 1994; **8**: 2831-2841 [PMID: 7995521 DOI: 10.1101/gad.8.23.2831]

39 **DeHart SL**, Heikens MJ, Tsai S. Jagged2 promotes the development of natural killer cells and the establishment of functional natural killer cell lines. *Blood* 2005; **105**: 3521-3527 [PMID: 15650053 DOI: 10.1182/blood-2004-11-4237]

P- Reviewer: Bartova E, Komada M, Loreni F **S- Editor**: Gou SX
L- Editor: A **E- Editor:** Lu YJ

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
doi:10.5315/wjh.v3.i3.105

World J Hematol 2014 August 6; 3(3): 105-114
ISSN 2218-6204 (online)

OBSERVATIONAL STUDY

Age is an independent adverse prognostic factor for overall survival in acute myeloid leukemia in Japan

Hiroaki Ohnishi, Osamu Imataki, Yasunori Kawachi, Makoto Ide, Kimihiro Kawakami, Masato Waki, Hidetaka Takimoto, Yasuo Hoshijima, Tetsuya Fukumoto, Kensuke Matsumoto, Fusako Waki, Akihito Matsuoka, Takamichi Shintani, Makiko Uemura, Shigeyuki Yokokura, Teruhisa Taoka, Takuya Matsunaga

Hiroaki Ohnishi, Osamu Imataki, Tetsuya Fukumoto, Kensuke Matsumoto, Fusako Waki, Akihito Matsuoka, Takamichi Shintani, Makiko Uemura, Shigeyuki Yokokura, Takuya Matsunaga, Department of Internal Medicine, Division of Endocrinology and Metabolism, Hematology, Rheumatology and Respiratory Medicine, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan
Hiroaki Ohnishi, Yasunori Kawachi, Makoto Ide, Department of Internal Medicine, Division of Hematology, Takamatsu Red Cross Hospital, Kagawa 760-0017, Japan
Kimihiro Kawakami, Masato Waki, Department of Internal Medicine, Division of Hematology, Kagawa Prefectural Central Hospital, Kagawa 760-8557, Japan
Hidetaka Takimoto, Department of Internal Medicine, Division of Hematology, Kagawa Rosai Hospital, Kagawa 763-8502, Japan
Yasuo Hoshijima, Department of Internal Medicine, Division of Hematology, Takamatsu Municipal Hospital, Kagawa 760-8538, Japan
Teruhisa Taoka, Department of Internal Medicine, Division of Hematology, Sakaide City Hospital, Kagawa 762-0031, Japan
Author contributions: Ohnishi H and Imataki O contributed equally to this work; Ohnishi H, Imataki O and Matsunaga T conceived and designed the study, analyzed and interpreted the data, wrote the paper; Kawachi Y, Ide M, Kawakami K, Waki M, Takimoto H, Hoshijima Y, Fukumoto T, Matsumoto K, Waki F, Matsuoka A, Shintani T, Uemura M and Yokokura S acquired the data, revised the paper; all authors approved the final version of the report.
Correspondence to: Osamu Imataki, MD, Department of Internal Medicine, Division of Hematology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. oima@med.kagawa-u.ac.jp
Telephone: +81-87-8912145 Fax: +81-87-8912147
Received: December 11, 2013 Revised: April 20, 2014
Accepted: May 28, 2014
Published online: August 6, 2014

Abstract

AIM: To elucidate risk factors for survival of elderly acute myeloid leukemia (AML) patients in a real-world practice by observational study.

METHODS: We conducted a population-based study in 213 adult and elderly AML patients (127 males and 86 females) in Kagawa Prefecture, Japan. To construct this cohort, we gathered all data for patients diagnosed with AML at 7 hospitals in Kagawa between 2006 and 2010. The primary end point was overall survival (OS) after AML diagnosis. Unadjusted Kaplan-Meier survival plots were used to determine OS in the overall cohort. Multivariate analysis was used to determine the independent adverse prognostic factors for OS, with the covariates of interest including age, gender, race/ethnicity, CCI, education, median income, metropolitan statistical area size and history of myelodysplastic syndrome.

RESULTS: The average population of Kagawa during the study period was 992489, and the incidence of AML was 4.26 per 100000 person-years. A total of 197 patients with non-acute promyelocytic leukemia (non-APL) (119 males and 78 females) were also included. The median age of non-APL patients was 70 years (average 67, range 24-95). The 5-year OS rate was 21.1%. Subsequent analysis by age group showed that the survival rate declined with age; the 5-year OS rates of non-APL patients younger than 64 years, 65-74 years, and older than 75 years were 41.5%, 14.1%, and 8.9%, respectively. Multivariate analysis revealed that unfavorable risk karyotype, older age, poor performance status (PS) (3-4), lack of induction chemotherapy, and antecedent haematological disease were independent prognostic predictors. In the subgroup analysis, we also found that older patients with non-APL had lower complete remission rates and higher early death rates than younger patients, irrespective of PS. However, intensive chemotherapy was a significant predictor for longer survival

not only in the patients < 75 years of age, but also in those over 75 with PS 0-2.

CONCLUSION: Age would contribute considerable life expectancy to indicate induction chemotherapy with eligible dose of cytotoxic drugs for a favorable case even in advanced elderly.

Key words: Acute myeloid leukemia; Elderly; Adverse prognostic factor; Overall survival; Population-based study

Core tip: The prevalence of acute myeloid leukemia (AML) is increasing among elderly patients in Japan. Our population-based observational study revealed that age was an independent prognostic factor in a real-world practice for the treatment of AML patients. Although we found that AML patients older than 75 years had lower complete remission rates and higher early death rates than patients younger than 75 years, an appropriately intensified induction chemotherapy would be helpful to prolong the survival of elderly AML patients with better performance status (PS) (1-2). The intensity of chemotherapy should thus be adjusted according to age and PS.

Ohnishi H, Imataki O, Kawachi Y, Ide M, Kawakami K, Waki M, Takimoto H, Hoshijima Y, Fukumoto T, Matsumoto K, Waki F, Matsuoka A, Shintani T, Uemura M, Yokokura S, Taoka T, Matsunaga T. Age is an independent adverse prognostic factor for overall survival in acute myeloid leukemia in Japan. *World J Hematol* 2014; 3(3): 105-114 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i3/105.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i3.105

INTRODUCTION

Elderly acute myeloid leukemia (AML) patients often have several comorbidities and poor performance status (PS) at the time of diagnosis, and may be intolerant to intensive chemotherapy, making them poor candidates for intensive induction chemotherapy[1-4]. Compared to young adult AML patients, elderly AML patients also have higher frequencies of adverse prognostic factors such as unfavorable risk karyotype and secondary AML (therapy-related AML)[5], leading to a poorer prognosis[6-8].

Juliusson *et al*[9] analyzed a population-based cohort of patients aged ≥ 16 years in the Swedish National Acute Leukemia Registry. They found that onset of AML may occur at any age, but is most common in the elderly population, with the highest incidence in individuals aged 80-85 years. The median age of onset was 72 years (range 16-97): 71 years for males and 72 years for females. A number of prospective clinical trials have studied treatments for young adult patients with non-acute promyelocytic leukemia (non-APL)[10-13]. These trials showed that the 5-year overall survival (OS) rate increased to 35%-48% in patients treated with induction chemotherapy using idarubicin and cytosine arabinoside. However, these trials excluded patients with poor PS (3-4) and elderly patients. Considering the distribution of the age onset of AML, these clinical trials included only a small proportion of the total AML population, and the results therefore do not accurately reflect treatment options for the overall AML population.

To accurately evaluate the overall AML population, several retrospective population-based studies have been conducted in Sweden[9,14], the United Kingdom[15,16], and the United States[17]. These studies found relatively low 5-year OS rates of 10%-20%[16,18,19]. Older age and poor PS were reported to be adverse prognostic factors for OS in these population-based studies, but not in prospective clinical trials. However, the retrospective population-based studies did not include multivariate analyses[9,18]. It is therefore still unclear whether older age and poor PS are independent adverse prognostic factors for OS in patients with AML.

In this study, multivariate analysis identified older age, lack of induction chemotherapy, poor PS (3-4), antecedent hematological disease, and unfavorable risk karyotype as independent prognostic factors for poor OS. We also found that older patients had lower complete remission (CR) rates and higher early death rates than younger patients, irrespective of PS. This analysis provides data describing the overall AML population.

MATERIALS AMD METHODS

Patients and survey methods

We performed a multicenter observational study of adult AML patients aged ≥ 17 years from seven institutions within Kagawa Prefecture (Kagawa Rosai Hospital, Takamatsu Municipal Hospital, Sakaide City Hospital, Kagawa Prefectural Central Hospital, Takamatsu Red Cross Hospital, Mitoyo General Hospital, and Kagawa University Hospital) between January 1, 2006 and December 31, 2010. Data were collected from the medical records. Diagnosis of AML was made according to the criteria of the French-American-British classification. We excluded patients with a previous diagnosis of myelodysplastic syndrome (MDS). Antecedent haematological disease was defined as benign hematological disease other than MDS. The potential effects of consolidation therapy and hematopoietic stem cell transplantation are beyond the scope of this analysis, and patients were not censored at the time of transplantation or any other treatment in the survival analysis. This study was approved by the Institutional Review Boards of Kagawa Rosai Hospital, Takamatsu Municipal Hospital, Sakaide City Hospital, Kagawa Prefectural Central Hospital, Takamatsu Red Cross Hospital, Mitoyo General Hospital, and Kagawa University Hospital.

Non-APL karyotype classifications

The karyotypes of non-APL patients were grouped according to the criteria of the National Comprehensive Cancer Network (NCCN) clinical practice guidelines[20], the Southwest Oncology Group (SWOG) classification[21], and the Cancer and Leukemia Group B (CALGB) classification[22].

Initial therapy regimens

Induction therapy regimens were chosen by each treating physician based on available clinical data and local standards of care, but not on karyotype. None of the patients were enrolled in clinical trials.

APL patients with a white blood cell (WBC) count of $< 3.0 \times 10^9$ per liter and a blast plus promyelocyte count of $< 1.0 \times 10^9$ per liter were started on oral all-*trans*-retinoic acid (ATRA) (45 mg/m^2 per day) alone until the start of consolidation therapy. Patients with a WBC count between 3.0×10^9 per liter and 10.0×10^9 per liter or a blast plus promyelocyte count of $\geqslant 1.0 \times 10^9$ per liter were started on oral ATRA until the start of consolidation therapy plus idarubicin (IDR) (12 mg/m^2 per day by 30-min infusion, days 1-2) plus cytarabine (Ara-C) (80 mg/m^2 per day by continuous infusion, days 1-5). Patients with a WBC count of $\geqslant 10.0 \times 10^9$ per liter were started on oral ATRA until the start of consolidation therapy plus IDR (days 1-3) plus Ara-C (100 mg/m^2 per day by continuous infusion, days 1-5).

Non-APL patients were treated with one of 15 regimens, which we categorized as intensive chemotherapy, less intensive chemotherapy, or best supportive care. The intensive chemotherapy regimens were: (1) full dose IDR (12 mg/m^2 per day by 30-min infusion, days 1-3) plus Ara-C (100 mg/m^2 per day by continuous infusion, days 1-7); (2) 80% dose IDR (days 1-3) plus Ara-C (days 1-7); (3) full dose daunorubicin (DNR) (50 mg/m^2 per day by 30-min infusion, days 1-5) plus Ara-C (days 1-7); (4) 80% dose DNR (days 1-5) plus Ara-C (days 1-7); (5) full dose IDR (days 1-3) plus enocitabine (BHAC) (200 mg/m^2 per day by 180-min infusion, days 1-8); (6) 80% dose IDR (days 1-3) plus BHAC (days 1-8); (7) full dose DNR (days 1-5) plus BHAC (days 1-8); (8) 80% dose DNR (days 1-5) plus BHAC (200 mg/m^2 per day by 180-min infusion, days 1-8). The less intensive chemotherapy regimens were: (1) less than 80% dose IDR (days 1-3) plus Ara-C (days 1-7); (2) less than 80% dose DNR (days 1-5) plus Ara-C (days 1-7); (3) less than 80% dose IDR (days 1-3) plus BHAC (days 1-8); (4) less than 80% dose DNR (days 1-5) plus BHAC (days 1-8); (5) CAG [Ara-C (10 mg/m^2 twice a day by subcutaneous injection, days 1-14) plus aclarubicin (ACR) (14 mg/m^2 per day by 30-min infusion, days 1-14) plus granulocyte colony-stimulating factor (G-CSF) (200 g/m^2 per day by subcutaneous injection, days 1-14)]; (6) CA [Ara-C (10 mg/m^2 twice a day by subcutaneous injection, days 1-14) plus ACR (14 mg/m^2 per day by 30-min infusion, days 1-14)]; (7) Low dose Ara-C (10 mg/m^2 twice a day by subcutaneous injection, days 1-14).

Statistical analysis

The χ^2 test was used to analyze the significance of differences between two or three groups (Microsoft Excel 2010, version 14.0; Microsoft Corporation Japan, Tokyo). A *P* value less than 0.05 was considered statistically significant. All other data were analysed using JMP 7.0.1 (SAS Institute Japan, Tokyo, Japan). The Kaplan-Meier method was used to estimate probabilities of OS, and the log-rank test was used to analyse the significance of differences in OS between two or three groups. For the survival analysis, patients were censored at the time of the last follow-up. Multivariate analysis of prognostic factors for OS was performed using the Cox proportional hazards method. All prognostic factors were first analyzed using univariate analysis. Early death was defined as 8-wk mortality after the diagnosis or initiation of chemotherapy. Factors with a *P* value of less than 0.05 on univariate analysis were included in the multivariate analysis using a stepwise method.

RESULTS

A total of 219 patients were diagnosed with AML between January 1, 2006 and December 31, 2010 at the 7 participating institutions. Considering the average population of Kagawa Prefecture during the study period, the incidence of AML was 4.26 per 100000 person-years.

We focused on analyzing adult patients with AML. Six patients were excluded due to a lack of available clinical data, and the remaining 213 patients were included. These patients were 127 men and 86 women (male to female ratio 1.48) with a median age of 70 years (range 24-95). Thirty-five patients underwent allogeneic hematopoietic stem cell transplantation. The cohort included 16 APL patients and 197 non-APL patients. The estimated 5-year OS rate of the APL patients was 69.2% (95%CI: 55.5-82.9). The median follow-up period for APL survivors was 23.5 mo (range 0-56).

The non-APL patients had a median age of 70 years (range 24-95), including 74 patients (37.6%) aged $\leqslant 64$ years, 50 patients (25.6%) aged 65-74 years, and 73 patients (36.8%) aged $\geqslant 75$ years. Table 1 shows the characteristics of the non-APL patients. In our study some data were missing for each parameter. Therefore, the total numbers of patients in each parameter group-that is, the sum of each column-are sometimes different. Some clinical features varied among the different age groups. Overall, 93 patients (47.2%) had one or more features of myelodysplasia (not satisfy the diagnosis criteria for MDS). The frequency of myelodysplastic features was higher in patients aged $\geqslant 75$ years than in younger patients. Approximately half of the patients (48.3%) had Eastern Cooperative Oncology Group PS of 2-4. As age increased, the proportion of patients with good PS (0-1) decreased and that with poor PS (3-4) increased. The chromosomal karyotype is known to be the strongest predictor of prognosis, but the systems used to classify the karyotypes vary among studies. We divided our co-

hort into three karyotype groups: 5.1%-6.6% of patients were classified as having a favourable risk karyotype, 22.8%-29.9% of patients were classified as having an unfavourable risk karyotype, and category not recognized. A favorable risk karyotype was more frequent in patients aged ≤ 64 years than patients aged ≥ 65 years. The serum lactase dehydrogenase (LDH) level was lower in patients aged 65-74 years than in the other age groups (≤ 64 years and ≥ 75 years); there were significantly more patients with a normal LDH level (< 250 U/L) and fewer patients with an increased LDH level (> 500 U/L) in the group aged 65-74 years than in the other age groups. Twenty-nine patients (14.7%) had renal dysfunction with a serum creatinine level of > 1.3 mg/dL, and 48 patients (24.3%) had an infection at the time of diagnosis. Intensive induction chemotherapy was administered to 102 patients (51.7%) in total, including 71.6% of patients aged ≤ 64 years, 46.0% of patients aged 65-74 years, and 35.6% of patients aged ≥ 75 years.

In non-APL patients, the estimated 5-year OS rate was 21.1% (95%CI: 1.7-40.5) (Figure 1A). The median follow-up period among non-APL survivors was 32 mo (range 1.0-59.5). Analysis by age group showed that the 5-year OS rate decreased with increasing age. In non-APL patients aged ≤ 64 years, 65-74 years, and ≥ 75 years, the 5-year OS rates were 41.5% (95%CI: 34.5-48.5), 14.1% (95%CI: 8.8-19.4), and 8.9% (95%CI: 4.1-13.7), respectively; and the median survival times were 19, 10 and 7 mo, respectively (Figure 1B). In addition, poor PS (3-4), lack of induction chemotherapy or less intensive induction chemotherapy, presence of antecedent hematological disease (except for MDS), and unfavourable risk karyotype according to the NCCN, SWOG, or CALGB classifications adversely affected the OS rate (Figure 1C-H). Multivariate analysis revealed that older age, poor PS (3-4), lack of induction chemotherapy, presence of antecedent hematological disease, and unfavourable risk karyotype according to any karyotype classification were adverse prognostic factors (Table 2). Detailed information regarding karyotype categories according to the NCCN, SWOG, and CALGB classifications is shown in Table 3.

The rates of CR and early death (within 8 wk of diagnosis) according to PS in different age groups are shown in Figure 2. The proportion of patients with poor PS (3-4) increased with age (Figure 2A). Early death rates were related to both age and PS (Figure 2B). Older patients had higher early deaths rates than younger patients, and patients with poor PS had higher early death rates than patients with good PS. CR rates were also related to both age and PS (Figure 2C). Older patients had lower CR rates than younger patients, and patients with poor PS had lower CR rates than patients with good PS.

DISCUSSION

This study was limited to AML patients in a specific area of Japan, unlike the large scale study conducted by Juliusson *et al*[9], which included data from the whole of Sweden. However, Kagawa Prefecture is surrounded by ocean and mountain ranges, and residents almost never seek treatment for malignancies elsewhere due to the inconvenience of travelling. This study has almost complete capture of the patient population. We therefore consider that the present results were highly representative of the patient representation. Furthermore, the median and interquartile range of age, and incidence of AML, were similar between this study and the Swedish study by Juliusson *et al*[9]. This similarity in the distributions of patients between the two reports indicates that the present study was a reliable population-based study. In Japan, all population-based studies of this sort are conducted under a strict registration system which is facilitated by a nation-wide organization. In all of the cases in the present cohort, a primary physician had reached consensus in referring the patient to a general community hospital due to a haematological malignancy.

Dores *et al*[17] reported that there were no differences in the age of onset or the incidence of AML among non-Hispanic whites, Hispanic whites, Blacks, and Asians/Pacific Islanders in the United States. They also reported that the frequencies of APL, a subtype of AML, differed among these subpopulations, accounting for 6.1%, 14.2%, 9.3%, and 7.0% of the four groups, respectively. Nakase *et al*[23] reported that although the frequency of t(8;21) AML was higher in the Japanese population than the Australian population (33.1% *vs* 15.3%, $P < 0.05$), there was no difference in the frequency of APL between these populations (14.8% *vs* 11.1%). However, these data may not be generalizable as the patients selected for the study were all diagnosed at a single hospital. The frequency of APL in our cohort was 7.5%, which is similar to the frequencies reported among Australians[23], Asians/Pacific Islanders and non-Hispanic white[17], higher than the frequency reported in the Swedish study[9], and lower than the frequencies reported among both Hispanic whites and Blacks in the United States[17], suggesting that the frequency of APL differs among races.

Several studies[7,8,24,25] have reported on differences between elderly and young adult AML patients in terms of host factors such as physiological functions and biological factors such as characteristics of AML cells. Pollyea *et al*[8] reported that outcomes in elderly AML patients were affected by host factors such as decreased drug metabolism, compromised immune defence systems, increased frequency of poor PS, increased frequency of hemorrhagic complications, and increased frequency of psychiatric medications. They also demonstrated that AML cells in elderly patients had more immature morphology and expressed higher levels of the multidrug resistance gene MDR1 than AML cells in young adult patients. Dombert *et al*[7] showed that the frequencies of myelodysplasia and unfavourable risk karyotype were higher in elderly AML patients than young adult AML patients. Our study showed similar results, with elderly patients having higher frequencies of poor PS, myelodysplasia, and unfavourable risk karyotype compared with young adult patients. These data suggest that our study results are a reliable reflection of the overall AML population.

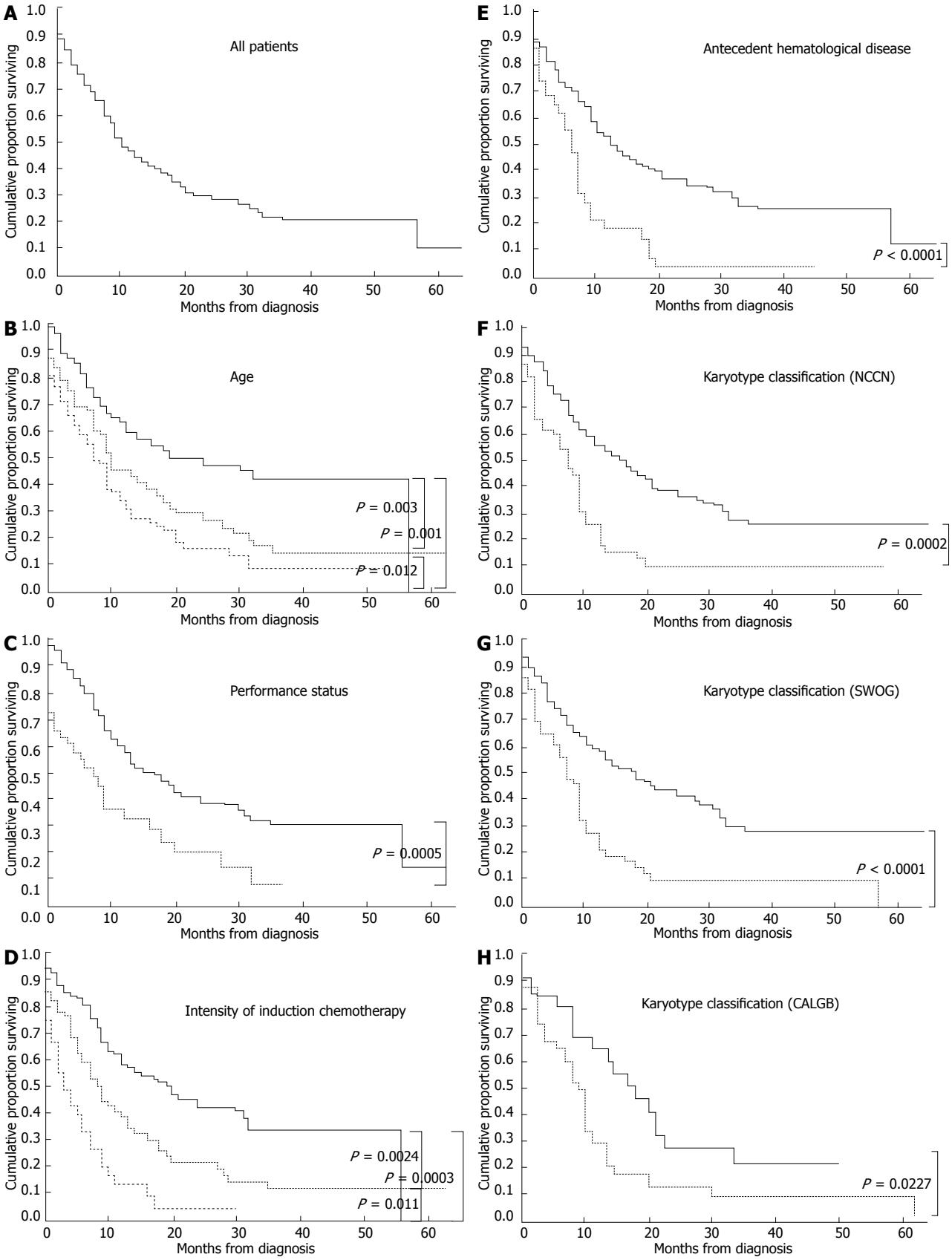


Figure 1 Kaplan-Meier estimate of overall survival in patients with non-acute promyelocytic leukemia. A: All patients; B: Patients categorised by age group. Black line, ≤ 64 years; dotted line, 65-74 years; grey line, ≥75 years; C: Patients categorised by performance status (PS). Black line, PS 0-2; dotted line, PS 3-4; D: Patients categorised by intensity of induction chemotherapy. Black line, intensive chemotherapy; dotted line, less intensive chemotherapy; grey line, best supportive care; E: Patients categorised by antecedent hematological disease. Black line, without antecedent hematological disease; dotted line, with antecedent haematological disease; F: Patients categorised according to the National Comprehensive Cancer Network (NCCN) clinical practice guidelines karyotype classification. Black line, favourable and intermediate risk karyotypes; dotted line, unfavourable risk karyotypes; G: Patients categorised according to the Southwest Oncology Group (SWOG) karyotype classification. Black line, favourable and intermediate risk karyotypes; dotted line, unfavourable risk karyotypes; H: Patients categorised according to the Cancer and Leukemia Group B (CALGB) karyotype classification. Black line, favourable and intermediate risk karyotypes; dotted line, unfavourable risk karyotypes.

Table 1 Characteristics of non-acute promyelocytic leukemia patients

Parameter	Category	Number of patients								
		All patients (n = 197)		Age in year						
				⩽ 64 (n = 74)		65-74 (n = 50)		⩾ 75 (n = 73)		
Gender	Female	78	39.60%	30	40.50%	19	38.00%	29	39.70%	
	Male	119	60.40%	44	59.50%	31	62.00%	44	60.30%	
FAB classification	M0	9	4.60%	3	4.10%	0	0.00%	6	8.20%	
	M1	24	12.20%	10	13.50%	4	8.00%	10	13.70%	
	M2	100	50.80%	34	45.90%	27	54.00%	39	53.40%	
	M4	24	12.20%	11	14.90%	7	14.00%	6	8.20%	
	M5	10	5.10%	5	6.80%	3	6.00%	2	2.70%	
	M6	13	6.60%	3	4.10%	4	8.00%	6	8.20%	
	M7	4	2.00%	2	2.70%	1	2.00%	1	1.40%	
Myelodysplasia	Yes	93	47.20%	32	43.20%	20	40.00%	41	56.20%	[a]
	No	100	50.80%	40	54.10%	29	58.00%	31	42.50%	
Performance status	0-1	98	49.70%	45	60.80%	28	56.00%	25	34.20%	
	2	46	23.40%	13	17.60%	8	16.00%	25	34.20%	
	3-4	49	24.90%	14	18.90%	13	26.00%	22	30.10%	
Karyotype risk category										
NCCN	F	13	6.60%	9	12.20%	2	4.00%	2	2.70%	[a]
	I	122	61.90%	43	58.10%	27	54.00%	52	71.20%	
	U	46	23.40%	16	21.60%	19	38.00%	11	15.10%	
SWOG	F	10	5.10%	7	9.50%	2	4.00%	1	1.40%	[a]
	I	97	49.20%	35	47.30%	23	46.00%	39	53.40%	
	U	59	29.90%	20	27.00%	21	42.00%	18	24.70%	
	others	15	7.60%	6	8.10%	2	4.00%	7	9.60%	
CALGB	F	13	6.60%	9	12.20%	3	6.00%	1	1.40%	[a]
	I	100	50.80%	33	44.60%	26	52.00%	41	56.20%	
	U	45	22.80%	15	20.30%	16	32.00%	14	19.20%	
	others	39	19.80%	17	23.10%	5	10.00%	17	23.20%	
Antecedent	No	158	80.20%	60	81.10%	37	74.00%	61	83.60%	
hematologic disease	Yes	36	18.30%	13	17.60%	11	22.00%	12	16.40%	
Prior chemotherapy	No	174	88.30%	65	87.80%	41	82.00%	68	93.20%	
	Yes	19	9.60%	7	9.50%	7	14.00%	5	6.80%	
Laboratory findings										
WBC (× 10^3/mL)	< 100	178	90.40%	65	87.80%	48	96.00%	65	89.00%	
	⩾ 100	16	8.10%	7	9.50%	1	2.00%	8	11.00%	
Hemoglobin (g/dL)	< 8.0	93	47.20%	36	48.60%	23	46.00%	34	46.60%	
	⩾ 8.0	101	51.30%	36	48.60%	26	52.00%	39	53.40%	
Platelet (× 10^4/mL)	< 5.0	85	43.10%	29	39.20%	23	46.00%	33	45.20%	
	5.0-10.0	64	32.50%	25	33.80%	20	40.00%	19	26.00%	
	⩾ 10.0	45	22.80%	18	24.30%	6	12.00%	21	28.80%	
% Blast in blood	< 20	89	45.20%	28	37.80%	29	58.00%	32	43.80%	
	20-50	43	21.80%	17	23.00%	11	22.00%	15	20.50%	
	> 50	59	29.90%	27	36.50%	8	16.00%	24	32.90%	
% Blast in marrow	< 50	98	49.70%	33	44.60%	30	60.00%	35	47.90%	
	⩾ 50	84	42.60%	35	47.30%	18	36.00%	31	42.50%	
LDH (IU/L)	< 250	58	29.40%	15	20.30%	24	48.00%	19	26.00%	[a]
	250-500	75	38.10%	27	36.50%	14	28.00%	34	46.60%	
	> 500	59	29.90%	29	39.20%	11	22.00%	19	26.00%	
Creatinine (mg/dL)	⩽ 1.3	162	82.20%	66	89.20%	41	82.00%	55	75.30%	
	> 1.3	29	14.70%	5	6.80%	8	16.00%	16	21.90%	
Infection at	No	146	74.10%	52	70.20%	39	78.00%	55	75.30%	[a]
induction therapy	Yes	48	24.30%	20	27.00%	10	20.00%	18	24.60%	
Intensity of	Intensive	102	51.70%	53	71.60%	23	46.00%	26	35.60%	[a]
induction therapy	Less-intensive	56	28.40%	13	17.50%	19	38.00%	24	32.80%	
	BSC	39	19.70%	8	10.80%	8	16.00%	23	31.50%	

[a]P < 0.05 *vs* total. F: Favorable; I: Intermediate; U: Unfavorable; WBC: White blood cell; NCCN: National Comprehensive Cancer Network; SWOG: Southwest Oncology Group; CALGB: Cancer and Leukemia Group B; LDH: Lactase dehydrogenase.

The estimated 5-year OS rate of our non-APL patients was 21.1%, which is similar to the 5-year OS rates reported by retrospective population-based studies conducted in Sweden[9,26,27], the United Kingdom[16], and the United States[17,28,29], but lower than the 5-year OS rates reported by prospective clinical trials for young adult non-APL patients[10-13]. When the data of all AML patients are analysed in a population-based study, patients with poor PS, organ dysfunction, documented infection, and severe comorbidities are included; this lowers the

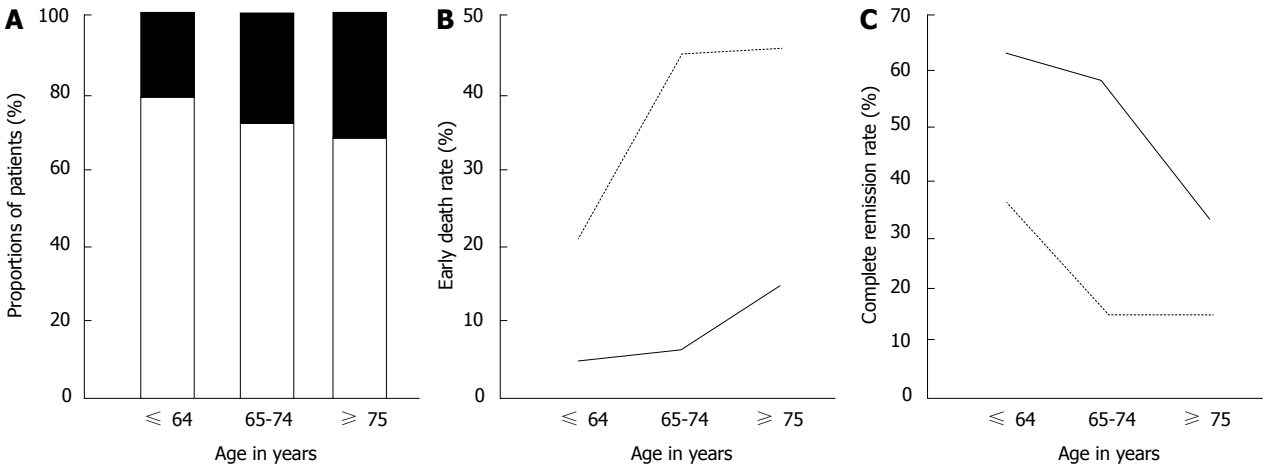


Figure 2 Early death rate and complete remission rate according to performance status in different age groups. A: Proportions of patients with good and poor performance status (PS) in different age groups. Grey, PS 0-2; black, PS 3-4; B: Early death rate according to PS in different age groups. Black line, PS 0-2; dotted line, PS 3-4; C: Complete remission rate according to PS in different age groups. Black line, PS 0-2; dotted line, PS 3-4.

Table 2 Multivariate analysis of prognostic factors affecting survival in non-acute promyelocytic leukemia patient

Risk factors			
Chromosomal abnormality	**NCCN**	**SWOG**	**CALGB**
	HR (95%CI) *P*	**HR (95%CI) *P***	**HR (95%CI) *P***
Age in years	0.031	0.043	0.026
≤ 64	1	1	1
65-74	1.33 (1.06-1.58)	1.39 (1.13-1.64)	1.40 (1.15-1.66)
≥ 75	1.88 (1.59-2.04)	1.82 (1.60-2.05)	1.91 (1.67-2.13)
Performance status	0.012	0.028	0.019
0-2	1	1	1
3-4	1.94 (1.15-3.26)	1.80 (1.06-3.05)	1.88 (1.11-3.20)
Intensity of induction therapy	0.041	0.029	0.027
Intensive, less-intensive	1	1	1
Best supportive care	1.74 (2.02-2.96)	1.80 (1.06-3.07)	1.83 (1.07-3.14)
Antecedent hematological disease	0.007	0.007	0.004
No	1	1	1
Yes	1.92 (1.20-3.08)	1.90 (1.19-3.05)	2.03 (1.25-3.27)
Chromosomal abnormality	0.001	< 0.001	< 0.001
Favorable, intermediate	1	1	1
Unfavorable	1.98 (1.31-2.99)	1.44 (0.55-3.78)	1.56 (0.63-3.87)

NCCN: National Comprehensive Cancer Network practice guidelines in oncology- v.2.2010; SWOG: Southwest Oncology Group; CALGB: Cancer and Leukemia Group B.

overall long-term OS rates compared with the survival rates in prospective clinical trials of young adult AML patients without these conditions.

Juliusson *et al*[9] analysed and compared the CR rates of patient cohorts grouped by PS and found that the CR rate decreases as age increases, indicating that PS and age are independent adverse prognostic factors. Our data are consistent with these findings. However, they did not conduct multivariate analysis to determine adverse prognostic factors associated with OS in non-APL patients. Our multivariate analysis identified older age, poor PS (3-4), lack of induction chemotherapy, presence of antecedent haematological disease, and unfavourable risk karyotype as independent adverse prognostic factors for OS. In prospective clinical trials for young adult patients with non-APL, good PS and no organ dysfunction, the presence of antecedent hematological disease and unfavourable risk karyotype were found to be independent adverse prognostic factors for OS[10-12]. Our retrospective population-based study yielded similar results, indicating that the presence of antecedent hematological disease and unfavourable risk karyotype are adverse prognostic factors for OS in all non-APL patients. It has been postulated that the frequencies of myelodysplasia, poor PS, and unfavourable risk karyotype are higher in elderly non-APL patients than young adult non-APL patients, leading to poorer long-term survival in elderly patients[6-8]. As elderly patients and those with poor PS are excluded from prospective clinical trials, population-based studies are necessary to determine whether older age and poor PS are adverse prognostic factors for OS. As described above, a study in Sweden found that older

Table 3 Detailed information regarding karyotype categories according to the National Comprehensive Cancer Network, Southwest Oncology Group, and Cancer and Leukemia Group B classifications

Category	Favorable	Intermediate	Unfavorable	Category not recognized
NCCN	t(8;21)	+8, t(9;11), -X, -Y, -6, +1, +4, +7, +11, +13, +21, del(9), del(20), add(12), add(16), add(17), inv(3), t(1;16), t(3;21), t(8;18), t(8;20), t(11;16), t(11;17)	–5, del(5q), -7, non-t(9;11) abn11q23, inv(3), t(9;22), complex karyotype ⩾ 3	-
SWOG	t(8;21)	-Y, +8	abn(3q), -5, -7, t(9;22), abn(9q), abn(11q), abn(17p), abn(20q), abn(21q), complex karyotype ≥ 3	t(9;11), -X, -6, +1, +4, +7, +11, +13, +21, add(12), add(16), inv(3), t(1;16), t(3;21), t(8;18), t(8;20)
CALGB	t(8;21), del(9q)	-Y, del(5q), t(9;11), +11, del(11q), abn(12p), +13, del(20q), +21	inv(3), -7, +8, complex karyotype ≥ 3	-X, -6, +1, +4, +7, add(12), add(16), add(17), t(1;16), t(3;21), t(8;18), t(8;20), t(11;16), t(11;17)

NCCN: National Comprehensive Cancer Network; SWOG: Southwest Oncology Group; CALGB: Cancer and Leukemia Group B.

age and poor PS independently affected prognosis in terms of the CR rate and early death rate, but these results were not obtained through statistical analyses[9]. It therefore could not be ruled out that factors such as myelodysplasia and unfavourable risk karyotype were related to older age and influenced the results. Our multivariate analysis shows for the first time that older age is an independent adverse prognostic factor for OS.

This study has several limitations: (1) it is a retrospective study; (2) the number of patients is small; (3) the study includes Japanese patients only; (4) analyses of the data regarding comorbidities and expression of MDR1 on AML cells at the start of treatment could not be performed; and (5) analyses of haemorrhagic and infectious complications and their severity could not be performed.

We propose the following three reasons why older age is an independent adverse prognostic factor for OS in non-APL patients, leading to poor prognosis: (1) epigenetic changes to genes affect the pharmacokinetics of anticancer drugs; (2) preclinical organ dysfunction may not be reflected in the findings of blood tests and functional investigations; and (3) other unknown factors.

It is necessary to perform further large-scale, prospective, population-based observational studies, which take the various parameters that change with age into consideration, in order to definitively determine the adverse prognostic factors associated with older age. Age would contribute a considerable life expectance to indicate induction chemotherapy with eligible dose of cytotoxic drugs for a favorable case even if advanced elderly.

ACKNOWLEDGMENTS

The authors thank Marguerite Elgin of Edanz Group Japan for editorial assistance.

COMMENTS

Background

Elderly acute myeloid leukemia (AML) patients have higher frequencies of myelodysplasia, unfavorable risk karyotype, and poor performance status (PS), which are the leading causes of poor long-term prognosis, compared with young adult AML patients. Elderly patients and those with poor PS are often excluded from prospective clinical trials for AML therapies. In such cases, the results of clinical practice may differ from the results reported in a clinical trial. Therefore, it is necessary to include a full cohort of consecutive patients diagnosed with AML in order to elucidate the real-world outcome for patients suffering from AML.

Research frontiers

In the field of medical oncology, it remains controversial whether age itself is an independent prognostic factor for prognosis. Generally it is axiomatic that older patients are more likely to have a poorer PS and have more underlying diseases, both of which result in morbid prognosis. Thus, the age and coexistence of related prognostic factors can be confounding. However, biological aging is not always associated with cognitive dysfunctions, and recent studies in geriatric oncology have aggregated sufficient evidence that geriatric assessment for chemotherapy is independent from age.

Innovations and breakthroughs

These multivariate analysis shows for the first time that older age is an independent adverse prognostic factor for overall survival (OS). This was validated in several chromosomal risk categories, *i.e.*, NCCN, SWOG, and CALGB. Though this cohort was limited to a local community in Japan, our results are expected to change the realistic planning of practical treatments for very elderly AML patients, who are not usually assessed in clinical trials.

Applications

The clinical outcome of patients over age 65 with AML is poor. However, intensive chemotherapy is a significant predictor for longer survival not only in patients younger than 75, but also in patients over age 75 with PS 0-2. Based on these results, patients with AML over 75 years of age could be candidates for intensive or less-intensive induction chemotherapy to obtain a better remission rate and further survival.

Terminology

The population-based study is an observational study for longitudinally registered patients without any medical interventions. The population-based cohort is set up to investigate whole populations in order to avoid intentional bias. It is crucial that the cohort be representative of a defined population. The population-based study offers three advantages: (1) it can illustrate the distributions, prevalence, and treatment outcome of the disease; (2) it can assess the risk factors for disease in a realistic manner; and (3) it can carry out unbiased evaluations of relations including confounders. Therefore, the authors believe that the present population-based study has reached a robust conclusion about whether advanced age and poor PS are adverse prognostic factors for OS.

Peer review

Ohnishi H *et al* reported for the first time that older age is an independent adverse prognostic factor for overall survival in AML patients through a population-based study cohorting 213 adult AML patients, by using multivariate analysis. Overall, This is a well-written and cafefully discussed paper.

REFERENCES

1 **Yanada M**, Naoe T. Acute myeloid leukemia in older adults. *Int J Hematol* 2012; **96**: 186-193 [PMID: 22791510 DOI: 10.1007/s12185-012-1137-3]

2 **Appelbaum FR**, Gundacker H, Head DR, Slovak ML, Will-

man CL, Godwin JE, Anderson JE, Petersdorf SH. Age and acute myeloid leukemia. *Blood* 2006; **107**: 3481-3485 [PMID: 16455952 DOI: 10.1182/blood-2005-09-3724]
3 **Hiddemann W**, Kern W, Schoch C, Fonatsch C, Heinecke A, Wörmann B, Büchner T. Management of acute myeloid leukemia in elderly patients. *J Clin Oncol* 1999; **17**: 3569-3576 [PMID: 10550156]
4 **Löwenberg B**, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med* 1999; **341**: 1051-1062 [PMID: 10502596 DOI: 10.1056/NEJM199909303411407]
5 **Grimwade D**, Walker H, Harrison G, Oliver F, Chatters S, Harrison CJ, Wheatley K, Burnett AK, Goldstone AH; Medical Research Council Adult Leukemia Working Party. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood* 2001; **98**: 1312-1320 [PMID: 11520776 DOI: 10.1182/blood.V98.5.1312]
6 **Estey E**. Acute myeloid leukemia and myelodysplastic syndromes in older patients. *J Clin Oncol* 2007; **25**: 1908-1915 [PMID: 17488990 DOI: 10.1200/JCO.2006.10.2731]
7 **Dombret H**, Raffoux E, Gardin C. Acute myeloid leukemia in the elderly. *Semin Oncol* 2008; **35**: 430-438 [PMID: 18692693 DOI: 10.1053/j.seminoncol.2008.04.013]
8 **Pollyea DA**, Kohrt HE, Medeiros BC. Acute myeloid leukaemia in the elderly: a review. *Br J Haematol* 2011; **152**: 524-542 [PMID: 21314823 DOI: 10.1111/j.1365-2141.2010.08470.x]
9 **Juliusson G**, Antunovic P, Derolf A, Lehmann S, Möllgård L, Stockelberg D, Tidefelt U, Wahlin A, Höglund M. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. *Blood* 2009; **113**: 4179-4187 [PMID: 19008455 DOI: 10.1182/blood-2008-07-172007]
10 **Ohtake S**, Miyawaki S, Fujita H, Kiyoi H, Shinagawa K, Usui N, Okumura H, Miyamura K, Nakaseko C, Miyazaki Y, Fujieda A, Nagai T, Yamane T, Taniwaki M, Takahashi M, Yagasaki F, Kimura Y, Asou N, Sakamaki H, Handa H, Honda S, Ohnishi K, Naoe T, Ohno R. Randomized study of induction therapy comparing standard-dose idarubicin with high-dose daunorubicin in adult patients with previously untreated acute myeloid leukemia: the JALSG AML201 Study. *Blood* 2011; **117**: 2358-2365 [PMID: 20693429 DOI: 10.1182/blood-2010-03-273243]
11 **Miyawaki S**, Ohtake S, Fujisawa S, Kiyoi H, Shinagawa K, Usui N, Sakura T, Miyamura K, Nakaseko C, Miyazaki Y, Fujieda A, Nagai T, Yamane T, Taniwaki M, Takahashi M, Yagasaki F, Kimura Y, Asou N, Sakamaki H, Handa H, Honda S, Ohnishi K, Naoe T, Ohno R. A randomized comparison of 4 courses of standard-dose multiagent chemotherapy versus 3 courses of high-dose cytarabine alone in postremission therapy for acute myeloid leukemia in adults: the JALSG AML201 Study. *Blood* 2011; **117**: 2366-2372 [PMID: 21190996 DOI: 10.1182/blood-2010-07-295279]
12 **Berman E**, Heller G, Santorsa J, McKenzie S, Gee T, Kempin S, Gulati S, Andreeff M, Kolitz J, Gabrilove J. Results of a randomized trial comparing idarubicin and cytosine arabinoside with daunorubicin and cytosine arabinoside in adult patients with newly diagnosed acute myelogenous leukemia. *Blood* 1991; **77**: 1666-1674 [PMID: 2015395]
13 **Berman E,** Wiernik P, Vogler R, Vélez-Gárcia E, Bartolucci A, Whaley FS. Long-term follow-up of three randomized trials comparing idarubicin and daunorubicin as induction therapies for patients with untreated acute myeloid leukemia. *Cancer* 1997; **80** (11 Suppl): 2181-5 [DOI: 10.1002/(SICI)1097-0142(19971201)80: 11 <2181: : AID-CNCR3>3.0.CO; 2-L]
14 **Mauritzson N**, Johansson B, Albin M, Billström R, Ahlgren T, Mikoczy Z, Nilsson PG, Hagmar L, Mitelman F. A single-center population-based consecutive series of 1500 cytogenetically investigated adult hematological malignancies: karyotypic features in relation to morphology, age and gender. *Eur J Haematol* 1999; **62**: 95-102 [PMID: 10052712 DOI: 10.1111/j.1600-0609.1999.tb01728.x]
15 **Taylor PR**, Reid MM, Stark AN, Bown N, Hamilton PJ, Proctor SJ. De novo acute myeloid leukaemia in patients over 55-years-old: a population-based study of incidence, treatment and outcome. Northern Region Haematology Group. *Leukemia* 1995; **9**: 231-237 [PMID: 7532766]
16 **Bhayat F**, Das-Gupta E, Smith C, McKeever T, Hubbard R. The incidence of and mortality from leukaemias in the UK: a general population-based study. *BMC Cancer* 2009; **9**: 252 [PMID: 19630999 DOI: 10.1186/1471-2407-9-252]
17 **Dores GM**, Devesa SS, Curtis RE, Linet MS, Morton LM. Acute leukemia incidence and patient survival among children and adults in the United States, 2001-2007. *Blood* 2012; **119**: 34-43 [PMID: 22086414 DOI: 10.1182/blood-2011-04-347872]
18 **Phekoo KJ**, Richards MA, Møller H, Schey SA; South Thames Haematology Specialist Committee. The incidence and outcome of myeloid malignancies in 2,112 adult patients in southeast England. *Haematologica* 2006; **91**: 1400-1404 [PMID: 17018393]
19 **Pulte D**, Gondos A, Brenner H. Improvements in survival of adults diagnosed with acute myeloblastic leukemia in the early 21st century. *Haematologica* 2008; **93**: 594-600 [PMID: 18322250 DOI: 10.3324/haematol.12304]
20 **NCCN.** Guideline. Available from: URL: http: //www.nccn.org/professionals/physician_gls/f_guidelines.asp
21 **Slovak ML**, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A, Paietta E, Willman CL, Head DR, Rowe JM, Forman SJ, Appelbaum FR. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood* 2000; **96**: 4075-4083 [PMID: 11110676]
22 **Byrd JC**, Mrózek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, Pettenati MJ, Patil SR, Rao KW, Watson MS, Koduru PR, Moore JO, Stone RM, Mayer RJ, Feldman EJ, Davey FR, Schiffer CA, Larson RA, Bloomfield CD. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* 2002; **100**: 4325-4336 [PMID: 12393746 DOI: 10.1182/blood-2002-03-0772]
23 **Nakase K**, Bradstock K, Sartor M, Gottlieb D, Byth K, Kita K, Shiku H, Kamada N. Geographic heterogeneity of cellular characteristics of acute myeloid leukemia: a comparative study of Australian and Japanese adult cases. *Leukemia* 2000; **14**: 163-168 [PMID: 10637492 DOI: 10.1038/sj.leu.2401638]
24 **Scholl S**, Theuer C, Scheble V, Kunert C, Heller A, Mügge LO, Fricke HJ, Höffken K, Wedding U. Clinical impact of nucleophosmin mutations and Flt3 internal tandem duplications in patients older than 60 yr with acute myeloid leukaemia. *Eur J Haematol* 2008; **80**: 208-215 [PMID: 18081718 DOI: 10.1111/j.1600-0609.2007.01019.x]
25 **Whitman SP**, Maharry K, Radmacher MD, Becker H, Mrózek K, Margeson D, Holland KB, Wu YZ, Schwind S, Metzeler KH, Wen J, Baer MR, Powell BL, Carter TH, Kolitz JE, Wetzler M, Moore JO, Stone RM, Carroll AJ, Larson RA, Caligiuri MA, Marcucci G, Bloomfield CD. FLT3 internal tandem duplication associates with adverse outcome and gene- and microRNA-expression signatures in patients 60 years of age or older with primary cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *Blood* 2010; **116**: 3622-3626 [PMID: 20656931 DOI: 10.1182/blood-2010-05-283648]
26 **Juliusson G**, Billström R, Gruber A, Hellström-Lindberg E, Höglunds M, Karlsson K, Stockelberg D, Wahlin A, Aström M, Arnesson C, Brunell-Abrahamsson U, Carstensen J, Fredriks-

son E, Holmberg E, Nordenskjöld K, Wiklund F. Attitude towards remission induction for elderly patients with acute myeloid leukemia influences survival. *Leukemia* 2006; **20**: 42-47 [PMID: 16327841 DOI: 10.1038/sj.leu.2404004]

27 **Palk K**, Luik E, Varik M, Viigimaa I, Vaht K, Everaus H, Wennström L, Stockelberg D, Safai-Kutti S, Holmberg E, Kutti J. The incidence and survival of acute de novo leukemias in Estonia and in a well-defined region of western Sweden during 1997-2001: a survey of patients aged & gt; or=65 years. *Cancer Epidemiol* 2010; **34**: 24-28 [PMID: 20071253 DOI: 10.1016/j.canep.2009.12.004]

28 **McClune BL**, Weisdorf DJ, Pedersen TL, Tunes da Silva G, Tallman MS, Sierra J, Dipersio J, Keating A, Gale RP, George B, Gupta V, Hahn T, Isola L, Jagasia M, Lazarus H, Marks D, Maziarz R, Waller EK, Bredeson C, Giralt S. Effect of age on outcome of reduced-intensity hematopoietic cell transplantation for older patients with acute myeloid leukemia in first complete remission or with myelodysplastic syndrome. *J Clin Oncol* 2010; **28**: 1878-1887 [PMID: 20212255 DOI: 10.1200/JCO.2009.25.4821]

29 **Oran B**, Weisdorf DJ. Survival for older patients with acute myeloid leukemia: a population-based study. *Haematologica* 2012; **97**: 1916-1924 [PMID: 22773600 DOI: 10.3324/haematol.2012.066100]

P- Reviewer: Chen SS, Nosaka T, Yokota T **S- Editor**: Song XX
L- Editor: A **E- Editor:** Lu YJ

World Journal of Hematology

Online Submissions: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
doi:10.5315/wjh.v3.i3.115

World J Hematol 2014 August 6; 3(3): 115-117
ISSN 2218-6204 (online)

CASE REPORT

Unusual cytogenetic abnormalities associated with Philadelphia chromosome

Sanjeev Kumar Sharma, Anil Handoo, Dharma Choudhary, Nitin Gupta

Sanjeev Kumar Sharma, Anil Handoo, Dharma Choudhary, Nitin Gupta, Department of Hemato-oncology and Bone Marrow Transplant, BL Kapur Superspeciality Hospital, New Delhi-05, India

Author contributions: Sharma SK designed and wrote the paper; Handoo A provided the laboratory data; Choudhary D and Gupta N provided the clinical data.

Correspondence to: Sanjeev Kumar Sharma, MD, DM, Department of Hemato-oncology and Bone Marrow Transplant, BL Kapur Superspeciality Hospital, Pusa Road, New Delhi-05, India. sksanjeev13@yahoo.com

Telephone: +91-996-8756303 Fax: +91-996-8756303

Received: December 25, 2013 Revised: February 8, 2014
Accepted: July 12, 2014
Published online: August 6, 2014

Abstract

Cytogenetic abnormalities are the hallmark of leukemias. We report here two cases of unusual cytogenetic abnormalities associated with Philadelphia chromosome, one with mixed phenotypic acute leukemia showing monosomy 7 and t(9;22) (q34;q11.2) and the other with chronic myeloid leukemia and additional translocation involving chromosomes 10 and 13. Both patients achieved complete remission following imatinib based treatment.

Key words: Philadelphia chromosome; Cytogenetic abnormalities

Core tip: Cytogenetic abnormalities are the hallmark of leukemias. We report here two cases of unusual cytogenetic abnormalities associated with Philadelphia chromosome, one with mixed phenotypic acute leukemia showing monosomy 7 and t(9;22) (q34;q11.2) and the other with chronic myeloid leukemia and additional translocation involving chromosomes 10 and 13. Both patients achieved complete remission following imatinib based treatment.These two cases highlight the novel additional cytogenetic abnormalities associated with Ph chromosome.

Sharma SK, Handoo A, Choudhary D, Gupta N. Unusual cytogenetic abnormalities associated with Philadelphia chromosome. *World J Hematol* 2014; 3(3): 115-117 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i3/115.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i3.115

INTRODUCTION

Cytogenetic abnormalities are the hallmark of leukemias. Translocation (9;22) is the characteristic feature of chronic myeloid leukemia and is also seen in variable number of patients with acute lymphoblastic leukemia. Monosomy 7 is seen in patients with myelodysplastic syndrome, acute myeloid leukemia and few cases of acute lymphoblastic leukemia. Mixed phenotypic acute leukemia (MPAL) is commonly associated with t(9;22) and sometimes with monosomy or deletion 7, but combined cytogenetic abnormality involving t(9;22) and monosomy 7 has rarely been reported in MPAL. We report here two cases, one of MPAL showing monosomy 7 and t(9;22) (q34;q11.2) and the other with chronic myeloid leukemia and additional translocation involving chromosomes 10 and 13. Both patients achieved complete remission following imatinib based treatment.

CASE REPORT

Case 1

A 27 years old man was admitted with weakness and low grade fever for 20 d. On examination he was found to have pallor without lymphadenopathy or hepato-splenomegaly. Hemogram showed hemoglobin 5.8 g/dL, total leukocyte count 62 × 10^9/L with 60% blasts and platelet count 170 × 10^9/L. Flowcytometry revealed two distinct

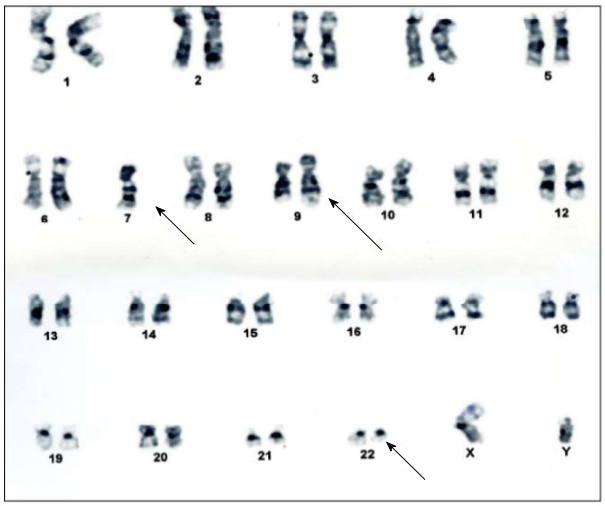


Figure 1 Karyotyping of the patient with mixed phenotype acute leukemia showing (arrows) 45, XY, monosomy 7, t(9;22) (q34;q11.2)[18]/46,XY[2].

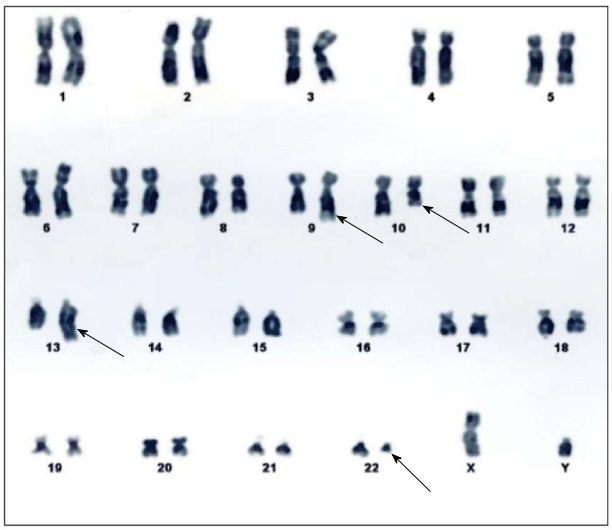


Figure 2 Karyotyping of the patient with chronic myeloid leukemia showing (arrows) 46 XY, t(9;22)(q34;11.2), t(10;13)(q23;q34)[20].

clusters of cells, one expressing CD34, HLD-DR, MPO, CD117, CD13 and CD33. The other cluster of blasts expressed cytoplasmic CD79a, CD19 and CD10. The blasts were negative for surface and cytoplasmic CD3, CD5, CD20 and CD16. The patient was diagnosed as a case of MPAL (Blymphoid/myeloid). Karyotyping revealed 45,XY, monosomy7, t(9;22)(q34;q11.2)[18]/46,XY[2] (Figure 1). RT-PCR for BCR-ABL detected genomic breakpoint at e13a2 corresponding to p210. Patient was treated with hyper-CVAD chemotherapy along with imatinib, which resulted in complete remission.

Case 2

A 50 years old man presented with complaints of mild weakness for 3 wk. His hemoglobin was 10.5 g/dL, total leukocyte count 46.7 × 10^9/L and platelet count 3.6 × 10^9/L, differential count showed neutrophils 62%, myelocytes 10%, metamyelocytes 8%, lymphocytes 12%, monocytes 3%, eosinophils 2% and basophils 3%. Leukocyte alkaline phosphatase was low. Karyotyping revealed 46 XY, t(9;22)(q34;11.2), t(10;13)(q23;q34)[20] (Figure 2). Real-time polymerase chain reaction for BCR-ABL was positive for p210. He was diagnosed as chronic myeloid leukemia-chronic phase and treated with imatinib. He achieved complete hematological remission in 2 mo.

DISCUSSION

Philadelphia (Ph) chromosome results from reciprocal translocation of chromosome 9 and 22. This translocation leads to the generation of a chimeric gene that results from the fusion of the *ABL* gene on chromosome 9 with the BCR gene on chromosome 22. MPAL is a rare leukemia arising from a hematopoietic pluripotent stem cell with a frequency of 0.5%-1%[1,2]. In a study by Matutes *et al*[2], comprising 100 patients of MPAL, cytogenetics revealed t(9;22) in 20%, 11q23/MLL-rearrangements in 8%, complex in 32%, aberrant in 27% and normal in 13% karyotypes. Three cases of CML[3,4] and one case of T-cell/myeloid MPAL[5] associated with del 7, t(9;22)(q34;q11) have been reported. Deletion/monosomy 7 is associated with poor prognosis in AML and t(9;22) confers a bad prognosis in ALL. Additional chromosomal abnormalities in CML may appear in about 5% of cases[6-8]. In a study by Luatti *et al*[9], 21 patients (5.6%) had additional chromosomal abnormalities; the overall cytogenetic and molecular response rates in these patients were significantly lower. None of these patients had translocations involving chromosome 10 and 13. Our patient with MPAL demonstrated characteristic bilineage leukemia and showed complete remission following hyper-CVAD plus imatinib therapy. The patient with CML also responded to imatinib. These two cases highlight the novel additional cytogenetic abnormalities associated with Ph chromosome. Whether this association of Philadelphia chromosome with these additional cytogenetic abnormalities adversely affect the prognosis needs to be evaluated. Though some studies have shown poor outcome with additional chromosomal abnormalities[9], our patients showed good initial response to imatinib based therapy.

COMMENTS

Case characteristics

A 27 years old man was admitted with weakness and low grade fever for 20 d. A 50 years old man presented with complaints of mild weakness for 3 wk.

Clinical diagnosis

Case 1: Hemogram showed hemoglobin 5.8 g/dL, total leukocyte count 62 × 10^9/L with 60% blasts and platelet count 170 × 10^9/L. Case 2: His hemoglobin was 10.5 g/dL, total leukocyte count 46.7 × 10^9/L and platelet count 3.6 × 10^9/L, differential count showed neutrophils 62%, myelocytes 10%, metamyelocytes 8%, lymphocytes 12%, monocytes 3%, eosinophils 2% and basophils 3%.

Laboratory diagnosis

Real-time polymerase chain reaction for BCR-ABL was positive for p210.

Treatment

Patient was treated with hyper-CVAD chemotherapy along with imatinib, which

resulted in complete remission.

Peer review

The manuscript demonstrates good initial response to imatinib based therapy of two rare cases of leukemia patients one with mixed phenotypic acute leukemia bilineage (B-lymphoid/myeloid) and the second with CML bearing an additional to t(9;22) chromosomal abnormality.

REFERENCES

1 **Borowitz M**, Bene MC, Harris NL, Porwit A, Matutes E. Acute leukemias of ambiguous lineage. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. World Health Organization classification of tumors. Pathology and genetics of tumors of hematopoietic and lymphoid tissues. Lyon, France: IARC Press, 2008: 150-155

2 **Matutes E**, Pickl WF, Van't Veer M, Morilla R, Swansbury J, Strobl H, Attarbaschi A, Hopfinger G, Ashley S, Bene MC, Porwit A, Orfao A, Lemez P, Schabath R, Ludwig WD. Mixed-phenotype acute leukemia: clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification. *Blood* 2011; **117**: 3163-3171 [PMID: 21228332 DOI: 10.1182/blood-2010-10-314682]

3 **Fischer K**, Fröhling S, Scherer SW, McAllister Brown J, Scholl C, Stilgenbauer S, Tsui LC, Lichter P, Döhner H. Molecular cytogenetic delineation of deletions and translocations involving chromosome band 7q22 in myeloid leukemias. *Blood* 1997; **89**: 2036-2041 [PMID: 9058725]

4 **Christodoulidou F**, Silver RT, Macera MJ, Verma RS. Disappearance of a highly unusual clone, 46,XY,del(7)(p12),t(9;22)(q34; q11) in chronic myeloid leukemia after treatment with recombinant interferon and cytosine arabinoside. *Cancer Genet Cytogenet* 1992; **64**: 174-177 [PMID: 1486569]

5 **Monma F**, Nishii K, Ezuki S, Miyazaki T, Yamamori S, Usui E, Sugimoto Y, Lorenzo V F, Katayama N, Shiku H. Molecular and phenotypic analysis of Philadelphia chromosome-positive bilineage leukemia: possibility of a lineage switch from T-lymphoid leukemic progenitor to myeloid cells. *Cancer Genet Cytogenet* 2006; **164**: 118-121 [PMID: 16434313 DOI: 10.1016/j.cancergencyto.2005.06.021]

6 **Marktel S**, Marin D, Foot N, Szydlo R, Bua M, Karadimitris A, De Melo VA, Kotzampaltiris P, Dazzi F, Rahemtulla A, Olavarria E, Apperley JF, Goldman JM. Chronic myeloid leukemia in chronic phase responding to imatinib: the occurrence of additional cytogenetic abnormalities predicts disease progression. *Haematologica* 2003; **88**: 260-267 [PMID: 12651263]

7 **Sokal JE**, Gomez GA, Baccarani M, Tura S, Clarkson BD, Cervantes F, Rozman C, Carbonell F, Anger B, Heimpel H. Prognostic significance of additional cytogenetic abnormalities at diagnosis of Philadelphia chromosome-positive chronic granulocytic leukemia. *Blood* 1988; **72**: 294-298 [PMID: 3164637]

8 **Kantarjian HM**, Smith TL, McCredie KB, Keating MJ, Walters RS, Talpaz M, Hester JP, Bligham G, Gehan E, Freireich EJ. Chronic myelogenous leukemia: a multivariate analysis of the associations of patient characteristics and therapy with survival. *Blood* 1985; **66**: 1326-1335 [PMID: 3864497]

9 **Luatti S**, Castagnetti F, Marzocchi G, Baldazzi C, Gugliotta G, Iacobucci I, Specchia G, Zanatta L, Rege-Cambrin G, Mancini M, Abruzzese E, Zaccaria A, Grimoldi MG, Gozzetti A, Ameli G, Capucci MA, Palka G, Bernasconi P, Palandri F, Pane F, Saglio G, Martinelli G, Rosti G, Baccarani M, Testoni N. Additional chromosomal abnormalities in Philadelphia-positive clone: adverse prognostic influence on frontline imatinib therapy: a GIMEMA Working Party on CML analysis. *Blood* 2012; **120**: 761-767 [PMID: 22692507 DOI: 10.1182/blood-2011-10-384651]

P- Reviewer: Demonacos C, Huang SA **S- Editor**: Gou SX
L- Editor: A **E- Editor:** Lu YJ

ISSN 2218-6204 (online)

World Journal of *Hematology*

World J Hematol 2014 November 6; 3(4): 118-137

Volume End

Published by Baishideng Publishing Group Inc

World Journal of Hematology

Contents

Quarterly Volume 3 Number 4 November 6, 2014

REVIEW

World Journal of Hematology
Volume 3 Number 4 November 6, 2014

Contents

NAME OF JOURNAL
World Journal of Hematology

ISSN
ISSN 2218-6204 (online)

LAUNCH DATE
June 6, 2012

FREQUENCY
Quarterly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Hematology
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
November 6, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/2218-6204/g_info_20100722173604.htm.

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Hematology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5315/wjh.v3.i4.118

World J Hematol 2014 November 6; 4(3): 118-127
ISSN 2218-6204 (online)

REVIEW

Follicular helper T lymphocytes in health and disease

Cecilia Parodi, María Noel Badano, Nora Galassi, Ana Coraglia, Patricia Baré, Alejandro Malbrán, María Marta de Elizalde de Bracco

Cecilia Parodi, Nora Galassi, Ana Coraglia, Patricia Baré, María Marta de Elizalde de Bracco, Department of Immunology, Instituto de Medicina Experimental (IMEX)– CONICET-Academia Nacional de Medicina (ANM) Pacheco de Melo 3081, Ciudad Autónoma de Buenos Aires 1425, Argentina
María Noel Badano, Patricia Baré, María Marta de Elizalde de Bracco, Department of Virology, Instituto de Investigaciones Hematológicas Mariano Castex (IIHEMA)-ANM, Pacheco de Melo 3081, Ciudad Autónoma de Buenos Aires 1425, Argentina
Alejandro Malbrán, Unidad de Alergia e Inmunología Clínica, Av. Pte. Roque Saenz Peña 1160, Ciudad Autónoma de Buenos Aires C1431GHA, Buenos Aires, Argentina
Author contributions: Parodi C, Badano MN, Galassi N and Coraglia A contributed equally to this work; Baré P participated in the discussion of TFH and viral diseases; Malbrán A participated in the discussion of TFH in the context of autoimmunity and immunodeficiency; de Elizalde de Bracco MM designed and wrote the manuscript.
Supported by Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, PIP Nos. 0032 and 11220120100619CO
Correspondence to: María Marta de Elizalde de Bracco, PhD, Chief, Department of Immunology, Instituto de Medicina Experimental (IMEX)-CONICET, Academia Nacional de Medicina (ANM), Pacheco de Melo 3081, Ciudad Autónoma de Buenos Aires 1425, Argentina. mebracco@hematologia.anm.edu.ar
Telephone: +54-11-48055759 Fax: +54-11-48039475
Received: May 7, 2014 Revised: July 12, 2014
Accepted: September 18, 2014
Published online: November 6, 2014

Abstract

A correct antibody response requires the participation of both B and T lymphocytes and antigen presenting cells. In this review we address the role of follicular helper T lymphocytes (TFH) in this reaction. We shall focus on the regulation of their development and function in health and disease. TFH can be characterized on the basis of their phenotype and the pattern of secretion of cytokines. This fact is useful to study their participation in the generation of antibody deficiency in primary immunodeficiency diseases such as common variable immunodeficiency, X-linked hyper IgM syndrome or X-linked lymphoproliferative disease. Increased numbers of TFH have been demonstrated in several autoimmune diseases and are thought to play a role in the development of autoantibodies. In chronic viral infections caused by the human immunodeficiency virus, hepatitis B or C virus, increased circulating TFH have been observed, but their role in the protective immune response to these agents is under discussion. Likewise, an important role of TFH in the control of some experimental protozoan infections has been proposed, and it will be important to assess their relevance in order to design effective vaccination strategies.

Key words: Follicular helper T (TFH) lymphocytes; TFH development; Chemokine (C-X-C motif) receptor 5; Interleukin-21; Programmed cell death-1/Programmed cell death ligand 1 (PDL-1) or PDL-2; Primary immunodeficiencies; Autoimmunity; Chronic viral infections; Protozoan infections

Core tip: Follicular helper T lymphocytes (TFH) are essential to establish a correct and protective humoral immune response. Correct regulation of their development and differentiation is necessary to achieve a normal antibody response. They can be characterized by their phenotype and function. It has been proposed that their role is important in the generation of immunodeficiency or autoimmunity, as well as in the control of chronic viral or protozoan infections. This review comments recent advances in human TFH research that may be useful in order to design adequate therapeutic or vaccination strategies.

Parodi C, Badano MN, Galassi N, Coraglia A, Baré P, Malbrán A, de Elizalde de Bracco MM. Follicular helper T lymphocytes in health and disease. *World J Hematol* 2014; 3(4): 118-127 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i4/118.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i4.118

INTRODUCTION

The assembly of a correct antibody response requires the participation of B and T lymphocytes, as well as that of antigen presenting cells from the myeloid lineage. It involves a complex system of interactions and regulatory mechanisms. Failure of this equilibrium at any level disturbs and impairs the generation of an efficient, long term antibody response.

A subset of helper T cells, follicular helper T lymphocytes (TFH) is necessary to provide help to B lymphocytes in the process of antibody synthesis and maturation. TFH encompass a heterogeneous group of cells with distinct gene expression profile and function[1]. Without TFH the protective antibody responses are largely diminished. Primary immune deficient patients with genetic defects that affect the synthesis of molecules essential for TFH generation or function, such as the inducible costimulator (ICOS) or the signaling adaptor SLAM-associated protein (SAP), lack an efficient antibody response and may suffer recurrent infections that compromise their health and survival[2,3]. Excessive or dysregulated TFH can also result in the generation of autoantibodies and are associated to autoimmune diseases[4,5].

In this review we shall describe the nature and function of this T cell subset and we will focus on its role in the generation of immune deficiency or autoimmunity in humans. We will also address the importance of TFH in the assembly of an efficient humoral response for the control of chronic diseases caused by different infectious viral agents, *e.g.*, human immunodeficiency virus (HIV), hepatitis B virus (HBV) or C virus (HCV), as well as parasites or protozoa.

TFH PHENOTYPE AND FUNCTION

$CD4^+$ T helper (Th) cells present in B cell follicles have been recognized as an important subset of helper T lymphocytes necessary for the assembly of the antibody response involving T-B cooperation and B cell memory[1,6,7]. TFH have a typical phenotype, appropriate transcription factors and exhibit surface molecules essential for helper function. They secrete interleukins (ILs) that promote growth, differentiation and class switching of B cells (IL-4, IL-10 and IL-21). Plasticity is a main characteristic of TFH. Thus, TFH can also express many transcription factors thought to be master regulators of T helper cell lineages, as GATA binding protein 3 (GATA-3) and the T-box transcription factor (T-bet)[7].

Antigen presentation by dendritic cells (DC) is necessary to initiate TFH commitment[8-10]. As a consequence of this initial encounter, TFH express achaete-scute homologue 2 (Ascl2)[11], B cell lymphoma 6 (Bcl-6), chemokine (C-X-C motif) receptor 5 (CXCR5) and ICOS triggering the TFH differentiation program[10,12,13]. These events take place outside the B-cell follicle in the absence of B cells[10,13,14]. SAP-deficient $CD4^+$ T cells, which fail to sustain prolonged interaction with B cells, but interact normally with antigen-presenting DC, upregulate Bcl-6 and CXCR5 following activation[8,9,15]. Late cognate interactions with activated B cells are required to complete and sustain full differentiation of TFH[15]. However, B cell-mediated antigen presentation can be overcome when antigen in excess is presented by DC[8,9]. Apparently, when provision of antigen is limited, B cells are more efficient than DC to capture antigen through their high affinity antigen-B cell receptor[10]. Therefore, antigen availability would dictate the transition of initially DC-primed-TFH towards B-cell primed-TFH as the differentiation program progresses in the interfollicular zone (Figure 1). The importance of DC in the induction of a full TFH response relies both on their ability to migrate to the B cell follicles through the upregulation of CXCR5 and downregulation of the chemokine (C-C motif) receptor 7 (CCR7) (providing a favorable spatial location for DC-B cell-TFH interactions), but also on their ability to release DC-derived cytokines that are necessary for TFH development[15,16].

In addition to their presence in B cell follicles, TFH circulating counterparts have been identified in the blood stream[1] and share many of the phenotypic and functional characteristics of TFH residing in the follicles. The phenotypic hallmark of TFH is the surface expression of the chemokine receptor CXCR5, which enables their migration into B cell follicles, in response to the specific chemokine ligand CXCL13-rich follicular areas.

Deficiency of CXCR5 affects the antibody response. It impairs the germinal center (GC) response, reducing the frequency of GC B lymphocytes and isotype-switched antibody-secreting cells. ICOS is necessary for the induction of CXCR5 and for an efficient GC reaction[2]. In the absence of CXCR5, T cells cannot migrate to the follicles, but migration is not an absolute requirement for the formation of GC. CD40 ligand (CD40L), SAP and ICOS are other molecules expressed by TFH that are essential to ensure their ability to provide help to B cells[17].

An increased expression of CXCR5, ICOS, the inhibitory receptor programmed cell death-1 (PD-1) and SAP characterize the TFH phenotype, as well as the downregulation of CCR7 and the IL-7 receptor (CD127)[1,6].

The cytokine secreting profile of TFH includes the production of high amounts of IL-21. IL-10, IL-4 and IL-6 are also produced by TFH. All these cytokines are involved in the generation of an adequate antibody response by promoting growth differentiation and class switching of B cells. These characteristics of TFH have been demonstrated both in humans and in mice. Table 1 summarizes this information for human TFH.

As a group, TFH are heterogeneous. Despite the definition of a basic TFH profile, TFH have an inherent plasticity and they may convert to other cell subsets. Likewise, forkhead box P3 ($Foxp3^+$) regulatory T cells that express CXCR5 and Bcl-6 (TFR) and migrate to human tonsils or murine lymphoid tissue have been described[18]. They are closely related by their phenotype to classic TFH and derive from T regulatory (Treg) cells[19]. In humans $CXCR5^+$ $CD4^+$ T cells are a circulating pool of memory

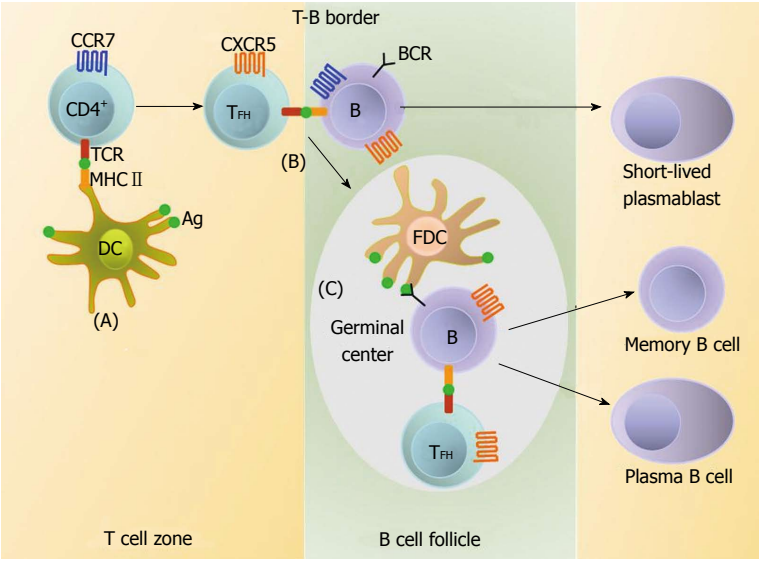


Figure 1 Follicular helper T cells and the differentiation program of B lymphocytes. A: Naïve CD4+ T cells are activated following recognition of antigen (Ag) presented by dendritic cells (DC) in T cell zones. Upon antigen activation and co-stimulation by DC, nascent TFH upregulate CXC-chemokine receptor 5 (CXCR5), downregulate CC-chemokine receptor 7 (CCR7) and migrate towards B cell follicles; B: At the T-B border TFH contact antigen-activated B cells that move to the T-cell zone after upregulating CCR7. TFH deliver help to B cells resulting in their differentiation into short-lived extrafollicular plasmablasts or their migration into B cell-follicles to form germinal centers (GCs); C: Within GC, TFH promotes the B cell differentiation into long-lived plasma cells and memory B cells. TFH: Follicular helper T lymphocytes; FDC: Follicle dendritic cell; BCR: B cell receptor; MHC: Major histocompatibility complex; TCR: T cell receptor.

Table 1 Follicular helper T lymphocytes markers

Marker	Human TFH		Naïve CD4+ T cell	Activated Non-TFH CD4+
	TFH	GC TFH		
CXCR5	+	++	-	-
Ascl2	?	++	?	?
Bcl-6	+	++	-	-
Blimp-1	-	-	+/-	++/variable
PD-1	+	++	-	Variable
ICOS	+	++	-	Variable
SAP	+	++	+	+
IL-21	+	++	-	Variable
IL-4	-/+	++	-	Th2+
CCR7	-/+	-	++	Variable

TFH: Follicular helper T lymphocytes; GC: Germinal center; CXCR5: Chemokine (C-X-C motif) receptor 5; Ascl2: Achaete-scute homologue 2; Bcl-6: B cell lymphoma 6; Blimp-1: B lymphocyte-induced maturation protein 1; PD-1: Programmed cell death-1; ICOS: Inducible costimulator; SAP: Signaling adaptor SLAM-associated protein; IL: Interleukin; CCR7: Chemokine (C-C motif) receptor 7; Th: T helper.

cells that comprises three CD4+ T helper subsets: Th1 TFH expressing CXCR5, CXCR3 and the transcription factor T-bet in the absence of CCR6; Th2 TFH expressing CXCR5 and the transcription factor GATA-3 in the absence of both CCR6 and CXCR3 and Th17 TFH expressing CXCR5, CCR6 and the transcription factor RORγt in the absence of CXCR3 (Table 2). These subsets of TFH have different helping abilities. While Th2 TFH and Th17 TFH can help naïve B cells to produce IgM, IgG and IgA, Th1 TFH cannot[1].

Furthermore, a subgroup of CXCR5+ CD4+ circulating lymphocytes with low CCR7 and high PD-1 expression have been identified as an early memory subset of TFH, which upon antigen exposure differentiates into mature TFH capable to provide a prompt protective antibody response[20].

REGULATION OF TFH DEVELOPMENT

TFH differentiation may be divided into two phases: the priming and the maintaining stages. Priming depends on antigen-presenting signaling of DC, while maintaining is related to sustained B cell-T cell interaction and the consequent signaling events. While most studies have pointed out the role of the transcription factor Bcl-6 as an initiator of the TFH differentiation program during the priming stage, recent work by Liu *et al*[11] demonstrated that Ascl2, another transcription factor, is crucial for TFH development and function. Ascl2 is a basic helix-loop-helix (bHLH) transcription factor[21]. It directly regulates TFH-related genes and inhibits Th1 and Th17 signature genes. Upregulation of Ascl2 precedes that of Bcl-6, indicating that Ascl2 and not Bcl-6 may be the initial trigger for the TFH differentiation program.

Large amounts of Bcl-6 expressed by TFH can be counterbalanced by the repressor B lymphocyte-induced maturation protein 1 (Blimp-1). While Bcl-6 favors the development of TFH *in vivo*, Blimp-1 regulates the function of Bcl-6 and inhibits the generation of TFH. Bcl-6 controls GC B cell differentiation by regulating cell cycle genes, regulating DNA damage response genes and suppressing a host of signaling pathways, including B cell receptor (BCR) signaling[22]. It is a member of the BTB-POZ (bric-a-bric, tramtrack, broad complex-poxvirus zinc finger) family of transcriptional repressors. These repressors directly bind to specific DNA sequences through their zinc-finger DNA binding domains with the BTB-POZ domain mediating transcriptional repression[23]. In both GC B cells and TFH, Bcl-6 controls TFH differentiation by regulating genes separate from those it controls in B cells [22]. Molecular crosstalk between GC B cells and TFH influences the survival, proliferation and differentiation of each cell type[24]. In addition to promoting the expression of TFH signature genes, Bcl-6 represses *Prdm1* (the gene encoding the transcriptional repressor Blimp-1). Bcl-6 antagonism of Blimp-1 is one of the key mechanisms by which Bcl-6 inhibits non-TFH differentiation. Bcl-6-dependent suppression of Blimp-1 activity (by removal of the Blimp-1 "brake") may favor the differen-

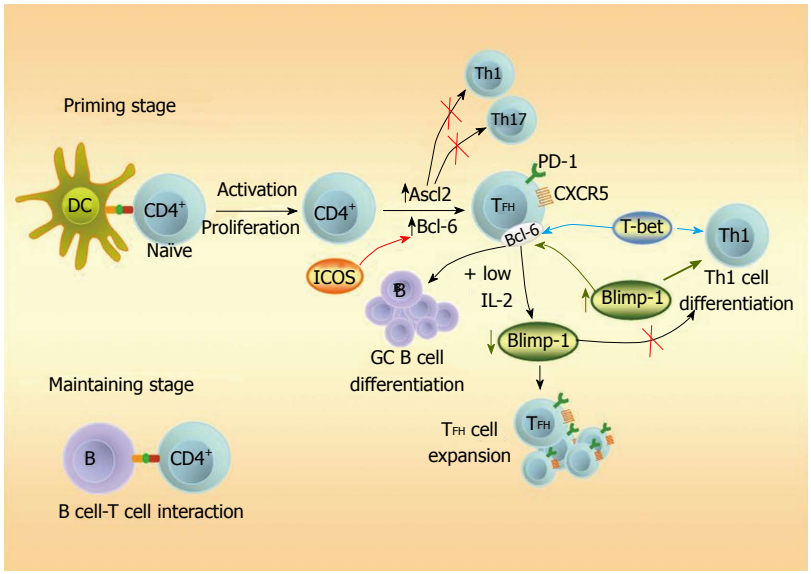


Figure 2 Regulation of Follicular helper T cell development. After antigen-presenting signaling of dendritic cells (DC) to CD4+ T cells during priming, achaete-scute homologue 2 (Ascl2) and B cell lymphoma 6 (Bcl-6) induced by the inducible costimulator (ICOS), trigger for TFH differentiation program and inhibit Th1 and Th17 genes. Bcl-6 also controls germinal center (GC) B cell differentiation. B lymphocyte-induced maturation protein 1 (Blimp-1) and the T-box transcription factor (T-bet) regulate the function of Bcl-6 and favor the induction of a Th1 profile. Under low interleukin 2 (IL-2) conditions, excess of Bcl-6 counteracts Blimp-1 allowing expansion of the TFH and reduction of the Th1 programs of differentiation. Initial priming is sufficient to acquire the TFH markers but cognate B cells are needed for the subsequent maintenance stage. TFH: Follicular helper T lymphocytes; CXCR5: Chemokine (C-X-C motif) receptor 5; Th: T helper.

Table 2 Heterogeneity of follicular helper T lymphocytes in relation to other T helper cells

	Markers				
	CD4	CXCR5	CXCR3	CCR6	Foxp3
Th1 TFH	+	+	+	-	-
Th2 TFH	+	+	-	-	-
Th17 TFH	+	+	-	+	-
TFR TFH	+	+	-	-	+

TFH: Follicular helper T lymphocytes; Th: T helper; CXCR5: Chemokine (C-X-C motif) receptor 5; CXCR3: Chemokine (C-X-C motif) receptor 3; CCR6: Chemokine (C-C motif) receptor 6; Foxp3: Forkhead box P3; TFR: Foxp3+ CXCR5+ Bcl-6+ regulatory T cells.

tiation program of Th cells towards the induction of TFH effectors[25].

As Ascl2[11], Bcl-6 is responsible for the repression of a subgroup of signature genes in effector Th1 cells. It has been shown that Bcl-6 can interact with T-bet[26], which is required for establishment of a Th1 gene expression profile[27]. Under low IL-2 conditions the Bcl-6/T-bet ratio increases and excess Bcl-6 represses *Prdm1* and counteracts Blimp-1-mediated inhibition of the TFH signature genes, allowing for expansion of the TFH and reduction of the Th1 programs of differentiation[26]. At the priming stage Bcl-6 expression is induced in CD4+ T cells independent of CD40 or SAP signaling, while ICOS provides a critical early signal to induce Bcl-6 transcription[15].

Both Ascl2[11] and Bcl-6 upregulate CXCR5 expression on T cells during priming and this facilitates their entry to the T/B border. This initial DC integrin-dependent priming is sufficient to acquire the TFH markers (CXCR5, PD-1, high levels of Bcl-6), but cognate B cells are needed for the subsequent maintenance and survival stage[28] (Figure 2).

THE ROLE OF CYTOKINES IN TFH DEVELOPMENT AND FUNCTION

IL-21 has been recognized as an essential factor determining the maintenance of TFH. It is secreted by TFH and has an autocrine effect on them. Through interaction with the IL-21 receptor expressed on B lymphocytes, it promotes survival and proliferation of GC B cells. It has also direct effects on CD4+ non-TFH T cells (Th17)[29] and may induce Bcl-6 in TFH[30]. However, IL-21 requirement does not exclusively determine TFH differentiation, as IL-21-/- and IL-21R-/- mice can develop TFH[31]. The combined absence of both IL-21 and IL-6 abrogates TFH [6]. IL-6 and IL-21 redundantly contribute to TFH differentiation, but in the absence of other triggers as ICOS, these cytokine signals are insufficient for the instruction of TFH differentiation[6].

In addition, IL-21 has been shown to prime human naïve B cells to respond to IL-2 by enhancing their differentiation into plasmablasts. This mechanism operates through STAT3 (signal transducer and activator of transcription 3) signaling and provides an adjunctive role to IL-21-induced B cell differentiation[32].

PD-1 AND ITS LIGANDS HAVE A CRITICAL ROLE IN THE ASSEMBLY OF THE HUMORAL RESPONSE

PD-1, a member of the CD28 family of costimulatory molecules, is highly expressed in TFH, while most human B cells do not express it[33]. In general, engagement of PD-1 by its ligands (Programmed cell death ligand 1 -PD-L1- or Programmed cell death 1 ligand 2 -PD-L2-, belonging to the B7 family) inhibits T cell proliferation and cytokine induction and leads to downregulation of T cell responses[34].

The role of the PD-1/PD-L1 or PD-L2 axis in the generation of an adequate antibody response has been highlighted by Good-Jacobson *et al*[35]. Though PD-1 is commonly thought as a marker of "exhaustion", TFH cannot be considered as exhausted because they secrete abundant IL-21 and other cytokines during humoral response. In the absence of an operative PD-1/PD-L1

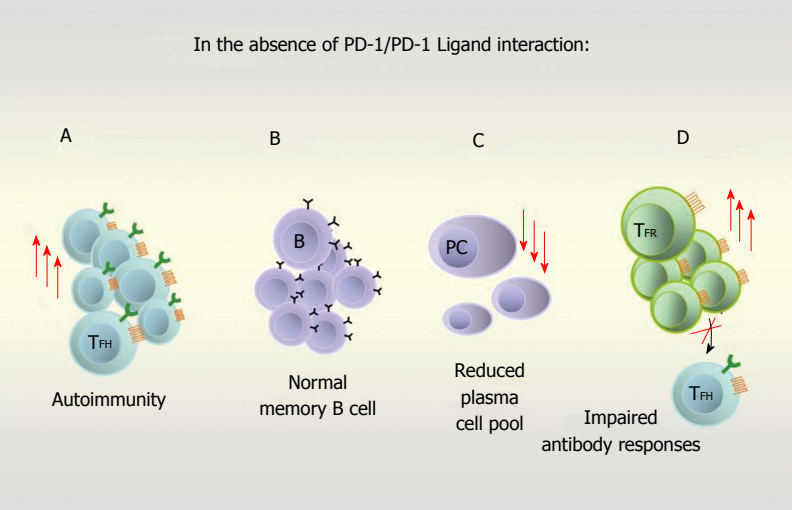


Figure 3 Inhibitory receptor programmed cell death 1, its ligands and their role in humoral response. In the absence of an operative programmed cell death 1 (PD-1)/PD-1 Ligand axis, follicular helper T lymphocytes (TFH) increase and autoimmunity may develop (A); memory B cells are formed normally (B); reduced plasma cells (PC) are found (C); Foxp3$^+$ CXCR5$^+$ Bcl-6$^+$ regulatory T cells (TFR) increase and have higher suppressive ability on TFH function leading to impaired antibody responses (D). PD-1: Programmed cell death-1; CXCR5: Chemokine (C-X-C motif) receptor; Bcl: B cell lymphoma.

Table 3 Follicular helper T lymphocytes in autoimmune diseases

Disease	TFH	Ref.
SLE	Increased CXCR5 and function	[46,47,48]
Myasthenia gravis	Increased CXCR5 and function	[49]
RA	Increased CXCR5 and function	[1,50]
Juvenile Dermatomyositis	Increased CXCR5 and function Mainly TFH with Th17 and Th2-like profile	[1]
ATD	Increased CXCR5, IL-21 high	[51]
Multiple Sclerosis	Increased CXCR5 IL-21 and IL-21R in neurons	[52,53]
Sjögren's syndrome	Increased CXCR5 TFH with Th17 and Th2-like profile IL-17?	[54,55]

TFH: Follicular helper T lymphocytes; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis; ATD: Autoimmune thyroid disease; CXCR5: Chemokine (C-X-C motif) receptor 5; IL: Interleukin; Th: T helper.

or PD-L2 axis, TFH numbers increase and autoimmunity may develop. Memory B cells are formed normally but the plasma cell pool that depends on the late stage of the GC response is reduced[36]. There are conflicting reports about the function of the PD-1 pathway in controlling the humoral response. While some studies report attenuated antibody responses in conditions where the PD-1/PD-L1 and PD-L2 interactions were prevented[35,37], others observed heightened humoral responses[38]. Recent work by Sage *et al*[19], in which the contributions of TFH devoid of contaminating TFR could be analyzed[19] has clarified this question. In the absence of PD-1 and its ligand PD-L1, TFR were increased and had higher suppressive ability on TFH function, leading to impaired antibody responses. Thus, there is a dynamic control of antibody production by the balance between TFH and TFR cells and this equilibrium is tuned by PD-1/PD-L1 and PD-L2 interactions (Figure 3).

TFH AND IMMUNODEFICIENCY

Defects in humoral immune response lead to immunodeficiencies, such as common variable immunodeficiency (CVID), X-linked hyper IgM syndrome (HIGM) or X-linked lymphoproliferative disease (XLP)[39]. Since ICOS, CD40L and SAP are highly expressed in TFH, their role in the development of the humoral defects that characterize these diseases has been explored. In ICOS deficiency, which is a very rare condition, ICOS mutations are associated with CVID[40]. ICOS deficiency leads to a reduction of CXCR5$^+$ T cells (including TFH and TFR)[2]. However, most CVID patients do not have ICOS mutations and in these patients circulating CXCR5$^+$ CD4$^+$ T cells are not reduced[41]. In HIGM patients, lack of CD40L causes generalized dysfunction of CD4$^+$ T cells and inability to induce immunoglobulin switching[42]. It had been shown that peripheral CXCR5$^+$ T cells from XLP patients were unable to support immunoglobulin synthesis *in vitro*[43,44] and this led to the suggestion that TFH were not functional in XLP. In fact, absence of SAP affects the stability of the TFH B cell conjugates, necessary for the completion of an effective GC reaction and T-B cell cooperation[45]. However circulating TFH in XLP patients could be induced to express ICOS, CD40L, IL-4, IL-10 and IL-21 upon stimulation, although the kinetics of expression was different to that of normal TFH[46]. Nevertheless, the humoral response was impaired and the number of memory B lymphocytes was reduced in these patients[47], leading to persistent hypogammaglobulinemia.

TFH AND AUTOIMMUNITY

TFH emit instructive signals to B cells that favor the formation and maintenance of GC. Unwanted antibody responses may come together with the normal defensive antibody response against infectious agents, and in this scenario TFH may play a crucial role. Several studies have addressed the contribution of TFH to the generation of autoimmune diseases both in murine models and in humans[39,48]. Evidence involving TFH in the generation of an autoantibody response has accumulated, in particular in systemic lupus erythematosus (SLE), both in humans and in mouse models (sanroque mice) as well as in other

autoimmune conditions. A deficit in the process of selection of GC B cells has been pointed out in SLE patients. GC are abundant in secondary lymphoid organs in SLE mice[49]. In human SLE, GC are overactive and it has been reported that expansion of TFH is causally related to the abundance of GC that form in the absence of foreign antigen, to the production of anti-double-stranded DNA antibodies and to end organ disease[49]. Although circulating TFH are expanded in sanroque mice and in SLE patients, their abundance appears to be a stable phenotype and not a marker of disease activity. A summary of reports on TFH activity or TFH role associated to autoimmune diseases is shown in Table 3. Increased numbers of circulating TFH have been reported associated to increased autoantibody titers in patients with SLE[49-51], myasthenia gravis[52,53], rheumatoid arthritis and juvenile dermatomyositis[1,54], autoimmune thyroid disease[55], multiple sclerosis[56,57] or Sjögren's syndrome patients[58,59]. TFH numbers increase correlating with titers of autoantibodies and the severity of end-organ involvement.

Autoimmune manifestations are encountered in many patients with CVID. In contrast to other patients with autoimmune manifestations, and no CVID, circulating immunoglobulin levels and plasma antibody titers were low in these patients, but co-existed with elevated circulating TFH[41]. Expansion of TFH may play a key role in breakdown of the peripheral tolerance of autoreactive B cells. These cells, which are normally deleted during the GC reaction, may escape from the tolerance checkpoints due to the abundance of the survival help signals provided by excessive TFH[60].

TFH IN VIRAL DISEASES

The role of TFH in HIV infection is not completely clear. Despite profound depletion of $CD4^+$ T cells during HIV infection, both the bulk TFH and HIV-specific TFH populations are expanded in HIV-infected patients[61]. This expansion correlates with changes observed in the B cell compartment, such as the increased frequencies of GC B cells and plasma cells and the decreased frequency of memory B cells[61,62].

Furthemore, the increase of TFH associates with hypergammaglobulinemia in HIV-infected patients. However, the majority of these antibodies are not able to neutralize the virus. Even though there is an expansion of TFH in HIV-infected individuals, it seems that these cells are unable to provide appropriate B cell help[62]. On the other hand, a resting peripheral blood memory population of $CXCR5^+$ $PD\text{-}1^+$ $CXCR3^-$ $CD4^+$ T cells has been identified in rare HIV individuals that are able to generate broadly neutralizing antibodies. It has also been demonstrated that the frequency of this cell population correlates with the development of broadly neutralizing antibodies[63]. Lastly, it has been proven that TFH can be infected by HIV. Furthermore, it was suggested that these cells are a major reservoir that contributes to viral persistence[64].

High frequency of peripheral blood TFH is also found in HBV-infected individuals[65,66]. It has been reported that treatment with adefovir dipivoxil reduces the frequency of TFH and the concentrations of hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg), but increases the concentrations of serum anti-HBsAg and "e" antigen antibodies (HBsAb, HBeAb), IL-2 and IFN-γ in drug-responding patients, although the precise relationship between the frequency of peripheral blood $CD4^+$ $CXCR5^+$ TFH and these parameters requires further investigation[66].

Peripheral blood TFH have also been associated with hypergammaglobulinemia in HBV-infected patients[67].

HCV-infected patients also show an increased percentage of peripheral blood TFH. This high percentage of TFH was associated with low levels of HCV viremia [68].

Even more, a study shows that liver TFH cells can be useful to predict the success of virological response following interferon-based treatment in HCV-infected patients. Tripodo *et al*[69] reported that the absolute number of liver TFH is lower in non-responders, intermediate in responding-relapsed patients and achieves a maximum in sustained virological response patients.

TFH IN PROTOZOAN DISEASES

Reports about the involvement of TFH within human infections caused by obligate intracellular parasites are still required. We will focus on the findings achieved using experimental protozoan infection in mice models to study the function of different factors, receptors and cytokines involved in pathways related to TFH.

It is well known that experimental infections with *Toxoplasma (T.) gondii* display a model of Th1 cell response induction[70]. This model was useful to evaluate if TFH represented a temporary "state" of differentiation rather than a distinct lineage parallel to other subsets[71]. Also, to confirm the action of T-bet as a suppressor of both TFH and humoral responses *in vivo*[71]. The generation of parasite-induced Th1 responses by *T. gondii*, also served to understand the association of the TFH marker ICOS with T helper cytokine production *in vivo*. $ICOS^+$ $CD4^+$ T helper cells produce a variety of different effector cytokines and their pattern depends on the infection challenge. Infection with *T. gondii* leads to IFN-γ production, while $ICOS^+$ $CD4^+$ T cells from the nematode Schistosoma mansoni (an inducer of Th2 responses) is associated with IL-10 secretion[72].

According to these findings, experimental models using *Leishmania (L.) major* also demonstrated that ICOS is a critical regulator of both Th1 and Th2 immune responses *in vivo*[73]. Chronic infection with *L. major*, a model of prominent T-B cell interaction, was also used to evaluate the contribution of IRF-4 (member of IFN-regulatory factor family) to the interaction of TFH and GC B cells. Bolling *et al*[74] demonstrated the implication of this factor, since $IRF4^{-/-}$ mice lacked GC formation, differentiation of GC B cells and lymph node $CD4^+$ T cells from these mice expressed reduced amounts of the TFH-related molecules ICOS, IL-21 and Bcl-6. *L. major*

infection model also helped to demonstrate the relation of TFH and IL-4. All the IL-4 secreting cells in lymph nodes during infection with this parasite were TFH and these cells were distinct from conventional Th2 cells based on phenotype, location and function[75].

Besides, analysis of the consequences of *in vivo* blockade of T cell inhibitory receptors indirectly associated with TFH were performed using blood-stage *Plasmodium* (*P.*) *yoelii* infection in mice. Butler *et al*[76] demonstrated that blockade of *PD-L1* and *LAG-3* (lymphocyte-activation gene 3) receptors led to improved parasite control associated with enhanced TFH numbers and substantial induction of plasma cell differentiation.

Experimental models in which mice were co-infected with *L. major* and *L. amazonensis* demonstrated that those mice that healed the lesions had more GC, more isotype switched GC B cells and more memory B cells than those who did not. A productive B cell response was required for healing a co-infection with these protozoans in this model[77].

The development of TFH was also assessed in order to find strategies to enhance the efficacy of recombinant protein subunit vaccines using lipid-based nanoparticles (NPs). In this context, Moon *et al*[78] studied the impact of NP delivery on immune responses elicited by a candidate *P. vivax* subunit vaccine. They found that prolonged antigen presentation by this vaccine contributed to expand TFH and promote GC induction. The $CD4^+$ TFH subset provided critical cytokines and signals required to initiate somatic hypermutation and affinity maturation of B cells[79], achieving broad antibody responses.

This information indicates that there is an association between protozoan infections, TFH and their related cytokines, receptors and B responses in the context of experimental mice models. Leishmaniasis, malaria, toxoplasmosis and other parasitic infections seriously affect humans. Reports about the implication of TFH and humoral responses are needed to better understand mechanisms involved in the progression and outcome of these diseases.

CONCLUSION

Clearly, our research on TFH demonstrates that they are essential for the generation of a long-lasting humoral response. Their role in the assembly of the GC reaction explains why their dysfunction or their inability to interact correctly with B cells leads to immunodeficiency, to autoimmunity or to inefficient management of infectious diseases. It will be necessary to understand how the regulation of their function may be modified or restored in order to revert TFH deficiency or over activity, as well as to design adequate strategies for antibody production in vaccination programs.

ACKNOWLEDGMENTS

The authors are grateful to Dr. N. E. Riera and M. Felippo for help throughout this study.

REFERENCES

1 **Morita R**, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, Foucat E, Dullaers M, Oh S, Sabzghabaei N, Lavecchio EM, Punaro M, Pascual V, Banchereau J, Ueno H. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011; **34**: 108-121 [PMID: 21215658 DOI: 10.1016/j.immuni.2010.12.012]

2 **Bossaller L**, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, Durandy A, Baumann U, Schlesier M, Welcher AA, Peter HH, Warnatz K. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. *J Immunol* 2006; **177**: 4927-4932 [PMID: 16982935]

3 **Cannons JL**, Yu LJ, Jankovic D, Crotty S, Horai R, Kirby M, Anderson S, Cheever AW, Sher A, Schwartzberg PL. SAP regulates T cell-mediated help for humoral immunity by a mechanism distinct from cytokine regulation. *J Exp Med* 2006; **203**: 1551-1565 [PMID: 16754717]

4 **Craft JE**. Follicular helper T cells in immunity and systemic autoimmunity. *Nat Rev Rheumatol* 2012; **8**: 337-347 [PMID: 22549246 DOI: 10.1038/nrrheum.2012.58]

5 **Yu D**, Vinuesa CG. Multiple checkpoints keep follicular helper T cells under control to prevent autoimmunity. *Cell Mol Immunol* 2010; **7**: 198-203 [PMID: 20364160 DOI: 10.1038/cmi.2010.18]

6 **Crotty S**. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011; **29**: 621-663 [PMID: 21314428 DOI: 10.1146/annurev-immunol-031210-101400]

7 **Cannons JL**, Lu KT, Schwartzberg PL. T follicular helper cell diversity and plasticity. *Trends Immunol* 2013; **34**: 200-207 [PMID: 23395212 DOI: 10.1016/j.it.2013.01.001]

8 **Deenick EK**, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, Tangye SG. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity* 2010; **33**: 241-253 [PMID: 20691615 DOI: 10.1016/j.immuni.2010.07.015]

9 **Goenka R**, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, Laufer TM. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol* 2011; **187**: 1091-1095 [PMID: 21715693 DOI: 10.4049/jimmunol.1100853]

10 **Ballesteros-Tato A**, Randall TD. Priming of T follicular helper cells by dendritic cells. *Immunol Cell Biol* 2014; **92**: 22-27 [PMID: 24145854 DOI: 10.1038/icb.2013.62]

11 **Liu X**, Chen X, Zhong B, Wang A, Wang X, Chu F, Nurieva RI, Yan X, Chen P, van der Flier LG, Nakatsukasa H, Neelapu SS, Chen W, Clevers H, Tian Q, Qi H, Wei L, Dong C. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature* 2014; **507**: 513-518 [PMID: 24463518 DOI: 10.1038/nature12910]

12 **Poholek AC**, Hansen K, Hernandez SG, Eto D, Chandele A, Weinstein JS, Dong X, Odegard JM, Kaech SM, Dent AL, Crotty S, Craft J. In vivo regulation of Bcl6 and T follicular helper cell development. *J Immunol* 2010; **185**: 313-326 [PMID: 20519643 DOI: 10.4049/jimmunol.0904023]

13 **Baumjohann D**, Okada T, Ansel KM. Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. *J Immunol* 2011; **187**: 2089-2092 [PMID: 21804014 DOI: 10.4049/jimmunol.1101393]

14 **Kerfoot SM**, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, Haberman AM. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* 2011; **34**: 947-960 [PMID: 21636295 DOI: 10.1016/j.immuni.2011.03.024]

15 **Choi YS**, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, Lao C, Crotty S. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 2011; **34**: 932-946 [PMID: 21636296 DOI: 10.1016/j.immuni.2011.03.023]

16 **Nurieva RI**, Podd A, Chen Y, Alekseev AM, Yu M, Qi X, Huang H, Wen R, Wang J, Li HS, Watowich SS, Qi H, Dong C, Wang D. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* 2012; **287**: 11234-11239 [PMID: 22318729 DOI: 10.1074/jbc.M111.324046]

17 **Chen M**, Guo Z, Ju W, Ryffel B, He X, Zheng SG. The development and function of follicular helper T cells in immune responses. *Cell Mol Immunol* 2012; **9**: 375-379 [PMID: 22659733 DOI: 10.1038/cmi.2012.18]

18 **Chung Y**, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, Wang YH, Lim H, Reynolds JM, Zhou XH, Fan HM, Liu ZM, Neelapu SS, Dong C. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* 2011; **17**: 983-988 [PMID: 21785430 DOI: 10.1038/nm.2426]

19 **Sage PT**, Francisco LM, Carman CV, Sharpe AH. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* 2013; **14**: 152-161 [PMID: 23242415 DOI: 10.1038/ni.2496]

20 **He J**, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, Sun X, Vandenberg K, Rockman S, Ding Y, Zhu L, Wei W, Wang C, Karnowski A, Belz GT, Ghali JR, Cook MC, Riminton DS, Veillette A, Schwartzberg PL, Mackay F, Brink R, Tangye SG, Vinuesa CG, Mackay CR, Li Z, Yu D. Circulating precursor CCR7(lo)PD-1(hi) CXCR5$^+$ CD4$^+$ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* 2013; **39**: 770-781 [PMID: 24138884 DOI: 10.1016/j.immuni.2013.09.007]

21 **van der Flier LG**, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, Begthel H, van den Born M, Guryev V, Oving I, van Es JH, Barker N, Peters PJ, van de Wetering M, Clevers H. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* 2009; **136**: 903-912 [PMID: 19269367 DOI: 10.1016/j.cell.2009.01.031]

22 **Crotty S**, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 2010; **11**: 114-120 [PMID: 20084069 DOI: 10.1038/ni.1837]

23 **Basso K**, Dalla-Favera R. BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. *Adv Immunol* 2010; **105**: 193-210 [PMID: 20510734 DOI: 10.1016/S0065-2776(10)05007-8]

24 **Nutt SL**, Tarlinton DM. Germinal center B and follicular helper T cells: siblings, cousins or just good friends? *Nat Immunol* 2011; **12**: 472-477 [PMID: 21739669]

25 **Johnston RJ**, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, Dent AL, Craft J, Crotty S. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 2009; **325**: 1006-1010 [PMID: 19608860 DOI: 10.1126/science.1175870]

26 **Oestreich KJ**, Mohn SE, Weinmann AS. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol* 2012; **13**: 405-411 [PMID: 22406686 DOI: 10.1038/ni.2242]

27 **Szabo SJ**, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000; **100**: 655-669 [PMID: 10761931]

28 **Cannons JL**, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, Wakeland EK, Germain RN, Schwartzberg PL. Optimal germinal center responses require a multistage T cell: B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* 2010; **32**: 253-265 [PMID: 20153220 DOI: 10.1016/j.immuni.2010.01.010]

29 **Nurieva R**, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 2007; **448**: 480-483 [PMID: 17581589]

30 **Nurieva RI**, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang YH, Dong C. Bcl6 mediates the development of T follicular helper cells. *Science* 2009; **325**: 1001-1005 [PMID: 19628815 DOI: 10.1126/science.1176676]

31 **Linterman MA**, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, Verma NK, Smyth MJ, Rigby RJ, Vinuesa CG. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med* 2010; **207**: 353-363 [PMID: 20142429 DOI: 10.1084/jem.20091738]

32 **Berglund LJ**, Avery DT, Ma CS, Moens L, Deenick EK, Bustamante J, Boisson-Dupuis S, Wong M, Adelstein S, Arkwright PD, Bacchetta R, Bezrodnik L, Dadi H, Roifman CM, Fulcher DA, Ziegler JB, Smart JM, Kobayashi M, Picard C, Durandy A, Cook MC, Casanova JL, Uzel G, Tangye SG. IL-21 signalling via STAT3 primes human naive B cells to respond to IL-2 to enhance their differentiation into plasmablasts. *Blood* 2013; **122**: 3940-3950 [PMID: 24159173 DOI: 10.1182/blood-2013-06-506865]

33 **Gunn MD**, Tangemann K, Tam C, Cyster JG, Rosen SD, Williams LT. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci USA* 1998; **95**: 258-263 [PMID: 9419363]

34 **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034 [PMID: 11015443]

35 **Good-Jacobson KL**, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* 2010; **11**: 535-542 [PMID: 20453843 DOI: 10.1038/ni.1877]

36 **Good-Jacobson KL**, Shlomchik MJ. Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. *J Immunol* 2010; **185**: 3117-3125 [PMID: 20814029 DOI: 10.4049/jimmunol.1001155]

37 **Hamel KM**, Cao Y, Wang Y, Rodeghero R, Kobezda T, Chen L, Finnegan A. B7-H1 expression on non-B and non-T cells promotes distinct effects on T- and B-cell responses in autoimmune arthritis. *Eur J Immunol* 2010; **40**: 3117-3127 [PMID: 21061440 DOI: 10.1002/eji.201040690]

38 **Hams E**, McCarron MJ, Amu S, Yagita H, Azuma M, Chen L, Fallon PG. Blockade of B7-H1 (programmed death ligand 1) enhances humoral immunity by positively regulating the generation of T follicular helper cells. *J Immunol* 2011; **186**: 5648-5655 [PMID: 21490158 DOI: 10.4049/jimmunol.1003161]

39 **Shekhar S**, Yang X. The darker side of follicular helper T cells: from autoimmunity to immunodeficiency. *Cell Mol Immunol* 2012; **9**: 380-385 [PMID: 22885524 DOI: 10.1038/cmi.2012.26]

40 **Warnatz K**, Bossaller L, Salzer U, Skrabl-Baumgartner A, Schwinger W, van der Burg M, van Dongen JJ, Orlowska-Volk M, Knoth R, Durandy A, Draeger R, Schlesier M, Peter HH, Grimbacher B. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. *Blood* 2006; **107**: 3045-3052 [PMID: 16384931]

41 **Coraglia A**. B immunological memory in X-linked lymphoproliferative disease (XLP) and in common variable immunodeficiency (CVID) [Doctoral Thesis]. Argentina: University of Buenos Aires, 2012

42 **Korthäuer U**, Graf D, Mages HW, Brière F, Padayachee M, Malcolm S, Ugazio AG, Notarangelo LD, Levinsky RJ, Kroczek RA. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* 1993; **361**: 539-541 [PMID: 7679206]

43 **Ma CS**, Hare NJ, Nichols KE, Dupré L, Andolfi G, Roncarolo MG, Adelstein S, Hodgkin PD, Tangye SG. Impaired hu-

moral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by CD4+ T cells. *J Clin Invest* 2005; **115**: 1049-1059 [PMID: 15761493]
44 **Ma CS**, Deenick EK. Human T follicular helper (Tfh) cells and disease. *Immunol Cell Biol* 2014; **92**: 64-71 [PMID: 24145858 DOI: 10.1038/icb.2013.55]
45 **Qi H**, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlie germinal centre formation. *Nature* 2008; **455**: 764-769 [PMID: 18843362 DOI: 10.1038/nature07345]
46 **Coraglia A**, Felippo M, Schierloh P, Malbran A, de Bracco MM. CD4+ T Lymphocytes with follicular helper phenotype (T(FH)) in patients with SH2D1A deficiency (XLP). *Clin Immunol* 2011; **141**: 357-364 [PMID: 21996454 DOI: 10.1016/j.clim.2011.09.007]
47 **Malbran A**, Belmonte L, Ruibal-Ares B, Baré P, Massud I, Parodi C, Felippo M, Hodinka R, Haines K, Nichols KE, de Bracco MM. Loss of circulating CD27+ memory B cells and CCR4+ T cells occurring in association with elevated EBV loads in XLP patients surviving primary EBV infection. *Blood* 2004; **103**: 1625-1631 [PMID: 14604960]
48 **Vinuesa CG**, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, Yu D, Domaschenz H, Whittle B, Lambe T, Roberts IS, Copley RR, Bell JI, Cornall RJ, Goodnow CC. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* 2005; **435**: 452-458 [PMID: 15917799]
49 **Simpson N**, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, Vyse TJ, Roncador G, Huttley GA, Goodnow CC, Vinuesa CG, Cook MC. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* 2010; **62**: 234-244 [PMID: 20039395 DOI: 10.1002/art.25032]
50 **Grammer AC**, Slota R, Fischer R, Gur H, Girschick H, Yarboro C, Illei GG, Lipsky PE. Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions. *J Clin Invest* 2003; **112**: 1506-1520 [PMID: 14617752]
51 **Terrier B**, Costedoat-Chalumeau N, Garrido M, Geri G, Rosenzwajg M, Musset L, Klatzmann D, Saadoun D, Cacoub P. Interleukin 21 correlates with T cell and B cell subset alterations in systemic lupus erythematosus. *J Rheumatol* 2012; **39**: 1819-1828 [PMID: 22859347 DOI: 10.3899/jrheum.120468]
52 **Drachman DB**. Myasthenia gravis. *N Engl J Med* 1994; **330**: 1797-1810 [PMID: 8190158]
53 **Luo C**, Li Y, Liu W, Feng H, Wang H, Huang X, Qiu L, Ouyang J. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. *J Neuroimmunol* 2013; **256**: 55-61 [PMID: 23305745 DOI: 10.1016/j.jneuroim.2012.12.001]
54 **Ma J**, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, Wu W, Chen J, Tong J, Yang M, Jiao Z, Xu H, Lu L, Wang S. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* 2012; **2012**: 827480 [PMID: 22649468 DOI: 10.1155/2012/827480]
55 **Zhu C**, Ma J, Liu Y, Tong J, Tian J, Chen J, Tang X, Xu H, Lu L, Wang S. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. *J Clin Endocrinol Metab* 2012; **97**: 943-950 [PMID: 22188745 DOI: 10.1210/jc.2011-2003]
56 **Romme Christensen J**, Börnsen L, Ratzer R, Piehl F, Khademi M, Olsson T, Sørensen PS, Sellebjerg F. Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression. *PLoS One* 2013; **8**: e57820 [PMID: 23469245 DOI: 10.1371/journal.pone.0057820]
57 **Tzartos JS**, Craner MJ, Friese MA, Jakobsen KB, Newcombe J, Esiri MM, Fugger L. IL-21 and IL-21 receptor expression in lymphocytes and neurons in multiple sclerosis brain. *Am J Pathol* 2011; **178**: 794-802 [PMID: 21281812 DOI: 10.1016/j.ajpath.2010.10.043]
58 **Li XY**, Wu ZB, Ding J, Zheng ZH, Li XY, Chen LN, Zhu P. Role of the frequency of blood CD4(+) CXCR5(+) CCR6(+) T cells in autoimmunity in patients with Sjögren's syndrome. *Biochem Biophys Res Commun* 2012; **422**: 238-244 [PMID: 22575453 DOI: 10.1016/j.bbrc.2012.04.133]
59 **Szabo K**, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol* 2013; **147**: 95-104 [PMID: 23578551 DOI: 10.1016/j.clim.2013.02.024]
60 **King C**, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* 2008; **26**: 741-766 [PMID: 18173374 DOI: 10.1146/annurev.immunol.26.021607.090344]
61 **Lindqvist M**, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, Flanders MD, Cutler S, Yudanin N, Muller MI, Davis I, Farber D, Hartjen P, Haag F, Alter G, Schulze zur Wiesch J, Streeck H. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* 2012; **122**: 3271-3280 [PMID: 22922259 DOI: 10.1172/JCI64314]
62 **Cubas RA**, Mudd JC, Savoye AL, Perreau M, van Grevenynghe J, Metcalf T, Connick E, Meditz A, Freeman GJ, Abesada-Terk G, Jacobson JM, Brooks AD, Crotty S, Estes JD, Pantaleo G, Lederman MM, Haddad EK. Inadequate T follicular cell help impairs B cell immunity during HIV infection. *Nat Med* 2013; **19**: 494-499 [PMID: 23475201 DOI: 10.1038/nm.3109]
63 **Locci M**, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, Su LF, Cubas R, Davis MM, Sette A, Haddad EK, Poignard P, Crotty S. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity* 2013; **39**: 758-769 [PMID: 24035365 DOI: 10.1016/j.immuni.2013.08.031]
64 **Perreau M**, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, De Leval L, Graziosi C, Pantaleo G. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J Exp Med* 2013; **210**: 143-156 [PMID: 23254284 DOI: 10.1084/jem.20121932]
65 **Hu TT**, Song XF, Lei Y, Hu HD, Ren H, Hu P. Expansion of circulating TFH cells and their associated molecules: involvement in the immune landscape in patients with chronic HBV infection. *Virol J* 2014; **11**: 54 [PMID: 24655429 DOI: 10.1186/1743-422X-11-54]
66 **Feng J**, Lu L, Hua C, Qin L, Zhao P, Wang J, Wang Y, Li W, Shi X, Jiang Y. High frequency of CD4+ CXCR5+ TFH cells in patients with immune-active chronic hepatitis B. *PLoS One* 2011; **6**: e21698 [PMID: 21750724 DOI: 10.1371/journal.pone.0021698]
67 **Ma Z**, Xie Y, Wang Y, Ma L, He Y, Zhang Y, Lian J, Guo Y, Jia Z. Peripheral blood $CD4^+$; $CXCR5^+$; follicular helper T cells are related to hyperglobulinemia of patients with chronic hepatitis B. *Xibao Yufenzi Mianyixue Zazhi* 2013; **29**: 515-518;521 [PMID: 23643274]
68 **Feng J**, Hu X, Guo H, Sun X, Wang J, Xu L, Jiang Z, Xu B, Niu J, Jiang Y. Patients with chronic hepatitis C express a high percentage of CD4(+)CXCR5(+) T follicular helper cells. *J Gastroenterol* 2012; **47**: 1048-1056 [PMID: 22426636 DOI: 10.1007/s00535-012-0568-1]
69 **Tripodo C**, Petta S, Guarnotta C, Pipitone R, Cabibi D, Colombo MP, Craxì A. Liver follicular helper T-cells predict the achievement of virological response following interferon-based treatment in HCV-infected patients. *Antivir Ther* 2012; **17**: 111-118 [PMID: 22267475 DOI: 10.3851/IMP1957]
70 **Jankovic D**, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, Wynn TA, Kamanaka M, Flavell RA, Sher A. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med* 2007; **204**: 273-283 [PMID: 17283209]
71 **Nakayamada S**, Kanno Y, Takahashi H, Jankovic D, Lu

KT, Johnson TA, Sun HW, Vahedi G, Hakim O, Handon R, Schwartzberg PL, Hager GL, O'Shea JJ. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity* 2011; **35**: 919-931 [PMID: 22195747 DOI: 10.1016/j.immuni.2011.11.012]

72 **Bonhagen K**, Liesenfeld O, Stadecker MJ, Hutloff A, Erb K, Coyle AJ, Lipp M, Kroczek RA, Kamradt T. ICOS+ Th cells produce distinct cytokines in different mucosal immune responses. *Eur J Immunol* 2003; **33**: 392-401 [PMID: 12645936]

73 **Greenwald RJ**, McAdam AJ, Van der Woude D, Satoskar AR, Sharpe AH. Cutting edge: inducible costimulator protein regulates both Th1 and Th2 responses to cutaneous leishmaniasis. *J Immunol* 2002; **168**: 991-995 [PMID: 11801630]

74 **Bollig N**, Brüstle A, Kellner K, Ackermann W, Abass E, Raifer H, Camara B, Brendel C, Giel G, Bothur E, Huber M, Paul C, Elli A, Kroczek RA, Nurieva R, Dong C, Jacob R, Mak TW, Lohoff M. Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci USA* 2012; **109**: 8664-8669 [PMID: 22552227 DOI: 10.1073/pnas.1205834109]

75 **Reinhardt RL**, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* 2009; **10**: 385-393 [PMID: 19252490 DOI: 10.1038/ni.1715]

76 **Butler NS**, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, Waldschmidt TJ, Crompton PD, Harty JT. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nat Immunol* 2012; **13**: 188-195 [PMID: 22157630 DOI: 10.1038/ni.2180]

77 **Gibson-Corley KN**, Boggiatto PM, Bockenstedt MM, Petersen CA, Waldschmidt TJ, Jones DE. Promotion of a functional B cell germinal center response after Leishmania species co-infection is associated with lesion resolution. *Am J Pathol* 2012; **180**: 2009-2017 [PMID: 22429963 DOI: 10.1016/j.ajpath.2012.01.012]

78 **Moon JJ**, Suh H, Li AV, Ockenhouse CF, Yadava A, Irvine DJ. Enhancing humoral responses to a malaria antigen with nanoparticle vaccines that expand Tfh cells and promote germinal center induction. *Proc Natl Acad Sci USA* 2012; **109**: 1080-1085 [PMID: 22247289 DOI: 10.1073/pnas.1112648109]

79 **Breitfeld D**, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Förster R. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 2000; **192**: 1545-1552 [PMID: 11104797]

P- Reviewer: Arvind C, Song JX **S- Editor**: Yu J **L- Editor**: A
E- Editor: Wu HL

World Journal of Hematology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.5315/wjh.v3.i4.128

World J Hematol 2014 November 6; 3(4): 128-137
ISSN 2218-6204 (online)

REVIEW

Granulysin and its clinical significance as a biomarker of immune response and NK cell related neoplasms

Masayuki Nagasawa, Kazuyuki Ogawa, Kinya Nagata, Norio Shimizu

Masayuki Nagasawa, Department of Hematology, Oncology, and Immunology, Tokyo Bay Urayasu/Ichikawa Medical Center, Chiba 279-0001, Japan
Kazuyuki Ogawa, Kinya Nagata, Bio Medical Laboratories Inc., Research and Development Center, Saitama 602-0841, Japan
Norio Shimizu, Tokyo Medical and Dental University, Post Graduate School, Department of Virology, Tokyo 113-8519, Japan
Author contributions: Nagasawa M designed the report; Ogawa K and Nagata K performed the protein analysis; Shimizu N established and provided the cell lines; Nagasawa M analyzed the data and wrote the paper.
Supported by The Grant-in-Aid for Scientific Research from Ministry of Education, Science and Culture Japan, 24591541 to Nagasawa M
Correspondence to: Masayuki Nagasawa, MD, PhD, Department of Hematology, Oncology, and Immunology, Tokyo Bay Urayasu/Ichikawa Medical Center, 3-4-32, Todaijima, Urayasu-city, Chiba 279-0001, Japan. mnagasawa.ped@tmd.ac.jp
Telephone: +81-47-3513101 Fax: +81-47-3526237
Received: October 8, 2013 Revised: February 18, 2014
Accepted: February 20, 2014
Published online: November 6, 2014

Abstract

Granulysin is a cytotoxic granular protein that was identified from human T cells by using the gene subtraction method in 1987. Based on its amino acid homology, granulysin belongs to the saposin-like protein family. The bioactive 9-kDa form of granulysin is processed from the 15-kDa pro-product in the cytoplasmic granules. It is expressed in CD8-positive αβT cells 5 d after mitogenic stimulation and constitutively in natural killer (NK) cells and γδT cells, although regulation of its expression has not yet been precisely determined. The 9-kDa granulysin form has anti-microbial activity against microorganisms such as bacteria, fungi, mycobacteria and parasites, as well as tumoricidal activity against some tumors at 1-10 μmol/L concentrations. Granulysin is secreted in both Ca-dependent and -independent manners. In sera, only the 15-kDa form is detectable and is expected to be a biomarker for immune potency, acute viral infection, anti-tumor immune reaction, acute graft *vs* host disease, and NK cell associated neoplasm.

Key words: Granulysin; Saposin-like protein family; Natural killer cell; Cytotoxic T lymphocyte

Core tip: Granulysin is a cytotoxic granular protein expressed in cytotoxic T cells, natural killer (NK) cells and γδT cells, and has anti-microbial activity against microorganisms such as bacteria, fungi, mycobacteria and parasites, as well as tumoricidal activity against some tumors. It is secreted constitutively and in a trigger-dependent manner. Clinically, serum granulysin is a unique biomarker for immune response, immune capacity and NK cell related neoplasms.

Nagasawa M, Ogawa K, Nagata K, Shimizu N. Granulysin and its clinical significance as a biomarker of immune response and NK cell related neoplasms. *World J Hematol* 2014; 3(4): 128-137 Available from: URL: http://www.wjgnet.com/2218-6204/full/v3/i4/128.htm DOI: http://dx.doi.org/10.5315/wjh.v3.i4.128

INTRODUCTION

Granulysin is a cytotoxic granular protein that was identified in human T cells by using the gene subtraction method in 1987[1]. While granulysin is not expressed in resting αβT cells, it is constitutively expressed in natural killer (NK) cells and γδT cells. In contrast to other cytotoxic granular proteins, such as perforin and granzyme, granulysin is expressed in αβT cells later following antigenic stimulation (Figure 1). In this review, we summarize the structure, the *in vivo* and *in vitro* functions, and the regulation of expression of granulysin. Furthermore, we

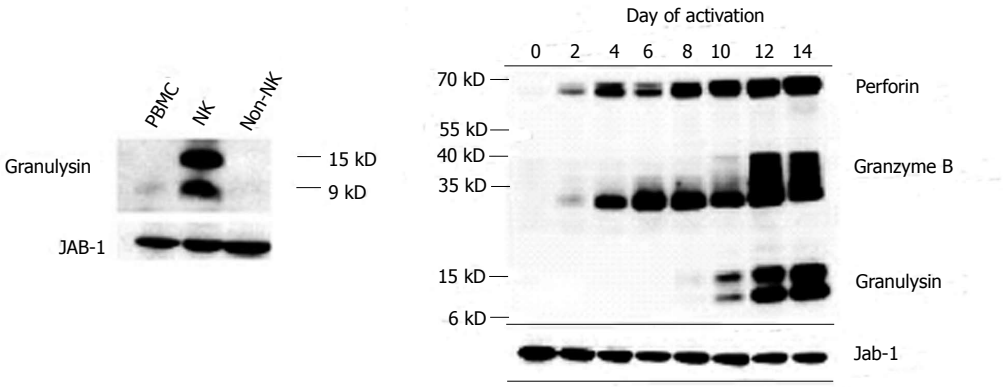


Figure 1 Expression of granulysin after T cell activation analyzed by western blotting. Granulysin is expressed later compared to perforin and granzyme B after T cell activation. In natural killer (NK) cells, granulysin is constitutively expressed. Jab-1 (c-Jun activation domain-binding protein-1) is used as internal control. PBMC: Peripheral blood mononuclear cell.

Table 1 Saposin-like protein family members and their proposed functions

Family member	Function	Identity to 9-kDa granulysin (amino acid)[1]	Similarity to 9-kDa granulysin (amino acid)[2]
Saposin A	Sphingolipid hydrolase activator	21	46
Saposin B	Sphingolipid hydrolase activator	19	50
Saposin C	Sphingolipid hydrolase activator	19	53
Saposin D	Sphingolipid hydrolase activator	20	46
Pulmonary surfactant protein B	Lipid organization in pulmonary surfactant	19	53
Acyloxyacyl hydrolase	Phagocytic cell lipase	22	50
Acyloxyacyl hydrolase	Sphingolipid hydrolase	13	42
Amoebapore A	Pore-forming Entamoeba histolytica granule protein	16	47
Amoebapore B	Pore-forming Entamoeba histolytica granule protein	13	42
Amoebapore C	Pore-forming Entamoeba histolytica granule protein	18	47
NK-lysin	Lytic porcine T cell and NK cell granule protein	35	66
Granulysin	Lytic human T cell and NK cell granule protein	100	100

[1]Identity denotes the percentage of identical amino acids; [2]Similarity denotes amino acids that share chemical properties, for example, charge or hydrophobicity. NK: Natural killer.

present results examining granulysin as a biomarker and discuss future investigations with granulysin.

STRUCTURE AND FUNCTION

Two isoforms of granulysin with molecular weights of 15-kDa and 9-kDa, respectively, have been identified and the biologically active 9-kDa isoform is derived from the 15-kDa isoform by intracellular processing (Figure 1). Based on amino acid sequence homology, the 9-kDa granulysin protein belongs to the saposin-like protein (SAPLIP) family containing the sphingolipid hydrolase activators of the central nervous system (Table 1)[2,3]. The gene is located at chromosome 2p11.2 in humans and homologues have been identified in pig, horse and cow. The absence of a homologous gene in rodents (mice) makes it difficult to investigate its physiological significance using rodent models[4,5]. Recently, Huang *et al*[6] and Liu *et al*[7] characterized a mouse model in which the human granulysin gene was introduced. This chimeric mouse model may be useful for the advanced functional analysis of granulysin in the future. Granulysin has cytotoxic activity similar to other SAPLIP family proteins such as amoebapore A, B, C (*Entamoeba histolytica* pore-forming protein) and NK-lysin (a porcine lytic granule protein)[8].

Crystal structure analysis of granulysin suggests that it consists of five α-helices (Figure 2). Although a physiological cell surface receptor for granulysin has not yet been identified, it is speculated that granulysin folds into a structure in which the positively charged active site interacts with negatively charged sites on bacterial or tumor cells and exhibits its cytotoxic activity. It is hypothesized that granulysin molecules aggregate on the target cell surface in an electric charge energy-dependent manner, and they rotate in the direction from α-helix1 to α-helix2 to α-helix3, pierce the cell membrane, and enter the cell[9,10]. Whereas synthetic peptides consisting of α-helix2 and α-helix3 kill bacterial and tumor cells, peptides consisting of α-helix3 alone kill bacterial, but not tumor cells. Substitution of cysteine residues in α-helix2 and α-helix3 with serine residues deprives the synthetic peptides of cytotoxic activity for human tumor cells, and replacing arginine residues with glutamine residues also abolishes its activity. When the cysteine residue is in the non-reduced state, the cytotoxic activity for tumor cells is lost while the cytotoxic activity for bacteria remains unaffected[10,11] (Figure 2B), suggesting that the reduced cysteine residue is essential for the cytotoxic activity for tumor cells. Substitution of D-amino acids 32-42 with L-amino acids maintains the same cytotoxic activity but induces resis-

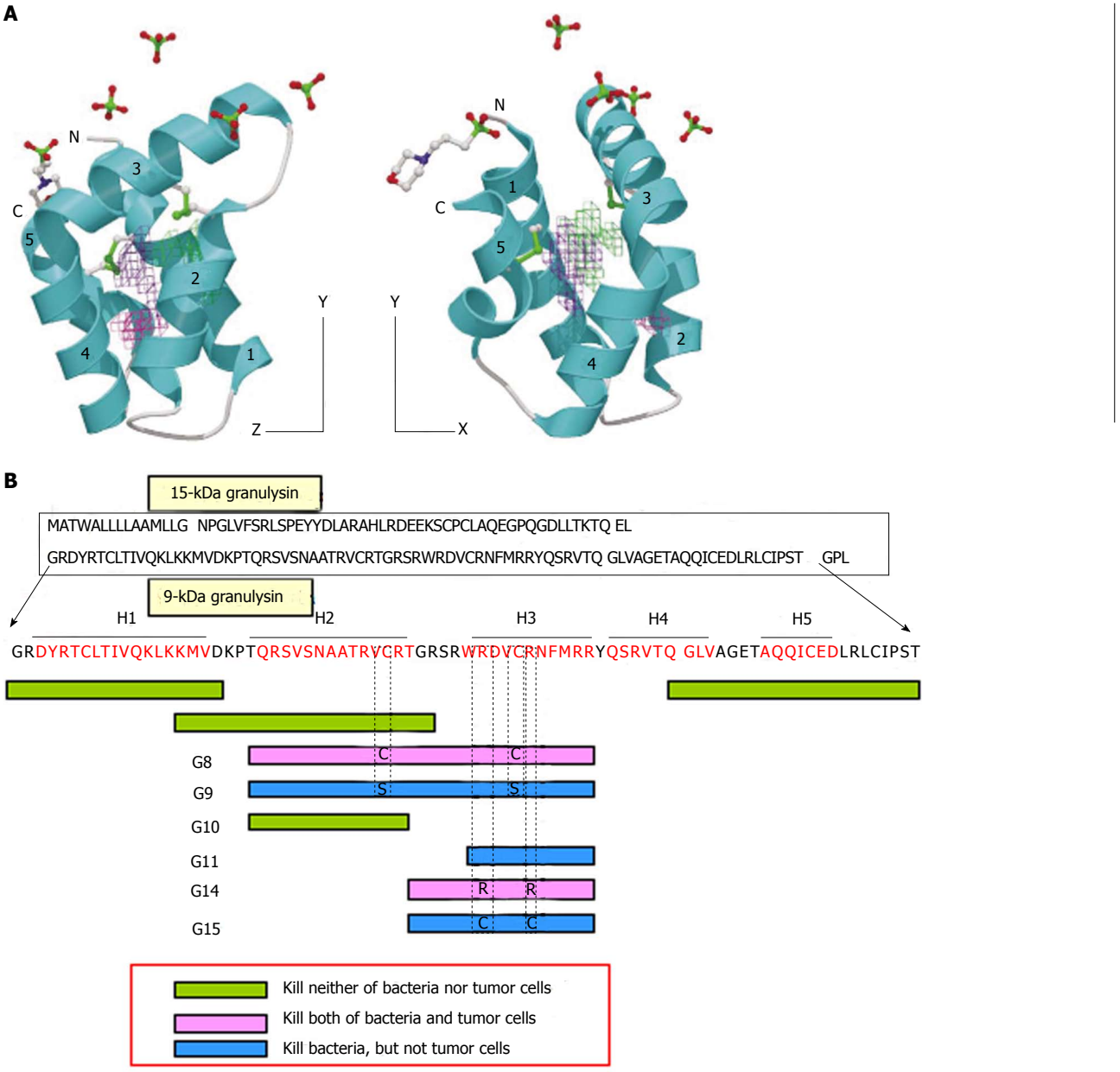


Figure 2 Granulysin. A: 3-D structure model of 9-kDa granulysin. Granulysin consists of five a-helices. Cytotoxic active site ranges between helix-2 and helix-3, in which positive electric charges are located[9]; B: Scheme of cytotoxic active site in granulysin. Amino acid sequence of granulysin and its biologically active site are illustrated. See STRUCTURE AND FUNCTION in the text for detailed explanation.

tance to inactivation by trypsin and the serum. These observations raise the possibility for the development of new synthetic peptides with cytotoxic activity, specifically for bacteria or for the development of biologically active peptides that can act for a long time *in vivo*[13].

EXPRESSION AND CYTOTOXIC ACTIVITY

Granulysin is expressed by activated cytotoxic T lymphocytes (CTL), mainly by CD8-positive T lymphocytes and some CD4-positive T lymphocytes[1,14]; it is also expressed by NK cells and γδT cells constitutively[15,16]. B cells and granular leukocytes do not express granulysin, but monocytes may express granulysin when activated. There is also a report indicating that granulysin was expressed in a megakaryocyte cell line, but whether it is expressed in platelets remains unclear[17].

Granulysin is synthesized as 15-kDa protein in the cytoplasm. The N-terminal amino acid sequence is thought to contain a transportation signal that directs granulysin to a cell granule. Some of the amino acids at the N- and C-termini are removed by unknown mechanisms within the cell granule to produce the active 9-kDa protein[14]. When the pH within the cytosomal granules is increased due to the presence of the H^+-ATPase inhibitor concanamycin A, processing to the 9-kDa protein is inhibited. Furthermore, against artificial cell membranes, the membrane injury activity of the 9-kDa granulysin is markedly reduced at pH 6.4 or lower. This most likely

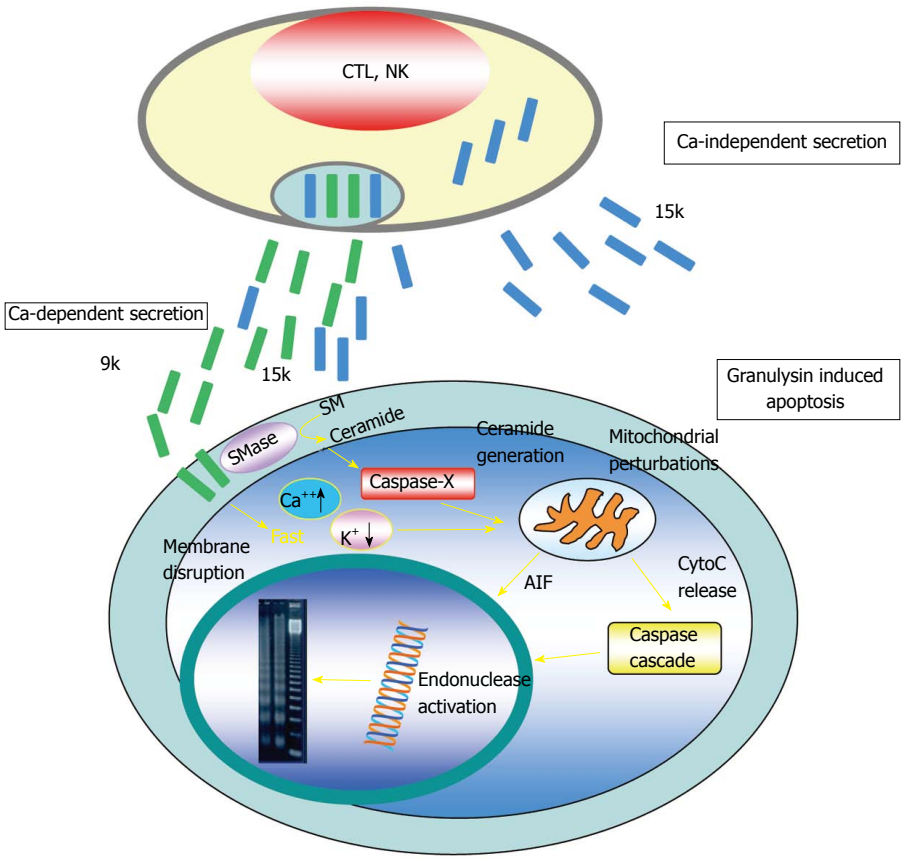


Figure 3 Schematic model of how granulysin kills target cell. (Cited from ref.[48] revised by author). NK: Natural killer; AIF: Apoptosis-inducing factor; CTL: Cytotoxic T lymphocytes.

explains why active 9-kDa granulysin does not cause autolysis in cytotoxic granules[18-20]. The CTL and NK cell granules have similar amounts of both the 15-kDa and the 9-kDa molecules, but while the 9-kDa molecules stay within the cytotoxic granules, the 15-kDa molecules are secreted constantly (Figure 3). Most of the 15-kDa molecules are thought to be secreted *via* an alternative pathway without entering the cytotoxic granules. Since the 15-kDa molecule does not have cytotoxic activity, its physiological role is currently not understood. Recently, it has been reported that 15-kDa granulysin induces differentiation of monocytes to dendritic cells and may modulate the immune response[21]. However, the 15-kDa molecule is detectable in serum and its potential significance as a biomarker has been recently reported.

9-kDa granulysin is released when co-cultured with target K562 cell and its release is prohibited by depletion of calcium, indicating Ca-dependent and trigger-dependent excretion of 9-kDa granulysin (see GRANULYSIN AS A BIOMARKER).

The 9-kDa molecule can kill gram-negative and gram-positive bacteria, fungi, parasitic worms, acid-fast bacilli and malarial parasites directly, but not intracellular parasites in the absence of perforin. Some studies also suggest that granulysin cannot enter the cytoplasm of the parasite in the absence of perforin[22].

Hata *et al*[23] reported that granulysin inhibits the growth of the varicella virus and induces apoptosis in infected cells. Granulysin-expressing CD4-positive T lymphocytes also kill *Cryptococcus neoformans*. Recently, Ochoa *et al*[24] reported that CD4-positive T lymphocytes infiltrating the lesions in leprosy patients express granulysin and are associated with control of the leprosy bacillus. Granulysin also has been reported to possess cytotoxic activity against some tumor cells[25]. The cytotoxic effects of granulysin against Jurkat cells are mediated by the entry of extracellular calcium into the cell after cell membrane destruction by granulysin, thereby inducing the release of intracellular calcium. Intracellular potassium (K) is reduced by a calcium-dependent K pump. This results in injury to the mitochondria and inhibits oxidative phosphorylation. With the release of cytochrome c and apoptosis-inducing factor (AIF) from the mitochondria, caspases are activated within several minutes and apoptosis is induced. This model of apoptosis induction by granulysin is evidenced by the fact that inhibition of the calcium-dependent K pump *via* suppression of intracellular calcium release inhibits apoptosis induction. In addition, granulysin also induces late caspase activation through an alternative pathway by activating membrane sphingomyelinase and inducing ceramide formation[9,10,26,27] (Figure 3).

The 9-kDa granulysin also has pro-inflammatory functions similar to defensins and acts as a chemotactic factor for CD-4 positive and CD8-positive T lymphocytes and monocytes. This chemotactic activity is affected at 10 nM concentrations of granulysin, which is much lower than that required for its cytotoxic activity (1-10 μmol/L). It is speculated that granulysin acts through a

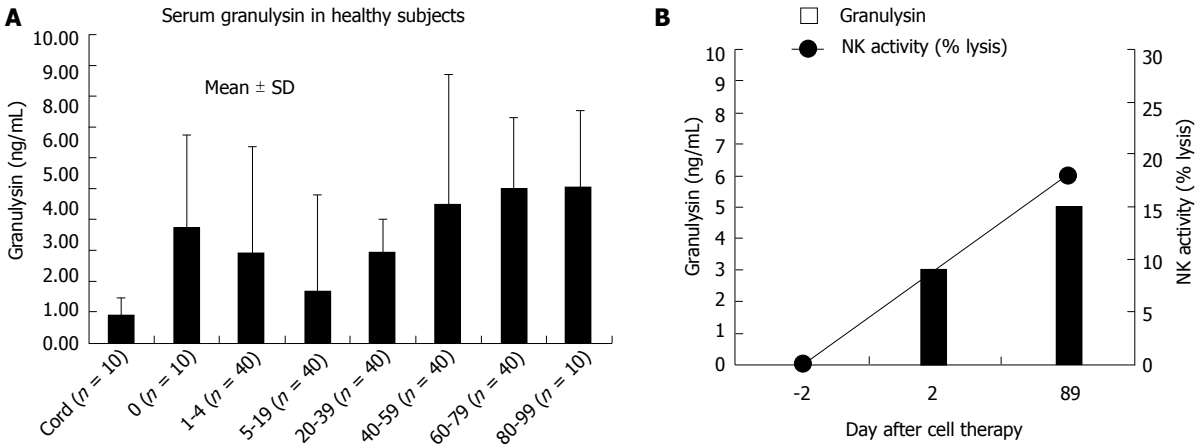


Figure 4 Serum granulysin in healthy subjects (A, see GRANULYSIN AS A BIOMARKER in the text) and relationship with natural killer cell activity (B). Serum granulysin is increased along with the recovery of natural killer (NK) cell activity in infant with combined immunodeficiency after cell therapy.

G protein conjugate receptor because the chemotaxis can be inhibited with pertussis toxin, but the details of the receptor are as yet unknown. The 9-kDa granulysin acts on monocytes and a cell line with monocytic-lineage (U937), and induces RANTES, monocyte chemotactic protein (MCP) 1, MCP-3, Macrophage inflammatory protein-1α, Interleukin (IL)-10, IL-1, IL-6 and interferon (IFN)-α[28].

REGULATION OF GRANULYSIN EXPRESSION

In comparison to its physiological functions, the regulation of granulysin expression remains to be elucidated.

The binding sites for activator protein-1 (AP-1), CCAAT/enhancer binding protein β (C/EBPβ) and nuclear factor kappa B (NF-κB) have been identified in the promoter region of the granulysin gene. Using the reporter assay system in which the monocyte-lineage cell line THP-1 is stimulated with *Acholeplasma laidlawii* (*A. laidlawii*) (mycoplasma), Kida *et al*[29,30] reported that two AP-1 binding sites (from -277 to -271 bp and from -96 to -86 bp) and the C/EBPβ binding site (from -1003 to -990 bp) are important for regulation of transcription, and that the former acts positively while the latter acts negatively. In the system described above, although *A. laidlawii* stimulation activated NF-κB through toll-like receptor 2 (TLR2) and the p50 homodimer bound to the NF-κB region, there was no influence on granulysin transcription[30].

NK cells express granulysin and IL-2 receptor β and γ chain constitutively. The expression of granulysin mRNA and protein was not altered after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, IL-2 or IFN-α[31]. Expression of granulysin was increased in CD8-positive T lymphocytes five days after antigen stimulation as mentioned above. Endsley *et al*[32] reported that CD4-positive T lymphocytes did not express granulysin even after PMA and ionomycin stimulation, whereas Zheng *et al*[33] reported that CD4-positive T lymphocytes expressed granulysin in the presence of IL-2 through PI3K and STAT5 activation, although anti-CD3 stimulation alone did not induce granulysin expression[33]. Transient activation of STAT5 occurred 30 to 60 min after IL-2 stimulation, following which a reactivation of STAT5 was observed after 3 d that induced IL-2 receptor β expression. Consequent interaction of IL-2 with IL-2 receptor β activated PI3K and induced granulysin[34]. Granulysin expression is inhibited by the anti-IL-2 receptor β antibody but not by the anti-IL-2 receptor α antibody, indicating the importance of IL-2 receptor α in inducing granulysin expression. Evidence for STAT5-controlled expression of granulysin also comes from the observation that patients with HIV infection have an increased susceptibility to *Cryptococcus neoformans*, which is probably due to insufficient activation of STAT5 and PI3K in CD4-positive T lymphocytes, resulting in reduced expression of granulysin[33].

Scherer *et al*[35] examined the expression of granulysin mRNA after stimulation with tuberculin purified protein derivative (PPD) in lymphocytes from bovine immunized with Bacille de Calmette et Guerin (BCG)[35]. Compared to non-immunized bovine controls, granulysin mRNA was increased more than 50 times in CD8-positive T lymphocytes 12 h after immunization and 48 h after immunization in CD4-positive T lymphocytes. Furthermore, whereas the mRNAs of perforin, IFN-γ and Fas-ligand in CD4-positive T lymphocytes increased after PMA + ionomycin stimulation, as well as after PPD stimulation, granulysin mRNA was not enhanced after PMA + ionomycin stimulation, corroborating the previous observation by Endsley *et al*[32].

GRANULYSIN AS A BIOMARKER

As mentioned above, the 15-kDa and 9-kDa granulysin forms exist at approximately a 1:1 ratio in cells. The precise mechanism of this conversion and its regulation is unknown. The non-active 15-kDa precursor of granulysin is secreted constantly, but the active 9-kDa form is released in a calcium-dependent manner. Based on the observation that the 9-kDa form is not detected in the culture medium even after *in vitro* stimulation, it

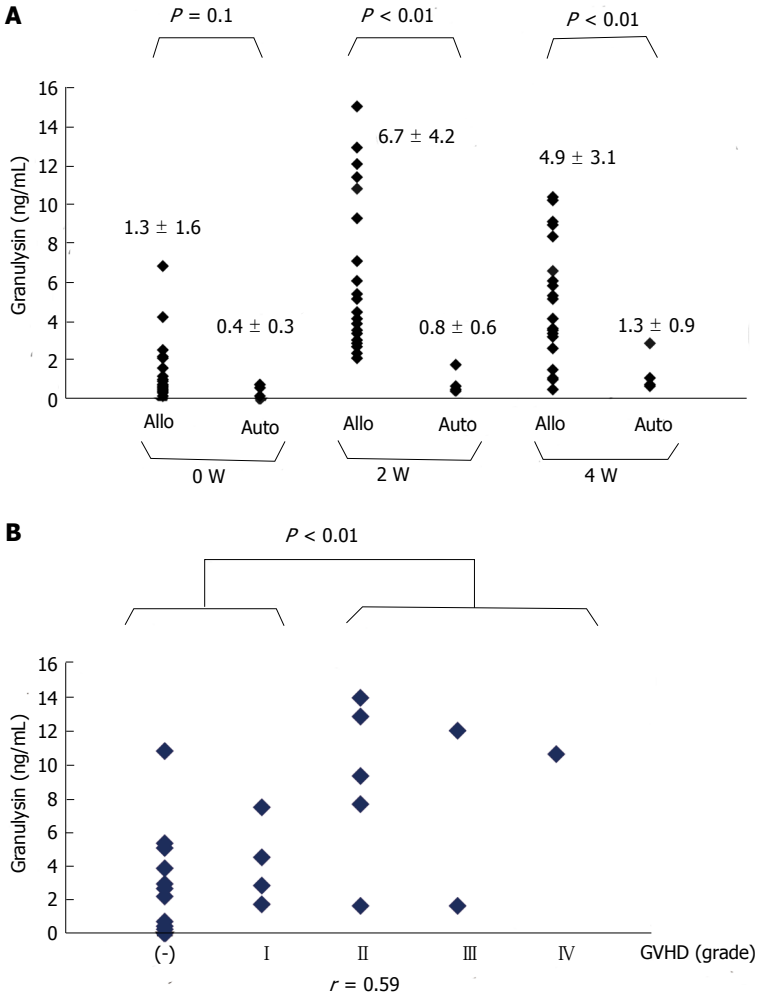


Figure 5 Trend of serum granulysin. A: In patients with allo-hematopoietic stem cell transplantation (HSCT) (n = 21) and auto-HSCT (n = 5). Serum granulysin is elevated 2 wk after allogeneic hematopoietic stem cell transplantation, but not autologous hematopoietic stem cell transplantation; B: With the grade of graft-versus-host disease (GVHD). Serum granulysin and the grade of GVHD were plotted and their correlation coefficient was calculated (r = 0.59). The serum granulysin level of patients with grade 2 or more is significantly higher than that of patients with grade 1 or no GVHD.

is possible that the active form is immediately adsorbed, consumed or destroyed. By contrast, the 15-kDa form is easily detected in the culture medium and serum and is increased after *in vitro* stimulation[36]. This indicates that the 9-kDa and 15-kDa forms are released together after stimulation, but only the 15-kDa form is detected. Any increase in the release of the 9-kDa form is therefore estimated to arise indirectly from the increased amount of the 15-kDa form, since inhibition of cellular secretion using Brefeldin A increased the intracellular levels of granulysin in CTL and NK cells but did not affect intracellular perforin and granzyme levels[36].

Granulysin as a biomarker in cell-mediated immunity

To estimate the levels of serum granulysin in healthy subjects, a novel, highly sensitive Enzyme Linked Immuno-Sorbent Assay method was used (Figure 4A). Levels of granulysin gradually increase with aging and are extremely low in umbilical cord blood. These levels reflect the levels of constitutively secreted granulysin and can be correlated either with NK cell activity or the number of NK cells and γδT cells, which constitutively express granulysin[36]. It is well known that NK activity increases with ageing until the age of 40 and decreases thereafter. The discrepancy between granulysin level and NK activity after the age of 40-50 is not well explained. One possibility is that the ratio of conversion from 15-kDa to 9-kDa changes after the age of 40. We have no data concerning this issue, which remains to be investigated.

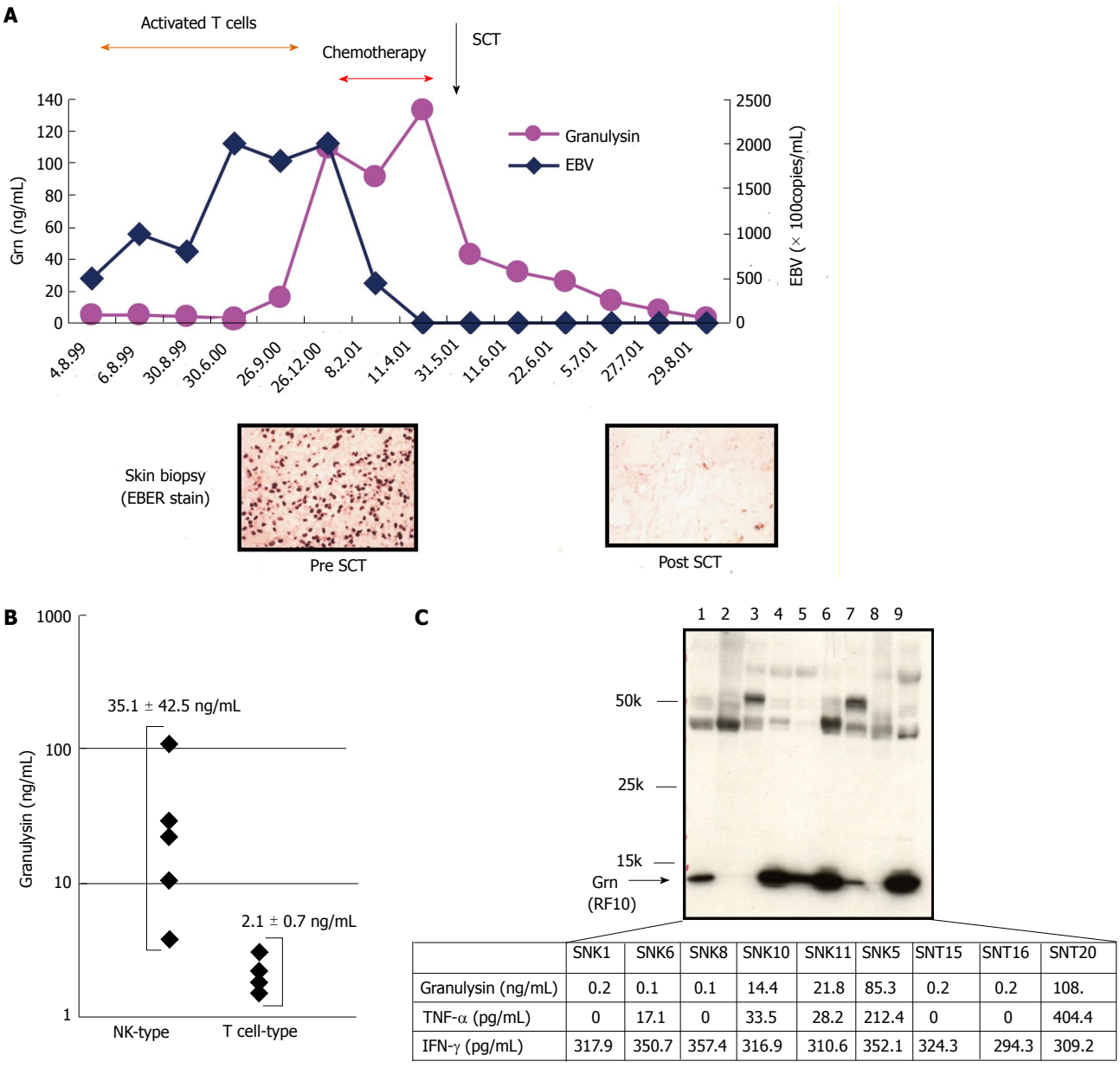
In infants with severe immunodeficiency without NK cells, serum granulysin was undetectable and became measurable when a cell-mediated immunity function was restored by hematopoietic stem cell transplantation (unpublished data). After transfusion of autologous *in vitro*-activated T cells back into a patient with incompetent cell-mediated immunity, levels of serum granulysin were increased along with the recovery of NK activity (Figure 4B)[36]. These observations indicate that serum granulysin is useful as a new biomarker for evaluation of cell-mediated immunity.

Granulysin as a biomarker in acute virus infection

Infectious mononucleosis is an acute disease resulting from primary Epstein-Barr (EB) virus infection, in which activated CD8-positive CTLs are increased in the peripheral blood. Increased CD8-positive CTLs are reactive and cytotoxic against EBV-infected B lymphocytes. Serum granulysin is markedly increased during an acute phase of infectious mononucleosis and becomes normalized in convalescence[36].

Granulysin as a biomarker of hemophagocytic lymphohistiocytosis

Hemophagocytic lymphohistiocytosis is a histiocytosis-



	SNK1	SNK6	SNK8	SNK10	SNK11	SNK5	SNT15	SNT16	SNT20
Granulysin (ng/mL)	0.2	0.1	0.1	14.4	21.8	85.3	0.2	0.2	108.
TNF-α (pg/mL)	0	17.1	0	33.5	28.2	212.4	0	0	404.4
IFN-γ (pg/mL)	317.9	350.7	357.4	316.9	310.6	352.1	324.3	294.3	309.2

Figure 6 Serum granulysin. A: Clinical course and trend of serum granulysin in a natural killer (NK) cell type chronic active EB virus infection (CAEBV) patient. For detailed explanation, see GRANULYSIN AS A BIOMARKER, 6: Granulysin in NK cell-related tumors or neoplasm in the text; B: Serum granulysin in patients with NK type and T cell type CAEBV, serum granulysin in patients with NK type (n = 5) and T cell type (n = 4) chronic active EB virus infection. Only in NK type CAEBV, serum granulysin is significantly elevated. Serum granulysin in patients with NK type (n = 5) and T cell type (n = 4) chronic active EB virus infection. Only in NK type CAEBV, serum granulysin is significantly elevated. C: Expression of granulysin and cytokine production in EB virus infected cell lines (SNK1,5,6,10,11: NK cell type, SNT8,15,16,20: γδT cell type). Western blotting was performed by using a monoclonal antibody, RF10 which reacts with 15-kDa but not 9-kDa granulysin. TNF-a and IFN-g in the culture supernatant were assayed by ELISA method. TNE: Tumor necrosis factor; INF: Interferon.

related disease characterized clinically by fever, pancytopenia, hepatosplenomegaly and hyperlipidemia. T cells are strongly activated during the acute phase of hemophagocytic lymphohistiocytosis (HLH) and levels of Th1 cytokines, such as IL-12, IL-18 and IFN-γ, are abnormally high, which leads to the abnormal activation of macrophages. Serum ferritin and soluble IL-2 receptor (sIL2R) have been reported as clinical markers of HLH. The treatment of HLH includes immunosuppressive therapy, anti-cancer drug chemotherapy and hematopoietic stem cell transplantation in severe cases. We measured serum granulysin in 24 HLH patients prior to treatment and reported that levels of granulysin were extremely high during the acute phase of HLH. Since serum granulysin levels decreased in parallel with disease regression following therapy, granulysin seems to be useful as a novel biomarker of HLH[37].

Granulysin as a biomarker of tumor immunity

Kishi *et al*[38] examined intracellular levels of granulysin and perforin in NK cells of cancer-bearing patients and healthy subjects by flow cytometry. Levels of intracellular granulysin were significantly decreased in cancer-bearing patients, while those of intracellular perforin were not

changed compared to healthy subjects[38]. Spontaneous regression of neuroblastoma has been observed frequently in infants younger than one year old. We previously reported a case study of an infant with neuroblastoma IVS who showed dramatic spontaneous regression. During the regression, serum granulysin and IFN-γ levels were transiently and markedly elevated[39]. Although interpretation of these observations is difficult, it seems that serum granulysin is related to tumor immunity and could be a novel biomarker of tumor immunity.

Granulysin as a biomarker in acute graft-versus-host disease

Elevated granulysin mRNA levels have been reported in infiltrating cells of acutely rejected kidneys from renal transplant patients[40]. To examine whether serum granulysin is a marker of acute graft-*vs*-host disease (GVHD) in hematopoietic stem cell transplantation (HSCT), we first isolated alloantigen-specific CTLs and confirmed that serum granulysin was released in an allospecific manner *in vitro*. Next, we examined serum granulysin in autologous and allogeneic hematopoietic stem cell transplantation cases. Serum granulysin was significantly and transiently increased in allogeneic HSCT 2 wk after SCT (6.7 ± 4.2 ng/mL), but not in autologous HSCT (0.8 ± 0.6 ng/mL) (Figure 5A). We also examined and found a significant correlation in the severity of acute GVHD and levels of granulysin (Figure 5B). Efficacy of soluble IL-2 receptor (sIL2R) has been reported as a biomarker of acute GVHD[41]. However, there were cases in which the change of sIL-2R levels and the symptoms of GVHD did not correlate in clinical settings. As per our observations, sIL-2R correlated well with serum granulysin during the first two months after HSCT, but serum granulysin reflected GVHD symptoms much better than sIL-2R thereafter. This discrepancy seems interesting in understanding the complicated pathology of GVHD and highlights the utility of serum granulysin as a biomarker that is distinct from sIL-2R for acute GVHD.

Granulysin in NK cell-related tumors or neoplasms

αβT cells express granulysin only after being activated and/or on maturation to CTLs. However, as mentioned above, granulysin is expressed constitutively in NK cells and γδT cells. Based on the foregoing observations, we examined the possibility of evaluating granulysin as a marker for NK-related tumors. Chronic active EB virus infection (CAEBV) is a disease with poor prognosis, presenting with fever, mosquito hypersensitivity, lymphadenopathy and hepatosplenomegaly, in which T cells or NK cells infected with EB virus are detected in the peripheral blood, and is usually classified as either the NK cell type or the T cell type. Interestingly, CD4-positive αβT cells are infected with the T cell type of EB virus. NK cell type CAEBV has been named hydroa vacciniforme because it is characterized clinically by varicelliform eruptions characterized histologically by infiltrating EB virus-positive cells. CAEBV frequently progresses to hemophagocytic syndrome or malignant lymphoma after a chronic clinical course. Figure 6A shows the levels of serum granulysin and blood EB viral genome in a patient with NK cell type CAEBV during a long-term clinical course. Serum granulysin and blood EB viral genome increased with progress of the disease. While blood EB viral genome decreased in response to chemotherapy, serum granulysin levels normalized only after allogeneic hematopoietic stem cell transplantation. A comparison of serum granulysin levels in NK cell type and T cell type CAEBV patients indicated that serum granulysin was significantly increased only in NK cell type patients (Figure 6B). Expression of granulysin was also confirmed by analyzing NK cell and γδT cell lines established from CAEBV patients[42]. CD4-positive αβT cell lines have not yet been established, but examination of a tumor tissue from a patient who presented with an EB virus-positive, CD4-positive lymphoma over the course of CAEBV[43], did not reveal any expression of granulysin (unpublished observations). Interestingly, cell lines with granulysin expression also showed enhanced TNF-α production, although the levels of INF-γ production were the same (Figure 6C). Culturing in the presence of the NF-κB inhibitor did not affect the expression of granulysin in these cell lines (unpublished observation). Sekiguchi *et al*[44] reported that serum granulysin was significantly increased in patients with aggressive NK cell leukemia[44]. Granulysin has also been implicated in the cell death of keratinocytes in Stevens-Johnson syndrome and toxic epidermal necrolysis[45]. Iwai *et al*[46] reported that histological examination of granulysin expression is useful for distinguishing Stevens-Johnson syndrome/toxic epidermal necrolysis from erythema multiforme major.

FUTURE DIRECTIONS

CTL and NK cells secrete the 15-kDa precursor of granulysin constitutively, whereas they secrete both the 15-kDa precursor and the active 9-kDa granulysin forms when exerting cytotoxic activity. Only the 15-kDa form can be detected in sera or culture media, because the active 9-kDa form may be adsorbed, consumed or destroyed rapidly. This characteristic is quite different from that of other cytotoxic granular proteins such as perforin and granzyme, and makes granulysin a unique biomarker of cell-mediated immunity, tumor immunity, infection and GVHD. Structural analysis of granulysin provides the potential for the development of new innovative agents by designing novel analogous proteins using biomolecular technology. The effectiveness of a granulysin-DNA vaccine for tuberculosis in mice models has been recently reported[47]. While many unknowns remain concerning granulysin regulation and function, the combination of novel biotechnological methods will make it possible to develop novel immune, anti-cancer and anti-infection treatment strategies. One difficulty for granulysin research comes from the fact that there is no homologous gene for granulysin in mice. Although granulysin was discovered in 1987, a new report that granulysin is associated with the onset of Stevens-Johnson syndrome

has refreshed interest in granulysin research. The clinical analysis of granulysin as a biomarker has only just begun and it is expected that new findings will be obtained in the future through both basic and clinical studies.

REFERENCES

1 **Jongstra J**, Schall TJ, Dyer BJ, Clayberger C, Jorgensen J, Davis MM, Krensky AM. The isolation and sequence of a novel gene from a human functional T cell line. *J Exp Med* 1987; **165**: 601-614 [PMID: 2434598 DOI: 10.1084/jem.165.3.601]

2 **Munford RS**, Sheppard PO, O'Hara PJ. Saposin-like proteins (SAPLIP) carry out diverse functions on a common backbone structure. *J Lipid Res* 1995; **36**: 1653-1663 [PMID: 7595087]

3 **Leippe M**. Ancient weapons: NK-lysin, is a mammalian homolog to pore-forming peptides of a protozoan parasite. *Cell* 1995; **83**: 17-18 [PMID: 7553868 DOI: 10.1016/0092-8674(95)90229-5]

4 **Andreu D**, Carreño C, Linde C, Boman HG, Andersson M. Identification of an anti-mycobacterial domain in NK-lysin and granulysin. *Biochem J* 1999; **344** Pt 3: 845-849 [PMID: 10585872]

5 **Davis EG**, Sang Y, Rush B, Zhang G, Blecha F. Molecular cloning and characterization of equine NK-lysin. *Vet Immunol Immunopathol* 2005; **105**: 163-169 [PMID: 15797485 DOI: 10.1016/j.vetimm.2004.12.007]

6 **Huang LP**, Lyu SC, Clayberger C, Krensky AM. Granulysin-mediated tumor rejection in transgenic mice. *J Immunol* 2007; **178**: 77-84 [PMID: 17182542]

7 **Liu B**, Liu S, Qu X, Liu J. Construction of a eukaryotic expression system for granulysin and its protective effect in mice infected with Mycobacterium tuberculosis. *J Med Microbiol* 2006; **55**: 1389-1393 [PMID: 17005788 DOI: 10.1099/jmm.0.46706-0]

8 **Andersson M**, Gunne H, Agerberth B, Boman A, Bergman T, Sillard R, Jörnvall H, Mutt V, Olsson B, Wigzell H. NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. *EMBO J* 1995; **14**: 1615-1625 [PMID: 7737114]

9 **Anderson DH**, Sawaya MR, Cascio D, Ernst W, Modlin R, Krensky A, Eisenberg D. Granulysin crystal structure and a structure-derived lytic mechanism. *J Mol Biol* 2003; **325**: 355-365 [PMID: 12488100 DOI: 10.1016/S0022-2836(02)01234-2]

10 **Kaspar AA**, Okada S, Kumar J, Poulain FR, Drouvalakis KA, Kelekar A, Hanson DA, Kluck RM, Hitoshi Y, Johnson DE, Froelich CJ, Thompson CB, Newmeyer DD, Anel A, Clayberger C, Krensky AM. A distinct pathway of cell-mediated apoptosis initiated by granulysin. *J Immunol* 2001; **167**: 350-356 [PMID: 11418670]

11 **Wang Z**, Choice E, Kaspar A, Hanson D, Okada S, Lyu SC, Krensky AM, Clayberger C. Bactericidal and tumoricidal activities of synthetic peptides derived from granulysin. *J Immunol* 2000; **165**: 1486-1490 [PMID: 10903754]

12 **Ernst WA**, Thoma-Uszynski S, Teitelbaum R, Ko C, Hanson DA, Clayberger C, Krensky AM, Leippe M, Bloom BR, Ganz T, Modlin RL. Granulysin, a T cell product, kills bacteria by altering membrane permeability. *J Immunol* 2000; **165**: 7102-7108 [PMID: 11120840]

13 **Hamamoto K**, Kida Y, Zhang Y, Shimizu T, Kuwano K. Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. *Microbiol Immunol* 2002; **46**: 741-749 [PMID: 12516770 DOI: 10.1111/j.1348-0421.2002.tb02759.x]

14 **Peña SV**, Hanson DA, Carr BA, Goralski TJ, Krensky AM. Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. *J Immunol* 1997; **158**: 2680-2688 [PMID: 9058801]

15 **Obata-Onai A**, Hashimoto S, Onai N, Kurachi M, Nagai S, Shizuno K, Nagahata T, Matsushima K. Comprehensive gene expression analysis of human NK cells and CD8(+) T lymphocytes. *Int Immunol* 2002; **14**: 1085-1098 [PMID: 12356674 DOI: 10.1093/intimm/dxf086]

16 **Spada FM**, Grant EP, Peters PJ, Sugita M, Melián A, Leslie DS, Lee HK, van Donselaar E, Hanson DA, Krensky AM, Majdic O, Porcelli SA, Morita CT, Brenner MB. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *J Exp Med* 2000; **191**: 937-948 [PMID: 10727456 DOI: 10.1084/jem.191.6.937]

17 **Kitamura N**, Koshiba M, Horie O, Ryo R. Expression of granulysin mRNA in the human megakaryoblastic leukemia cell line CMK. *Acta Haematol* 2002; **108**: 13-18 [PMID: 12145461 DOI: 10.1159/000063061]

18 **Hanson DA**, Kaspar AA, Poulain FR, Krensky AM. Biosynthesis of granulysin, a novel cytolytic molecule. *Mol Immunol* 1999; **36**: 413-422 [PMID: 10449094 DOI: 10.1016/S0161-5890(99)00063-2]

19 **Kataoka T**, Shinohara N, Takayama H, Takaku K, Kondo S, Yonehara S, Nagai K. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J Immunol* 1996; **156**: 3678-3686 [PMID: 8621902]

20 **Uellner R**, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, Morgan BP, Podack E, Waterfield MD, Griffiths GM. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *EMBO J* 1997; **16**: 7287-7296 [PMID: 9405358 DOI: 10.1093/emboj/16.24.7287]

21 **Clayberger C**, Finn MW, Wang T, Saini R, Wilson C, Barr VA, Sabatino M, Castiello L, Stroncek D, Krensky AM. 15 kDa granulysin causes differentiation of monocytes to dendritic cells but lacks cytotoxic activity. *J Immunol* 2012; **188**: 6119-6126 [PMID: 22586033]

22 **Stenger S**, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melián A, Bogdan C, Porcelli SA, Bloom BR, Krensky AM, Modlin RL. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 1998; **282**: 121-125 [PMID: 9756476 DOI: 10.1126/science.282.5386.121]

23 **Hata A**, Zerboni L, Sommer M, Kaspar AA, Clayberger C, Krensky AM, Arvin AM. Granulysin blocks replication of varicella-zoster virus and triggers apoptosis of infected cells. *Viral Immunol* 2001; **14**: 125-133 [PMID: 11398808]

24 **Ochoa MT**, Stenger S, Sieling PA, Thoma-Uszynski S, Sabet S, Cho S, Krensky AM, Rollinghoff M, Nunes Sarno E, Burdick AE, Rea TH, Modlin RL. T-cell release of granulysin contributes to host defense in leprosy. *Nat Med* 2001; **7**: 174-179 [PMID: 11175847 DOI: 10.1038/84620]

25 **Peña SV**, Krensky AM. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. *Semin Immunol* 1997; **9**: 117-125 [PMID: 9194222 DOI: 10.1006/smim.1997.0061]

26 **Okada S**, Li Q, Whitin JC, Clayberger C, Krensky AM. Intracellular mediators of granulysin-induced cell death. *J Immunol* 2003; **171**: 2556-2562 [PMID: 12928406]

27 **Gamen S**, Hanson DA, Kaspar A, Naval J, Krensky AM, Anel A. Granulysin-induced apoptosis. I. Involvement of at least two distinct pathways. *J Immunol* 1998; **161**: 1758-1764 [PMID: 9712041]

28 **Deng A**, Chen S, Li Q, Lyu SC, Clayberger C, Krensky AM. Granulysin, a cytolytic molecule, is also a chemoattractant and proinflammatory activator. *J Immunol* 2005; **174**: 5243-5248 [PMID: 15843520]

29 **Kida Y**, Kuwano K, Zhang Y, Arai S. Acholeplasma laidlawii up-regulates granulysin gene expression via transcription factor activator protein-1 in a human monocytic cell line, THP-1. *Immunology* 2001; **104**: 324-332 [PMID:

11722647]
30 **Kida Y**, Shimizu T, Kuwano K. Opposing roles of activator protein-1 and CCAAT/enhancer binding protein beta in the regulation of inducible granulysin gene expression in a human monocytic cell line, THP-1. *Immunology* 2002; **107**: 507-516 [PMID: 12460196]
31 **Mori S**, Jewett A, Cavalcanti M, Murakami-Mori K, Nakamura S, Bonavida B. Differential regulation of human NK cell-associated gene expression following activation by IL-2, IFN-alpha and PMA/ionomycin. *Int J Oncol* 1998; **12**: 1165-1170 [PMID: 9538144]
32 **Endsley JJ**, Hogg A, Shell LJ, McAulay M, Coffey T, Howard C, Capinos Scherer CF, Waters WR, Nonnecke B, Estes DM, Villarreal-Ramos B. Mycobacterium bovis BCG vaccination induces memory CD4+ T cells characterized by effector biomarker expression and anti-mycobacterial activity. *Vaccine* 2007; **25**: 8384-8394 [PMID: 17996992 DOI: 10.1016/j.vaccine.2007.10.011]
33 **Zheng CF**, Ma LL, Jones GJ, Gill MJ, Krensky AM, Kubes P, Mody CH. Cytotoxic CD4+ T cells use granulysin to kill Cryptococcus neoformans, and activation of this pathway is defective in HIV patients. *Blood* 2007; **109**: 2049-2057 [PMID: 17038537 DOI: 10.1182/blood-2006-03-009720]
34 **Zheng CF**, Jones GJ, Shi M, Wiseman JC, Marr KJ, Berenger BM, Huston SM, Gill MJ, Krensky AM, Kubes P, Mody CH. Late expression of granulysin by microbicidal $CD4^+$ T cells requires PI3K- and STAT5-dependent expression of IL-2Rbeta that is defective in HIV-infected patients. *J Immunol* 2008; **180**: 7221-7229 [PMID: 18490721]
35 **Capinos Scherer CF**, Endsley JJ, de Aguiar JB, Jacobs WR, Larsen MH, Palmer MV, Nonnecke BJ, Ray Waters W, Mark Estes D. Evaluation of granulysin and perforin as candidate biomarkers for protection following vaccination with Mycobacterium bovis BCG or M. bovisDeltaRD1. *Transbound Emerg Dis* 2009; **56**: 228-239 [PMID: 19389081]
36 **Ogawa K**, Takamori Y, Suzuki K, Nagasawa M, Takano S, Kasahara Y, Nakamura Y, Kondo S, Sugamura K, Nakamura M, Nagata K. Granulysin in human serum as a marker of cell-mediated immunity. *Eur J Immunol* 2003; **33**: 1925-1933 [PMID: 12884856 DOI: 10.1002/eji.200323977]
37 **Nagasawa M**, Ogawa K, Imashuku S, Mizutani S. Serum granulysin is elevated in patients with hemophagocytic lymphohistiocytosis. *Int J Hematol* 2007; **86**: 470-473 [PMID: 18192122 DOI: 10.1007/BF02984011]
38 **Kishi A**, Takamori Y, Ogawa K, Takano S, Tomita S, Tanigawa M, Niman M, Kishida T, Fujita S. Differential expression of granulysin and perforin by NK cells in cancer patients and correlation of impaired granulysin expression with progression of cancer. *Cancer Immunol Immunother* 2002; **50**: 604-614 [PMID: 11807624 DOI: 10.1007/s002620100228]
39 **Nagasawa M**, Kawamoto H, Tsuji Y, Mizutani S. Transient increase of serum granulysin in a stage IVs neuroblastoma patient during spontaneous regression: case report. *Int J Hematol* 2005; **82**: 456-457 [PMID: 16533752 DOI: 10.1532/IJH97.05091]
40 **Sarwal MM**, Jani A, Chang S, Huie P, Wang Z, Salvatierra O, Clayberger C, Sibley R, Krensky AM, Pavlakis M. Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation. *Hum Immunol* 2001; **62**: 21-31 [PMID: 11165712 DOI: 10.1016/S0198-8859(00)00228-7]
41 **Nagasawa M**, Isoda T, Itoh S, Kajiwara M, Morio T, Shimizu N, Ogawa K, Nagata K, Nakamura M, Mizutani S. Analysis of serum granulysin in patients with hematopoietic stem-cell transplantation: its usefulness as a marker of graft-versus-host reaction. *Am J Hematol* 2006; **81**: 340-348 [PMID: 16628730]
42 **Nagasawa M**, Ogawa K, Nagata K, Shimizu N. Serum granulysin as a possible biomarker of natural killer cell neoplasms. *Br J Haematol* 2010; **148**: 812-814 [PMID: 19912220]
43 **Nagasawa M**, Hirai K, Mizutani S, Okawa H, Yata J. EBV infection induced transformation of benign T lymphoproliferative state in patient with chronic active EBV infection into malignant lymphoma: implication of EBV infection as additive oncogenic factor in tumorigenesis. *Leuk Res* 1999; **23**: 1071-1078 [PMID: 10576513]
44 **Sekiguchi N**, Asano N, Ito T, Momose K, Momose M, Ishida F. Elevated serum granulysin and its clinical relevance in mature NK-cell neoplasms. *Int J Hematol* 2012; **96**: 461-468 [PMID: 22890551]
45 **Chung WH**, Hung SI, Yang JY, Su SC, Huang SP, Wei CY, Chin SW, Chiou CC, Chu SC, Ho HC, Yang CH, Lu CF, Wu JY, Liao YD, Chen YT. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. *Nat Med* 2008; **14**: 1343-1350 [PMID: 19029983]
46 **Iwai S**, Sueki H, Watanabe H, Sasaki Y, Suzuki T, Iijima M. Distinguishing between erythema multiforme major and Stevens-Johnson syndrome/toxic epidermal necrolysis immunopathologically. *J Dermatol* 2012; **39**: 781-786 [PMID: 22458564]
47 **Kita Y**, Hashimoto S, Nakajima T, Nakatani H, Nishimatsu S, Nishida Y, Kanamaru N, Kaneda Y, Takamori Y, McMurray D, Tan EV, Cang ML, Saunderson P, Dela Cruz EC, Okada M. Novel therapeutic vaccines [(HSP65 + IL-12)DNA-, granulysin- and Ksp37-vaccine] against tuberculosis and synergistic effects in the combination with chemotherapy. *Hum Vaccin Immunother* 2013; **9**: 526-533 [PMID: 23249609]
48 **Clayberger C**, Krensky AM. Granulysin. *Curr Opin Immunol* 2003; **15**: 560-565 [PMID: 14499265]

P- Reviewer: Kitagawa M, Murdaca G **S- Editor:** Wen LL
L- Editor: Roemmele A **E- Editor:** Wu HL

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com