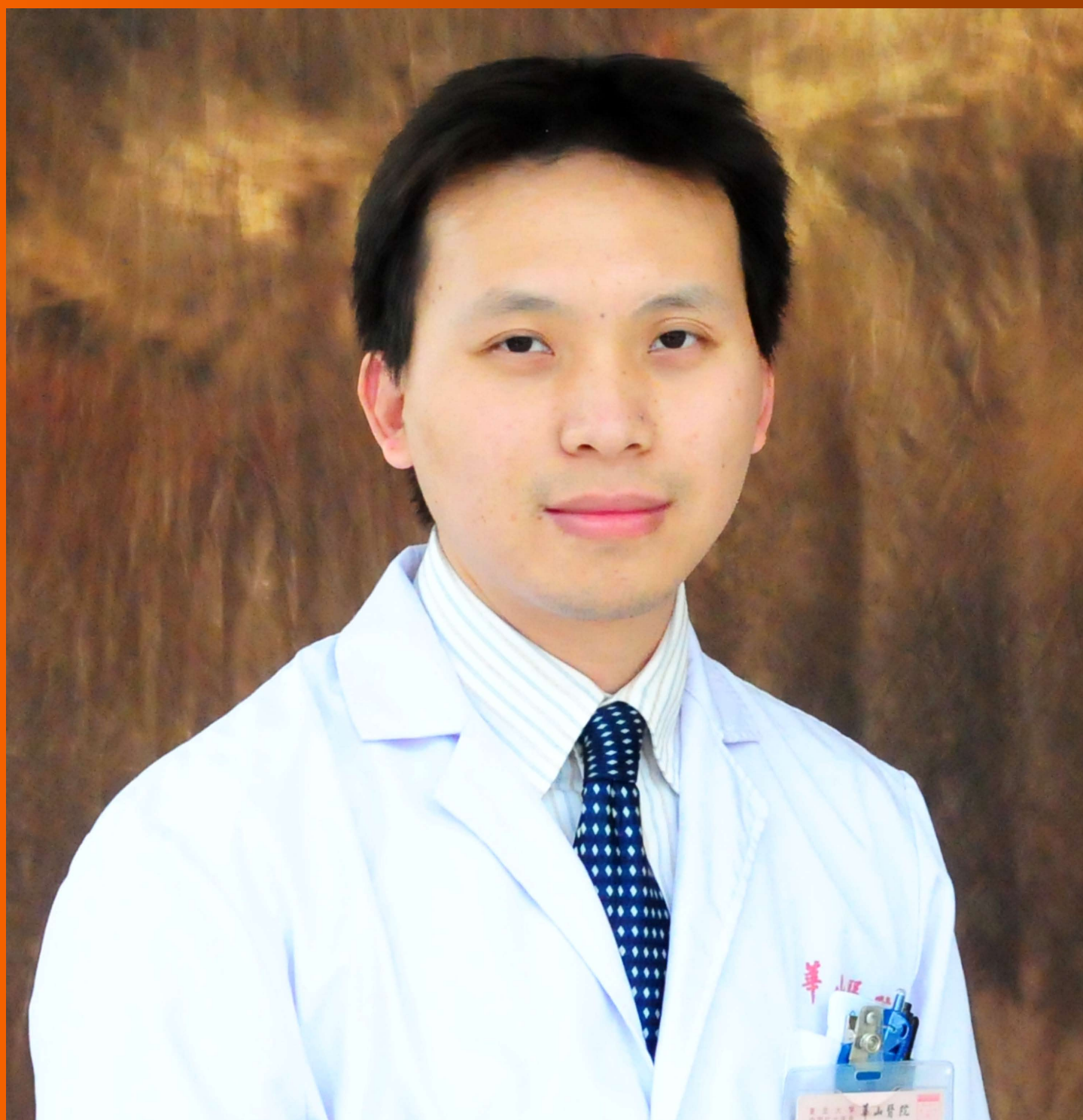


World Journal of *Gastroenterology*

World J Gastroenterol 2018 April 7; 24(13): 1373-1490



**REVIEW**

- 1373 Dissecting the molecular pathophysiology of drug-induced liver injury

Ye H, Nelson LJ, Gómez del Moral M, Martínez-Naves E, Cubero FJ

MINIREVIEWS

- 1386 Thrombocytopenia after liver transplantation: Should we care?

Takahashi K, Nagai S, Safwan M, Liang C, Ohkohchi N

ORIGINAL ARTICLE**Basic Study**

- 1398 Systems pharmacology approach reveals the antiinflammatory effects of *Ampelopsis grossedentata* on dextran sodium sulfate-induced colitis

Chen YL, Zhang YL, Dai YC, Tang ZP

Retrospective Cohort Study

- 1410 Potential triggering factors of acute liver failure as a first manifestation of autoimmune hepatitis-a single center experience of 52 adult patients

Buechter M, Manka P, Heinemann FM, Lindemann M, Baba HA, Schlattjan M, Canbay A, Gerken G, Kahraman A

- 1419 *Helicobacter pylori* infection in subjects negative for high titer serum antibody

Toyoshima O, Nishizawa T, Arita M, Kataoka Y, Sakitani K, Yoshida S, Yamashita H, Hata K, Watanabe H, Suzuki H

Retrospective Study

- 1429 Impact of postoperative TNM stages after neoadjuvant therapy on prognosis of adenocarcinoma of the gastro-oesophageal junction tumours

Thomaschewski M, Hummel R, Petrova E, Knief J, Wellner UF, Keck T, Bausch D

- 1440 Mild drinking habit is a risk factor for hepatocarcinogenesis in non-alcoholic fatty liver disease with advanced fibrosis

Kimura T, Tanaka N, Fujimori N, Sugiura A, Yamazaki T, Joshita S, Komatsu M, Umemura T, Matsumoto A, Tanaka E

- 1451 Prognostic significance of combined preoperative fibrinogen and CA199 in gallbladder cancer patients

Xu WY, Zhang HH, Yang XB, Bai Y, Lin JZ, Long JY, Xiong JP, Zhang JW, Sang XT, Zhao HT

- 1464 Fecal microbial dysbiosis in Chinese patients with inflammatory bowel disease

Ma HQ, Yu TT, Zhao XJ, Zhang Y, Zhang HJ

Prospective Study

- 1478** Hepatocellular carcinoma or interferon-based therapy history attenuates sofosbuvir/ribavirin for Japanese genotype 2 hepatitis C virus

Yada M, Miyazaki M, Tanaka K, Masumoto A, Motomura K

CASE REPORT

- 1486** Gilbert syndrome combined with prolonged jaundice caused by contrast agent: Case report

Qian JD, Hou FQ, Wang TL, Shao C, Wang GQ

Contents

World Journal of Gastroenterology
Volume 24 Number 13 April 7, 2018

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Feng Yang, MD, PhD, Associate Professor, Doctor, Surgeon, Pancreatic Surgery, Huashan Hospital, Fudan University, Shanghai 200040, China

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents[®]/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch[®]), Journal Citation Reports[®], Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2017 edition of Journal Citation Reports[®] cites the 2016 impact factor for *WJG* as 3.365 (5-year impact factor: 3.176), ranking *WJG* as 29th among 79 journals in gastroenterology and hepatology (quartile in category Q2).

EDITORS FOR THIS ISSUE

Responsible Assistant Editor: *Xiang Li*
Responsible Electronic Editor: *Yan Huang*
Proofing Editor-in-Chief: *Lian-Sheng Ma*

Responsible Science Editor: *Xue-Jiao Wang*
Proofing Editorial Office Director: *Ze-Mao Gong*

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Damian Garcia-Olmo, MD, PhD, Doctor, Professor, Surgeon, Department of Surgery, Universidad Autonoma de Madrid; Department of General Surgery, Fundacion Jimenez Diaz University Hospital, Madrid 28040, Spain

Stephen C Strom, PhD, Professor, Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Stockholm 141-86, Sweden

Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach,

CA 90822, United States

EDITORIAL BOARD MEMBERS
All editorial board members resources online at <http://www.wjgnet.com/1007-9327/editorialboard.htm>

EDITORIAL OFFICE
Ze-Mao Gong, Director
World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: <http://www.f6publishing.com/helpdesk>
<http://www.wjgnet.com>

PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk: <http://www.f6publishing.com/helpdesk>
<http://www.wjgnet.com>

PUBLICATION DATE
April 7, 2018

COPYRIGHT
© 2018 Baishideng Publishing Group Inc. Articles published by this Open-Access journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license.

SPECIAL STATEMENT
All articles published in journals owned by the Baishideng Publishing Group (BPG) represent the views and opinions of their authors, and not the views, opinions or policies of the BPG, except where otherwise explicitly indicated.

INSTRUCTIONS TO AUTHORS
Full instructions are available online at <http://www.wjgnet.com/bpg/gerinfo/204>

ONLINE SUBMISSION
<http://www.f6publishing.com>

Dissecting the molecular pathophysiology of drug-induced liver injury

Hui Ye, Leonard J Nelson, Manuel Gómez del Moral, Eduardo Martínez-Naves, Francisco Javier Cubero

Hui Ye, Eduardo Martínez-Naves, Francisco Javier Cubero, Department of Immunology, Ophthalmology and ORL, Complutense University School of Medicine, Madrid 28040, Spain

Hui Ye, Eduardo Martínez-Naves, Francisco Javier Cubero, 12 de Octubre Health Research Institute (imas12), Madrid 28041, Spain

Leonard J Nelson, Institute for BioEngineering (Human Liver Tissue Engineering, Faraday Building, The University of Edinburgh, The King Buildings, Mayfield Road, Edinburgh EH9 3 JL, Scotland, United Kingdom

Manuel Gómez del Moral, Department of Cell Biology, Complutense University School of Medicine, Madrid 28040, Spain

ORCID number: Hui Ye (0000-0002-7894-2992); Leonard J Nelson (0000-0002-4197-4843); Manuel Gómez del Moral (0000-0002-0642-8142); Eduardo Martínez-Naves (0000-0001-8136-9042); Francisco Javier Cubero (0000-0003-1499-650X).

Author contributions: Ye H and Cubero FJ outlined the review, wrote the manuscript and designed figures; Nelson LJ, Gómez del Moral M and Martínez-Naves E corrected the manuscript, checked English language and provided fundamental guidance.

Supported by the Spanish Ministerio de Economía y Competitividad (MINECO), No. RYC2014-15242 and No. SAF2016-78711 to Martínez-Naves E and Cubero FJ. Martínez-Naves E and Cubero FJ are part of the UCM group "Lymphocyte Immunobiology", Ref. 920631 (imas12-associated, Ref. IBL-6); Chinese Scholarship Council fellow to Ye H.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Francisco Javier Cubero, BSc, MSc, PhD, Assistant Professor, Department of Immunology, Ophthalmology and ORL, Complutense University School of Medicine, Plaza de Ramón y Cajal s/n, Madrid 28040, Spain. fcubero@ucm.es
Telephone: +34-91-3941385
Fax: +34-91-3941641

Received: January 27, 2018

Peer-review started: January 28, 2018

First decision: February 10, 2018

Revised: February 16, 2018

Accepted: February 26, 2018

Article in press: February 26, 2018

Published online: April 7, 2018

Abstract

Drug-induced liver injury (DILI) has become a major topic in the field of Hepatology and Gastroenterology. DILI can be clinically divided into three phenotypes: hepatocytic, cholestatic and mixed. Although the clinical manifestations of DILI are variable and the pathogenesis complicated, recent insights using improved preclinical models, have allowed a better understanding of the mechanisms that trigger liver damage. In this review, we will discuss the pathophysiological mechanisms underlying DILI. The toxicity of the drug eventually induces hepatocellular damage through multiple molecular pathways, including direct hepatic toxicity and innate and adaptive immune responses. Drugs or their metabolites, such as the common analgesic, acetaminophen, can cause direct hepatic toxicity through accumulation of reactive oxygen species and mitochondrial dysfunction. The innate and adaptive immune responses play also a very important role in the occurrence of idiosyncratic DILI. Furthermore, we examine common forms of hepatocyte death and their association with the activation of specific signaling pathways.

Key words: Signaling pathways; Acetaminophen; Drug-

induced liver injury; Cell death; Reactive oxygen species

© **The Author(s) 2018.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Drug-induced liver injury (DILI) represents a broad spectrum of clinical manifestations, and is generally divided into two subtypes: intrinsic and idiosyncratic hepatotoxicity. Drugs and their reactive metabolites covalently bind to mitochondria and cause direct hepatic toxicity through accumulation of oxidative stress (ROS and RNS), endoplasmic reticulum stress and mitochondrial dysfunction, ultimately leading to cell death. The innate and adaptive immune responses also play an important role in the occurrence of idiosyncratic immunological reactions towards the drugs. In this review, we discuss the pathophysiological mechanisms underlying DILI, specific signaling pathways and the common forms of hepatocyte death.

Ye H, Nelson LJ, Gómez del Moral M, Martínez-Naves E, Cubero FJ. Dissecting the molecular pathophysiology of drug-induced liver injury. *World J Gastroenterol* 2018; 24(13): 1373-1385 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1373.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1373>

INTRODUCTION

Drug-induced liver injury (DILI) is the most common cause of acute liver failure (ALF) in the United States and Europe^[1], and is a leading reason for drug withdrawal and the high attrition rates in drug development (Table 1). In addition, the incidence of DILI has continued to rise and is therefore recognized as a major public health concern^[2]. DILI is one of the most common and serious adverse drug reactions (ADRs)^[3], and is defined as a chemical insult resulting in injury to the liver^[4]. It can be triggered by the parent drug and/or its metabolites, or as a reaction of hypersensitivity to the compound. A wide variety of drugs can cause DILI, including anti-tumor chemotherapy drugs, anti-tuberculosis drugs, antipyretic analgesics, immunosuppressive agents, hypoglycaemic therapies, or anti-bacterial, anti-fungal and antiviral drugs. DILI leads to multiple presentations in the clinic, including elevated liver enzymes, hepatitis, hepatocellular necrosis, cholestasis, fatty liver and liver cirrhosis. Occasionally, the clinical symptoms are not specific and they are indistinguishable from other hepatic disorders. In some patients, liver injury is easily detected by blood tests. The wide range of clinical manifestation, the complication of aetiology and the lack of effective tests make its diagnosis and treatment particularly challenging.

DILI is generally divided into two subtypes according to the hepatotoxicity of the drug: "intrinsic" hepatotoxicity and "idiosyncratic" hepatotoxicity. The

former refers to dose-dependent hepatotoxicity that is predictable in humans or animal models, while idiosyncratic DILI (iDILI) is an unpredictable injury that cannot be explained by the known pharmacological properties. Recently, the screening of new drugs has become more stringent and the monitoring of ADRs improved. Problems associated with DILI have become a major driver in the development of new medications, and for the withdrawal, restriction or project termination of existing drugs and drug compound candidates. In developed countries, iDILI is less common, occurring only very rarely among treated patients, while intrinsic hepatotoxicity is still a main cause of DILI^[5,6]. The pathogenesis of DILI is a complex process that has recently attracted much attention. Some researchers recently proposed a new hypothesis, providing a clear framework and direction for the further study of DILI^[7]. According to this hypothesis, drug-induced liver injury can be divided into three steps: an initial insulting stimulation causes the mitochondrial dysfunction, and ultimately leads to cell death. However, until now, the exact mechanism remains unclear. For the purpose of preventing DILI and improving clinical management, the study of the pathogenesis of DILI is particularly important. In this article, we will review and discuss recent progress towards understanding the underlying mechanisms triggering DILI.

DIRECT HEPATIC TOXICITY

The liver plays an important role in the metabolism of drugs or exogenous toxicants, and the majority of drugs are biologically transformed in the liver. The pathological state of the liver can affect drug metabolism, thus changing the efficacy and the ADRs, whilst the metabolic products of drugs can cause liver damage.

The cytochrome P450s (CYP) are a superfamily of iron porphyrin proteins, which are key factors involved in drug oxidative and reduction reactions. Through the P450s, drugs are metabolized and can form ions, oxygen free radicals and other active substances. The balance between toxic formation and detoxification is essential for DILI. Toxins are inactivated by the detoxification phase I - III pathways of the liver. However, once the amount of toxins exceeds the capacity of the hepatic detoxification function, drugs and their reactive metabolites impact cell function - leading to liver cell damage - eventually causing apoptotic or necrotic cell death. At present, the most frequently studied drug, which causes intrinsic DILI, is acetaminophen (APAP), which is also known as paracetamol^[8].

Upon rapid absorption, APAP is mainly metabolized *via* Phase- I reactions (sulfation or glucuronidation) and then excreted into the urine. APAP toxicity is caused mainly by the excess formation of the reactive intermediate, N-acetyl-p-benzoquinone imine (NAPQI)^[9], as a result of CYP (predominately CYP2E1 and CYP1A2) metabolism. Under normal circumstances, NAPQI

Table 1 The incidence of drug-induced liver injury

Country	France	Iceland	South Korea	Spain	United Kingdom	United States	Sweden
DILI incidence (%)	0.139	0.191	0.12	0.03	0.007-0.013	0.10-1.50	0.023

DILI: Drug-induced liver injury.

is detoxified by rapid conjugation with the hepatic glutathione (GSH) and excreted into the bile, thus, APAP usage is nontoxic. Following overdose, APAP saturates both the sulfation and the glucuronidation pathways^[10], enhanced NAPQI production depletes mitochondrial GSH, and the excess NAPQI then reacts with sulfhydryl groups of proteins to form protein adducts^[11]. The interaction of NAPQI with target DNA and proteins in the mitochondria and the formation of protein adducts is thought to be critical for the development of hepatic toxicity^[12,13], leading to oxidative stress, mitochondrial dysfunction^[14,15] and mitogen-activated protein kinase (MAPK) activation (Figure 1). Specific targets in the mitochondria, including glutathione peroxidase (GPx) and the alpha subunit of adenosine triphosphate (ATP) synthase, participate in adduct formation, which was identified using proteomic approaches^[16]. Furthermore, some drugs lead to the obstruction of the bile duct and mediate inhibition of hepatobiliary transporter systems^[17]. Bile salt export pump (BSEP) is an efflux transporter of bile acids (BAs) transport and responsible for the clearance of drugs from liver and the secretion of bile salts into bile. The inhibition of BSEP expression has profound effects on bile acid homeostasis^[18]. The cytotoxic bile acids accumulating in the liver results in liver cell damage, and potentially cirrhosis^[17].

Oxidative and nitrosative stress

Oxidative stress is the result of the generation of ROS, which are a by-product of normal metabolism and have roles in cell signaling and homeostasis. Some DILI-causing drugs increase ROS accumulation through a variety of mechanisms^[19]. Iron overload also amplifies oxidative stress as a catalyst for ROS formation *via* the Fenton reaction, in which H₂O₂ splits into hydroxyl radicals (OH[•]) and hydroxide (OH⁻) (Figure 2). Free radical metabolites participate in the redox process and are capable of inducing cell damage by covalently binding to macromolecules^[20]. Moreover, radical species can oxidize essential cell components and result in mutations in genomic and mitochondrial DNA (p21, p53) and tumor generation.

The role of lipid peroxidation (LPO) remains controversial in APAP hepatotoxicity, and is often considered to be involved in cell death^[21]. However, APAP overdose causes severe liver damage but a minor increase in the levels of LPO in normal animals^[22]. Thus it seems that lipid peroxidation is not a critical event in APAP-induced hepatotoxicity. The cell injury induced by LPO requires not only oxidant formation but also impairment of the

antioxidant defense systems. Additionally, LPO can be a consequence of tissue injury rather than the cause^[23].

Given a toxic dose of APAP, histological necrosis is evident in the liver at 4 h, and tyrosine nitration occurs, indicating peroxynitrite formation^[24]. Enhanced production of superoxide radicals (O₂^{•-}) reacts with nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS), forming peroxynitrite (ONOO⁻)^[25]. Since the O₂^{•-} anion scarcely passes through the hepatocyte cell membrane, this process occurs exclusively within the mitochondria. The highly reactive and potent oxidant ONOO⁻ also causes nitration of protein tyrosine residues^[26] which induces damage to mitochondrial DNA and the opening of the mitochondrial membrane pore.

Mitochondrial oxidative stress alone is not sufficient to ultimately trigger mitochondrial membrane permeability transition (MPT) and induce cell death. A group of protein kinases known as the mitogen-activated protein kinases (MAPKs), one of the most actively studied kinases or signaling pathways, participates in this process. Conventional studies have shown that MAPK pathways include many proteins such as the extracellular signal-related kinases (ERK), c-Jun N-terminal kinases (JNKs) and p38^[27]. The JNK genes, JNK1 and JNK2, are expressed in the liver. A dysregulation of JNK1 and JNK2 protein expression is characteristic in both human and murine models of DILI, and is a potential therapeutic target^[28]. JNK activation occurs early after APAP overdose and is sustained during the process, inducing hepatocyte death. JNK activation has been found in both hepatocytes and infiltrating cells, and is mediated by MAP kinase kinases (MAP2K)^[29], which in turn are phosphorylated and activated by MAP kinase kinase kinases (MAP3K). The apoptosis signal-regulating kinase-1 (ASK1) is involved in APAP-induced JNK elevation^[30] and activated by the dissociation with thioredoxin-1 (Trx-1). The mixed-lineage kinase-3 (MLK3), a member of serine/threonine protein kinases family, mediates the initial phase of JNK activation^[31]. ASK1 and MLK belong to the MAP3K group and different MAP3K group members function cooperatively in the response to oxidative stress. The role of MAP3K in JNK regulation still requires to be further investigated; whilst the activation of JNK can also be influenced by the dose of APAP^[32]. The fact that RIP3-deficiency prevented oxidant stress suggests that RIPK3 acted upstream of JNK activation^[33]. After JNK activation and phosphorylation in the cytosol, JNK binds to the Sab protein on the outer mitochondrial membrane^[34,35], leading to the inactivation of p-Src

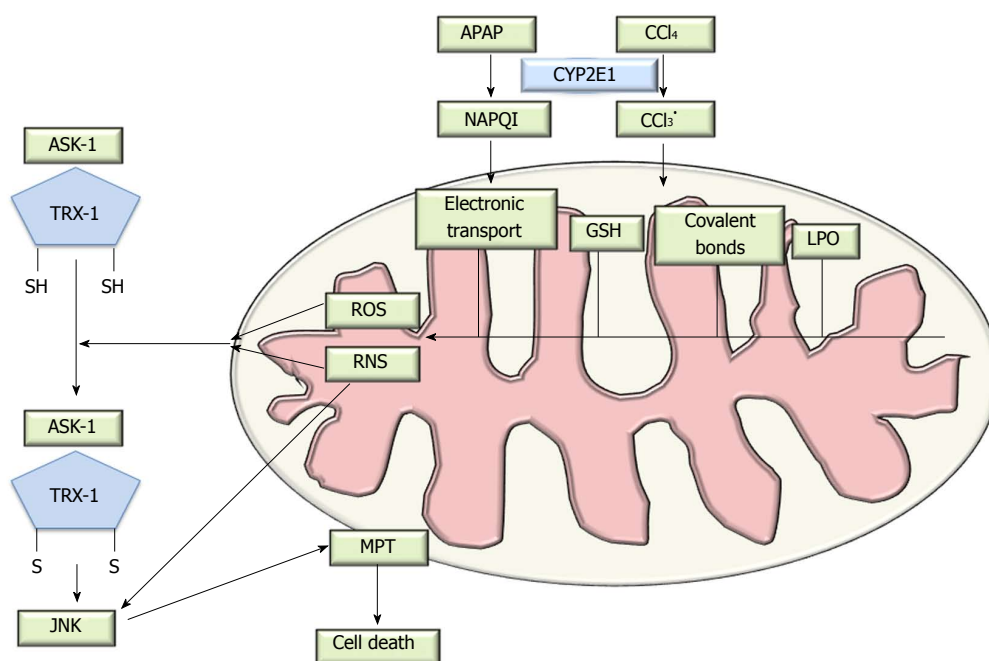


Figure 1 Pathophysiology of drug-induced liver injury. Schematic representation of paracetamol toxicology. Metabolism of acetaminophen (APAP) or carbon tetrachloride (CCl_4) catalyzed by CYP2E1 enzyme causes the generation of an intermediate reactive compound which causes covalent bonds, glutathione (GSH) depletion and increased in oxidative stress. Thioredoxin-1 (Trx-1) normally binds the N-terminal domain of Apoptosis signal-regulating kinase 1 (ASK1) and inhibits kinase activity. Reactive oxygen species (ROS) accumulation oxidizes and consequently removes Trx-1 from Trx-ASK1 complexes, leading to activation of ASK1 and subsequent apoptosis signalling cascade. Then c-Jun N-terminal kinases (JNK) translocates into the mitochondria and alters of the mitochondrial membrane potential, which triggers cell death. DILI: Drug-induced liver injury; MPT: Membrane permeability transition; LPO: Lipid peroxidation; NAPQI: N-acetyl-p-benzoquinone imine.

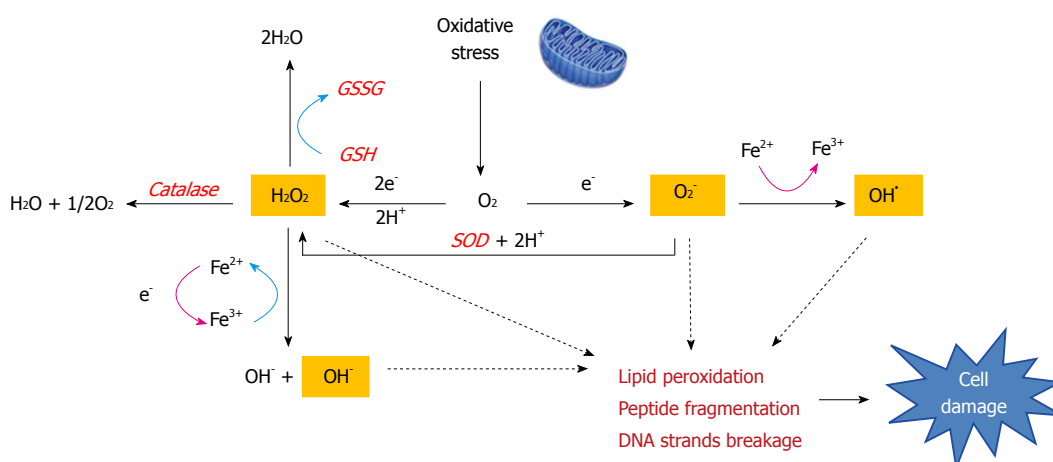


Figure 2 The Fenton reaction in liver disease. Oxidative stress produces large amounts of reactive compounds and cytotoxic free radicals (H_2O_2 , $\text{O}_2^{\cdot-}$ and OH^{\cdot}). The Fenton reaction generates hydroxyl radicals (OH^{\cdot}) from hydrogen peroxide (H_2O_2) and superoxide ($\text{O}_2^{\cdot-}$) catalyzed by iron. This reaction occurs in cells and free radicals can attack the double bonds of non-saturated phospholipids in cell membranes which eventually degrade the structural integrity of cell membranes, impair enzymatic function and cause cross-linking of proteins or strand breaks in DNA. Cells also have an antioxidant enzyme system (catalase, GSH or SOD) is meant to neutralize free radicals and prevent damage.

on the inner mitochondrial membrane, which inhibits electron transport and increases ROS generation and further mitochondrial injury^[36]. Ultimately, the pJNK translocates to the mitochondria and results in downstream signaling events^[34].

Mitochondrial dysfunction

Mitochondrial dysfunction is the main cause of hepatocellular necrosis. The amplification of mitochondrial

oxidative stress can reduce the synthesis of mitochondrial proteins and increase mitochondrial permeability transition. The induction of MPT increases mitochondrial membrane permeability allowing the exit of molecules less than 1500 Daltons^[37], which causes mitochondria to become further depolarized, thus reducing the proton gradient leading to the collapse of the mitochondrial membrane potential (MMP). The mitochondria then swell, rupture and release proteins from the inter-

membrane space^[38], a sequence implicated in cell death pathways such as apoptosis^[39,40]. ROS are also produced because of the opening of the MPT pore, in turn, exaggerating oxidative stress and inducing DNA damage. Furthermore, the β -oxidation respiratory chain is compromised, and the process of ATP production is disrupted, resulting in reduced energy^[41].

Endoplasmic reticulum stress

Various cellular stresses such as ROS or alteration in the cellular calcium (Ca^{2+}) concentration can impair protein folding and initiate the endoplasmic reticulum (ER) stress, which plays a critical role in APAP-induced hepatotoxicity^[42]. Efficient protein folding in the ER requires tight coupling between the subunits of new proteins in the ER lumen and the ER folding capacity^[43]. If the demand for protein folding increases, unfolded or misfolded proteins in the lumen also increase. ER stress is induced late after APAP intoxication (500 mg/kg) in murine models, and becomes significant 12 h following APAP administration^[44]. The mechanisms by which APAP induces ER stress are poorly understood. One hypothesis is the alteration in the microsomes secondary to NAPQI generation. It has been reported that APAP induces an oxidative shift of the ER oxidoreductases, Erp72 and protein disulfide isomerase (PDI) in liver microsomes^[45]. Furthermore, NAPQI can covalently bind to several microsomal proteins such as PDI and calreticulin, which have a significant role in protein folding in the ER, thus inducing ER stress. Another hypothesis suggests that ER stress might be due to ROS overproduction and mitochondrial dysfunction^[46], including loss of the MMP and increase in intracellular Ca^{2+} concentration. Inhibition of BSEP results in not only cholestasis in some cases, but importantly, *via* bile acid retention, causes mitochondrial and ER stress, which may amplify injury or sensitize hepatocytes to other injury mechanisms^[47].

iDILI

iDILI is a rare ADR^[48], and occurs with a variable latency to onset, usually after several weeks or months of continuous treatment with the offending drug but, more importantly, it is unpredictable^[49]. The incidence of iDILI ranges from 1/1000 to 1/200000^[50], depending on the agent. The diagnosis of iDILI relies on the exclusion of other causes of liver injury and detailed medical history. The mechanisms of iDILI have not yet been elucidated. Although it is thought that iDILI is not dose-related, recent studies have supported the prediction of dose-response to some extent^[51]. In general, it is associated with host condition, behavioural factors and drug exposure. Amongst behavioural factors, excessive alcohol consumption and smoking are very common triggers of iDILI. The host factors include genetic and non-genetic-derived iDILI. For example, genetically, it is

considered that iDILI is caused by the deficiency or low activity of drug-metabolizing enzymes and an abnormal immune response. Non-genetic types include existing disease states, pregnancy, age and host gender. In some iDILI reactions, the same mechanisms of intrinsic DILI are involved: ROS, mitochondrial dysfunction and altered bile acid homeostasis. The typical drugs are tacrine and stavudine. Additionally, in some iDILI, after exposure to certain drugs, neoantigens are produced in the liver and can mobilize the immune cells and result in idiosyncratic immunological reactions towards the drugs.

The innate immune response

As a result of hepatocyte damage, iDILI triggers the inflammatory reaction, which involves the innate immune system. The innate immune system in the human liver is mainly composed of Kupffer cells (KCs), neutrophils, monocytes and natural killer cells/natural killer T cells (NK/NKT cells)^[52,53]. In recent years, increasingly studies have confirmed that the innate immune system participates in the pathogenesis of iDILI, but the specific mechanism is still on the controversy. The main hypothesis is that neoantigen stimulates the cells of the innate immune system and creates inflammation by binding to Toll-like receptors (TLRs), scavenger receptors (SCRs) and mannitol receptors (MRs) of macrophages. In patients with iDILI, a large number of macrophages are mobilized in the blood and assemble around the damaged hepatocytes *via* adhesion factors. The proliferation of macrophages is also seen in the bone marrow^[54]. The depletion of KC reduces the expression of IL-10, IL-6 and other mediators, and increases APAP-induced liver injury. Overall, the activation of KC is beneficial because the anti-inflammatory effects outweigh potential toxic effects^[55]. Antigens derived from damage associated molecular patterns (DAMPs)^[56] act as signals to activate innate immune cells. High mobility group box 1 protein (HMGB1) is one of the previously identified DAMPs. HMGB1 induces the infiltration of neutrophils, associates with TLRs and promotes the release of cytokines such as $\text{TNF}\alpha$, $\text{IFN}\gamma$ and IL-1, thereby activating the KC^[57] and aggravating iDILI. In addition, the controversy surrounds the role of NK/NKT cells. Some researchers believe that NK/NKT cells ameliorate the liver injury caused by drugs through secreting $\text{IFN}\gamma$, IL-4 and other cytokines. However, some authors have reported no significant differences in the expression level of protective cytokines from the liver of NKT-cell-deficient mice^[58]. In addition, the released cytokines and chemokines can enhance the adaptive immune response through a variety of mechanisms.

The adaptive immune response

The finding that the liver injury recurs promptly after

the iDILI patient is re-exposed to the offending drug, reflects the involvement of an adaptive immune response. This is in fact predictable, given that the antigen-specific immunocytes still remain in the body. During the process of drug metabolism, drug metabolite covalently binds to hepatic protein or modified proteins expressed on the surface of hepatocytes and form protein haptens (essentially an incomplete antigen). The hapten is released after hepatocyte death or damage and presented by antigen-presenting cells (APCs) in complex with major histocompatibility complex (MHC) class II molecules to cluster of differentiation 4 (CD4⁺) T cells. When recognized as 'foreign' by T cells and following binding to T-cell receptors of CD4⁺T cells, it then activates cluster of differentiation 8 (CD8⁺) T cytotoxic cells *via* secretion of TNF α and IFN γ . CD8⁺T cytotoxic cells mediate cytotoxic reactions through FasL or perforin and induce hepatocellular apoptosis. The anaesthetic drug Halothane exactly triggers this mechanism. Under normal conditions, hapten alone is not sufficient to activate the immune response, therefore activation of the adaptive immune system requires other cell/ tissue threatening events. This is termed the 'Hypothesis of danger signalling'. Indeed, it has been shown that the presence of an inflammatory background is associated with increased susceptibility to iDILI^[59,60]. Infection and inflammation act as the danger signal and further augment the immune response by cell death or cytokine release^[61]. However, the specific mechanisms still need further study.

The "hapten" hypothesis is a dominant mechanism proposed for the creation of neoantigens after drug exposure^[62]. More recently, the 'pharmacological interaction' or 'p-i' model suggests a new hypothesis for activating T-cell-mediated immune responses^[63]. The drug directly binds to either the T-cell receptor (TCR) or human leucocyte antigen (HLA) without intracellular antigen processing^[64-66], and activates T cells in a peptide-independent manner^[67]. This hypothesis might also explain the rapid reaction of T-cells after drug exposure *in vitro*, which is inconsistent with the time-course of antigen processing *in vivo*. Classic drugs that are considered to respond in this way are sulfamethoxazole, lamotrigine and carbamazepine.

The immune genetic polymorphism

A genome-wide association study (GWAS) proved that iDILI is associated with the HLA region on chromosome 6^[68,69]. The HLA polymorphism results in the human body to be more prone to produce adaptive immune responses to certain drugs^[70]. HLA genotyping of 75 amoxicillin-clavulanate hepatotoxicity cases in Spain has also demonstrated phenotype-specific HLA association^[71]. Abacavir, a human immunodeficiency virus reverse transcriptase inhibitor, induces multi-organ toxicity exclusively in patients carrying the HLA-B*57:01 allele^[72]. A GWAS on flucloxacillin hepatotoxicity

(FLUX-DILI) has revealed a strong association with the HLA-B*57:01 allele^[73]. Flucloxacillin is an effective antimicrobial drug against staphylococcal infections and widely used in Europe and Australia. The incidence of cholestatic hepatitis, which is induced by the use of flucloxacillin, is estimated to be 8.5 per 100000 in the first 1 to 45 d after start of treatment^[74]. However, the incidence in the HLA-B*57:01 allele carriers raises more than 3-fold, indicating the HLA-B*57:01 have an added effect on FLUX-DILI^[75].

Immune tolerance

Hepatocyte stress can be detected in the majority of individuals exposed to the insulting drug, especially at high concentrations. However, injury occurs in only a very small number of individuals. Although the liver is considered itself to be an immune-tolerant organ, the variation of susceptibility to the ensuing stress response(s) still exists; only in individuals with low tolerance, will DILI occur. The tolerance phenomenon due to liver immunity can be explained by: apoptosis of activated T cells, immune deviation and immune active suppression^[53]. Antigen-specific CD8⁺ T-cell populations accumulate transiently within the liver before apoptosis^[76]. It is possible that the liver can induce apoptosis of activated T cells through toxic molecules or the deprivation of survival signals^[77]. Hepatocytes can attract apoptotic T cells because specific markers are expressed in the membrane and are recognized by KCs or other cells in the liver. During the hepatic immune responses, there is immune deviation occurring. Klugewitz *et al*^[78], reported that the liver sinusoidal endothelial cells (LSECs) can selectively inhibit T helper-1 (Th1) cells and reduce the production of INF- γ , but LSECs can also activate T helper-2 (Th2) cells leading to an increase in IL-4 secretion. The third mechanism is the result of the unique composition of tolerogenic APCs in the liver. The tolerogenic APCs within the liver include LSECs, KCs, and hepatic dendritic cells (DCs). Although recognized as APCs, these cells are incapable of stimulating antigen-specific T-cell responses^[79]. On the contrary, they trap and interact with the inactive T cells in the liver sinusoid, thereby promoting tolerance. LSECs can act as APCs to some extent, but CD4⁺ or CD8⁺ T cells activated by them cannot further differentiate into Th1 cells or cytotoxic cells. Moreover, hepatic KCs can also suppress T-cell activation through the production of prostaglandins^[80].

SIGNAL TRANSDUCTION AND HEPATOCYTE DEATH

The traditionally recognized forms of cell death are apoptosis and necrosis: apoptosis is a highly regulated and controlled cell death process that does not cause inflammation, while necrosis is a traumatic mode of

cell death that induces inflammation and can promote tissue fibrosis^[81]. Recently, increasing evidence has shown that there is a specific subtype of necrosis, termed necroptosis^[82]. Autophagy was first observed by Keith R Porter and his student in 1962^[83] and has become a controversial topic in the occurrence of DILI, which is not only a protective pathway but also associated with cell death (discussed below).

Apoptosis

Apoptosis is a process of programmed cell death, which maintains physiological homeostasis in the normal human liver^[84]. Characteristic apoptotic morphology includes cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay. Apoptosis can be initiated through two pathways: the intrinsic pathway (also called the mitochondrial pathway), and the extrinsic pathway. The intrinsic pathway is activated by intracellular signals generated when cells are stressed and depends on the release of proteins from the mitochondrial intermembrane space. The extrinsic pathway is activated by extracellular ligands binding to cell-surface death receptors (DRs). Both pathways induce cell death by activating executioner caspases (caspase 3 and 7) or enzymes that degrade protein (*e.g.*, non-caspases, cathepsins, calpains, granzymes, and the proteasome complex, also have roles in mediating and promoting cell death).

Death receptors belong to the TNF family, comprising TNF receptor (TNFR), FAS and TNF-related apoptosis-inducing ligand receptor (TRAIL-R)^[85]. The most widely expressed on the hepatocellular membrane are CD95 (APO-1/FAS) and TNFR1 (CD120a). When DRs are engaged by their ligands, the death domains of the receptors are oligomerized and form a membrane-bound supramolecular structure termed death-inducing signaling complex (DISC), including TNFR-associated death domain (TRADD), receptor interacting protein kinase-1 (RIPK1), cellular inhibitor of apoptosis 1 and 2 (cIAP1 and 2) and TNFR-associated factor 2 (TRAF2) or TRAF5^[86], thereby recruiting caspase-8^[87], and transducing a downstream signal cascade resulting in apoptosis^[88]. The intrinsic pathway is commonly triggered *via* Bid, a protein of the B-cell lymphoma 2 (Bcl-2) family. Caspase-8 mediates the cleavage of Bid and cleaved Bid (tBid) would translocate to mitochondria, lead to mitochondrial outer membrane permeabilization (MOMP) *via* Bax and Bak and induce cytochrome C release to the cytoplasm, which binds to Apaf-1, forming a complex with caspase-9. The activation of procaspase-9 initiates the caspase cascade, promoting cell death. Several intracellular factors can activate this pathway, including ER stress and P53 activation^[89]. ER stress induces an intrinsic cell death pathway termed lipoapoptosis mediated by JNK activation, whereas p53 induces apoptosis through regulation of specific target genes such as Bax. In the

liver, the extrinsic and intrinsic pathways are linked, because hepatocytes require mitochondrial amplification activating caspase-3 for cell death execution^[90].

Necrosis

Conventionally necrosis is thought to be 'unprogrammed' cell death caused by factors external to the cell or tissue, such as infection, virus, toxins, drugs or trauma. This results in the loss of cell membrane integrity with an uncontrolled release of cellular constituents into the extracellular space, thus eliciting an inflammatory response in the surrounding tissue^[91]. The typical features of necrosis include depletion of ATP, ion imbalance and mitochondrial dysfunction. Similar to the intrinsic pathway of apoptosis, mitochondrial injury is the key factor of early-stage necrosis. The change of cell size and the formation of membrane "blebs" are reversible, but once MPT is changed and cellular constituents are released, the cascade is irreversible, and leads to cell rupture. Hepatocellular necrosis also requires the participation of proteases, one of which is calpain-mediating necrosis. Furthermore, recent work has demonstrated that necrosis can be regulated by MPT inhibitor or caspase inhibitors^[92]. RIPK3-mediated mitochondrial fission seems to be also a feature of APAP-induced hepatocyte necrosis. Drp1 translocates to the mitochondria mediated by RIPK3, polymerizes and constricts mitochondria to facilitate organelle division^[33].

Necroptosis

Necroptosis is a "programmed" form of necrosis, which incorporates features of necrosis and apoptosis (Figure 3)^[93]. Necroptosis shares the upstream pathway with apoptosis, and leads to cellular leakage, as seen in necrosis. Necroptosis can lead to cell death without the facilitation of caspase, in the presence of caspase inhibitors^[93]. The typical signaling pathway of necroptosis is mediated by TNF super family member receptor. TNF α can stimulate its receptor TNFR1, and the TNFR-associated death protein TRADD signals to RIPK1 - which recruits RIPK3, to form the necrosome through the interaction of RIP-homology interaction motif (RHIM). RIPK3 then activates mixed lineage kinase domain like pseudokinase (MLKL) by phosphorylation, and p-MLKL subsequently drives oligomerization of MLKL, allowing MLKL to insert into and permeabilize plasma membranes and organelles^[94]. The pro-inflammatory factors are then released and elicit immune responses ensue. The role of necroptosis in APAP-derived DILI is still controversial. Although TNF receptor signalling pathway is the best studied initiating event for necroptosis, there are multiple mechanisms to trigger this mode of cell death and further studies are needed to identify potential activators^[95]. Many studies showed that Nec-1, an inhibitor of RIPK1, protects against APAP hepatotoxicity *in vivo* and *in vitro*^[33,96]. However, RIPK3 and MLKL seem to be dispensable in APAP-derived DILI, whilst RIPK1 is essential for APAP

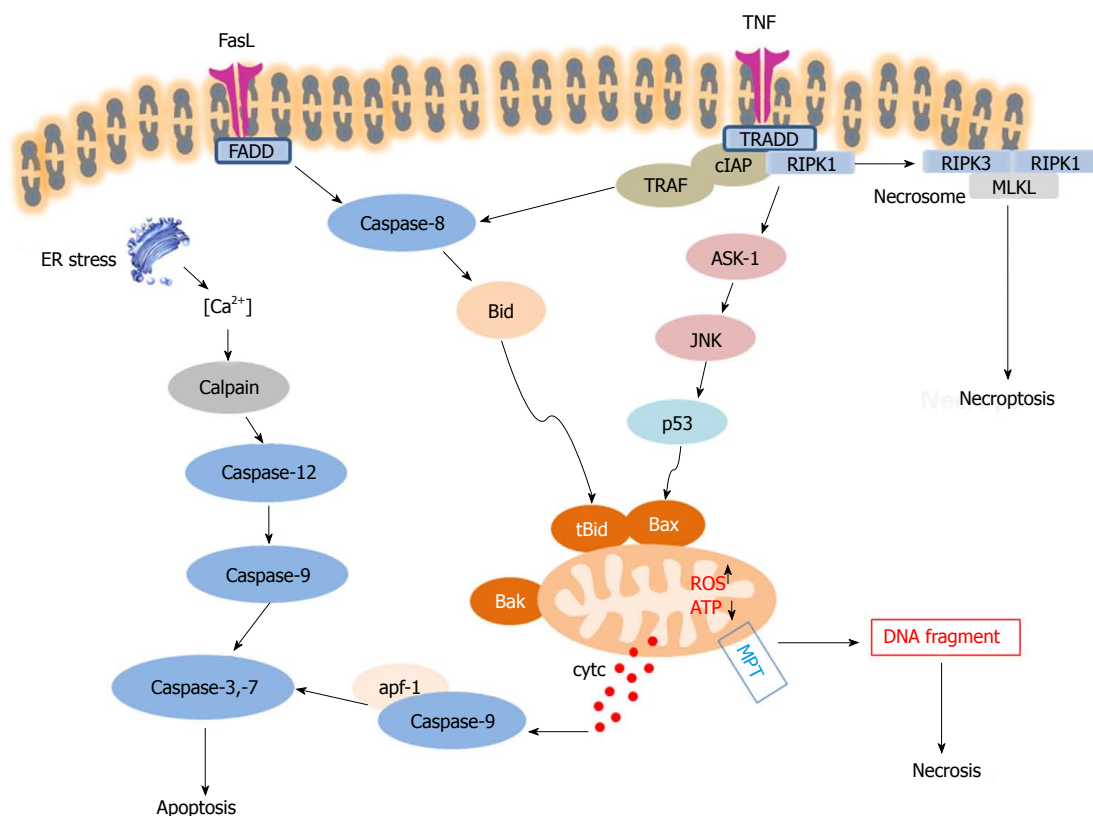


Figure 3 Schematic overview of three different modes of cell death: apoptosis, necrosis and necroptosis. When FasL or Tumor necrosis factor- α (TNF- α) bind to their death receptors (DRs), death domains of DRs are oligomerized and form death-inducing signalling complex (DISC), which recruits caspase-8. Active caspase 8 cleaves Bid into cleaved Bid (tBid), which translocates to mitochondria and cooperates with Bax. Meanwhile, JNKs are activated by Mitogen-activated protein kinases (MAPKs) and pJNK also translocates to the mitochondria via binding to the Sab protein. ROS accumulation and ATP depletion in the mitochondria aggravate mitochondrial damage and induce membrane permeability transition (MPT), resulting in release of cytochrome C, in turn promoting the activation of caspase-9 and caspase-3. Activated caspase-3 then leads to hepatocyte apoptosis. The extrinsic and intrinsic pathways of hepatocyte apoptosis are linked, because hepatocytes require mitochondrial amplification activating caspase-3 for cell death execution. The mitochondrial injury and MPT are also key factors in cascade of events leading to necrosis. Necroptosis shares the upstream pathway with apoptosis. When cellular inhibitors of apoptosis (cIAPs) are depleted, Receptor interacting protein kinase (RIPK)1 and RIPK3 interact with each other via membrane permeability transition (RHIM) domains to form the necrosome, and further recruit and phosphorylate MLKL to initiate necroptosis.

toxicity via JNK activation^[97].

Autophagy

Autophagy functions in a wide variety of physiological and pathophysiological roles as a complex, destructive mechanism of the cell that disassembles unnecessary or dysfunctional components^[98]. Lysosomes are responsible for intracellular autophagy and the degradation of the cell. Autophagy is observable with the formation of autophagosomes, which are double-membrane vesicles originating from rough ER and contain part of the cytoplasm, the organelles and the proteins need to be degraded. Then autophagosomes fuse with lysosomes and initiate the orderly degradation and recycling of cellular components^[99]. In disease, autophagy is considered to be an adaptive response to stress, which promotes survival and plays a vital role in cellular reconstruction. Ni *et al.*^[100] found that autophagy is important for the regulation of APAP protein adducts levels in hepatocytes, and this selective autophagic removal is mediated by ubiquitin and p62^[100,101]. It

is thought that autophagy activated by 5'-adenosine monophosphate-activated protein kinase (AMPK), can lead to adiponectin accumulation, which, in turn, removes damaged mitochondria, thereby ameliorating oxidative stress and hepatotoxicity^[102]. And Parkin-induced mitophagy is also a mechanism of protection against APAP-induced liver injury and necrosis by negatively regulating JNK activity^[36,103] and Mcl-1 degradation and increasing hepatocyte proliferation^[104]. However, chronic deletion and acute knockdown of Parkin have different regulation towards mitophagy and liver injury, the mechanism of which is still unidentified and need further study^[104]. Autophagy has emerged as an exciting new field in DILI and warrants further investigation.

CLINICAL PERSPECTIVES

The clinical manifestations of DILI are usually non-specific. Many patients may have no significant symptoms and only present with elevations in the level of

hepatic biochemical indexes. For about the last half century, the traditional serum biomarkers for detecting DILI in clinics are alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TBIL). However, elevations in these biomarkers take place when hepatocyte injury has already occurred and cannot be used to identify a potential for DILI. In recent years, with the further understanding of the mechanisms of DILI, several new biomarkers have been reported, including apoptosis-related caspase cleaved keratin 18 (cCK18)^[105], necrosis-related HMGB1^[106,107] and microRNA (especially microRNA-122)^[108,109], specific mitochondrial injury biomarker glutamate dehydrogenase (GLDH)^[110], biomarkers reflecting cholestasis (e.g. BAs) as well as genetic biomarkers reflecting the susceptibility to DILI, such as the genetic polymorphisms of HLA, drug metabolizing enzymes and drug transport proteins^[2]. MicroRNA-122 and GLDH have been proposed as more sensitive and specific biomarkers of liver injury than ALT^[111]. APAP-protein adducts and NAPQI are specific biomarkers of APAP-mediated DILI^[112]. And apolipoprotein-A1 and haptoglobin have significant predictive values for the prediction of recovery in DILI patients^[113]. Some of these biomarkers are already being used in early clinical trials. Though current biomarker are not specific to DILI and their value for clinical use still needs to be widely verified, their addition to conventional measurements could soon transform DILI prediction and detection, thereby promoting earlier treatment.

CONCLUSION

The liver works as a central detoxifying organ towards xenobiotics and chemicals. However, during the process of biotransformation to less toxic substances, molecules that can induce liver injury through various pathways are produced. The pathogenesis of DILI is very complex, and the occurrence of DILI is the consequence of multiple factors. Generally, important mechanisms involved in drug-induced hepatic injury can be divided into: (1) reactive metabolite formation *via* metabolism; (2) covalent binding between cellular components with drug; (3) reactive oxygen species generation in the cells; (4) activation of signal transduction pathways that modulate cell death or survival; and (5) cellular mitochondrial damage^[114]. The common forms of hepatocyte death include apoptosis, necrosis, necroptosis and autophagy. The clinical characteristics of DILI are variable, and no specific laboratory tests are predictable for DILI, thereby presenting a major challenge for clinical diagnosis and treatment. Research on the molecular mechanisms underlying DILI will contribute greatly to early-stage screening of new drugs, predicting hepatotoxicity, and the monitoring of drug side-effects, eventually reducing the incidence of DILI, but clinical translation of the

numerous mechanisms remains a challenge, requiring a considerable investment.

REFERENCES

- 1 Lee WM. Drug-induced acute liver failure. *Clin Liver Dis* 2013; **17**: 575-586, viii [PMID: 24099019 DOI: 10.1016/j.cld.2013.07.001]
- 2 Yu YC, Mao YM, Chen CW, Chen JJ, Chen J, Cong WM, Ding Y, Duan ZP, Fu QC, Guo XY, Hu P, Hu XQ, Jia JD, Lai RT, Li DL, Liu YX, Lu LG, Ma SW, Ma X, Nan YM, Ren H, Shen T, Wang H, Wang JY, Wang TL, Wang XJ, Wei L, Xie Q, Xie W, Yang CQ, Yang DL, Yu YY, Zeng MD, Zhang L, Zhao XY, Zhuang H; Drug-induced Liver Injury (DILI) Study Group; Chinese Society of Hepatology (CSH); Chinese Medical Association (CMA). CSH guidelines for the diagnosis and treatment of drug-induced liver injury. *Hepatol Int* 2017; **11**: 221-241 [PMID: 28405790 DOI: 10.1007/s12072-017-9793-2]
- 3 Miguel A, Azevedo LF, Araújo M, Pereira AC. Frequency of adverse drug reactions in hospitalized patients: a systematic review and meta-analysis. *Pharmacoepidemiol Drug Saf* 2012; **21**: 1139-1154 [PMID: 22761169 DOI: 10.1002/pds.3309]
- 4 Feldman M, Friedman LS, Brandt LJ. Sleisenger and Fordtran's Gastrointestinal and Liver Disease. In: Chitturi S TN, Farrell GC, editor Hepatic drug metabolism and liver disease caused by drugs. Philadelphia, 2016
- 5 Bell LN, Chalasani N. Epidemiology of idiosyncratic drug-induced liver injury. *Semin Liver Dis* 2009; **29**: 337-347 [PMID: 19826967 DOI: 10.1055/s-0029-1240002]
- 6 Chalasani NP, Hayashi PH, Bonkovsky HL, Navarro VJ, Lee WM, Fontana RJ; Practice Parameters Committee of the American College of Gastroenterology. ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. *Am J Gastroenterol* 2014; **109**: 950-66; quiz 967 [PMID: 24935270 DOI: 10.1038/ajg.2014.131]
- 7 Vinken M, Maes M, Vanhaecke T, Rogiers V. Drug-induced liver injury: mechanisms, types and biomarkers. *Curr Med Chem* 2013; **20**: 3011-3021 [PMID: 23746274]
- 8 Park K, Williams DP, Naisbitt DJ, Kitteringham NR, Pirmohamed M. Investigation of toxic metabolites during drug development. *Toxicol Appl Pharmacol* 2005; **207**: 425-434 [PMID: 15996699 DOI: 10.1016/j.taap.2005.02.029]
- 9 Jaeschke H, Knight TR, Bajt ML. The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. *Toxicol Lett* 2003; **144**: 279-288 [PMID: 12927346]
- 10 Xie Y, McGill MR, Cook SF, Sharpe MR, Winefield RD, Wilkins DG, Rollins DE, Jaeschke H. Time course of acetaminophen-protein adducts and acetaminophen metabolites in circulation of overdose patients and in HepaRG cells. *Xenobiotica* 2015; **45**: 921-929 [PMID: 25869248 DOI: 10.3109/00498254.2015.1026426]
- 11 McGill MR, Lebofsky M, Norris HR, Slawson MH, Bajt ML, Xie Y, Williams CD, Wilkins DG, Rollins DE, Jaeschke H. Plasma and liver acetaminophen-protein adduct levels in mice after acetaminophen treatment: dose-response, mechanisms, and clinical implications. *Toxicol Appl Pharmacol* 2013; **269**: 240-249 [PMID: 23571099 DOI: 10.1016/j.taap.2013.03.026]
- 12 McGill MR, Williams CD, Xie Y, Ramachandran A, Jaeschke H. Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. *Toxicol Appl Pharmacol* 2012; **264**: 387-394 [PMID: 22980195 DOI: 10.1016/j.taap.2012.08.015]
- 13 Hu J, Ramshesh VK, McGill MR, Jaeschke H, Lemasters JJ. Low Dose Acetaminophen Induces Reversible Mitochondrial Dysfunction Associated with Transient c-Jun N-Terminal Kinase Activation in Mouse Liver. *Toxicol Sci* 2016; **150**: 204-215 [PMID: 26721299 DOI: 10.1093/toxsci/kfv319]
- 14 Jaeschke H, Williams CD, Ramachandran A, Bajt ML. Acetaminophen hepatotoxicity and repair: the role of sterile inflammation and innate immunity. *Liver Int* 2012; **32**: 8-20 [PMID: 21745276 DOI: 10.1111/j.1478-3231.2011.02501.x]

- 15 **Jaeschke H**, Xie Y, McGill MR. Acetaminophen-induced Liver Injury: from Animal Models to Humans. *J Clin Transl Hepatol* 2014; **2**: 153-161 [PMID: 26355817 DOI: 10.14218/JCTH.2014.00014]
- 16 **Qiu Y**, Benet LZ, Burlingame AL. Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. *J Biol Chem* 1998; **273**: 17940-17953 [PMID: 9651401]
- 17 **Pauli-Magnus C**, Meier PJ. Hepatobiliary transporters and drug-induced cholestasis. *Hepatology* 2006; **44**: 778-787 [PMID: 17006912 DOI: 10.1002/hep.21359]
- 18 **Qiu X**, Zhang Y, Liu T, Shen H, Xiao Y, Bournier MJ, Pratt JR, Thompson DC, Marathe P, Humphreys WG, Lai Y. Disruption of BSEP Function in HepaRG Cells Alters Bile Acid Disposition and Is a Susceptible Factor to Drug-Induced Cholestatic Injury. *Mol Pharm* 2016; **13**: 1206-1216 [PMID: 26910619 DOI: 10.1021/acs.molpharmaceut.5b00659]
- 19 **Gómez-Lechón MJ**, Tolosa L, Donato MT. Metabolic activation and drug-induced liver injury: in vitro approaches for the safety risk assessment of new drugs. *J Appl Toxicol* 2016; **36**: 752-768 [PMID: 26691983 DOI: 10.1002/jat.3277]
- 20 **Srivastava A**, Maggs JL, Antoine DJ, Williams DP, Smith DA, Park BK. Role of reactive metabolites in drug-induced hepatotoxicity. *Handb Exp Pharmacol* 2010; **196**: 165-194 [PMID: 20020263 DOI: 10.1007/978-3-642-00663-0_7]
- 21 **Negre-Salvayre A**, Auge N, Ayala V, Basaga H, Boada J, Brenke R, Chapple S, Cohen G, Feher J, Grune T, Lengyel G, Mann GE, Pamplona R, Poli G, Portero-Otin M, Riahi Y, Salvayre R, Sasson S, Serrano J, Shamni O, Siems W, Siow RC, Wiswedel I, Zarkovic K, Zarkovic N. Pathological aspects of lipid peroxidation. *Free Radic Res* 2010; **44**: 1125-1171 [PMID: 20836660 DOI: 10.3109/10715762.2010.498478]
- 22 **Knight TR**, Fariss MW, Farhood A, Jaeschke H. Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. *Toxicol Sci* 2003; **76**: 229-236 [PMID: 12944590 DOI: 10.1093/toxsci/kfg220]
- 23 **Jaeschke H**, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. *Drug Metab Rev* 2012; **44**: 88-106 [PMID: 22229890 DOI: 10.3109/03602532.2011.602688]
- 24 **Hinson JA**, Pike SL, Pumford NR, Mayeux PR. Nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of acetaminophen in mice. *Chem Res Toxicol* 1998; **11**: 604-607 [PMID: 9625727 DOI: 10.1021/tx9800349]
- 25 **Cover C**, Mansouri A, Knight TR, Bajt ML, Lemasters JJ, Pessayre D, Jaeschke H. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. *J Pharmacol Exp Ther* 2005; **315**: 879-887 [PMID: 16081675 DOI: 10.1124/jpet.105.088898]
- 26 **Radi R**, Peluffo G, Alvarez MN, Naviliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med* 2001; **30**: 463-488 [PMID: 11182518]
- 27 **Cargnello M**, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* 2011; **75**: 50-83 [PMID: 21372320 DOI: 10.1128/MMBR.00031-10]
- 28 **Cubero FJ**, Zoubek ME, Hu W, Peng J, Zhao G, Nevzorova YA, Al Masaoudi M, Bechmann LP, Boekschoten MV, Muller M, Preisinger C, Gassler N, Canbay AE, Luedde T, Davis RJ, Liedtke C, Trautwein C. Combined Activities of JNK1 and JNK2 in Hepatocytes Protect Against Toxic Liver Injury. *Gastroenterology* 2016; **150**: 968-981 [PMID: 26708719 DOI: 10.1053/j.gastro.2015.12.019]
- 29 **Tournier C**, Dong C, Turner TK, Jones SN, Flavell RA, Davis RJ. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev* 2001; **15**: 1419-1426 [PMID: 11390361 DOI: 10.1101/gad.888501]
- 30 **Nakagawa H**, Maeda S, Hikiba Y, Ohmae T, Shibata W, Yanai A, Sakamoto K, Ogura K, Noguchi T, Karin M, Ichijo H, Omata M. Deletion of apoptosis signal-regulating kinase 1 attenuates acetaminophen-induced liver injury by inhibiting c-Jun N-terminal kinase activation. *Gastroenterology* 2008; **135**: 1311-1321 [PMID: 18700144 DOI: 10.1053/j.gastro.2008.07.006]
- 31 **Sharma M**, Gadang V, Jaeschke A. Critical role for mixed-lineage kinase 3 in acetaminophen-induced hepatotoxicity. *Mol Pharmacol* 2012; **82**: 1001-1007 [PMID: 22918968 DOI: 10.1124/mol.112.079863]
- 32 **Xie Y**, Ramachandran A, Breckenridge DG, Liles JT, Lebofsky M, Farhood A, Jaeschke H. Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury. *Toxicol Appl Pharmacol* 2015; **286**: 1-9 [PMID: 25818599 DOI: 10.1016/j.taap.2015.03.019]
- 33 **Ramachandran A**, McGill MR, Xie Y, Ni HM, Ding WX, Jaeschke H. Receptor interacting protein kinase 3 is a critical early mediator of acetaminophen-induced hepatocyte necrosis in mice. *Hepatology* 2013; **58**: 2099-2108 [PMID: 23744808 DOI: 10.1002/hep.26547]
- 34 **Hanawa N**, Shinohara M, Saberi B, Gaarde WA, Han D, Kaplowitz N. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. *J Biol Chem* 2008; **283**: 13565-13577 [PMID: 18337250 DOI: 10.1074/jbc.M708916200]
- 35 **Win S**, Than TA, Han D, Petrovic LM, Kaplowitz N. c-Jun N-terminal kinase (JNK)-dependent acute liver injury from acetaminophen or tumor necrosis factor (TNF) requires mitochondrial Sab protein expression in mice. *J Biol Chem* 2011; **286**: 35071-35078 [PMID: 21844199 DOI: 10.1074/jbc.M111.276089]
- 36 **Saito C**, Lemasters JJ, Jaeschke H. c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. *Toxicol Appl Pharmacol* 2010; **246**: 8-17 [PMID: 20423716 DOI: 10.1016/j.taap.2010.04.015]
- 37 **Karch J**, Kwong JQ, Burr AR, Sargent MA, Elrod JW, Peixoto PM, Martinez-Caballero S, Osinska H, Cheng EH, Robbins J, Kinnally KW, Molkentin JD. Bax and Bak function as the outer membrane component of the mitochondrial permeability pore in regulating necrotic cell death in mice. *Elife* 2013; **2**: e00772 [PMID: 23991283 DOI: 10.7554/eLife.00772]
- 38 **Honda HM**, Korge P, Weiss JN. Mitochondria and ischemia/reperfusion injury. *Ann N Y Acad Sci* 2005; **1047**: 248-258 [PMID: 16093501 DOI: 10.1196/annals.1341.022]
- 39 **Masubuchi Y**, Suda C, Horie T. Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice. *J Hepatol* 2005; **42**: 110-116 [PMID: 15629515 DOI: 10.1016/j.jhep.2004.09.015]
- 40 **Ramachandran A**, Lebofsky M, Baines CP, Lemasters JJ, Jaeschke H. Cyclophilin D deficiency protects against acetaminophen-induced oxidant stress and liver injury. *Free Radic Res* 2011; **45**: 156-164 [PMID: 20942566 DOI: 10.3109/10715762.2010.520319]
- 41 **Will Y**, Dykens J. Mitochondrial toxicity assessment in industry—a decade of technology development and insight. *Expert Opin Drug Metab Toxicol* 2014; **10**: 1061-1067 [PMID: 25023361 DOI: 10.1517/17425255.2014.939628]
- 42 **Kalinek GM**, Thein P, Parsa A, Yorgason J, Luxford W, Urrutia R, Kalinec F. Acetaminophen and NAPQI are toxic to auditory cells via oxidative and endoplasmic reticulum stress-dependent pathways. *Hear Res* 2014; **313**: 26-37 [PMID: 24793116 DOI: 10.1016/j.heares.2014.04.007]
- 43 **Halperin L**, Jung J, Michalak M. The many functions of the endoplasmic reticulum chaperones and folding enzymes. *IUBMB Life* 2014; **66**: 318-326 [PMID: 24839203 DOI: 10.1002/iub.1272]
- 44 **Uzi D**, Barda L, Scaiewicz V, Mills M, Mueller T, Gonzalez-Rodriguez A, Valverde AM, Iwawaki T, Nahmias Y, Xavier R, Chung RT, Tirosh B, Shibolet O. CHOP is a critical regulator of acetaminophen-induced hepatotoxicity. *J Hepatol* 2013; **59**: 495-503 [PMID: 23665281 DOI: 10.1016/j.jhep.2013.04.024]
- 45 **Letelier ME**, López-Valladares M, Peredo-Silva L, Rojas-Sepúlveda D, Aracena P. Microsomal oxidative damage promoted by acetaminophen metabolism. *Toxicol In Vitro* 2011; **25**:

- 1310-1313 [PMID: 21569833 DOI: 10.1016/j.tiv.2011.04.022]
- 46 **Vineetha VP**, Soumya RS, Raghu KG. Phloretin ameliorates arsenic trioxide induced mitochondrial dysfunction in H9c2 cardiomyoblasts mediated via alterations in membrane permeability and ETC complexes. *Eur J Pharmacol* 2015; **754**: 162-172 [PMID: 25746422 DOI: 10.1016/j.ejphar.2015.02.036]
 - 47 **Morgan RE**, Trauner M, van Staden CJ, Lee PH, Ramachandran B, Eschenberg M, Afshari CA, Qualls CW Jr, Lightfoot-Dunn R, Hamadeh HK. Interference with bile salt export pump function is a susceptibility factor for human liver injury in drug development. *Toxicol Sci* 2010; **118**: 485-500 [PMID: 20829430 DOI: 10.1093/toxsci/kfq269]
 - 48 **Cosgrove BD**, Alexopoulos LG, Hang TC, Hendriks BS, Sorger PK, Griffith LG, Lauffenburger DA. Cytokine-associated drug toxicity in human hepatocytes is associated with signaling network dysregulation. *Mol Biosyst* 2010; **6**: 1195-1206 [PMID: 20361094 DOI: 10.1039/b926287c]
 - 49 **Fontana RJ**, Hayashi PH, Gu J, Reddy KR, Barnhart H, Watkins PB, Serrano J, Lee WM, Chalasani N, Stolz A, Davern T, Talwakar JA; DILIN Network. Idiosyncratic drug-induced liver injury is associated with substantial morbidity and mortality within 6 months from onset. *Gastroenterology* 2014; **147**: 96-108.e4 [PMID: 24681128 DOI: 10.1053/j.gastro.2014.03.045]
 - 50 **Goldman L**, Schafer AI. Goldman-Cecil medicine. In: WM L, editor 152-Toxin- and drug-induced liver disease. Philadelphia, 2012: 979-984
 - 51 **Uetrecht J**, Naisbitt DJ. Idiosyncratic adverse drug reactions: current concepts. *Pharmacol Rev* 2013; **65**: 779-808 [PMID: 23476052 DOI: 10.1124/pr.113.007450]
 - 52 **Tujios S**, Fontana RJ. Mechanisms of drug-induced liver injury: from bedside to bench. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 202-211 [PMID: 21386809 DOI: 10.1038/nrgastro.2011.22]
 - 53 **Ju C**, Reilly T. Role of immune reactions in drug-induced liver injury (DILI). *Drug Metab Rev* 2012; **44**: 107-115 [PMID: 22235834 DOI: 10.3109/03602532.2011.645579]
 - 54 **Fisher JE**, McKenzie TJ, Lillegard JB, Yu Y, Juskewitch JE, Nedredal GI, Brunn GJ, Yi ES, Malhi H, Smyrk TC, Nyberg SL. Role of Kupffer cells and toll-like receptor 4 in acetaminophen-induced acute liver failure. *J Surg Res* 2013; **180**: 147-155 [PMID: 23260383 DOI: 10.1016/j.jss.2012.11.051]
 - 55 **Jaeschke H**. Innate immunity and acetaminophen-induced liver injury: why so many controversies? *Hepatology* 2008; **48**: 699-701 [PMID: 18752320 DOI: 10.1002/hep.22556]
 - 56 **Martin-Murphy BV**, Holt MP, Ju C. The role of damage associated molecular pattern molecules in acetaminophen-induced liver injury in mice. *Toxicol Lett* 2010; **192**: 387-394 [PMID: 19931603 DOI: 10.1016/j.toxlet.2009.11.016]
 - 57 **Kubes P**, Mehal WZ. Sterile inflammation in the liver. *Gastroenterology* 2012; **143**: 1158-1172 [PMID: 22982943 DOI: 10.1053/j.gastro.2012.09.008]
 - 58 **Martin-Murphy BV**, Kominsky DJ, Orlicky DJ, Donohue TM Jr, Ju C. Increased susceptibility of natural killer T-cell-deficient mice to acetaminophen-induced liver injury. *Hepatology* 2013; **57**: 1575-1584 [PMID: 23150232 DOI: 10.1002/hep.26134]
 - 59 **Laverty HG**, Antoine DJ, Benson C, Chaponda M, Williams D, Kevin Park B. The potential of cytokines as safety biomarkers for drug-induced liver injury. *Eur J Clin Pharmacol* 2010; **66**: 961-976 [PMID: 20694460 DOI: 10.1007/s00228-010-0862-x]
 - 60 **Maiuri AR**, Breier AB, Gora LF, Parkins RV, Ganey PE, Roth RA. Cytotoxic Synergy Between Cytokines and NSAIDs Associated With Idiosyncratic Hepatotoxicity Is Driven by Mitogen-Activated Protein Kinases. *Toxicol Sci* 2015; **146**: 265-280 [PMID: 25953702 DOI: 10.1093/toxsci/kfv091]
 - 61 **Jiang J**, Mathijs K, Timmermans L, Claessen SM, Hecka A, Weusten J, Peters R, van Delft JH, Kleinjans JCS, Jennen DGJ, de Kok TM. Omics-based identification of the combined effects of idiosyncratic drugs and inflammatory cytokines on the development of drug-induced liver injury. *Toxicol Appl Pharmacol* 2017; **332**: 100-108 [PMID: 28733206 DOI: 10.1016/j.taap.2017.07.014]
 - 62 **Tailor A**, Faulkner L, Naisbitt DJ, Park BK. The chemical, genetic and immunological basis of idiosyncratic drug-induced liver injury. *Hum Exp Toxicol* 2015; **34**: 1310-1317 [PMID: 26614821 DOI: 10.1177/0960327115606529]
 - 63 **White KD**, Chung WH, Hung SI, Mallal S, Phillips EJ. Evolving models of the immunopathogenesis of T cell-mediated drug allergy: The role of host, pathogens, and drug response. *J Allergy Clin Immunol* 2015; **136**: 219-34; quiz 235 [PMID: 26254049 DOI: 10.1016/j.jaci.2015.05.050]
 - 64 **Zanni MP**, von Greylitz S, Schnyder B, Brander KA, Frutig K, Hari Y, Valitutti S, Pichler WJ. HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. *J Clin Invest* 1998; **102**: 1591-1598 [PMID: 9788973 DOI: 10.1172/JCI3544]
 - 65 **Zanni MP**, von Greylitz S, Schnyder B, Wendland T, Pichler WJ. Allele-unrestricted presentation of lidocaine by HLA-DR molecules to specific alphabeta+ T cell clones. *Int Immunol* 1998; **10**: 507-515 [PMID: 9620607]
 - 66 **Schnyder B**, Mauri-Hellweg D, Zanni M, Bettens F, Pichler WJ. Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *J Clin Invest* 1997; **100**: 136-141 [PMID: 9202065 DOI: 10.1172/JCI119505]
 - 67 **Schnyder B**, Burkhart C, Schnyder-Frutig K, von Greylitz S, Naisbitt DJ, Pirmohamed M, Park BK, Pichler WJ. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J Immunol* 2000; **164**: 6647-6654 [PMID: 10843725]
 - 68 **Spraggs CF**, Budde LR, Briley LP, Bing N, Cox CJ, King KS, Whittaker JC, Mooser VE, Preston AJ, Stein SH, Cardon LR. HLA-DQA1*02:01 is a major risk factor for lapatinib-induced hepatotoxicity in women with advanced breast cancer. *J Clin Oncol* 2011; **29**: 667-673 [PMID: 21245432 DOI: 10.1200/JCO.2010.31.3197]
 - 69 **Lucena MI**, Molokhia M, Shen Y, Urban TJ, Aithal GP, Andrade RJ, Day CP, Ruiz-Cabello F, Donaldson PT, Stephens C, Pirmohamed M, Romero-Gomez M, Navarro JM, Fontana RJ, Miller M, Groome M, Bondon-Guitton E, Conforti A, Stricker BH, Carvajal A, Ibanez L, Yue QY, Eichelbaum M, Floratos A, Pe'er I, Daly MJ, Goldstein DB, Dillon JF, Nelson MR, Watkins PB, Daly AK; Spanish DILI Registry; EUDRAGENE; DILIN; DILIGEN; International SAEC. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. *Gastroenterology* 2011; **141**: 338-347 [PMID: 21570397 DOI: 10.1053/j.gastro.2011.04.001]
 - 70 **Alfirevic A**, Gonzalez-Galarza F, Bell C, Martinsson K, Platt V, Bretland G, Evelyn J, Lichtenfels M, Cederbrant K, French N, Naisbitt D, Park BK, Jones AR, Pirmohamed M. In silico analysis of HLA associations with drug-induced liver injury: use of a HLA-genotyped DNA archive from healthy volunteers. *Genome Med* 2012; **4**: 51 [PMID: 22732016 DOI: 10.1186/gm350]
 - 71 **Stephens C**, López-Nevot MÁ, Ruiz-Cabello F, Ulzurrun E, Soriano G, Romero-Gómez M, Moreno-Casares A, Lucena MI, Andrade RJ. HLA alleles influence the clinical signature of amoxicillin-clavulanate hepatotoxicity. *PLoS One* 2013; **8**: e68111 [PMID: 23874514 DOI: 10.1371/journal.pone.0068111]
 - 72 **Song B**, Aoki S, Liu C, Susukida T, Ito K. An animal model of abacavir-induced HLA-mediated liver injury. *Toxicol Sci* 2018; **162**: 713-723 [PMID: 29319822 DOI: 10.1093/toxsci/kfy001]
 - 73 **Daly AK**, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, Daly MJ, Goldstein DB, John S, Nelson MR, Graham J, Park BK, Dillon JF, Bernal W, Cordell HJ, Pirmohamed M, Aithal GP, Day CP; DILIGEN Study; International SAE Consortium. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet* 2009; **41**: 816-819 [PMID: 19483685 DOI: 10.1038/ng.379]
 - 74 **Russmann S**, Kaye JA, Jick SS, Jick H. Risk of cholestatic liver disease associated with flucloxacillin and flucloxacillin prescribing habits in the UK: cohort study using data from the UK General Practice Research Database. *Br J Clin Pharmacol* 2005; **60**: 76-82 [PMID: 15963097 DOI: 10.1111/j.1365-2125.2005.02370.x]
 - 75 **Andrews E**, Armstrong M, Tugwood J, Swan D, Glaves P,

- Pirmohamed M, Aithal GP, Wright MC, Day CP, Daly AK. A role for the pregnane X receptor in flucloxacillin-induced liver injury. *Hepatology* 2010; **51**: 1656-1664 [PMID: 20222094 DOI: 10.1002/hep.23549]
- 76 **Holt MP**, Ju C. Mechanisms of drug-induced liver injury. *AAPS J* 2006; **8**: E48-E54 [PMID: 16584133 DOI: 10.1208/aapsj080106]
- 77 **Iwai Y**, Terawaki S, Ikegawa M, Okazaki T, Honjo T. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J Exp Med* 2003; **198**: 39-50 [PMID: 12847136 DOI: 10.1084/jem.20022235]
- 78 **Klugewitz K**, Blumenthal-Barby F, Schrage A, Knolle PA, Hamann A, Crispe IN. Immunomodulatory effects of the liver: deletion of activated CD4⁺ effector cells and suppression of IFN- γ -producing cells after intravenous protein immunization. *J Immunol* 2002; **169**: 2407-2413 [PMID: 12193708]
- 79 **Ju C**, McCoy JP, Chung CJ, Graf ML, Pohl LR. Tolerogenic role of Kupffer cells in allergic reactions. *Chem Res Toxicol* 2003; **16**: 1514-1519 [PMID: 14680364 DOI: 10.1021/tx0341761]
- 80 **You Q**, Cheng L, Kedl RM, Ju C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology* 2008; **48**: 978-990 [PMID: 18712788 DOI: 10.1002/hep.22395]
- 81 **Danial NN**, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; **116**: 205-219 [PMID: 14744432]
- 82 **Linkermann A**, Green DR. Necroptosis. *N Engl J Med* 2014; **370**: 455-465 [PMID: 24476434 DOI: 10.1056/NEJMra1310050]
- 83 **Ashford TP**, Porter KR. Cytoplasmic components in hepatic cell lysosomes. *J Cell Biol* 1962; **12**: 198-202 [PMID: 13862833]
- 84 **Wang J**, Yuan L, Xiao H, Xiao C, Wang Y, Liu X. Momordin Ic induces HepG2 cell apoptosis through MAPK and PI3K/Akt-mediated mitochondrial pathways. *Apoptosis* 2013; **18**: 751-765 [PMID: 23417763 DOI: 10.1007/s10495-013-0820-z]
- 85 **Iorga A**, Dara L, Kaplowitz N. Drug-Induced Liver Injury: Cascade of Events Leading to Cell Death, Apoptosis or Necrosis. *Int J Mol Sci* 2017; **18**: pii: E1018 [PMID: 28486401 DOI: 10.3390/ijms18051018]
- 86 **Ashkenazi A**, Salvesen G. Regulated cell death: signaling and mechanisms. *Annu Rev Cell Dev Biol* 2014; **30**: 337-356 [PMID: 25150011 DOI: 10.1146/annurev-cellbio-100913-013226]
- 87 **Reinehr R**, Häussinger D. CD95 death receptor and epidermal growth factor receptor (EGFR) in liver cell apoptosis and regeneration. *Arch Biochem Biophys* 2012; **518**: 2-7 [PMID: 22182753 DOI: 10.1016/j.abb.2011.12.004]
- 88 **Micheau O**, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 2003; **114**: 181-190 [PMID: 12887920]
- 89 **Shore GC**, Papa FR, Oakes SA. Signaling cell death from the endoplasmic reticulum stress response. *Curr Opin Cell Biol* 2011; **23**: 143-149 [PMID: 21146390 DOI: 10.1016/j.ceb.2010.11.003]
- 90 **Scaffidi C**, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; **17**: 1675-1687 [PMID: 9501089 DOI: 10.1093/emboj/17.6.1675]
- 91 **Festjens N**, Vanden Berghe T, Vandenabeele P. Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. *Biochim Biophys Acta* 2006; **1757**: 1371-1387 [PMID: 16950166 DOI: 10.1016/j.bbabi.2006.06.014]
- 92 **Schwab BL**, Guerini D, Didszun C, Bano D, Ferrando-May E, Fava E, Tam J, Xu D, Xanthoudakis S, Nicholson DW, Carafoli E, Nicotera P. Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis. *Cell Death Differ* 2002; **9**: 818-831 [PMID: 12107825 DOI: 10.1038/sj.cdd.4401042]
- 93 **Vanden Berghe T**, Linkermann A, Jouan-Lanhoutet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol* 2014; **15**: 135-147 [PMID: 24452471 DOI: 10.1038/nrm3737]
- 94 **Su L**, Quade B, Wang H, Sun L, Wang X, Rizo J. A plug release mechanism for membrane permeation by MLKL. *Structure* 2014; **22**: 1489-1500 [PMID: 25220470 DOI: 10.1016/j.str.2014.07.014]
- 95 **Vanlangenakker N**, Vanden Berghe T, Vandenabeele P. Many stimuli pull the necrotic trigger, an overview. *Cell Death Differ* 2012; **19**: 75-86 [PMID: 22075985 DOI: 10.1038/cdd.2011.164]
- 96 **Takemoto K**, Hatano E, Iwaisako K, Takeiri M, Noma N, Ohmae S, Toriguchi K, Tanabe K, Tanaka H, Seo S, Taura K, Machida K, Takeda N, Saji S, Uemoto S, Asagiri M. Necrostatin-1 protects against reactive oxygen species (ROS)-induced hepatotoxicity in acetaminophen-induced acute liver failure. *FEBS Open Bio* 2014; **4**: 777-787 [PMID: 25349782 DOI: 10.1016/j.fob.2014.08.007]
- 97 **Dara L**, Johnson H, Suda J, Win S, Gaarde W, Han D, Kaplowitz N. Receptor interacting protein kinase 1 mediates murine acetaminophen toxicity independent of the necrosome and not through necroptosis. *Hepatology* 2015; **62**: 1847-1857 [PMID: 26077809 DOI: 10.1002/hep.27939]
- 98 **Klionsky DJ**. Autophagy revisited: a conversation with Christian de Duve. *Autophagy* 2008; **4**: 740-743 [PMID: 18567941]
- 99 **Kobayashi S**. Choose Delicately and Reuse Adequately: The Newly Revealed Process of Autophagy. *Biol Pharm Bull* 2015; **38**: 1098-1103 [PMID: 26235572 DOI: 10.1248/bpb.b15-00096]
- 100 **Ni HM**, McGill MR, Chao X, Du K, Williams JA, Xie Y, Jaeschke H, Ding WX. Removal of acetaminophen protein adducts by autophagy protects against acetaminophen-induced liver injury in mice. *J Hepatol* 2016; **65**: 354-362 [PMID: 27151180 DOI: 10.1016/j.jhep.2016.04.025]
- 101 **Katsuragi Y**, Ichimura Y, Komatsu M. p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J* 2015; **282**: 4672-4678 [PMID: 26432171 DOI: 10.1111/febs.13540]
- 102 **Lin Z**, Wu F, Lin S, Pan X, Jin L, Lu T, Shi L, Wang Y, Xu A, Li X. Adiponectin protects against acetaminophen-induced mitochondrial dysfunction and acute liver injury by promoting autophagy in mice. *J Hepatol* 2014; **61**: 825-831 [PMID: 24882054 DOI: 10.1016/j.jhep.2014.05.033]
- 103 **Gunawan BK**, Liu ZX, Han D, Hanawa N, Gaarde WA, Kaplowitz N. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* 2006; **131**: 165-178 [PMID: 16831600 DOI: 10.1053/j.gastro.2006.03.045]
- 104 **Williams JA**, Ni HM, Haynes A, Manley S, Li Y, Jaeschke H, Ding WX. Chronic Deletion and Acute Knockdown of Parkin Have Differential Responses to Acetaminophen-induced Mitophagy and Liver Injury in Mice. *J Biol Chem* 2015; **290**: 10934-10946 [PMID: 25752611 DOI: 10.1074/jbc.M114.602284]
- 105 **Antoine DJ**, Williams DP, Kipar A, Jenkins RE, Regan SL, Sathish JG, Kitteringham NR, Park BK. High-mobility group box-1 protein and keratin-18, circulating serum proteins informative of acetaminophen-induced necrosis and apoptosis in vivo. *Toxicol Sci* 2009; **112**: 521-531 [PMID: 19783637 DOI: 10.1093/toxsci/kfp235]
- 106 **Antoine DJ**, Dear JW, Lewis PS, Platt V, Coyle J, Masson M, Thanacoody RH, Gray AJ, Webb DJ, Moggs JG, Bateman DN, Goldring CE, Park BK. Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. *Hepatology* 2013; **58**: 777-787 [PMID: 23390034 DOI: 10.1002/hep.26294]
- 107 **Antoine DJ**, Jenkins RE, Dear JW, Williams DP, McGill MR, Sharpe MR, Craig DG, Simpson KJ, Jaeschke H, Park BK. Molecular forms of HMGB1 and keratin-18 as mechanistic biomarkers for mode of cell death and prognosis during clinical acetaminophen hepatotoxicity. *J Hepatol* 2012; **56**: 1070-1079 [PMID: 22266604 DOI: 10.1016/j.jhep.2011.12.019]
- 108 **Wang K**, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, Hood LE, Galas DJ. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc Natl Acad Sci USA* 2009; **106**: 4402-4407 [PMID: 19246379 DOI: 10.1073/pnas.0813371106]
- 109 **Dear JW**, Antoine DJ, Starkey-Lewis P, Goldring CE, Park BK. Early detection of paracetamol toxicity using circulating liver microRNA and markers of cell necrosis. *Br J Clin Pharmacol* 2014; **77**: 904-905 [PMID: 23879521 DOI: 10.1111/bcp.12214]
- 110 **McGill MR**, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *J Clin Invest* 2012; **122**: 1574-1583 [PMID: 22378043 DOI: 10.1172/JCI59755]

- 111 **Church RJ**, Watkins PB. The transformation in biomarker detection and management of drug-induced liver injury. *Liver Int* 2017; **37**: 1582-1590 [PMID: 28386997 DOI: 10.1111/liv.13441]
- 112 **Fannin RD**, Russo M, O'Connell TM, Gerrish K, Winnike JH, Macdonald J, Newton J, Malik S, Sieber SO, Parker J, Shah R, Zhou T, Watkins PB, Paules RS. Acetaminophen dosing of humans results in blood transcriptome and metabolome changes consistent with impaired oxidative phosphorylation. *Hepatology* 2010; **51**: 227-236 [PMID: 19918972 DOI: 10.1002/hep.23330]
- 113 **Peta V**, Tse C, Perazzo H, Munteanu M, Ngo Y, Ngo A, Ramanujam N, Verglas L, Mallet M, Ratzu V, Thabut D, Rudler M, Thibault V, Schuppe-Koistinen I, Bonnefont-Rousselot D, Hainque B, Imbert-Bismut F, Merz M, Kullak-Ublick G, Andrade R, van Boemmel F, Schott E, Poynard T; Drug Induced Liver Injury- Groupe Hospitalier Pitié-Salpêtrière; Drug Induced Liver Group of the Injury Safer and Faster Evidence-based Translation consortium. Serum apolipoprotein A1 and haptoglobin, in patients with suspected drug-induced liver injury (DILI) as biomarkers of recovery. *PLoS One* 2017; **12**: e0189436 [PMID: 29287080 DOI: 10.1371/journal.pone.0189436]
- 114 **Han D**, Shinohara M, Ybanez MD, Saberi B, Kaplowitz N. Signal transduction pathways involved in drug-induced liver injury. *Handb Exp Pharmacol* 2010; **(196)**: 267-310 [PMID: 20020266 DOI: 10.1007/978-3-642-00663-0_10]

P- Reviewer: Higuera-de la Tijera F, Xiao J, Tanaka N
S- Editor: Gong ZM **L- Editor:** A **E- Editor:** Huang Y



Thrombocytopenia after liver transplantation: Should we care?

Kazuhiro Takahashi, Shunji Nagai, Mohamed Safwan, Chen Liang, Nobuhiro Ohkohchi

Kazuhiro Takahashi, Chen Liang, Nobuhiro Ohkohchi, Department of Surgery, Division of Gastroenterological and Hepatobiliary Surgery, and Organ Transplantation, University of Tsukuba, Tsukuba, Ibaraki 3058575, Japan

Shunji Nagai, Mohamed Safwan, Transplant and Hepatobiliary Surgery, Henry Ford Hospital, Detroit, ML 48202, United States

ORCID number: Kazuhiro Takahashi (0000-0003-1089-0644); Shunji Nagai (0000-0003-2612-8427); Mohamed Safwan (0000-0002-3299-9045); Chen Liang (0000-0002-4528-7303); Nobuhiro Ohkohchi (0000-0003-2779-1247).

Author contributions: Takahashi K, Nagai S, Safwan M, Liang C and Ohkohchi N contributed equally to this work; Takahashi K wrote the paper.

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other coauthors contributed their efforts in this manuscript.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Nobuhiro Ohkohchi, MD, PhD, Department of Surgery, Division of Gastroenterological and Hepatobiliary Surgery, and Organ Transplantation, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan. nokochi3@md.tsukuba.ac.jp
Telephone: +81-29-8533221
Fax: +81-29-8533222

Received: January 30, 2018

Peer-review started: January 31, 2018

First decision: February 11, 2018

Revised: March 6, 2018

Accepted: March 18, 2018

Article in press: March 18, 2018

Published online: April 7, 2018

Abstract

Transient thrombocytopenia is a common phenomenon after liver transplantation. After liver transplantation (LT), platelet count decreases and reaches a nadir on postoperative days 3-5, with an average reduction in platelet counts of 60%; platelet count recovers to preoperative levels approximately two weeks after LT. The putative mechanisms include haemodilution, decreased platelet production, increased sequestration, medications, infections, thrombosis, or combination of these processes. However, the precise mechanisms remain unclear. The role of platelets in liver transplantation has been highlighted in recent years, and particular attention has been given to their effects beyond hemostasis and thrombosis. Previous studies have demonstrated that perioperative thrombocytopenia causes poor graft regeneration, increases the incidence of postoperative morbidity, and deteriorates the graft and decreases patient survival in both the short and long term after liver transplantation. Platelet therapies to increase perioperative platelet counts, such as thrombopoietin, thrombopoietin receptor agonist, platelet transfusion, splenectomy, and intravenous immunoglobulin treatment might have a potential for improving graft survival, however clinical trials are lacking. Further studies are warranted to detect direct evidence on whether thrombocytopenia is the cause or result of poor-graft function and postoperative complications, and to determine who needs platelet therapies in order to prevent postoperative complications and thus improve post-transplant outcomes.

Key words: Thrombocytopenia; Liver regeneration; Platelet therapy; Platelet; Thrombopoietin receptor agonist; Intravenous immunoglobulin treatment; Liver transplantation

© The Author(s) 2018. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Transient thrombocytopenia is commonly seen after liver transplantation, and many studies have demonstrated that perioperative thrombocytopenia is associated with deterioration of the graft and decreased patient survival after liver transplantation. The role of platelets in liver transplantation has recently been highlighted, and particular attention has been given to their effects beyond hemostasis and thrombosis. Platelet therapies that increase platelet count, such as thrombopoietin, thrombopoietin receptor agonist, platelet transfusion, splenectomy, and intravenous immunoglobulin treatment, have a potential role for improving graft survival; however, clinical trials are still lacking, and further studies are warranted.

Takahashi K, Nagai S, Safwan M, Liang C, Ohkohchi N. Thrombocytopenia after liver transplantation: Should we care? *World J Gastroenterol* 2018; 24(13): 1386-1397 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1386.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1386>

INTRODUCTION

Platelets are anucleate cytoplasmic discs derived from megakaryocytes in the bone marrow^[1-3]. The normal life span of platelets is 8-10 d, and they are removed from circulation by sequestration in the spleen^[4]. Platelets contain three types of secretory granules: alpha granules, dense granules, and lysosomal granules. Each granule contains growth factors and cytokines, such as platelet-derived growth factor, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), serotonin, epidermal growth factor, and transforming growth factor- β ^[5,6]. Platelets have major roles in hemostasis, thrombosis, inflammation, and vascular biology and have recently been discovered to have additional functions in antimicrobial defense, angiogenesis, tissue repair and regeneration^[7-10].

Orthotopic liver transplantation (LT) is the treatment of choice for patients with end-stage liver disease and hepatocellular carcinoma within the Milan criteria^[11,12]. The short and long term outcomes of this procedure have dramatically improved as a result of innovations in both immune suppression and surgical techniques^[11]. The total number of adult LTs performed in the world was 27759 in 2015, of which living donor LT (LDLT) accounted for 21%^[13]. The number of transplant candidates on a waiting list has also steadily increased despite organ shortage being a worldwide issue. According to the 2015 annual report from the Scientific Registry of Transplant Recipients, the incidence of graft failure in the United States continues to decrease; in 2014, there were 6-mo graft failure rates of 7.8%

and 12.5% and 1-year rates of 10.3% and 15.1% in deceased donor LT (DDLT) and in LDLT, respectively^[14].

Post-transplant thrombocytopenia occurs in the majority of patients immediately after LT, with reported incidences of up to 90%^[15,16]. After LT, platelet count decreases and reaches a nadir on postoperative days (PODs) 3-5, with an average reduction in platelet counts of 60%; platelet count recovers to preoperative levels approximately two weeks after LT^[17]. Thrombocytopenia in the postoperative period is not simply an academic observation but can lead to catastrophic events, such as postoperative bleeding, cerebral hemorrhage, and infection, which eventually lead to graft failure and mortality. The putative mechanisms involved include decreased platelet production, increased platelet consumption, sequestration in the liver graft or spleen, dilution, medication, or a combination of these processes^[18-22]. However, the precise mechanism is still unknown. In this review, we aimed to describe the clinical and experimental evidence of the role of platelets in LT. This review differs from previous reviews in the following three points. First, we describe the role of platelets in LT specifically with a focus on "post-transplant thrombocytopenia". Second, the involvement of platelets in DDLT and LDLT are described separately, since they are different in many aspects including the graft quality, the length of ischemia, and the recovery of portal hypertension after LT. Third, we delve into the potential mechanisms of post-transplant thrombocytopenia. We report previous evidence with consideration for future perspectives.

PLATELETS AND DDLT

Post-transplant thrombocytopenia after DDLT has been reported since the advent of liver transplantation and has been described in many articles. It was first reported by Hutchison *et al.*^[23] in 1968 (Table 1). They reviewed 8 LT recipients who received DDLT at the University of Colorado, which included 2 auxiliary and 6 orthotopic LT. An acute drop in platelet count to less than $10 \times 10^3/\mu\text{L}$ was observed in most patients within the first three postoperative days. To better comprehend this phenomenon, they performed experimental LT in dogs and found platelets located in the space of Disse along with Kupffer cells, some of which were ingesting the platelets. They concluded that post-transplant thrombocytopenia was primarily caused by the mechanical entrapment of platelets in the grafts, which were then destroyed by the Kupffer cells. The next report of this phenomenon came after a twenty year interval and was described by Plevak *et al.*^[16]. They observed that platelet counts dropped from preoperative levels of $137 \times 10^3/\mu\text{L}$ to $72 \times 10^3/\mu\text{L}$ on POD 3. Using ¹¹¹In-labeled platelets, they demonstrated that transplant recipients showed a delayed recovery of platelet counts after LT.

Since then, several consecutive reports have been

Table 1 Reports of postoperative thrombocytopenia after liver transplant

Author	Year	Type	Number of patients	Results
Hutchison <i>et al</i> ^[23]	1968	DDLT	8	Platelet count change from $200\text{--}400 \times 10^3/\mu\text{L}$ to $67 \times 10^3/\mu\text{L}$ on POD 3
Plevak <i>et al</i> ^[16]	1988	DDLT	76	Platelet count change from $137 \times 10^3/\mu\text{L}$ to $72 \times 10^3/\mu\text{L}$ on POD 3
Munoz <i>et al</i> ^[15]	1989	DDLT	3	Three patients with severe postoperative thrombocytopenia were successfully treated with high-dose gamma-globulin
McCaughan <i>et al</i> ^[17]	1992	DDLT	53	Patients who died during their hospital stay had lower postoperative platelet counts at the nadir, and the day of the nadir tended to be delayed
Chatzipetrou <i>et al</i> ^[24]	1999	DDLT	541	A platelet nadir of $< 20 \times 10^3/\mu\text{L}$ was associated with allograft dysfunction, graft rejection and poorer patient and graft survival
Chang <i>et al</i> ^[25]	2000	DDLT	50	Fungal infection was frequent in patients with a platelet nadir of $< 30 \times 10^3/\mu\text{L}$
Ben Hamida <i>et al</i> ^[26]	2003	DDLT	161	Patients with a platelet count $< 50 \times 10^3/\mu\text{L}$ for three consecutive days showed a high mortality rate.
Nascimbene <i>et al</i> ^[19]	2007	DDLT	8	Infusion of high-dose gamma-globulins induced a prompt, complete and persistent resolution of postoperative severe thrombocytopenia in more than 70% of patients
Kim <i>et al</i> ^[47]	2010	LDLT	87	A total unit of platelet transfusion was significantly associated with graft regeneration
Lesurtel <i>et al</i> ^[28]	2014	DDLT	247	A platelet count of $< 60 \times 10^3/\mu\text{L}$ on POD 5 was related to poor graft survival within 90 d after LT
Sonny <i>et al</i> ^[27]	2015	DDLT	223	A preoperative platelet count of $< 45 \times 10^3/\mu\text{L}$ was related with short-term outcomes in patients ≥ 60 years old
Li <i>et al</i> ^[49]	2015	LDLT	234	Patients with an immediate postoperative platelet count of $< 68 \times 10^3/\mu\text{L}$ had a higher chance of developing EAD and severe complications
Takahashi <i>et al</i> ^[29]	2016	DDLT	975	A platelet count of $< 72.5 \times 10^3/\mu\text{L}$ on POD 5 was related to poor graft survival
Han <i>et al</i> ^[48]	2016	LDLT	441	An intraoperative platelet transfusion was also independently associated with enhanced graft regeneration at 14 ± 2 d after surgery
Pamecha <i>et al</i> ^[50]	2016	LDLT	120	A platelet count of $< 30 \times 10^3/\mu\text{L}$ on POD 3 was a strong predictor of complications and EAD
Gwiasda <i>et al</i> ^[31]	2017	DDLT	134	A higher preoperative platelet count was related to graft loss
Takahashi <i>et al</i> ^[30]	2017	DDLT	771	Persistent thrombocytopenia within 5 d after LT was related to progression of biliary anastomotic stricture
Akamatsu <i>et al</i> ^[51]	2017	LDLT	445	A Low platelet count on POD 3 was an independent predictor of grade IIIb/IV complications

LT: Liver transplant; DDLT: Deceased donor liver transplant; LDLT: Living donor liver transplant; POD: Postoperative day; EAD: Early allograft dysfunction.

published on this topic, and the majority of these studies indicate that postoperative thrombocytopenia may influence post-LT outcomes and have a negative impact on grafts and patient survival in the short and long term after LT^[17,19,24–27]. McCaughan *et al*^[17] reported a drop in postoperative platelet counts by 63%, which was the only independent predictor of short-term patient survival post-LT. In their analysis of a large cohort of 541 patients of DDLT in a single institute, Chatzipetrou *et al*^[24] described that a post-transplant platelet count nadir of $< 20 \times 10^3/\mu\text{L}$ was associated with allograft dysfunction, graft rejection and poorer patient and graft survival. Chang *et al*^[25] reported that the incidence of fungal infection was more frequent in patients with a platelet nadir of $< 30 \times 10^3/\mu\text{L}$, leading to higher mortality rates. Sonny *et al*^[27] focused on the elderly population and found that the length of intensive care unit (ICU) and hospital stays were longer in patients with preoperative platelet counts of $< 45 \times 10^3/\mu\text{L}$. Factors such as low preoperative platelet counts, massive intraoperative platelet transfusions, retransplantation, and poor general preoperative conditions such as the need

for dialysis, were found to be associated with post-transplant thrombocytopenia^[24,26]. For the treatment of post-transplant thrombocytopenia, Munoz *et al*^[15] and Nascimbene *et al*^[19] separately reported that an infusion of high-dose gamma-globulins could induce resolution of severe postoperative thrombocytopenia. However, they could not explain its mechanism of action.

Recently, some groups have focused on platelet counts particularly on POD 5, when platelet counts start to rise after the nadir. In 2014, Lesurtel *et al*^[28] proposed the 60-5 criteria, in which a platelet count of $< 60 \times 10^3/\mu\text{L}$ on POD 5 was an independent risk factor associated for severe postoperative complications, early graft failure, and patient mortality in the short term after DDLT. Takahashi *et al*^[29] focused on the long term impact after DDLT and noted that a platelet count of $< 72.5 \times 10^3/\mu\text{L}$ on POD 5 was a predictor for poor graft and patient survival. More recently, Takahashi *et al*^[30] described that low perioperative platelet counts within 5 d after DDLT were associated with biliary anastomotic stricture (BAS) with duct-to-duct biliary reconstruction. They found that persistent postoperative thrombocytopenia, which

was defined as platelet counts of $< 41 \times 10^3/\mu\text{L}$ and $< 53 \times 10^3/\mu\text{L}$ on POD 3 and POD 5, respectively, was an independent risk factor for BAS.

In contrast, Gwiasda *et al.*^[31] stated that higher preoperative platelet count was associated with graft loss because platelets contribute to reperfusion injury after graft ischemia. Further, Eldeen *et al.*^[32] described that recipients who experienced early hepatic arterial thrombosis (HAT) had higher preoperative platelet counts, but this was not associated with the development of late HAT.

Clinical studies suggest that increasing postoperative platelet counts might improve graft and patient survival after DDLT^[28-30]. This "protective effect of platelets" may be compatible with a study by Hisakura *et al.*^[33] who showed that thrombopoietin-mediated thrombocytosis protected the liver from damage after an extended hepatectomy in a pig model. However, transplant surgeons prefer relatively low postoperative platelet counts due to fear of HAT, and this preference has made it difficult to perform prospective trials.

PLATELETS AND LDLT

After LDLT, the liver undergoes two different processes, namely, liver regeneration and ischemia-reperfusion^[34-36]. The liver regeneration process after LDLT has been divided into three phases^[34]. The early phase is rapid regeneration, which occurs during the first two weeks and is associated with vascular engorgement and tissue edema. The second phase is volume decline, which may be attributed to the normalization of transient vascular engorgement or tissue edema at one to two months after hepatectomy. The third phase is a slow increase in volume, which occurs until the volume reaches a constant level^[34]. Partial liver grafts need rapid regeneration to meet the functional demands of recipients; otherwise, liver failure would occur, and the short- and long-term outcomes would be affected. Liver regeneration is orchestrated by the interplay of various cells and mediators, and platelets are understood to have a role as well^[37,38]. The role of platelets in accelerating liver regeneration after partial hepatectomy was first reported by Murata in 2004^[39], and Lesurtel *et al.*^[40] reported platelet-derived serotonin-mediated liver regeneration using transgenic mice. Since then, many studies have been reported in this field, and it was implicated by a Japanese group that liver regeneration after partial hepatectomy is promoted by platelets through three different mechanisms: (1) a direct effect on hepatocytes; (2) a cooperative effect with liver sinusoidal endothelial cells; and (3) a collaborative effect with Kupffer cells^[41-45]. On the other hand, in ischemia-reperfusion, platelets are generally considered to act in concert with activated Kupffer cells and leukocytes, and a triangular interaction between Kupffer cells, leukocytes, and platelets has been demonstrated to be the core mechanism of liver injury^[46]. Thus, platelets

have two ambivalent roles in LDLT.

Kim *et al.*^[47] were the first to report the positive role of platelets in LDLT in 2010 (Table 1). They investigated the relationships between clinical variables and liver graft regeneration rates in their study population of 87 recipients with adult-to-adult living donor recipients, all receiving right lobe grafts. They found that total units of platelet transfusion were significantly associated with graft regeneration. Han *et al.*^[48] studied the relationship between platelets and liver regeneration after LDLT. They described that intraoperative platelet transfusion was independently associated with enhanced graft regeneration at 14 ± 2 d after surgery without increasing morbidity and mortality rates. Furthermore, platelet count during the reperfusion phase was identified as a prognostic factor for graft regeneration. Li *et al.*^[49] focused on platelet count immediately after transplant and reported that patients with an immediate postoperative platelet count of $< 68 \times 10^3/\mu\text{L}$ had a higher chance of developing early allograft dysfunction and severe complications. Pamecha *et al.*^[50] demonstrated that a platelet count of $< 30 \times 10^3/\mu\text{L}$ on POD 3 was a strong predictor of major postoperative complications and was associated with early graft dysfunction, prolonged ascites, and sepsis. Akamatsu *et al.*^[51] also focused on POD 3 and described that a low platelet count on POD 3 was an independent predictor of grade IIIb/IV complications.

Only recently has post-transplant thrombocytopenia been reported to be associated with LDLT. Lower platelet counts lead to poor graft regeneration but lower incidences of ischemia-reperfusion injury in partial grafts. Overall higher platelets counts are beneficial because their impact on liver regeneration outweighs the associated risk of ischemia-reperfusion injury, most notably during the early post-LDLT period^[52]. Animal experiments to explain this phenomenon are still lacking, and basic studies and prospective clinical trials are warranted.

PLATELET FUNCTION AND ANTIPLATELET THERAPY AFTER LT

In patients with end-stage liver disease (ESLD), platelet function is often reported to be compromised^[53]. However, recent studies have demonstrated that platelet function in patients with ESLD was not as compromised as it was previously believed^[54]. A few observational studies that evaluated platelet function after LT have been reported in the past, but these studies involved a small number of patients. Himmelreich *et al.*^[55] reported decreased platelet aggregation immediately after reperfusion in 10 patients after DDLT. The authors considered that a dysfunction in platelet aggregation may have been a major cause of intraoperative bleeding^[55]. They also mentioned that administration of a small amount of University of Wisconsin (UW) solution into systemic circulation during reperfusion might further

decrease platelet function^[56]. Jüttner *et al*^[57] found marked depressed GP II b/IIIa and P-selectin expression in circulating platelets, and maximum aggregation of platelets was restored on the third day after reperfusion among patients with all types of underlying disease. Eyraud *et al*^[58] conducted platelet function testing with aggregometry using platelet-rich plasma obtained from 15 patients after DDLT. Compared with pre-transplant conditions, no significant difference was found in platelet function at 7 and 28 d after DDLT. From these reports, platelet function is temporally impaired immediately after LT but recovers in 3-7 d.

Regarding the use of antiplatelet therapy, some studies have indicated favorable effects on LT, including a reduced incidence of post-transplant hepatic arterial thrombosis^[59,60] and the prevention of progression of liver fibrosis after postoperative recurrence of hepatitis C^[61]. Antiplatelet therapy has also been described to prevent the recurrence of hepatocellular carcinoma after curative hepatectomy^[62] and to suppress hepatocellular carcinogenesis in patients with chronic hepatitis^[63,64]. However, most of these studies were performed at a single institution or were retrospective in nature. A randomized clinical trial should be undertaken to analyze the risks and benefits of the use of post-transplant antiplatelet therapy.

MECHANISM OF POST-TRANSPLANT THROMBOCYTOPENIA

Thrombocytopenia, which is defined by a platelet count of $< 150 \times 10^3/\mu\text{L}$, has been reported to occur in more than 70% of patients with cirrhosis^[65-67]. This disorder is considered to be a result of reduced synthesis of thrombopoietin (TPO) and increased sequestration by hypersplenism^[67]. In LT, post-transplantation thrombocytopenia can occur due to the following: hemodilution; decreased production of TPO; increased platelet sequestration in the liver graft or spleen; immunological reactions; platelet activation and consumption due to thrombosis, such as in disseminated intravascular coagulation (DIC), thrombotic microangiopathy, or venous thromboembolism; medication; infections; or a combination of these processes^[18-22].

Hemodilution

Hemodilution due to intensive use of blood products, colloids and crystalloids during the transplant procedure may lead to a drop in the platelet count immediately after surgery, but the platelet nadir usually occurs on days 3-4, which does not validate hemodilution as a potential cause of postoperative thrombocytopenia.

Preservation solution

A correlation between lower post-transplant thrombocytopenia and the use of UW solution was implicated by Williams *et al*^[68]. However, their study was an observational study that consisted of a small number of recipients, and the level of evidence was low^[69].

Sequestration in the spleen

Richards *et al*^[20] described that patients in a hyposplenic state exhibit the same pattern of post-transplant thrombocytopenia as those with intact splenic function. Nascimbene *et al*^[19] also noted that thrombocytopenia occurred regardless of the presence of hypersplenism. Thus, the spleen is not considered to be a major site of platelet consumption^[20].

Consumption in the liver graft by Kupffer cells

Sindram *et al*^[46] demonstrated that reperfusion of rat livers preserved for 24 h at a cold temperature resulted in rapid sequestration of platelets in the liver graft and platelet adherence to the sinusoidal lining, which induced apoptosis of the sinusoidal endothelial cells in concert with leukocytes and activated Kupffer cells. Cywes *et al*^[18] detected significantly increased adherence of platelets to the hepatic endothelium after reperfusion of the liver graft. Porte *et al*^[70] reported that thrombocytopenia started immediately after reperfusion, and sequestration of platelets was observed as platelets accumulated in the sinusoids and were phagocytized by Kupffer cells, which was similar to an earlier study. On the other hand, Takahashi *et al*^[41] suggested that platelets that accumulate in the liver graft have with contact with Kupffer cells and release growth factors, such as HGF, IGF-1, and VEGF, that protect the liver graft and lead to improved graft survival in patients with higher platelet counts after DDLT^[29].

Consumption at the liver graft with local thrombin generation

Richards *et al*^[20] found that the levels of fibrin and fibrinogen degradation products are elevated postoperatively, which they speculated was due to endothelial damage in the liver graft during the preservation period, which lead to hepatic sequestration due to local thrombin generation. Nobuoka *et al*^[71] focused on activity of a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs member 13 (ADAMTS13), which is produced by stellate cells. ADAMTS13 is an enzyme that specifically cleaves multimeric von Willebrand factor (vWF), which mediates the adhesion of platelets to the site of vascular damage. They described that decreased activity of ADAMTS13 accompanied by elevated vWF levels was associated with thrombocytopenia 2 wk after LDLT. They considered that prolonged thrombocytopenia after LDLT was due to decreased production of ADAMTS13 in the graft with local thrombin generation and platelet aggregation. Nakanuma *et al*^[72] found that platelet aggregation was mainly present at zone 3 in the liver graft as extravasated platelet aggregation (EPA), and peripheral platelet counts were lower after LDLT in the EPA-positive patients than the EPA-negative patients. They considered that EPA in the zone 3 caused the platelet consumption, activation, and degranulation, following the release of negative cytokines by platelets, and

might be involved in liver damage and poor outcomes after LDLT^[72].

TPO production

Serum TPO concentration is inversely related to platelet concentration in patients with hematopoietic disorders characterized by deceased megakaryocytes in the bone marrow^[5]. The level of expression of mRNA for TPO is high in the liver, indicating that the liver is the main source of TPO synthesis^[73].

Richards *et al.*^[74] reported that thrombocytopenia following LT was accompanied by an increased rate of thrombopoiesis in the early period after transplantation, shown by increased reticulated platelets following the platelet nadir. Peck-Radosavljevic *et al.*^[75] observed that serum thrombopoietin levels increased significantly on the first day after LT, which preceded the increase in reticulated platelets by 3 d and in peripheral platelets by 5 d. This delayed rise in platelet count was compatible with the time lag between the appearance of reticulated platelets and peripheral platelets after *in vivo* administration of a recombinant human thrombopoietin analogue, and rules out the impaired production of TPO as a possible cause of post-transplant thrombocytopenia. Usui *et al.*^[76] reported the TPO levels in the prolonged thrombocytopenic group were significantly decreased. They considered that prolonged post-transplant thrombocytopenia was secondary to a decrease in TPO production suggesting graft dysfunction.

Medication

Immunosuppressive medications (*e.g.*, azathioprine, mycophenolate mofetil, cyclosporine and tacrolimus), heparin, anti-thymocyte globulin (ATG), antiviral drugs (ganciclovir and valganciclovir), trimethoprim sulfamethoxazole, *etc.*, can cause thrombocytopenia after LT. Antimetabolites, such as azathioprine and mycophenolate mofetil, have myelosuppressive effects on the bone marrow in a dose-dependent manner^[77,78]. Nascimbene *et al.*^[19] performed bone marrow aspirates during the early post-LT period and noticed marked megakaryocytic hyperplasia in all cases, ruling out drug-induced myelosuppression as a cause of post-transplant thrombocytopenia. Calcineurin inhibitors, such as cyclosporine and tacrolimus, may cause thrombocytopenia. The presentation is similar to thrombotic thrombocytopenic purpura (TTP), in which renal dysfunction is accompanied by thrombocytopenia^[79-81]. Lee *et al.*^[79] described that the incidence of cyclosporine-induced TTP was low (incidence of 1%) and was seen only in the pediatric population, occurring at 2 to 30 wk after LT.

Heparin-induced thrombocytopenia (HIT) after LT has been reported in several articles^[22,82-85]. Kaneko *et al.*^[22] demonstrated that the percentage of heparin-induced thrombocytopenia (HIT) antibody-positive patients was low (incidence of 5.6%), and none of the patients developed HIT. Bakchoul *et al.*^[82] also described that HIT was clinically suspected in 16%

of recipients at a median of POD 6. However, only one of these patients was positive for anti-platelet factor 4/heparin IgG antibodies. ATG induces dose-dependent T-cell depletion by complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity, and apoptosis^[86,87]. ATG-induced platelet aggregation is a specific reaction responsible for thrombocytopenia. Platelet surface antigen-CD32 has been suggested to play a crucial role in ATG-induced thrombocytopenia^[88]. Ganciclovir and valganciclovir are suspected to have direct bone marrow toxicity. Gabardi *et al.*^[89] reported that the incidence of thrombocytopenia with low-dose valganciclovir, used as prophylaxis for cytomegalovirus (CMV) infections in post-transplant patients, was 24%. Trimethoprim sulfamethoxazole, when used as prophylaxis against *Pneumocystis jiroveci* pneumonia, causes drug-induced immune thrombocytopenia (ITP) by antibody formation^[90].

ITP and infection

Viral infections, including CMV, Epstein-Barr virus (EBV), parvovirus B19, herpes zoster, human herpes virus 8, and some donor-derived viral infections, can induce ITP^[91-97]. The early onset of ITP after LT occurs due to reactivation of CMV, EBV or varicella infection when patients are receiving high-dose immunosuppression. On the other hand, Taylor *et al.*^[21] reported 8 cases of ITP after LT (incidence of 0.7%), in which they could not find any evidence of infection. The majority of their patients developed ITP more than one year post-LT. Maar *et al.*^[98] described that recipients with CMV infection showed delayed thrombocytopenia, occurring later than 24 d after LT. They considered that CMV infection induced systemic endothelial activation with the expression of tissue factor on the endothelial cell surface and the release of vWF. These processes activate the clotting cascade and may augment platelet aggregation.

Considering that post-transplant thrombocytopenia mostly occurs during the early period after LT, sequestration in the new liver graft has the strongest potential to explain the temporal drop in platelet counts. However, the precise mechanism of sequestration is still unknown. Prolonged thrombocytopenia, which occurs more than one month after LT, may be attributed to other causes such as impaired TPO production due to graft dysfunction, viral infections, and medications.

PLATELETS AND TRANSFUSION

The median blood loss associated with LT has fallen dramatically with the development of surgical and anesthetic techniques. However, there are still a number of patients who require significant amounts of blood products perioperatively.

DDL T

In 1989, Miyata *et al.*^[99] described that there was positive correlation between the number of platelet

units transfused and endotoxin concentrations at the end of the anhepatic phase, which they considered to be the reason for increased pulmonary complications. de Boer *et al*^[100] demonstrated that intraoperative platelet transfusion was an independent risk factor for one- and five-year survival after DDLT. A subsequent report from Pereboom *et al*^[101] noted that platelet transfusion led to an increased one-year mortality from acute lung injury. More recently, Chin *et al*^[102] reported that graft survival was reduced significantly in patients receiving intraoperative platelet transfusions at one year, but not at 90 d, and considered that intraoperative transfusion and not thrombocytopenia was associated with a poor outcome after LT. They found a relationship between intraoperative platelet transfusion and postoperative septicemia as a cause of death. Nacoti *et al*^[103] focused on a pediatric population and found that platelet transfusion was an independent risk factor for developing major complications in the first year after DDLT. In contrast, Nixon *et al*^[104] found that there was no substantiated effect of platelet transfusion on survival after LT, due to their use of plateletpheresis. They insisted on using single-donor platelet transfusions rather than random donor platelet preparations, along with leucocyte reduction strategies.

LDLT

Authors from two different institutes in South Korea described that platelet transfusion after LDLT was a protective factor for graft regeneration and survival^[47,48]. Li *et al*^[105] described that although massive red blood transfusion led to poor long-term survival, higher postoperative infection rates and prolonged ICU stays, platelet transfusion was not a risk factor for long-term graft survival.

Thromboelastography

With the hope of limiting the use of blood products, some transplant centers use thromboelastography (TEG) to monitor and detect coagulopathies^[106]. TEG is a viscoelastic test that is performed on whole blood to analyze complete hemostasis, from platelet plug formation through coagulation and fibrinolysis. There is growing evidence to support the use of TEG as a technique to guide transfusion strategies for LT^[107-109]. Kang *et al*^[109] prospectively validated the use of TEG for reducing total blood product use. Lawson *et al*^[107] described that the maximum amplitude measured preoperatively by TEG had high predictability for intraoperative massive transfusion. Krzanicki *et al*^[110] performed a retrospective review of 124 DDLT recipients and found a higher incidence of a hypercoagulable state in patients with a background of cholestatic diseases and an intraoperative hypercoagulable state that was correlated with early HAT after LT. On the other hand, Wikkelsøe *et al*^[111] performed a systemic review and meta-analysis including a sequential analysis of

randomized clinical trials of a TEG/thromboelastometry-based algorithm compared with standard treatment in patients with cardiac surgery and LT, and found that the former had no impact on mortality, the amount of blood transfused, or the incidence of surgical reinterventions.

There was a significant difference in the impact of platelet transfusion between DDLT and LDLT, the former being negative, and the latter being positive. The reason for this difference could be due to graft type; partial grafts require postoperative liver regeneration. This result is compatible with a report from Matsuo *et al*^[44] that transfusion of platelet-rich plasma accelerated liver regeneration, including liver/body weight ratio and hepatocyte Ki-67 labeling index during the early phase after hepatectomy in a rat model. However, it is still unknown how platelets interact with other cells when under ischemia-reperfusion conditions. The precise mechanisms need to be clarified by animal experiments. TEG may be a good option to stratify the risk of perioperative transfusions. However, it is still debated whether the use of TEG is realistically efficient for predicting the need for transfusions.

PLATELET, LT AND SPLENECTOMY

Splenectomy is currently one of the therapeutic procedures for avoiding small-for-size syndrome, and it is a choice for preventing postoperative thrombocytopenia in LDLT^[112,113]. It has been indicated for the completion of post-transplant interferon therapy for hepatitis C virus (HCV) infection and ABO incompatible LT^[113,114]. However, due to recent advances in interferon (INF)-free direct-acting antivirals for HCV infection and rituximab induction for ABO incompatible transplantation, the necessity for splenectomy is currently decreasing^[114]. Partial splenic embolization is a minimally invasive treatment that can be performed as an additional treatment after LT; however, its efficacy may be insufficient, and serious complications, such as splenic abscess, splenic rupture, and venous thrombosis, have been reported^[115]. On the other hand, simultaneous splenectomy in DDLT is not usually performed based on historical reports of septic complications after LT^[116].

Marubashi *et al*^[112] revealed that 7 patients who underwent a simultaneous splenectomy showed remarkable increases in platelet counts 2 wk after LDLT and found that graft size was positively associated with post-transplant thrombocytopenia. Morimoto *et al*^[117] demonstrated that with simultaneous splenectomy, the platelet count increased to $> 100 \times 10^3/\mu\text{L}$ one month post-transplantation in recipients with HCV infection and achieved better sustained virological response after INF therapy. Additionally, a similar report by Chu *et al*^[114] noted that patients with simultaneous splenectomy had significantly higher platelet counts at 1 and 6 mo after transplantation, with a higher HCV anti-therapy completion rate. On the other hand, Ito *et al*^[116] observed that simultaneous splenectomy increased

platelet count more than 2 wk after LDLT but not during the early postoperative period. In addition, the incidence of reoperation for postoperative hemorrhage increased within the first week. The authors further demonstrated that simultaneous splenectomy was an independent predictor for postoperative lethal infectious complications. On the other hand, Takahashi *et al.*^[118] described the usefulness of pre-transplant splenectomy in pediatric recipients suffering from biliary atresia. After splenectomy, the platelet count was significantly elevated, with an improvement in the PT-INR and Model for End-Stage Liver Disease score. However, the complication rate for this procedure was relatively high.

The effect of splenectomy on restoring postoperative platelet counts during the early post-LT period may be delayed from the time when a higher platelet count is necessary. This issue may be resolved by pre-transplant splenectomy, which can elevate preoperative platelet counts. However, the decision to perform pre-transplant splenectomy should be given much care and consideration due to the poor general condition of the patients and their bleeding tendencies.

FUTURE PERSPECTIVES

TPO^[33,43,45,119,120], TPO receptor agonists^[121], artificial platelets^[122,123], and freeze-dried platelets^[124] are developing and beginning to be utilized in various clinical settings, and the importance of platelets is becoming more obvious. Additionally, the infusion of high-dose immunoglobulins may provide a safe, prompt, complete, and persistent resolution of severe post-transplant thrombocytopenia^[15,19]. These platelet therapies, splenectomy and intravenous immunoglobulin treatment may have potential as therapeutic strategies to resolve post-transplant thrombocytopenia, leading to improved graft and patient survival after LT. In particular in LDLT, these strategies may be able to prevent small-for-size syndrome by promoting liver regeneration^[47,48,112,118]. However, decreases in platelet count are sometimes falsely overestimated by automatic analyzers due to platelet aggregation. Therefore, manual counting to confirm a platelet reduction before initiating platelet therapies and monitoring precise platelet counts after therapy are necessary.

LIMITATIONS

We acknowledge there are limitations to this review. First, most studies are based on small retrospective series from single institutions. The reason for this is there is still no consensus regarding the role of platelets in LT (*i.e.*, "Are platelets a friend or foe in LT?"). This fact has led to difficulty in conducting multi-institutional prospective trials to clarify the role of platelets in LT. Second, it is still difficult to prove whether thrombocytopenia is a "result" or a "cause" of postoperative complications. Many studies describe post-

transplant thrombocytopenia as a phenomenon, but there has been no direct evidence that show whether thrombocytopenia is a cause or a result of poor graft function or complications. It is necessary to clarify this important point with basic animal experiments. Third, since thrombocytopenia is common after LT, it is still unclear how to determine which patients need platelet therapies to prevent postoperative complications and yield better outcomes. By conducting multi-institutional prospective trials, it is important to generate a standardized cut-off value to specify the target patients for platelet therapies.

CONCLUSION

We described convincing evidence of post-transplant thrombocytopenia and the role of platelets in LT and discussed future perspectives. The mechanisms of thrombocytopenia and its effect on postoperative outcomes are still not completely understood. Since platelets have both beneficial and detrimental effects on liver grafts, therapeutic strategies to increase perioperative platelet counts, such as the use of thrombopoietin, thrombopoietin receptor agonist, platelet transfusion, splenectomy, and intravenous immunoglobulin treatment, could be targeted to enhance the beneficial effects while minimizing potential detrimental effects.

REFERENCES

- 1 **Holinstat M.** Normal platelet function. *Cancer Metastasis Rev* 2017; **36**: 195-198 [PMID: 28667366 DOI: 10.1007/s10555-017-9677-x]
- 2 **Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H.** Platelets at work in primary hemostasis. *Blood Rev* 2011; **25**: 155-167 [PMID: 21496978 DOI: 10.1016/j.blre.2011.03.002]
- 3 **Broos K, De Meyer SF, Feys HB, Vanhoorelbeke K, Deckmyn H.** Blood platelet biochemistry. *Thromb Res* 2012; **129**: 245-249 [PMID: 22119499 DOI: 10.1016/j.thromres.2011.11.002]
- 4 **Holmsen H.** Platelet metabolism and activation. *Semin Hematol* 1985; **22**: 219-240 [PMID: 2994234]
- 5 **McNicol A, Israels SJ.** Platelet dense granules: structure, function and implications for haemostasis. *Thromb Res* 1999; **95**: 1-18 [PMID: 10403682]
- 6 **Polasek J.** Platelet secretory granules or secretory lysosomes? *Platelets* 2005; **16**: 500-501 [PMID: 16287618 DOI: 10.1080/09537100500169926]
- 7 **Elzey BD, Sprague DL, Ratliff TL.** The emerging role of platelets in adaptive immunity. *Cell Immunol* 2005; **238**: 1-9 [PMID: 16442516 DOI: 10.1016/j.cellimm.2005.12.005]
- 8 **Yamaguchi R, Terashima H, Yoneyama S, Tadano S, Ohkohchi N.** Effects of platelet-rich plasma on intestinal anastomotic healing in rats: PRP concentration is a key factor. *J Surg Res* 2012; **173**: 258-266 [PMID: 21074782 DOI: 10.1016/j.jss.2010.10.001]
- 9 **Mehta P.** Potential role of platelets in the pathogenesis of tumor metastasis. *Blood* 1984; **63**: 55-63 [PMID: 6360248]
- 10 **Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, Zhang Q, Lavalley C, McKeown T, Marshall AH, Ni H.** Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. *Crit Rev Clin Lab Sci* 2016; **53**: 409-430 [PMID: 27282765 DOI: 10.1080/10408363.2016.1200008]
- 11 **Meirelles Júnior RF, Salvalaggio P, Rezende MB, Evangelista**

- AS, Guardia BD, Matielo CE, Neves DB, Pandullo FL, Felga GE, Alves JA, Curvelo LA, Diaz LG, Rusi MB, Viveiros Mde M, Almeida MD, Pedrosa PT, Rocco RA, Meira Filho SP. Liver transplantation: history, outcomes and perspectives. *Einstein (Sao Paulo)* 2015; **13**: 149-152 [PMID: 25993082 DOI: 10.1590/S1679-45082015RW3164]
- 12 **Abbasoglu O**. Liver transplantation: yesterday, today and tomorrow. *World J Gastroenterol* 2008; **14**: 3117-3122 [PMID: 18506914 DOI: 10.3748/wjg.14.3117]
 - 13 **Carmona M**, Álvarez M, Marco J, Mahillo B. Organ Donation and Transplantation Activities 2015 Report. Global Observatory on Donation and Transplantation World Health Organization, 2017
 - 14 **Kim WR**, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB, Harper AM, Wainright JL, Snyder JJ, Israni AK, Kasiske BL. OPTN/SRTR 2015 Annual Data Report: Liver. *Am J Transplant* 2017; **17** Suppl 1: 174-251 [PMID: 28052604 DOI: 10.1111/ajt.14126]
 - 15 **Munoz SJ**, Carabasi AR, Moritz MJ, Jarrell BE, Maddrey WC. Postoperative thrombocytopenia in liver transplant recipients: prognostic implications and treatment with high dose of gamma-globulin. *Transplant Proc* 1989; **21**: 3545-3546 [PMID: 2662521]
 - 16 **Plevak DJ**, Halma GA, Forstrom LA, Dewanjee MK, O'Connor MK, Moore SB, Krom RA, Rettke SR. Thrombocytopenia after liver transplantation. *Transplant Proc* 1988; **20**: 630-633 [PMID: 3279654]
 - 17 **McCaughan GW**, Herkes R, Powers B, Rickard K, Gallagher ND, Thompson JF, Sheil AG. Thrombocytopenia post liver transplantation. Correlations with pre-operative platelet count, blood transfusion requirements, allograft function and outcome. *J Hepatol* 1992; **16**: 16-22 [PMID: 1484150]
 - 18 **Cywes R**, Mullen JB, Stratis MA, Greig PD, Levy GA, Harvey PR, Strasberg SM. Prediction of the outcome of transplantation in man by platelet adherence in donor liver allografts. Evidence of the importance of prepreservation injury. *Transplantation* 1993; **56**: 316-323 [PMID: 7689257]
 - 19 **Nascimbene A**, Iannacone M, Brando B, De Gasperi A. Acute thrombocytopenia after liver transplant: role of platelet activation, thrombopoietin deficiency and response to high dose intravenous IgG treatment. *J Hepatol* 2007; **47**: 651-657 [PMID: 17716776 DOI: 10.1016/j.jhep.2007.06.012]
 - 20 **Richards EM**, Alexander GJ, Calne RY, Baglin TP. Thrombocytopenia following liver transplantation is associated with platelet consumption and thrombin generation. *Br J Haematol* 1997; **98**: 315-321 [PMID: 9266927]
 - 21 **Taylor RM**, Bockenstedt P, Su GL, Marrero JA, Pellitier SM, Fontana RJ. Immune thrombocytopenic purpura following liver transplantation: a case series and review of the literature. *Liver Transpl* 2006; **12**: 781-791 [PMID: 16628698 DOI: 10.1002/lt.20715]
 - 22 **Kaneko J**, Sugawara Y, Tamura S, Togashi J, Matsui Y, Makuuchi M. Heparin-induced thrombocytopenia after liver transplantation. *Transplant Proc* 2008; **40**: 1518-1521 [PMID: 18589141 DOI: 10.1016/j.transproceed.2008.01.069]
 - 23 **Hutchison DE**, Genton E, Porter KA, Daloze PM, Huguet C, Brettschneider L, Groth CG, Starzl TE. Platelet changes following clinical and experimental hepatic homotransplantation. *Arch Surg* 1968; **97**: 27-33 [PMID: 4232038]
 - 24 **Chatzipetrou MA**, Tsaroucha AK, Weppeler D, Pappas PA, Kenyon NS, Nery JR, Khan MF, Kato T, Pinna AD, O'Brien C, Viciana A, Ricordi C, Tzakis AG. Thrombocytopenia after liver transplantation. *Transplantation* 1999; **67**: 702-706 [PMID: 10096525]
 - 25 **Chang FY**, Singh N, Gayowski T, Wagener MM, Mietzner SM, Stout JE, Marino IR. Thrombocytopenia in liver transplant recipients: predictors, impact on fungal infections, and role of endogenous thrombopoietin. *Transplantation* 2000; **69**: 70-75 [PMID: 10653383]
 - 26 **Ben Hamida C**, Lauzet JY, Rézaiguia-Delclaux S, Duvoux C, Cherqui D, Duvaldestin P, Stéphan F. Effect of severe thrombocytopenia on patient outcome after liver transplantation. *Intensive Care Med* 2003; **29**: 756-762 [PMID: 12677370 DOI: 10.1007/s00134-003-1727-x]
 - 27 **Sonny A**, Kelly D, Hammel JP, Albeldawi M, Zein N, Cywinski JB. Predictors of poor outcome among older liver transplant recipients. *Clin Transplant* 2015; **29**: 197-203 [PMID: 25528882 DOI: 10.1111/ctr.12500]
 - 28 **Lesurtel M**, Raptis DA, Melloul E, Schlegel A, Oberkofler C, El-Badry AM, Weber A, Mueller N, Dutkowski P, Clavien PA. Low platelet counts after liver transplantation predict early posttransplant survival: the 60-5 criterion. *Liver Transpl* 2014; **20**: 147-155 [PMID: 24123804 DOI: 10.1002/lt.23759]
 - 29 **Takahashi K**, Nagai S, Putchakayala KG, Safwan M, Li AY, Kane WJ, Singh PL, Collins KM, Rizzari MD, Yoshida A, Schnickel GT, Abouljoud MS. Prognostic impact of postoperative low platelet count after liver transplantation. *Clin Transplant* 2017; **31**: [PMID: 27992667 DOI: 10.1111/ctr.12891]
 - 30 **Takahashi K**, Nagai S, Putchakayala KG, Safwan M, Gosho M, Li AY, Kane WJ, Singh PL, Rizzari MD, Collins KM, Yoshida A, Abouljoud MS, Schnickel GT. Prediction of biliary anastomotic stricture after deceased donor liver transplantation: the impact of platelet counts - a retrospective study. *Transpl Int* 2017; **30**: 1032-1040 [PMID: 28605573 DOI: 10.1111/tri.12996]
 - 31 **Gwiada J**, Schrem H, Klemmner J, Kaltenborn A. Identifying independent risk factors for graft loss after primary liver transplantation. *Langenbecks Arch Surg* 2017; **402**: 757-766 [PMID: 28573420 DOI: 10.1007/s00423-017-1594-5]
 - 32 **Zahr Eldeen F**, Roll GR, Derosas C, Rao R, Khan MS, Gunson BK, Hodson J, Mergental H, Ferraz-Neto BH, Isaac J, Muiresan P, Mirza DF, Iqbal A, Perera MT. Preoperative Thromboelastography as a Sensitive Tool Predicting Those at Risk of Developing Early Hepatic Artery Thrombosis After Adult Liver Transplantation. *Transplantation* 2016; **100**: 2382-2390 [PMID: 27780186 DOI: 10.1097/TP.0000000000001395]
 - 33 **Hisakura K**, Murata S, Fukunaga K, Myronovych A, Tadano S, Kawasaki T, Kohno K, Ikeda O, Pak S, Ikeda N, Nakano Y, Matsuo R, Konno K, Kobayashi E, Saito T, Yasue H, Ohkohchi N. Platelets prevent acute liver damage after extended hepatectomy in pigs. *J Hepatobiliary Pancreat Sci* 2010; **17**: 855-864 [PMID: 20734209 DOI: 10.1007/s00534-010-0276-2]
 - 34 **Taki-Eldin A**, Zhou L, Xie HY, Zheng SS. Liver regeneration after liver transplantation. *Eur Surg Res* 2012; **48**: 139-153 [PMID: 22572792 DOI: 10.1159/000337865]
 - 35 **Hua ZY**, Song J, Cheng F, Yu Y, Gao Y, Yao A, Wang X. The effect of hepatocyte growth factor on the initiation phase of liver regeneration after cold ischemia in a rat model of small-for-size liver transplantation. *Hepatogastroenterology* 2012; **59**: 1548-1552 [PMID: 22683971 DOI: 10.5754/hge10485]
 - 36 **Haga J**, Shimazu M, Wakabayashi G, Tanabe M, Kawachi S, Fuchimoto Y, Hoshino K, Morikawa Y, Kitajima M, Kitagawa Y. Liver regeneration in donors and adult recipients after living donor liver transplantation. *Liver Transpl* 2008; **14**: 1718-1724 [PMID: 19025926 DOI: 10.1002/lt.21622]
 - 37 **Michalopoulos GK**. Liver regeneration. *J Cell Physiol* 2007; **213**: 286-300 [PMID: 17559071 DOI: 10.1002/jcp.21172]
 - 38 **Fausto N**, Campbell JS, Riehle KJ. Liver regeneration. *J Hepatol* 2012; **57**: 692-694 [PMID: 22613006 DOI: 10.1016/j.jhep.2012.04.016]
 - 39 **Murata S**, Ohkohchi N, Abe T, Enomoto Y, Doi H, Samomi S. Platelets promote G1-S progression of liver regeneration after hepatectomy. Congress of the European Society for Surgical Research 2004; 107-112
 - 40 **Lesurtel M**, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA. Platelet-derived serotonin mediates liver regeneration. *Science* 2006; **312**: 104-107 [PMID: 16601191 DOI: 10.1126/science.1123842]
 - 41 **Takahashi K**, Kozuma Y, Suzuki H, Tamura T, Maruyama T, Fukunaga K, Murata S, Ohkohchi N. Human platelets promote liver regeneration with Kupffer cells in SCID mice. *J Surg Res* 2013; **180**: 62-72 [PMID: 23260232 DOI: 10.1016/j.jss.2012.11.030]
 - 42 **Kawasaki T**, Murata S, Takahashi K, Nozaki R, Ohshiro Y, Ikeda

- N, Pak S, Myronovych A, Hisakura K, Fukunaga K, Oda T, Sasaki R, Ohkohchi N. Activation of human liver sinusoidal endothelial cell by human platelets induces hepatocyte proliferation. *J Hepatol* 2010; **53**: 648-654 [PMID: 20615569 DOI: 10.1016/j.jhep.2010.04.021]
- 43 **Murata S**, Ohkohchi N, Matsuo R, Ikeda O, Myronovych A, Hoshi R. Platelets promote liver regeneration in early period after hepatectomy in mice. *World J Surg* 2007; **31**: 808-816 [PMID: 17354025 DOI: 10.1007/s00268-006-0772-3]
- 44 **Matsuo R**, Nakano Y, Ohkohchi N. Platelet administration via the portal vein promotes liver regeneration in rats after 70% hepatectomy. *Ann Surg* 2011; **253**: 759-763 [PMID: 21475016 DOI: 10.1097/SLA.0b013e318211caf8]
- 45 **Myronovych A**, Murata S, Chiba M, Matsuo R, Ikeda O, Watanabe M, Hisakura K, Nakano Y, Kohno K, Kawasaki T, Hashimoto I, Shibasaki Y, Yasue H, Ohkohchi N. Role of platelets on liver regeneration after 90% hepatectomy in mice. *J Hepatol* 2008; **49**: 363-372 [PMID: 18602717 DOI: 10.1016/j.jhep.2008.04.019]
- 46 **Sindram D**, Porte RJ, Hoffman MR, Bentley RC, Clavien PA. Synergism between platelets and leukocytes in inducing endothelial cell apoptosis in the cold ischemic rat liver: a Kupffer cell-mediated injury. *FASEB J* 2001; **15**: 1230-1232 [PMID: 11344097]
- 47 **Kim J**, Yi NJ, Shin WY, Kim T, Lee KU, Suh KS. Platelet transfusion can be related to liver regeneration after living donor liver transplantation. *World J Surg* 2010; **34**: 1052-1058 [PMID: 20151125 DOI: 10.1007/s00268-010-0464-x]
- 48 **Han S**, Park HW, Song JH, Gwak MS, Lee WJ, Kim G, Lee SK, Ko JS. Association Between Intraoperative Platelet Transfusion and Early Graft Regeneration in Living Donor Liver Transplantation. *Ann Surg* 2016; **264**: 1065-1072 [PMID: 26720430 DOI: 10.1097/SLA.0000000000001526]
- 49 **Li L**, Wang H, Yang J, Jiang L, Yang J, Wang W, Yan L, Wen T, Li B, Xu M. Immediate Postoperative Low Platelet Counts After Living Donor Liver Transplantation Predict Early Allograft Dysfunction. *Medicine (Baltimore)* 2015; **94**: e1373 [PMID: 26313775 DOI: 10.1097/MD.0000000000001373]
- 50 **Pamecha V**, Mahansaria SS, Kumar S, Bharathy KG, Sasturkar SV, Sinha PK, Kumar N, Kumar V. Association of thrombocytopenia with outcome following adult living donor liver transplantation. *Transpl Int* 2016; **29**: 1126-1135 [PMID: 27429066 DOI: 10.1111/tri.12819]
- 51 **Akamatsu N**, Sugawara Y, Kanako J, Arita J, Sakamoto Y, Hasegawa K, Kokudo N. Low Platelet Counts and Prolonged Prothrombin Time Early After Operation Predict the 90 Days Morbidity and Mortality in Living-donor Liver Transplantation. *Ann Surg* 2017; **265**: 166-172 [PMID: 28009742 DOI: 10.1097/SLA.0000000000001634]
- 52 **Takahashi K**, Kurokawa T, Oshiro Y, Fukunaga K, Sakashita S, Ohkohchi N. Postoperative Decrease in Platelet Counts Is Associated with Delayed Liver Function Recovery and Complications after Partial Hepatectomy. *Tohoku J Exp Med* 2016; **239**: 47-55 [PMID: 27181573 DOI: 10.1620/tjem.239.47]
- 53 **Lisman T**, Leebeek FW, de Groot PG. Haemostatic abnormalities in patients with liver disease. *J Hepatol* 2002; **37**: 280-287 [PMID: 12127437]
- 54 **Lisman T**, Adelmeijer J, de Groot PG, Janssen HL, Leebeek FW. No evidence for an intrinsic platelet defect in patients with liver cirrhosis--studies under flow conditions. *J Thromb Haemost* 2006; **4**: 2070-2072 [PMID: 16836657 DOI: 10.1111/j.1538-7836.2006.02122.x]
- 55 **Himmelreich G**, Hundt K, Neuhaus P, Roissant R, Riess H. Decreased platelet aggregation after reperfusion in orthotopic liver transplantation. *Transplantation* 1992; **53**: 582-586 [PMID: 1549850]
- 56 **Himmelreich G**, Hundt K, Isenberg C, Bechstein WO, Neuhaus P, Riess H. Thrombocytopenia and platelet dysfunction in orthotopic liver transplantation. *Semin Thromb Hemost* 1993; **19**: 209-212 [PMID: 8362249 DOI: 10.1055/s-2007-994027]
- 57 **Jüttner B**, Brock J, Weissig A, Becker T, Studzinski A, Osthaus WA, Bornscheuer A, Scheinichen D. Dependence of platelet function on underlying liver disease in orthotopic liver transplantation. *Thromb Res* 2009; **124**: 433-438 [PMID: 19616824 DOI: 10.1016/j.thromres.2009.06.011]
- 58 **Eyraud D**, Gostian O, Gossem P. Does liver transplantation affect platelet function? *Euro J Anaesth* 2011; **28**: 87-88
- 59 **Vivarelli M**, La Barba G, Cucchetti A, Lauro A, Del Gaudio M, Ravaioli M, Grazi GL, Pinna AD. Can antiplatelet prophylaxis reduce the incidence of hepatic artery thrombosis after liver transplantation? *Liver Transpl* 2007; **13**: 651-654 [PMID: 17457885 DOI: 10.1002/lt.21028]
- 60 **Shay R**, Taber D, Pilch N, Meadows H, Tischer S, McGillicuddy J, Bratton C, Baliga P, Chavin K. Early aspirin therapy may reduce hepatic artery thrombosis in liver transplantation. *Transplant Proc* 2013; **45**: 330-334 [PMID: 23267805 DOI: 10.1016/j.transproceed.2012.05.075]
- 61 **Poujol-Robert A**, Boëlle PY, Conti F, Durand F, Duvoux C, Wendum D, Paradis V, Mackiewicz V, Chazouillères O, Corpechot C, Poupon R. Aspirin may reduce liver fibrosis progression: Evidence from a multicenter retrospective study of recurrent hepatitis C after liver transplantation. *Clin Res Hepatol Gastroenterol* 2014; **38**: 570-576 [PMID: 25130796 DOI: 10.1016/j.clinre.2014.07.004]
- 62 **Lee PC**, Yeh CM, Hu YW, Chen CC, Liu CJ, Su CW, Huo TI, Huang YH, Chao Y, Chen TJ, Lin HC, Wu JC. Antiplatelet Therapy is Associated with a Better Prognosis for Patients with Hepatitis B Virus-Related Hepatocellular Carcinoma after Liver Resection. *Ann Surg Oncol* 2016; **23**: 874-883 [PMID: 27541812 DOI: 10.1245/s10434-016-5520-9]
- 63 **Sitia G**, Aiolfi R, Di Lucia P, Mainetti M, Fiocchi A, Mingozzi F, Esposito A, Ruggeri ZM, Chisari FV, Iannacone M, Guidotti LG. Antiplatelet therapy prevents hepatocellular carcinoma and improves survival in a mouse model of chronic hepatitis B. *Proc Natl Acad Sci USA* 2012; **109**: E2165-E2172 [PMID: 22753481 DOI: 10.1073/pnas.1209182109]
- 64 **Sahasrabudde VV**, Gunja MZ, Graubard BI, Trabert B, Schwartz LM, Park Y, Hollenbeck AR, Freedman ND, McGlynn KA. Nonsteroidal anti-inflammatory drug use, chronic liver disease, and hepatocellular carcinoma. *J Natl Cancer Inst* 2012; **104**: 1808-1814 [PMID: 23197492 DOI: 10.1093/jnci/djs452]
- 65 **Giannini EG**. Review article: thrombocytopenia in chronic liver disease and pharmacologic treatment options. *Aliment Pharmacol Ther* 2006; **23**: 1055-1065 [PMID: 16611265 DOI: 10.1111/j.1365-2036.2006.02889.x]
- 66 **Afdhal N**, McHutchison J, Brown R, Jacobson I, Manns M, Poordad F, Weksler B, Esteban R. Thrombocytopenia associated with chronic liver disease. *J Hepatol* 2008; **48**: 1000-1007 [PMID: 18433919 DOI: 10.1016/j.jhep.2008.03.009]
- 67 **Peck-Radosavljevic M**. Thrombocytopenia in chronic liver disease. *Liver Int* 2017; **37**: 778-793 [PMID: 27860293 DOI: 10.1111/liv.13317]
- 68 **Williams R**, O'Grady JG. Liver transplantation: results, advances and problems. *J Gastroenterol Hepatol* 1990; **5** Suppl 1: 110-126 [PMID: 2103420]
- 69 **Kalayoglu M**, Sollinger HW, Stratta RJ, D'Alessandro AM, Hoffmann RM, Pirsch JD, Belzer FO. Extended preservation of the liver for clinical transplantation. *Lancet* 1988; **1**: 617-619 [PMID: 2894550]
- 70 **Porte RJ**, Blauw E, Knot EA, de Maat MP, de Ruiter C, Minke Bakker C, Terpstra OT. Role of the donor liver in the origin of platelet disorders and hyperfibrinolysis in liver transplantation. *J Hepatol* 1994; **21**: 592-600 [PMID: 7814807]
- 71 **Nobuoka Y**, Wada H, Mizuno S, Kishiwada M, Usui M, Sakurai H, Tabata M, Kobayashi T, Nobori T, Uemoto S, Isaji S. Prolonged thrombocytopenia after living donor liver transplantation is a strong prognostic predictor irrespective of splenectomy: the significance of ADAMTS13 and graft function [corrected]. *Int J Hematol* 2014; **99**: 418-428 [PMID: 24595551 DOI: 10.1007/s12185-014-1543-9]
- 72 **Nakanuma S**, Miyashita T, Hayashi H, Tajima H, Takamura H, Tsukada T, Okamoto K, Sakai S, Makino I, Kinoshita J, Nakamura K, Oyama K, Inokuchi M, Nakagawara H, Ninomiya I, Kitagawa H,

- Fushida S, Fujimura T, Ohta T. Extravasated platelet aggregation in liver zone 3 may correlate with the progression of sinusoidal obstruction syndrome following living donor liver transplantation: A case report. *Exp Ther Med* 2015; **9**: 1119-1124 [PMID: 25780397 DOI: 10.3892/etm.2015.2245]
- 73 **Shimada Y**, Kato T, Ogami K, Horie K, Kokubo A, Kudo Y, Maeda E, Sohma Y, Akahori H, Kawamura K. Production of thrombopoietin (TPO) by rat hepatocytes and hepatoma cell lines. *Exp Hematol* 1995; **23**: 1388-1396 [PMID: 7498368]
 - 74 **Richards EM**, Alexander GJ, Nichol JL, Baglin TP. Serum thrombopoietin levels following orthotopic liver transplantation. *Thromb Haemost* 1997; **78**: 1420-1421 [PMID: 9408033]
 - 75 **Peck-Radosavljevic M**, Wichlas M, Zacherl J, Stiegler G, Stohlawetz P, Fuchsjäger M, Kreil A, Metz-Schimmerl S, Panzer S, Steininger R, Mühlbacher F, Ferenci P, Pidlich J, Gangl A. Thrombopoietin induces rapid resolution of thrombocytopenia after orthotopic liver transplantation through increased platelet production. *Blood* 2000; **95**: 795-801 [PMID: 10648388]
 - 76 **Usui M**, Wada H, Mizuno S, Isaji S. Platelet activation and liver transplantation. *J Liver Dis* 2017; **6** [DOI: 10.4172/2167-0889.1000210]
 - 77 **Tredger JM**, Brown NW, Adams J, Gonde CE, Dhawan A, Rela M, Heaton N. Monitoring mycophenolate in liver transplant recipients: toward a therapeutic range. *Liver Transpl* 2004; **10**: 492-502 [PMID: 15048791 DOI: 10.1002/lt.10124]
 - 78 **Danesi R**, Del Tacca M. Hematologic toxicity of immunosuppressive treatment. *Transplant Proc* 2004; **36**: 703-704 [PMID: 15110637 DOI: 10.1016/j.transproceed.2004.03.016]
 - 79 **Lee CH**, Chen CL, Lin CC, Yang CH, Cheng YF, Wang MC, Eng HL, Liu PP, Chuang FR. Plasma exchange therapy for thrombotic thrombocytopenic purpura in pediatric patients with liver transplantation. *Transplant Proc* 2008; **40**: 2554-2556 [PMID: 18929799 DOI: 10.1016/j.transproceed.2008.07.011]
 - 80 **Nwaba A**, MacQuillan G, Adams LA, Garas G, Delriviere L, Augustson B, DeBoer B, Moody H, Jeffrey GP. Tacrolimus-induced thrombotic microangiopathy in orthotopic liver transplant patients: case series of four patients. *Intern Med J* 2013; **43**: 328-333 [PMID: 23441660 DOI: 10.1111/imj.12048]
 - 81 **Rerolle JP**, Akposso K, Lerolle N, Mougnot B, Ponnelle T, Rondeau E, Sraer JD. Tacrolimus-induced hemolytic uremic syndrome and end-stage renal failure after liver transplantation. *Clin Transplant* 2000; **14**: 262-265 [PMID: 10831087]
 - 82 **Bakchoul T**, Assfalg V, Zöllner H, Evert M, Novotny A, Matevossian E, Friess H, Hartmann D, Hron G, Althaus K, Greinacher A, Huser N. Anti-platelet factor 4/heparin antibodies in patients with impaired graft function after liver transplantation. *J Thromb Haemost* 2014; **12**: 871-878 [PMID: 24655935 DOI: 10.1111/jth.12569]
 - 83 **Bachmann R**, Bachmann J, Lange J, Nadalin S, Königsrainer A, Ladurner R. Incidence of heparin-induced thrombocytopenia type II and postoperative recovery of platelet count in liver graft recipients: a retrospective cohort analysis. *J Surg Res* 2014; **186**: 429-435 [PMID: 24100055 DOI: 10.1016/j.jss.2013.08.034]
 - 84 **Pannicke N**, Pollok JM, Kluge S, Petzoldt M. Heparin-induced thrombocytopenia associated with acute liver graft failure. *BMJ Case Rep* 2012; **2012**: [PMID: 23188860 DOI: 10.1136/bcr-2012-007323]
 - 85 **Hüser N**, Abfal V, Reim D, Novotny A, Thorban S, Cheng Z, Kornberg A, Friess H, Büchler P, Matevossian E. Heparin-induced thrombocytopenia (HIT II) in liver transplant recipients: a retrospective multivariate analysis of prognostic factors. *Transpl Int* 2012; **25**: 739-747 [PMID: 22548256 DOI: 10.1111/j.1432-2277.2012.01486.x]
 - 86 **Moicean AD**, Popp AM, Sinescu I. Thymoglobulin--new approaches to optimal outcomes. *J Med Life* 2009; **2**: 319-324 [PMID: 20112478]
 - 87 **Lazarchick J**, Russell R, Horn B. Anti-thymocyte globulin induced thrombocytopenia. *Ann Clin Lab Sci* 1990; **20**: 373-378 [PMID: 2073086]
 - 88 **Ankersmit HJ**, Roth GA, Moser B, Zuckermann A, Brunner M, Rosin C, Buchta C, Bielek E, Schmid W, Jensen-Jarolim E, Wolner E, Boltz-Nitulescu G, Volf I. CD32-mediated platelet aggregation in vitro by anti-thymocyte globulin: implication of therapy-induced in vivo thrombocytopenia. *Am J Transplant* 2003; **3**: 754-759 [PMID: 12780568]
 - 89 **Gabardi S**, Magee CC, Baroletti SA, Powelson JA, Cina JL, Chandraker AK. Efficacy and safety of low-dose valganciclovir for prevention of cytomegalovirus disease in renal transplant recipients: a single-center, retrospective analysis. *Pharmacotherapy* 2004; **24**: 1323-1330 [PMID: 15628830]
 - 90 **Hayashi M**, Strouse JJ, Veltri MA, Curtis BR, Takemoto CM. Immune thrombocytopenia due to Trimethoprim-Sulfamethoxazole; under-recognized adverse drug reaction in children? *Pediatr Blood Cancer* 2015; **62**: 922-923 [PMID: 25683320 DOI: 10.1002/pbc.25430]
 - 91 **Piano S**, Gatta A, Angeli P. Immune thrombocytopenic purpura and Kaposi's sarcoma in a liver transplant recipient. *Transpl Int* 2012; **25**: e50-e52 [PMID: 22417011 DOI: 10.1111/j.1432-2277.2011.01424.x]
 - 92 **Rosoff PM**, Tuttle-Newhall E, Treem WR. Successful treatment of immune thrombocytopenic purpura with anti-D antibody following a cadaveric liver transplant for hepatoblastoma. *Med Pediatr Oncol* 2003; **40**: 402-404 [PMID: 12692815 DOI: 10.1002/mpo.10243]
 - 93 **Assy N**, Rosenthal E, Hazani A, Etzioni A, Baruch Y. Human parvovirus B19 infection associated with idiopathic thrombocytopenic purpura in a child following liver transplantation. *J Hepatol* 1997; **27**: 934-936 [PMID: 9382984]
 - 94 **Singh N**, Gayowski T, Yu VL. Herpes zoster-associated idiopathic thrombocytopenic purpura in a liver transplant recipient: a case report and overview. *Transpl Int* 1995; **8**: 58-60 [PMID: 7888054]
 - 95 **Takatsuki M**, Uemoto S, Kurokawa T, Koshiha T, Inomata Y, Tanaka K. Idiopathic thrombocytopenic purpura after a living-related liver transplantation. *Transplantation* 1999; **67**: 479-481 [PMID: 10030298]
 - 96 **Gupta RK**, Gupta G, Chorasaya VK, Bag P, Shandil R, Bhatia V, Wadhawan M, Vij V, Kumar A. Dengue Virus Transmission from Living Donor to Recipient in Liver Transplantation: A Case Report. *J Clin Exp Hepatol* 2016; **6**: 59-61 [PMID: 27194898 DOI: 10.1016/j.jceh.2016.01.005]
 - 97 **Arnold JC**, Heilig B, Otto G, Kommerell B, Theilmann L. Cytomegalovirus-induced severe thrombocytopenia after liver transplantation. *Transplantation* 1993; **56**: 1286-1288 [PMID: 8249141]
 - 98 **Maar E**, Porte R, Harmsen M, Son W, Berg A, Slooff MJ, Nijsten M. Decreased platelet count precedes CMV antigenemia after liver transplantation. University of Groningen 2003; 129-138
 - 99 **Miyata T**, Yokoyama I, Todo S, Tzakis A, Selby R, Starzl TE. Endotoxaemia, pulmonary complications, and thrombocytopenia in liver transplantation. *Lancet* 1989; **2**: 189-191 [PMID: 2568522]
 - 100 **de Boer MT**, Christensen MC, Asmussen M, van der Hilst CS, Hendriks HG, Slooff MJ, Porte RJ. The impact of intraoperative transfusion of platelets and red blood cells on survival after liver transplantation. *Anesth Analg* 2008; **106**: 32-44, table of contents [PMID: 18165548 DOI: 10.1213/01.ane.0000289638.26666.ed]
 - 101 **Pereboom IT**, de Boer MT, Haagsma EB, Hendriks HG, Lisman T, Porte RJ. Platelet transfusion during liver transplantation is associated with increased postoperative mortality due to acute lung injury. *Anesth Analg* 2009; **108**: 1083-1091 [PMID: 19299765 DOI: 10.1213/ane.0b013e3181948a59]
 - 102 **Chin JL**, Hisamuddin SH, O'Sullivan A, Chan G, McCormick PA. Thrombocytopenia, Platelet Transfusion, and Outcome Following Liver Transplantation. *Clin Appl Thromb Hemost* 2016; **22**: 351-360 [PMID: 25430936 DOI: 10.1177/1076029614559771]
 - 103 **Nacoti M**, Cazzaniga S, Colombo G, Corbella D, Fazzi F, Fochi O, Gattoni C, Zambelli M, Colledan M, Bonanomi E. Postoperative complications in cirrhotic pediatric deceased donor liver transplantation: Focus on transfusion therapy. *Pediatr Transplant* 2017; **21** [PMID: 28681471 DOI: 10.1111/ptr.13020]
 - 104 **Nixon C**, Gunn K, Main T, Young Y, McCall J. Platelets and survival after liver transplantation. *Anesth Analg* 2009; **108**: 1354-1355; author reply 1355 [PMID: 19299817 DOI: 10.1213/ane.0b013e318197c7c7]

- 105 **Li C**, Mi K, Wen TF, Yan LN, Li B, Wei YG, Yang JY, Xu MQ, Wang WT. Risk factors and outcomes of massive red blood cell transfusion following living donor liver transplantation. *J Dig Dis* 2012; **13**: 161-167 [PMID: 22356311 DOI: 10.1111/j.1751-2980.2011.00570.x]
- 106 **Nogami K**. The utility of thromboelastography in inherited and acquired bleeding disorders. *Br J Haematol* 2016; **174**: 503-514 [PMID: 27264484 DOI: 10.1111/bjh.14148]
- 107 **Lawson PJ**, Moore HB, Moore EE, Stettler GR, Pshak TJ, Kam I, Silliman CC, Nydam TL. Preoperative thrombelastography maximum amplitude predicts massive transfusion in liver transplantation. *J Surg Res* 2017; **220**: 171-175 [PMID: 29180179 DOI: 10.1016/j.jss.2017.05.115]
- 108 **Trautman CL**, Palmer WC, Taner CB, Canabal JM, Getz T, Goldman A, Heckman MG, Diehl NN, Lee DD, Stancampiano FF. Thromboelastography as a Predictor of Outcomes Following Liver Transplantation. *Transplant Proc* 2017; **49**: 2110-2116 [PMID: 29149970 DOI: 10.1016/j.transproceed.2017.07.015]
- 109 **Kang YG**, Martin DJ, Marquez J, Lewis JH, Bontempo FA, Shaw BW Jr, Starzl TE, Winter PM. Intraoperative changes in blood coagulation and thrombelastographic monitoring in liver transplantation. *Anesth Analg* 1985; **64**: 888-896 [PMID: 3896028]
- 110 **Krzanicki D**, Sugavanam A, Mallett S. Intraoperative hypercoagulability during liver transplantation as demonstrated by thromboelastography. *Liver Transpl* 2013; **19**: 852-861 [PMID: 23696318 DOI: 10.1002/lt.23668]
- 111 **Wikkelsøe AJ**, Afshari A, Wetterslev J, Brok J, Moeller AM. Monitoring patients at risk of massive transfusion with Thrombelastography or Thromboelastometry: a systematic review. *Acta Anaesthesiol Scand* 2011; **55**: 1174-1189 [PMID: 22092122 DOI: 10.1111/j.1399-6576.2011.02534.x]
- 112 **Marubashi S**, Dono K, Miyamoto A, Takeda Y, Nagano H, Umeshita K, Monden M. Impact of graft size on postoperative thrombocytopenia in living donor liver transplant. *Arch Surg* 2007; **142**: 1054-1058 [PMID: 18025333 DOI: 10.1001/archsurg.142.11.1054]
- 113 **Li DW**, Du CY, Fan B, Huang P, Luo SQ, He Q. Impact of simultaneous splenectomy and orthotopic liver transplantation in patients with end-stage liver diseases and splenic hyperfunction. *Hepatobiliary Pancreat Dis Int* 2012; **11**: 489-493 [PMID: 23060393]
- 114 **Chu HC**, Hsieh CB, Hsu KF, Fan HL, Hsieh TY, Chen TW. Simultaneous splenectomy during liver transplantation augments anti-viral therapy in patients infected with hepatitis C virus. *Am J Surg* 2015; **209**: 180-186 [PMID: 24928331 DOI: 10.1016/j.amjsurg.2014.03.004]
- 115 **Rysmakhanov M**, Dorskali M, Taganova A, Kulmagambetov A, Smagulov A, Seidakhmetov A, Baigenzhin A, Dorskaliyev Z. Splenic Artery Embolization in Patients After Orthotopic Liver Transplant. *Exp Clin Transplant* 2015; **13** Suppl 3: 52-54 [PMID: 26640912 DOI: 10.6002/ect.tdtd2015.O43]
- 116 **Ito K**, Akamatsu N, Ichida A, Ito D, Kaneko J, Arita J, Sakamoto Y, Hasegawa K, Kokudo N. Splenectomy is not indicated in living donor liver transplantation. *Liver Transpl* 2016; **22**: 1526-1535 [PMID: 27253521 DOI: 10.1002/lt.24489]
- 117 **Morimoto H**, Ishiyama K, Ishifuro M, Ohira M, Ide K, Tanaka Y, Tahara H, Teraoka Y, Yamashita M, Abe T, Hashimoto S, Hirata F, Tanimine N, Saeki Y, Shimizu S, Sakai H, Yano T, Tashiro H, Ohdan H. Clinical efficacy of simultaneous splenectomy in liver transplant recipients with hepatitis C virus. *Transplant Proc* 2014; **46**: 770-773 [PMID: 24767345 DOI: 10.1016/j.transproceed.2013.12.034]
- 118 **Takahashi Y**, Matsuura T, Yanagi Y, Yoshimaru K, Taguchi T. The role of splenectomy before liver transplantation in biliary atresia patients. *J Pediatr Surg* 2016; **51**: 2095-2098 [PMID: 27720430 DOI: 10.1016/j.jpedsurg.2016.09.048]
- 119 **Murata S**, Hashimoto I, Nakano Y, Myronovych A, Watanabe M, Ohkohchi N. Single administration of thrombopoietin prevents progression of liver fibrosis and promotes liver regeneration after partial hepatectomy in cirrhotic rats. *Ann Surg* 2008; **248**: 821-828 [PMID: 18948810 DOI: 10.1097/SLA.0b013e31818584c7]
- 120 **Watanabe M**, Murata S, Hashimoto I, Nakano Y, Ikeda O, Aoyagi Y, Matsuo R, Fukunaga K, Yasue H, Ohkohchi N. Platelets contribute to the reduction of liver fibrosis in mice. *J Gastroenterol Hepatol* 2009; **24**: 78-89 [PMID: 18624898 DOI: 10.1111/j.1440-1746.2008.05497.x]
- 121 **Schipperus M**, Fijnheer R. New therapeutic options for immune thrombocytopenia. *Neth J Med* 2011; **69**: 480-485 [PMID: 22173361]
- 122 **Bode AP**, Fischer TH. Lyophilized platelets: fifty years in the making. *Artif Cells Blood Substit Immobil Biotechnol* 2007; **35**: 125-133 [PMID: 17364477 DOI: 10.1080/10731190600974962]
- 123 **Okamura Y**, Takeoka S, Eto K, Maekawa I, Fujie T, Maruyama H, Ikeda Y, Handa M. Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. *J Thromb Haemost* 2009; **7**: 470-477 [PMID: 19143920 DOI: 10.1111/j.1538-7836.2008.03269.x]
- 124 **Hoshi R**, Murata S, Matsuo R, Myronovych A, Hashimoto I, Ikeda H, Ohkohchi N. Freeze-dried platelets promote hepatocyte proliferation in mice. *Cryobiology* 2007; **55**: 255-260 [PMID: 17936259 DOI: 10.1016/j.cryobiol.2007.08.007]

P- Reviewer: Rodriguez-Peralvarez ML, Stanciu C **S- Editor:** Ma YJ
L- Editor: A **E- Editor:** Huang Y



Basic Study

Systems pharmacology approach reveals the antiinflammatory effects of *Ampelopsis grossedentata* on dextran sodium sulfate-induced colitis

You-Lan Chen, Ya-Li Zhang, Yan-Cheng Dai, Zhi-Peng Tang

You-Lan Chen, Ya-Li Zhang, Zhi-Peng Tang, Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

You-Lan Chen, Yan-Cheng Dai, Zhi-Peng Tang, Department of Gastroenterology, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

ORCID number: You-Lan Chen (0000-0002-4304-5693); Ya-Li Zhang (0000-0002-3538-9832); Yan-Cheng Dai (0000-0001-9919-4033); Zhi-Peng Tang (0000-0001-5695-8072).

Author contributions: Chen YL performed the majority of experiments and drafted the paper; Zhang YL participated in the treatment of animals; Dai YC analyzed the data; Tang ZP designed and coordinated the research; all authors have approved the final version of the article to be published.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (IACUC Protocol Approval Number: SZY201710004).

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this study.

Data sharing statement: Supplementary data accompanying this paper are included in the Supplementary information file.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and

the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Zhi-Peng Tang, MD, PhD, Professor, Director, Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, No. 725 South Wanping Road, Shanghai 200032, China. zhipengtang@sohu.com
Telephone: +86-21-64385700
Fax: +86-21-64385700

Received: January 27, 2018

Peer-review started: January 28, 2018

First decision: February 10, 2018

Revised: February 12, 2018

Accepted: March 3, 2018

Article in press: March 3, 2018

Published online: April 7, 2018

Abstract

AIM

To investigate the protective effects of *Ampelopsis grossedentata* (AMP) on dextran sulfate sodium (DSS)-induced colitis in mice based on systems pharmacology approach.

METHODS

Systems pharmacology approach was used to predict the active ingredients, candidate targets and the efficacy of AMP on ulcerative colitis (UC) using a holistic process of active compound screening, target fishing, network construction and analysis. A DSS-induced colitis model in C57BL/6 mice ($n = 10/\text{group}$) was constructed and treated with 5-aminosalicylic acid (100 mg/kg/d) and AMP (400 mg/kg/d) to confirm

the underlying mechanisms and effects of AMP on UC with western blot analyses, polymerase chain reaction, histological staining and immunohistochemistry.

RESULTS

The therapeutic effects of AMP against DSS-induced colitis were determined in the beginning, and the results showed that AMP significantly improved the disease in general observations and histopathology analysis. Subsequent systems pharmacology predicted 89 corresponding targets for the four candidate compounds of AMP, as well as 123 candidate targets of UC, and protein-protein interaction networks were constructed for the interaction of putative targets of AMP against UC. Enrichment analyses on TNF- α and RANKL/RANK, a receptor activator of NF- κ B signaling pathways, were then carried out. Experimental validation revealed that inflammation-related signaling pathways were activated in the DSS group, and AMP significantly suppressed DSS-induced high expression of IRAK1, TRAF6, I κ B and NF- κ B, and inhibited the elevated expression levels of TNF- α , IL-1 β , IL-6 and IL-8.

CONCLUSION

AMP could exert protective effects on UC *via* suppressing the IRAK1/TRAF6/NF- κ B-mediated inflammatory signaling pathways.

Key words: Ulcerative colitis; *Ampelopsis grossedentata*; Traditional Chinese medicine; Systems pharmacology; Inflammation

© The Author(s) 2018. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Ulcerative colitis (UC), as one of the major forms of inflammatory bowel disease, could lead to various intestinal and extra-intestinal manifestations, which brings a huge challenge to the health care system. Given studies have confirmed that the flavonoid bioactive compounds contained in *Ampelopsis grossedentata* (AMP) possess strong antiinflammatory activity, we examined the potential therapeutic effects of AMP on UC based on systems pharmacology. Results showed that AMP could suppress the inflammation-related signaling pathways in dextran sulfate sodium-induced colitis, indicating protective effects on UC, which might provide an effective natural therapy for the treatment and prevention of UC.

Chen YL, Zhang YL, Dai YC, Tang ZP. Systems pharmacology approach reveals the antiinflammatory effects of *Ampelopsis grossedentata* on dextran sodium sulfate-induced colitis. *World J Gastroenterol* 2018; 24(13): 1398-1409 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1398.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1398>

INTRODUCTION

Ulcerative colitis (UC), which is primarily characterized

by recurrent abdominal pain, diarrhea and bloody purulent stools^[1], is a chronic inflammatory condition of the intestine, with mucosal inflammation beginning in the rectum and extending continuously to part of or the entire colon. Inflammation in UC can lead to the occurrence of multifocal ulcers on the wall of the large intestine, causing nausea, cramps, diarrhea, pus, bleeding and fatigue. In general, patients with UC may develop varying degrees of extra-intestinal manifestations, which are attributed to the inflammatory cascade in the colorectum, including skin, mucosal, joint, ocular, hepatic and pulmonary disorders^[2,3]. Meanwhile, the increasing prevalence of UC brings a considerable challenge to health care systems worldwide^[4].

Given that UC is a kind of long-term disease with uncertain etiology, the aim of therapy is to induce and maintain clinical remission, defined as control of symptoms, endoscopic mucosal healing and avoidance of complications^[1,5]. In addition to dietary control, the available pharmacologic treatments include 5-aminosalicylates, steroids, thiopurines and biological agents^[6]. However, the routine medical treatments for UC are not fully curative, and investigations have shown that compared with standard care, the elevated cost-utility ratios of biologics reached up to \$456979 (in United States' dollars)^[7]. In addition, as UC mostly affects young people and takes a lifelong treatment, as well as has a low mortality^[8,9] that is not different from that in the healthy population, the disease poses an enormous economic burden on individuals, families and society. Thus, promising and novel therapeutic tactics are urgently needed to be explored and developed for UC.

Complementary and alternative medicine, especially Chinese herbal medicine, is widely used among UC patients. Recent investigations revealed that many natural compounds have significant protective efficacies in UC patients^[10]. *Ampelopsis grossedentata* (AMP), which contains a large amount of flavonoid active ingredients, has been widely consumed as a functional beverage and may be used consecutively as a supplementary option to the current standard treatment of UC. Dihydromyricetin, a major compound of AMP, has been reported to be highly distributed in the intestinal tract^[11], and has hepatoprotective^[12], insulin resistant^[13], antioxidation^[14], anticancer^[15] and antiinflammatory activities, which are produced by suppression of nuclear factor kappa-B (NF- κ B) activation^[16,17]. However, few researchers have investigated the underlying mechanisms of the potential protective effects of AMP against UC, although AMP contains much more flavonoids and deserves further study.

Considering the complex combination and multi-target interactions of Chinese herbal plants, it is quite difficult to conduct a systematic study of the effects of AMP on diseases using conventional methods. Whereas, systems pharmacology, an emerging systems-oriented approach which has been reported to reveal the

mechanism of a disease and link it to the chemical network of a drug^[18,19], provides new perspectives to predict the active ingredients and candidate targets through a holistic process of active compound screening, target fishing, network construction and analysis^[20,21]. To further investigate the potential mechanisms and effects of AMP on UC, a systems pharmacology analysis and animal experiments were conducted in this study.

MATERIALS AND METHODS

Systems pharmacology

The active compounds and their corresponding putative targets of AMP were identified by the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database. Known UC-related targets were obtained from the Genetic Association database, Therapeutic Target database and Online Mendelian Inheritance in Man database. Protein-protein interaction (PPI) networks were constructed for the interaction of putative targets of AMP against UC based on BisoGenet, and the degree centrality (DC) value, which represents the topological importance of a node in the intersection network, was used to filter the candidate targets. Finally, enrichment analysis was conducted using ClueGO, a Cytoscape plugin.

Animals

Male C57BL/6 mice aged 6 weeks were obtained from the SLAC Animal Laboratories (Shanghai, China). All mice were raised under standard conditions (room temperature, $24 \pm 2^\circ\text{C}$; humidity, 50%-60%; light, 12-h light/12-h dark cycle).

Colitis model construction and treatment

Experimental colitis was induced by administration of 3.5% (w/v) dextran sulfate sodium (DSS, 36-50 kDa; MP Biomedicals, United States) in drinking water provided ad libitum for 7 d. The DSS solution was changed every day. For each experiment, the mice were randomly divided into four groups ($n = 10/\text{group}$): the control group was given normal water only; the DSS group, 5-aminosalicylic acid (5-ASA) group and AMP group received a 3.5% DSS solution for 7 d, respectively. For treatment experiment, the control group and DSS group were administered normal saline intragastrically daily. The 5-ASA group was given 5-ASA solution (100 mg/kg/d) and the AMP group were administered AMP solution (400 mg/kg/d) intragastrically. The severity of colitis was calculated daily using the disease activity index^[22]; the disease activity index scores are shown in Supplementary Table 1. After treatment, blood was extracted by cardiac puncture under anesthesia. Intestinal tissues were fixed in 4% buffered formalin for hematoxylin & eosin/immunohistochemistry and the other tissues were snap-frozen for western blot/RT-PCR.

Histopathology and immunohistochemistry

Paraffin-embedded colon samples were stained with HE for histological evaluation, as well as were immunostained with anti-NF- κ B, anti-tumor necrosis factor- α (TNF- α) and anti-interleukin-1 beta (IL-1 β) for protein expression in intestinal tissues after being cut into 4 μm sections.

Western blot analysis

Protein extracts were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore, Germany). Protein expression was detected by western blot analysis. Primary antibodies used were rabbit monoclonal anti-NF- κ B, anti-inhibitor of NF- κ B (I κ B), anti-p-I κ B, anti-IL-1 receptor associated kinase (IRAK1), anti-TNF receptor-associated factor 2 (TRAF2) and anti-TNF receptor-associated factor 6 (TRAF6). All antibodies were purchased from Cell Signaling Technology (United States).

RNA isolation and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, United States). RT-PCR was carried out using SYBR Green PCR Master Mix (Toyobo, Japan) in an Eppendorf PCR system. The data were analyzed by the $\Delta\Delta\text{Ct}$ method and samples were normalized to β -actin. Primer sequences are shown in Supplementary Table 2.

Enzyme-linked immunosorbent assay

The levels of inflammation-related cytokines (TNF- α , IL-1 β , IL-6 and IL-8) were detected using mouse-specific ELISA kits (Dakewe Bio-engineering Co. Ltd., China), following the manufacturer's instructions.

Statistical analysis

All data are presented as the mean \pm SD. Statistical analyses were performed using the Student's t-test or one-way ANOVA for group comparisons, and $P < 0.05$ was considered statistically significant.

RESULTS

AMP improved the DSS-induced inflammatory response in acute colitis mice

As the flavonoid bioactive compound extracted from AMP could inhibit inflammatory response *in vitro* and *in vivo*^[16], the protective effects of AMP in DSS-induced colitis were examined in this study. Results presented a reduction of body weight together with an elevation of disease activity index scores under DSS stimulation (Figure 1A and B); meanwhile, treatment with 5-ASA and AMP prominently improved the body weight as well as the length of colon, with significant difference (Figure 1A and C). Additionally, histological analyses revealed more neutrophil infiltration, increased ulceration and

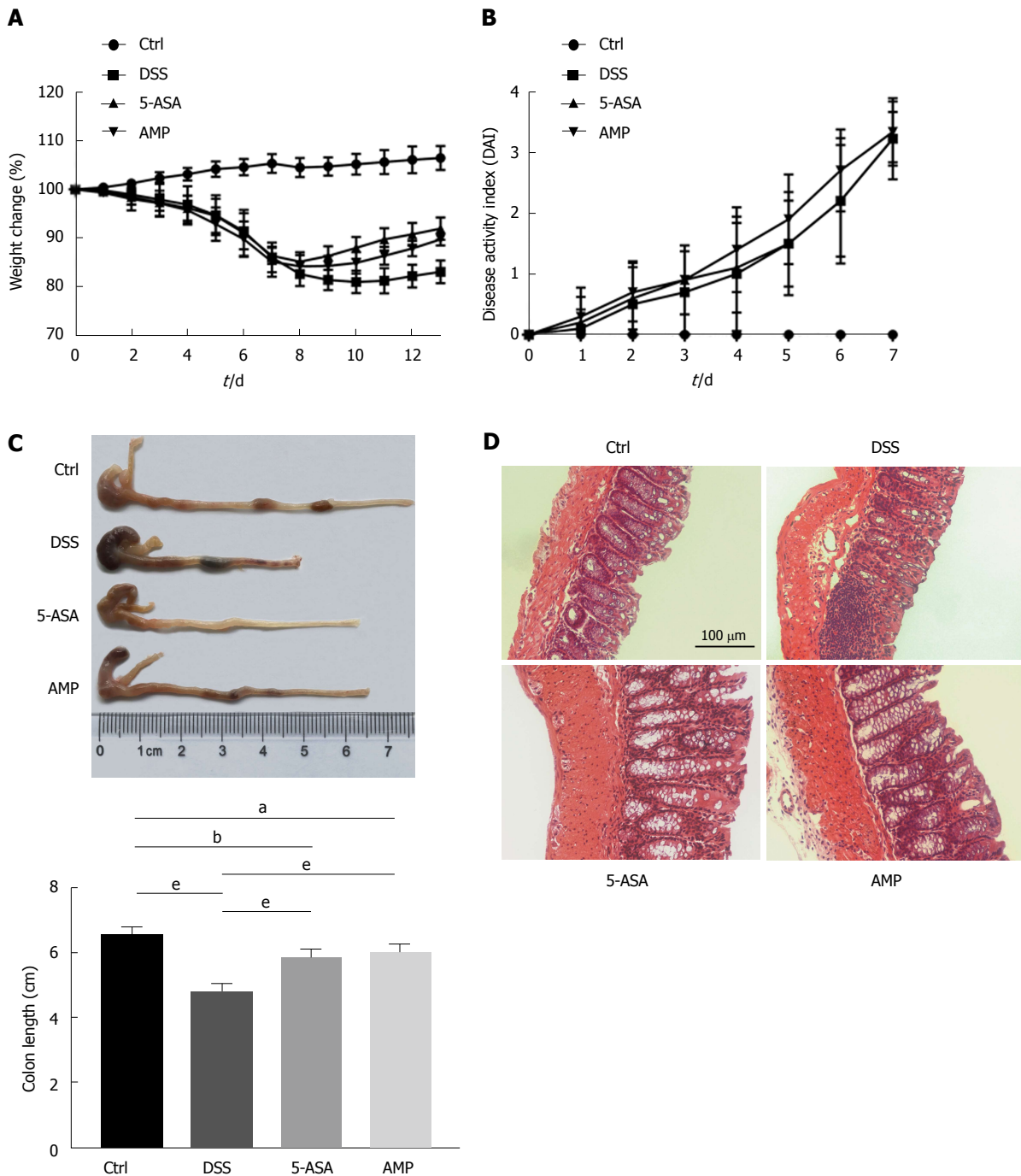


Figure 1 *Ampelopsis grossedentata* improved dextran sulfate sodium-induced inflammatory response in acute colitis mice. A: Body weight was measured every day; B: Disease activity index under DSS stimulation in mice; C: Length of colons and statistical graph; D: HE staining of colons. Data are presented as mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$. 5-ASA: 5-aminosalicylic acid; AMP: *Ampelopsis grossedentata*; DSS: Dextran sulfate sodium.

crypt loss in the colon in the DSS group compared with the control and treatment groups (Figure 1D), indicating a protective effect of AMP on experimental colitis.

Potential pharmacological mechanisms of AMP

Candidate compound screening and putative target prediction for AMP: Four candidate compounds of AMP were obtained from the TCMSP database, including dihydromyricetin, myricetin, quercetin and taxifolin (Figure 2A and Supplementary Table 3). Since recent research studies have demonstrated

strong antiinflammatory activities for all the selected compounds^[16,23-25], which may be crucially involved in the improvement of UC, putative target prediction was subsequently performed to confirm this hypothesis. Given that the comprehensive biological and pharmacological effects of Chinese herbal plants rely upon their complex compounds and multitarget interactions, a compound-target network was built based on massive open-source initiatives and free web-based tools. A total of 156 potential targets were collected from TCMSP, and 89 targets were ultimately

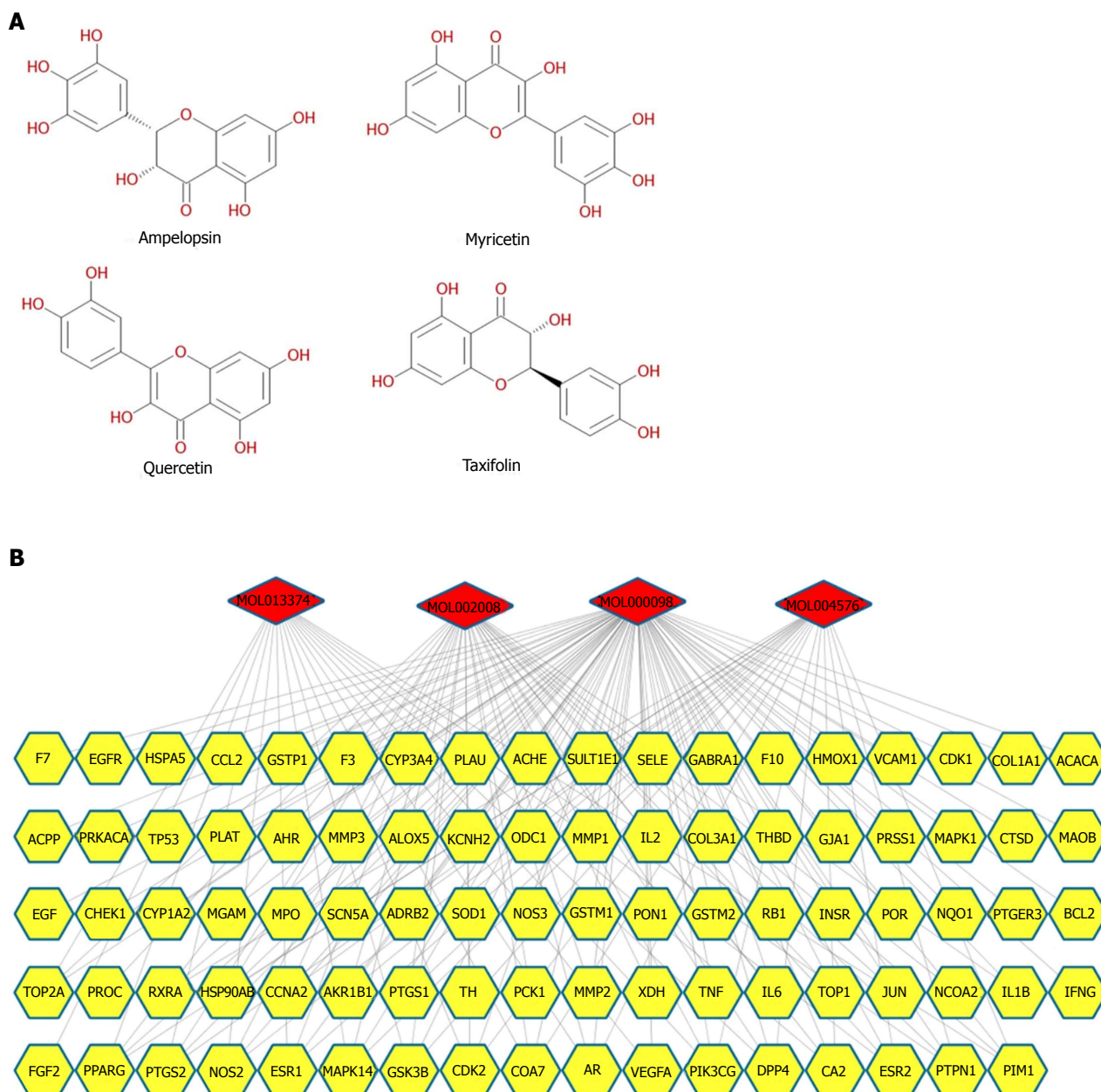


Figure 2 Candidate compound screening and putative target prediction for *ampelopsis grossedentata*. A: Chemical structures of active compounds for AMP; B: Compound-target network for AMP. AMP: *Ampelopsis grossedentata*.

included after filtering (Figure 2B and Supplementary Tables 4 and 5). Moreover, 67 targets overlapped in the 4 compounds, which indicated that most of the compounds of AMP hit multitargets to exert multifarious therapeutic effects.

PPI network constitution and identification of candidate targets for AMP against UC: To better understand the mechanisms of AMP in the treatment of UC, 123 candidate targets of UC were obtained from Genetic Association database, Therapeutic Target database and Online Mendelian Inheritance in Man database (Supplementary Table 6). As PPI maps represented physical interactions on a molecular level^[19], a putative-target network (4998 nodes and 126594

edges) and a known UC-related target network (3824 nodes and 91207 edges) were constructed based on PPI database (Figure 3A and B). Subsequently, to confirm the candidate targets of AMP against UC, an intersection of the above networks was conducted, which consisted of 2543 nodes and 70816 edges, and the DC values of 35 and 70 were computed by CytoNCA to identify the putative targets during the process (Figure 3C).

In order to further elucidate the possible effects of AMP on UC, the biological processes and signaling pathways were determined through Cytoscape software. The results showed that the biological processes were largely related to the nucleic acid metabolic process, positive regulation of macromolecule metabolic process, positive regulation of response to stimulus and regu-

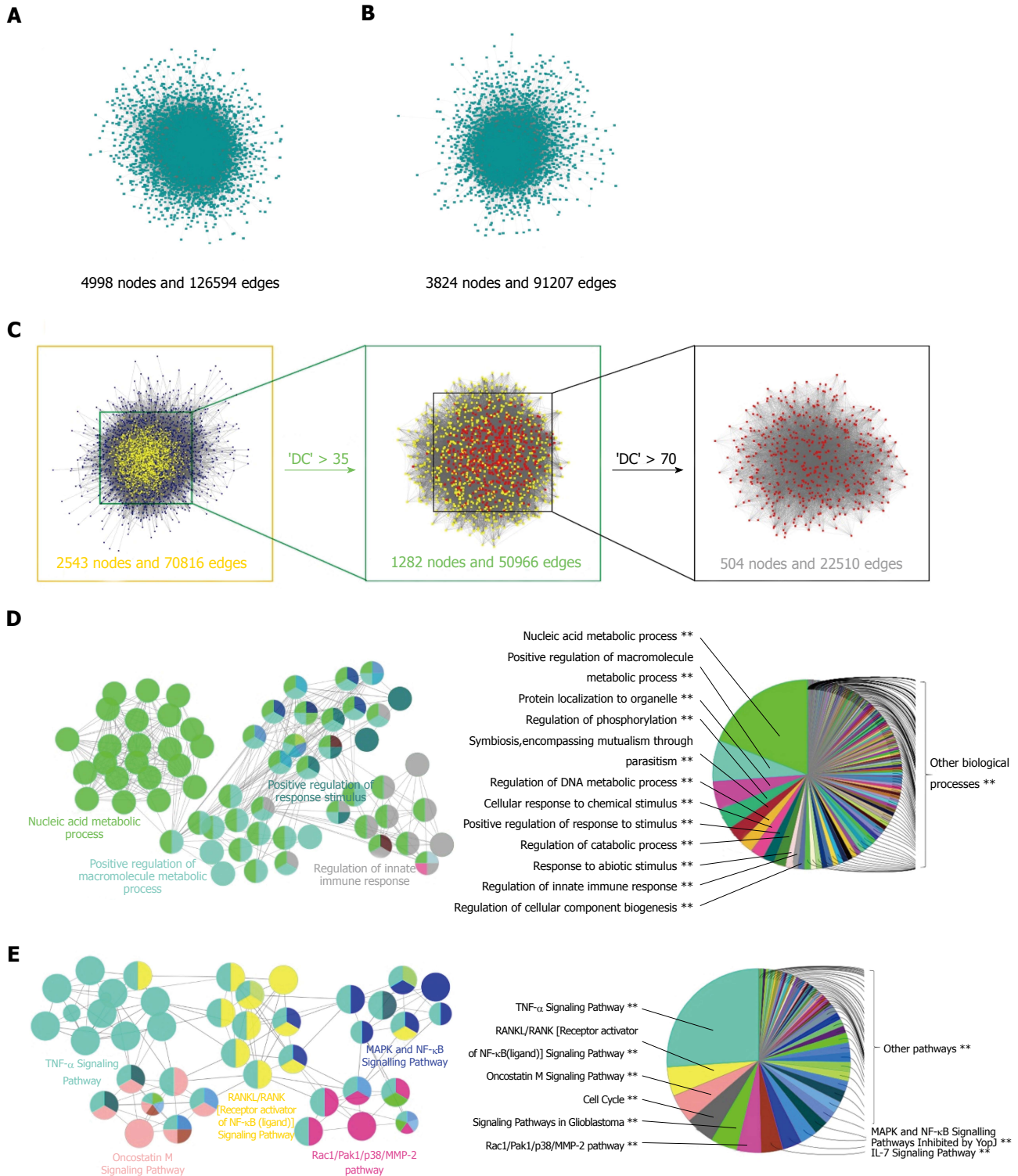


Figure 3 Protein-protein interaction network constitution and identification of candidate targets for *ampelopsis grossedentata* against ulcerative colitis. A: PPI network was constructed for the putative targets of AMP; B: PPI network was constructed for the known UC-related targets; C: Intersection networks for AMP against UC; D: Enrichment analysis of biological processes for AMP against UC; E: Enrichment analysis of signaling pathways for AMP against UC. AMP: *Ampelopsis grossedentata*; PPI: Protein-protein interaction; UC: Ulcerative colitis.

lation of the innate immune response (Figure 3D), and the signaling pathways were mainly involved with the TNF- α signaling pathway, RANKL/RANK (receptor activator of NF- κ B) signaling pathway, oncostatin M pathway, Rac1/Pak1/p38/MMP-2 pathway and MAPK/NF- κ B signaling pathway (Figure 3E). Based on these data, we proposed a hypothesis that the protective

mechanisms of AMP on UC were possibly related to inflammatory signaling pathways.

Experimental validation

AMP could suppress the inflammation-related signaling pathways in DSS-induced colitis: Since systems pharmacology demonstrated that TNF- α and

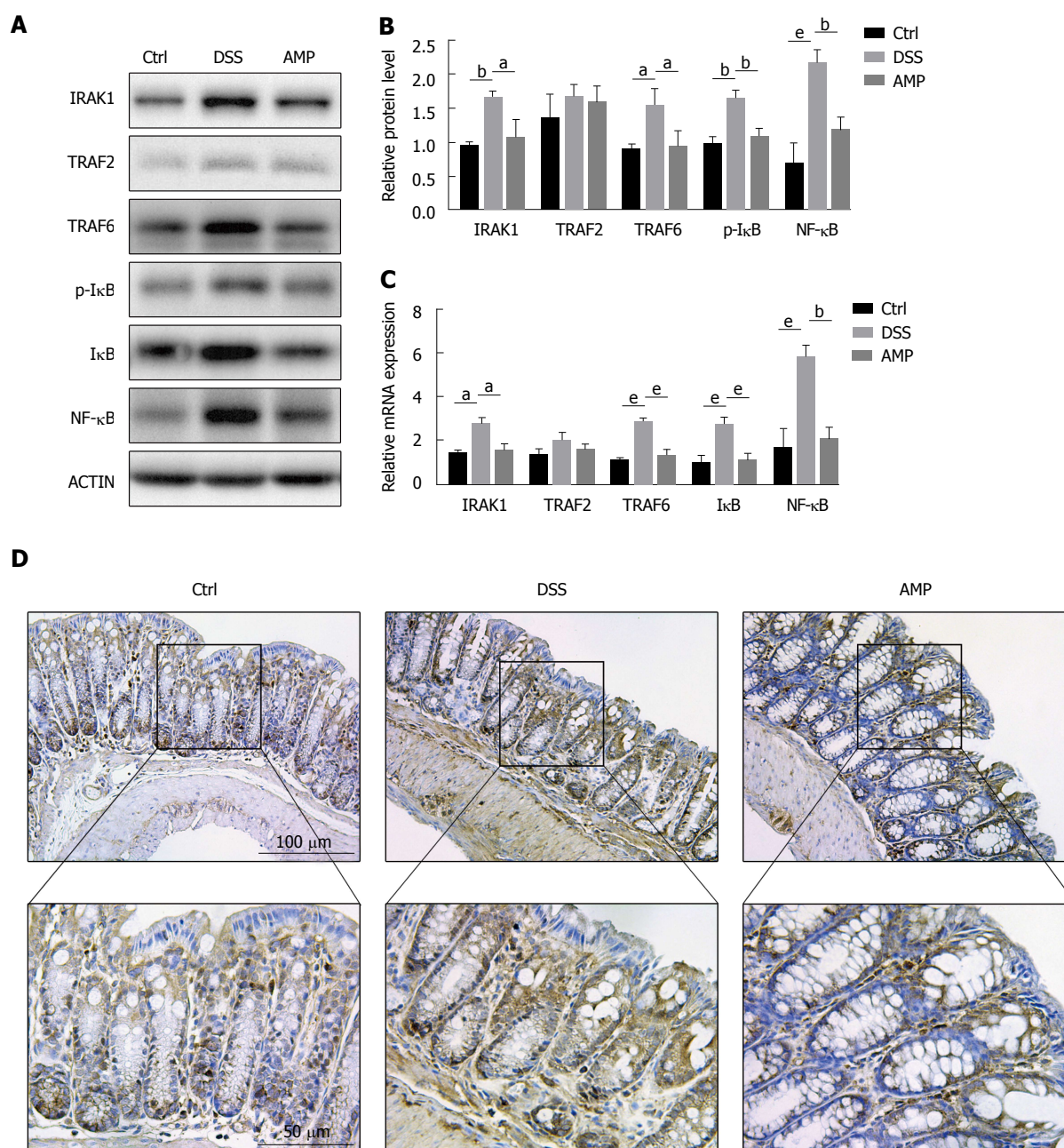


Figure 4 *Ampelopsis grossedentata* could suppress the inflammation-related signaling pathways in dextran sulfate sodium-induced colitis. A: Representative western blot analyses of inflammation-related proteins in colon tissues; B: Statistical graph of western blot analyses, relative protein levels of IRAK1, TRAF2, TRAF6 and NF-κB were determined by normalization to actin, and relative p-IκB level was determined by normalization to IκB; C: mRNA expression of inflammation-related genes in colon tissues, relative mRNA expression levels of IRAK1, TRAF2, TRAF6, IκB and NF-κB were determined by normalization to actin; D: Representative images of anti-NF-κB immunohistochemistry. Data are presented as mean ± SD. ^a $P < 0.05$, ^b $P < 0.01$, ^e $P < 0.001$. AMP: *Ampelopsis grossedentata*; DSS: Dextran sulfate sodium.

RANKL/RANK (receptor activator of NF-κB) signaling pathways were involved in the therapeutic effects of AMP on UC, western blot analyses were performed to evaluate TRAF2 and TRAF6, the downstream events of TNFR and RANK. Interestingly, outcomes presented a significant increase of TRAF6 expression in the DSS group as well as a mild increase of TRAF2 expression, compared with the control group, and AMP could improve this condition (Figure 4A and B). Thus, the upstream indicator of TRAF6, IRAK1 was determined next to investigate the further mechanisms in the

TRAF6-mediated inflammatory signaling pathway, and results showed a marked elevation in the DSS group and that AMP could alleviate this situation, suggesting that IL-1-mediated proinflammatory signaling might be involved in the antiinflammatory action of AMP in UC. Accordingly, the mRNA expression of TRAF6 and IRAK1 was enhanced, whereas the expression of TRAF2 was not (Figure 4C), indicating that AMP might exert antiinflammatory effects *via* IRAK1/TRAF6-mediated signaling pathway.

Given IκB was the shared core downstream event

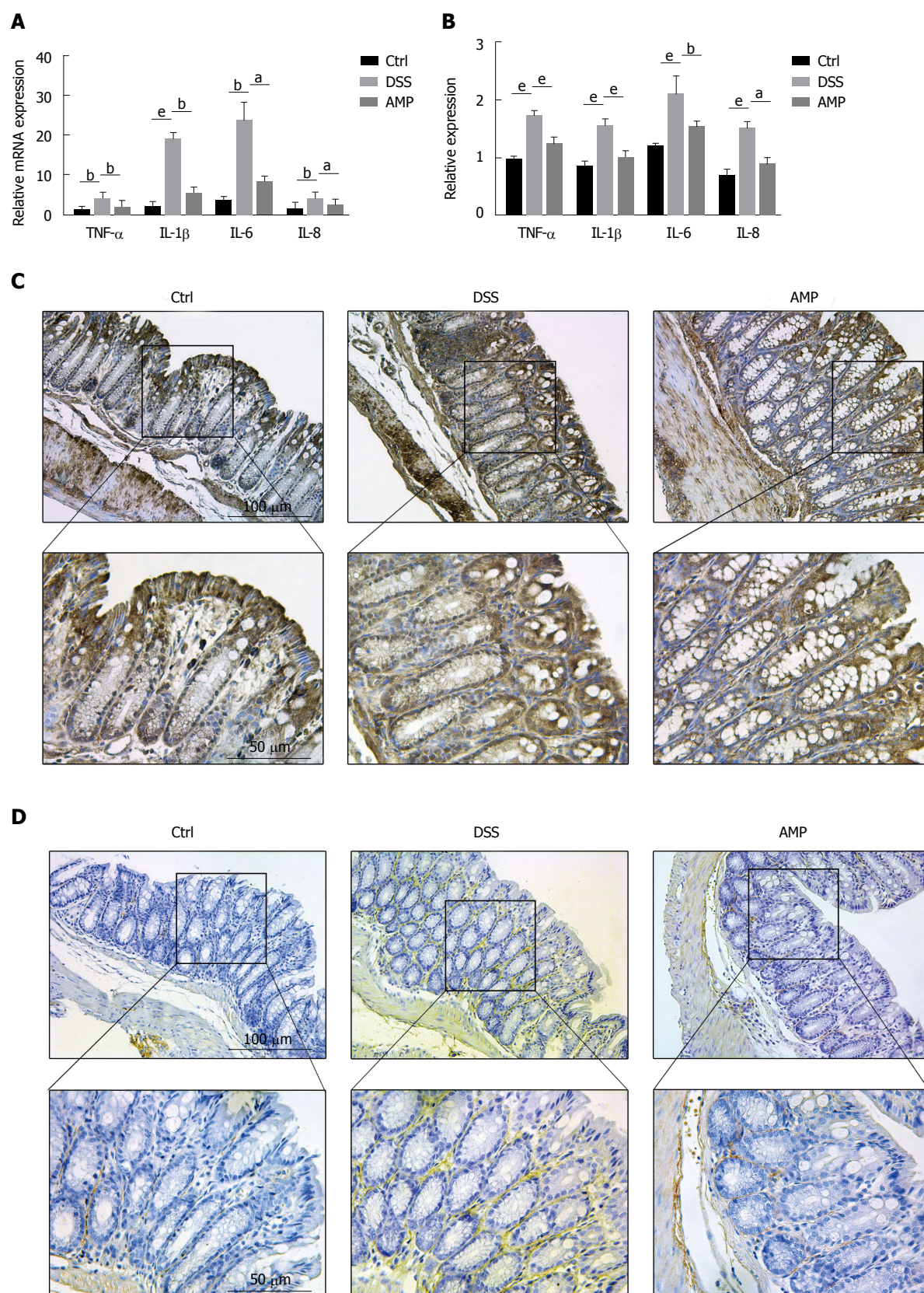


Figure 5 *Ampelopsis grossedentata* has protective effects on ulcerative colitis by inhibiting inflammation. A: mRNA expression of inflammation-related cytokines in colon tissues, with actin used as loading control; B: Relative expression levels of inflammation-related cytokines in serum examined by enzyme-linked immunosorbent assay; C: Representative images of anti-TNF- α immunohistochemistry; D: Representative images of anti-IL-1 β immunohistochemistry. Data are presented as mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$. AMP: *Ampelopsis grossedentata*; DSS: Dextran sulfate sodium; UC: Ulcerative colitis.

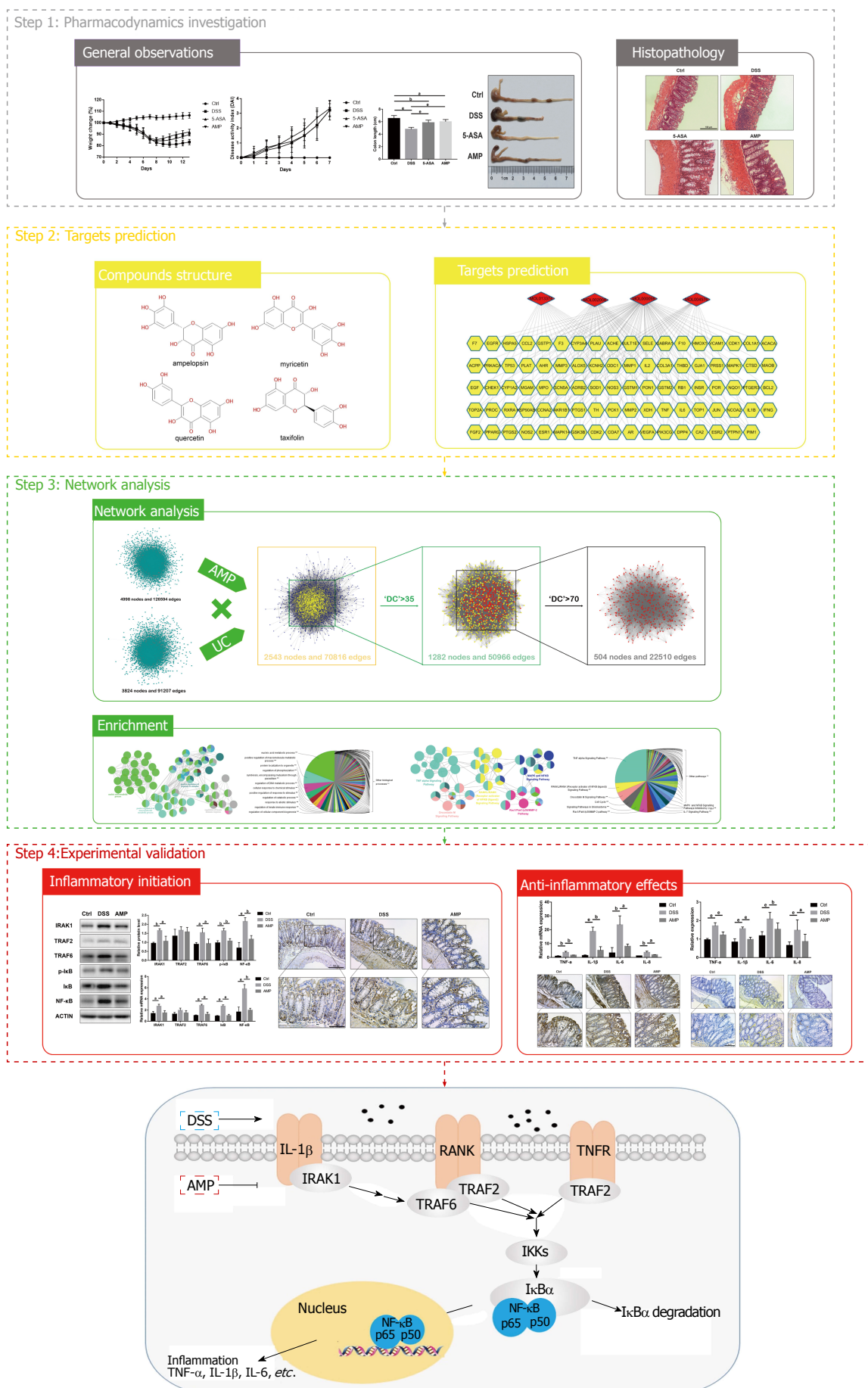


Figure 6 Schematic diagram of the research methodology and the proposed model of *ampelopsis grossedentata* acting on ulcerative colitis.

of TRAF2/6 and IRAK1, and phosphorylation of I κ B could release NF- κ B into the nucleus thus initiating the gene transcription of relevant proinflammatory signals to result in inflammatory responses in intestine, we determined the levels of p-I κ B and NF- κ B consequently. Western blot analyses manifested significant differences in p-I κ B/I κ B and NF- κ B between the AMP group and the DSS group, and these changes were also significant compared with the control group (Figure 4A and B). Meanwhile, parallel changes were seen with the PCR analyses (Figure 4C), suggesting that NF- κ B was activated in the DSS-induced colitis and was inhibited by AMP. Additionally, immunohistochemical estimation revealed that the protein expression of NF- κ B in inflamed colon tissue was significantly increased in the DSS group and was prevented in the AMP group (Figure 4D), confirming the previous findings.

AMP has protective effects on UC by inhibiting inflammation:

As NF- κ B was tightly associated with a great deal of inflammation-related genes, which could release a series of proinflammatory cytokines, including TNF- α and IL-1 β , thus activating the whole inflammatory feedback cycle process^[26,27], we measured the relative expression levels of TNF- α , IL-1 β , IL-6 and IL-8 with PCR and ELISA. Results presented that all the indicators were distinctly elevated under DSS stimulation, being alleviated with AMP treatment (Figure 5A and B). Furthermore, immunohistochemical estimation showed that the expression of secretory proteins TNF- α and IL-1 β were both prominently increased in the DSS group and were improved in the AMP group (Figure 5C and D). Taken together, all the findings indicated that AMP might exert protective effects on UC *via* suppressing the IRAK1/TRAF6/NF- κ B-mediated inflammatory signaling pathway.

DISCUSSION

UC, as one of the major forms of inflammatory bowel disease, represents an increased risk for progressing colorectal cancer^[28]. Recent investigations have confirmed that flavonoid bioactive compounds isolated from the edible herb AMP have strong antiinflammatory activities in macrophages^[16], and may provide effective natural therapies for the treatment and prevention of UC. Thus, a systems pharmacology approach was conducted in this study to explore the underlying pharmacological mechanisms of AMP on UC. The schematic diagram of the research methodology and the proposed model of AMP acting on UC was shown in Figure 6.

A DSS-induced experimental colitis model was constructed to verify the therapeutic effects, and results showed that AMP could significantly improve the general observations and histopathology analysis. Thus, systems pharmacology was performed subsequently to identify the active compounds and their corresponding targets, and results showed that four candidate

compounds obtained from the TCMSP database were ultimately included in this study. Meanwhile, another four compounds, including ambrein, apiin, ampeloptin and myricomplanoside, were obtained from the National Scientific Data Sharing Platform database and Traditional Chinese Medicine Database @Taiwan, but were excluded due to rare corresponding targets. Finally, a compound-target network was constructed for the four selected compounds and their corresponding targets, indicating synergistic as well as multifarious effects for each compound of AMP.

PPI maps were next constructed for the functional analysis, on the basis of putative-target networks of AMP and UC. Enrichment analyses presented a series of biological processes and signaling pathways, and a large number of those were supposed to be tightly associated with the inflammatory response, including the nucleic acid metabolic process, positive regulation of macromolecule metabolic process, positive regulation of response to stimulus and the regulation of innate immune response, together with the TNF- α signaling pathway, RANKL/RANK (receptor activator of NF- κ B) signaling pathway, oncostatin M pathway, Rac1/Pak1/p38/MMP-2 pathway and MAPK/NF- κ B signaling pathway. Thus, we proposed that the NF- κ B-related inflammatory signaling pathway, which was highly involved in all the above findings, might be a pivotal target in the treatment of AMP on UC.

NF- κ B, as a central transcription factor of inflammatory mediators and a key participant in innate and adaptive immune responses involved in the perpetuation of inflammatory cascade^[29], has been considered as the central molecular pathway for UC incidence^[27]. Recent investigations revealed that the activation of proinflammatory cytokines, such as TNF- α and IL-1 β , could induce the translocation of NF- κ B from the cytoplasm to the nucleus, leading to the expression of a variety of inflammatory genes^[30]. Given that inflammation was a major factor for the progression of UC^[31], we performed the experimental validation to verify the above assumption.

The TNF- α signaling pathway and RANKL/RANK (receptor activator of NF- κ B) signaling pathway were the most important parts in the signaling pathways obtained from enrichment analyses. TRAF2 and TRAF6, the downstream events of TNFR and RANK, were detected firstly, and results presented a significant response on TRAF6 but an insignificant response on TRAF2, suggesting the activation of TRAF6 in DSS-induced colitis. IRAK1, a protein kinase which is partially responsible for IL1-induced up-regulation of NF- κ B by combining with TRAF6 and was also detected in previous enrichment analyses^[32], was determined next to further explore the potential antiinflammatory mechanisms of AMP on UC. Outcomes presented a high expression of IRAK1 under DSS stimulation, indicating that IRAK1/TRAF6-mediated proinflammatory signaling might be participated in the antiinflammatory action of AMP on UC.

Thus, we validated the downstream proinflammatory signaling and the therapeutic effects, and results showed the activation of NF- κ B under DSS stimulation as well as the protective effects of AMP against UC in mice. Interestingly, in the DSS group compared with the control group, there was an increased expression of proinflammatory signaling pathways and also of the levels of I κ B; however, the increased expression of I κ B detected by PCR was in accordance with the western blot analyses and the p-I κ B/I κ B ratio was elevated ultimately, which might be due to the feedback regulation of activated NF- κ B. Together, all the findings revealed that AMP could exert protective effects on UC *via* suppressing the IRAK1/TRAF6/NF- κ B-mediated inflammatory signaling pathway.

Limitations should be acknowledged in regards to the fact that research studies on AMP have been rare, resulting in a lack of retrieved compounds, so that the active compounds of AMP might not be fully predicted. Furthermore, some selected compounds were also rejected for the absence of efficient corresponding targets, which might be due to the relevant databases still not being well-developed. Besides, since UC is a chronic inflammatory condition of the intestine combined with a great deal of other biological processes, which have also been detected in this study, further studies are necessary to explore the systematic effects of AMP on UC.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC), as a recurrent chronic inflammatory disease, greatly affects the quality of life of patients, which brings an enormous challenge for both individuals and society worldwide. However, the etiology of UC is still unknown and conventional medical treatments for UC are not fully curative. Thus, promising and novel therapeutic strategies are imperatively needed and should be explored for UC.

Research motivation

Inflammation is a major factor for the progression of UC, and studies have confirmed that a great deal of natural medicines are intended for inhibition of various chronic inflammation-associated diseases, such as *Ampelopsis grossedentata* (AMP). Thus, we explored the mechanisms of therapeutic effects of AMP on UC, and provided a valid complementary treatment to the standard therapy.

Research objectives

To investigate the underlying mechanisms of protective effects of AMP on dextran sulfate sodium (DSS)-induced colitis.

Research methods

As an emerging approach, systems pharmacology was performed in this study to explore the systematic effects of AMP on DSS-induced colitis by active compound screening, target fishing, network construction and analysis.

Research results

The study revealed an antiinflammatory effect of AMP against DSS-induced colitis based on systems pharmacology and animal experiments.

Research conclusions

AMP could exert beneficial effects on DSS-induced colitis *via* suppressing

inflammation-related signaling pathways.

Research perspectives

Since UC is an inflammatory condition combined with a large amount of other biological processes, we determined the inflammatory-related signaling pathways in this study. Other biological processes and signaling pathways as well as the systematic effects of AMP on UC were still unknown and need to be explored in further studies.

REFERENCES

- 1 McDowell C, Bhimji SS. Bowel, Inflammatory Disease (IBD). 2017 [PMID: 29262182]
- 2 Olpin JD, Sjoberg BP, Stilwill SE, Jensen LE, Rezvani M, Shaaban AM. Beyond the Bowel: Extraintestinal Manifestations of Inflammatory Bowel Disease. *Radiographics* 2017; **37**: 1135-1160 [PMID: 28548906 DOI: 10.1148/rg.2017160121]
- 3 Ott C, Schölmerich J. Extraintestinal manifestations and complications in IBD. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 585-595 [PMID: 23835489 DOI: 10.1038/nrgastro.2013.117]
- 4 M'Koma AE. Inflammatory bowel disease: an expanding global health problem. *Clin Med Insights Gastroenterol* 2013; **6**: 33-47 [PMID: 24833941 DOI: 10.4137/CGast.S12731]
- 5 Reinisch W, Van Assche G, Befrits R, Connell W, D'Haens G, Ghosh S, Michetti P, Ochsenkühn T, Panaccione R, Schreiber S, Silverberg MS, Sorrentino D, van der Woude CJ, Vermeire S, Panes J. Recommendations for the treatment of ulcerative colitis with infliximab: a gastroenterology expert group consensus. *J Crohns Colitis* 2012; **6**: 248-258 [PMID: 22325181 DOI: 10.1016/j.crohns.2011.11.001]
- 6 Bressler B, Marshall JK, Bernstein CN, Bitton A, Jones J, Leontiadis GI, Panaccione R, Steinhardt AH, Tse F, Feagan B; Toronto Ulcerative Colitis Consensus Group. Clinical practice guidelines for the medical management of nonhospitalized ulcerative colitis: the Toronto consensus. *Gastroenterology* 2015; **148**: 1035-1058.e3 [PMID: 25747596 DOI: 10.1053/j.gastro.2015.03.001]
- 7 Stawowczyk E, Kawalec P. A Systematic Review of the Cost-Effectiveness of Biologics for Ulcerative Colitis. *Pharmacoeconomics* 2017; **36**: 419-434 [PMID: 29260508 DOI: 10.1007/s40273-017-0601-6]
- 8 Bernstein CN, Ng SC, Lakatos PL, Moum B, Loftus EV Jr; Epidemiology and Natural History Task Force of the International Organization of the Study of Inflammatory Bowel Disease. A review of mortality and surgery in ulcerative colitis: milestones of the seriousness of the disease. *Inflamm Bowel Dis* 2013; **19**: 2001-2010 [PMID: 23624887 DOI: 10.1097/MIB.0b013e318281f3bb]
- 9 Ordás I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. *Lancet* 2012; **380**: 1606-1619 [PMID: 22914296 DOI: 10.1016/s0140-6736(12)60150-0]
- 10 Zhao L, Zhang S, He P. Mechanistic Understanding of Herbal Therapy in Inflammatory Bowel Disease. *Curr Pharm Des* 2017; **23**: 5173-5179 [PMID: 29032748 DOI: 10.2174/1381612823666171010124414]
- 11 Fan L, Tong Q, Dong W, Yang G, Hou X, Xiong W, Shi C, Fang J, Wang W. Tissue Distribution, Excretion, and Metabolic Profile of Dihydromyricetin, a Flavonoid from Vine Tea (*Ampelopsis grossedentata*) after Oral Administration in Rats. *J Agric Food Chem* 2017; **65**: 4597-4604 [PMID: 28534405 DOI: 10.1021/acs.jafc.7b01155]
- 12 Zeng X, Yang J, Hu O, Huang J, Ran L, Chen M, Zhang Y, Zhou X, Zhu J, Zhang Q, Yi L, Mi M. Dihydromyricetin Ameliorates Nonalcoholic Fatty Liver Disease by Improving Mitochondrial Respiratory Capacity and Redox Homeostasis Through Modulation of SIRT3 Signaling. *Antioxid Redox Signal* 2018; Epub ahead of print [PMID: 29310441 DOI: 10.1089/ars.2017.7172]
- 13 Le L, Jiang B, Wan W, Zhai W, Xu L, Hu K, Xiao P. Metabolomics reveals the protective of Dihydromyricetin on glucose homeostasis by enhancing insulin sensitivity. *Sci Rep* 2016; **6**: 36184 [PMID:

- 27796348 DOI: 10.1038/srep36184]
- 14 **Wang Y**, Wang W, Qiu E. Protection of oxidative stress induced apoptosis in osteosarcoma cells by dihydromyricetin through down-regulation of caspase activation and up-regulation of Bcl-2. *Saudi J Biol Sci* 2017; **24**: 837-842 [PMID: 28490955 DOI: 10.1016/j.sjbs.2016.12.004]
 - 15 **Wang Z**, Sun X, Feng Y, Liu X, Zhou L, Sui H, Ji Q, E Q, Chen J, Wu L, Li Q. Dihydromyricetin reverses MRP2-mediated MDR and enhances anticancer activity induced by oxaliplatin in colorectal cancer cells. *Anticancer Drugs* 2017; **28**: 281-288 [PMID: 27997436 DOI: 10.1097/cad.0000000000000459]
 - 16 **Wang R**, Pi J, Su X, Liu J, Zeng X, Wong I, Huang L, Zhou H, Cai J, Li T, Liu L. Dihydromyricetin suppresses inflammatory responses in vitro and in vivo through inhibition of IKK β activity in macrophages. *Scanning* 2016; **38**: 901-912 [PMID: 27487564 DOI: 10.1002/sca.21339]
 - 17 **Tang N**, Ma J, Wang KS, Mi C, Lv Y, Piao LX, Xu GH, Li X, Lee JJ, Jin X. Dihydromyricetin suppresses TNF- α -induced NF- κ B activation and target gene expression. *Mol Cell Biochem* 2016; **422**: 11-20 [PMID: 27686451 DOI: 10.1007/s11010-016-2799-6]
 - 18 **Fotis C**, Antoranz A, Hatziaivramidis D, Sakellaropoulos T, Alexopoulos LG. Network-based technologies for early drug discovery. *Drug Discov Today* 2017; **23**: 626-635 [PMID: 29294361 DOI: 10.1016/j.drudis.2017.12.001]
 - 19 **Bloomingdale P**, Nguyen VA, Niu J, Mager DE. Boolean network modeling in systems pharmacology. *J Pharmacokinet Pharmacodyn* 2018; **45**: 159-180 [PMID: 29307099 DOI: 10.1007/s10928-017-9567-4]
 - 20 **Pei F**, Li H, Henderson MJ, Titus SA, Jadhav A, Simeonov A, Cobanoglu MC, Mousavi SH, Shun T, McDermott L, Iyer P, Fioravanti M, Carlisle D, Friedlander RM, Bahar I, Taylor DL, Lezon TR, Stern AM, Schurdak ME. Connecting Neuronal Cell Protective Pathways and Drug Combinations in a Huntington's Disease Model through the Application of Quantitative Systems Pharmacology. *Sci Rep* 2017; **7**: 17803 [PMID: 29259176 DOI: 10.1038/s41598-017-17378-y]
 - 21 **Suh SY**, An WG. Systems Pharmacological Approach to the Effect of Bulsu-san Promoting Parturition. *Evid Based Complement Alternat Med* 2017; **2017**: 7236436 [PMID: 29234425 DOI: 10.1155/2017/7236436]
 - 22 **Chaudhary G**, Mahajan UB, Goyal SN, Ojha S, Patil CR, Subramanya SB. Protective effect of Lagerstroemia speciosa against dextran sulfate sodium induced ulcerative colitis in C57BL/6 mice. *Am J Transl Res* 2017; **9**: 1792-1800 [PMID: 28469784]
 - 23 **Zhang MJ**, Su H, Yan JY, Li N, Song ZY, Wang HJ, Huo LG, Wang F, Ji WS, Qu XJ, Qu MH. Chemopreventive effect of Myricetin, a natural occurring compound, on colonic chronic inflammation and inflammation-driven tumorigenesis in mice. *Biomed Pharmacother* 2018; **97**: 1131-1137 [PMID: 29136951 DOI: 10.1016/j.biopha.2017.11.018]
 - 24 **Zhu M**, Zhou X, Zhao J. Quercetin prevents alcohol-induced liver injury through targeting of PI3K/Akt/nuclear factor- κ B and STAT3 signaling pathway. *Exp Ther Med* 2017; **14**: 6169-6175 [PMID: 29285175 DOI: 10.3892/etm.2017.5329]
 - 25 **Kim A**, Nam YJ, Lee CS. Taxifolin reduces the cholesterol oxidation product-induced neuronal apoptosis by suppressing the Akt and NF- κ B activation-mediated cell death. *Brain Res Bull* 2017; **134**: 63-71 [PMID: 28710022 DOI: 10.1016/j.brainresbull.2017.07.008]
 - 26 **Vlantis K**, Wullaert A, Polykratis A, Kondylis V, Dannappel M, Schwarzer R, Welz P, Corona T, Walczak H, Weih F, Klein U, Kelliher M, Pasparakis M. NEMO Prevents RIP Kinase 1-Mediated Epithelial Cell Death and Chronic Intestinal Inflammation by NF- κ B-Dependent and -Independent Functions. *Immunity* 2016; **44**: 553-567 [PMID: 26982364 DOI: 10.1016/j.immuni.2016.02.020]
 - 27 **Eissa N**, Hussein H, Kermarrec L, Elgazzar O, Metz-Boutigue MH, Bernstein CN, Ghia JE. Chromofungin (CHR: CHGA47-66) is downregulated in persons with active ulcerative colitis and suppresses pro-inflammatory macrophage function through the inhibition of NF- κ B signaling. *Biochem Pharmacol* 2017; **145**: 102-113 [PMID: 28827109 DOI: 10.1016/j.bcp.2017.08.013]
 - 28 **Ullman TA**, Itzkowitz SH. Intestinal inflammation and cancer. *Gastroenterology* 2011; **140**: 1807-1816 [PMID: 21530747 DOI: 10.1053/j.gastro.2011.01.057]
 - 29 **DiDonato JA**, Mercurio F, Karin M. NF- κ B and the link between inflammation and cancer. *Immunol Rev* 2012; **246**: 379-400 [PMID: 22435567 DOI: 10.1111/j.1600-065X.2012.01099.x]
 - 30 **Matsuhisa K**, Watari A, Iwamoto K, Kondoh M, Yagi K. Lignosulfonic acid attenuates NF- κ B activation and intestinal epithelial barrier dysfunction induced by TNF- α /IFN- γ in Caco-2 cells. *J Nat Med* 2017; **72**: 448-455 [PMID: 29275476 DOI: 10.1007/s11418-017-1167-5]
 - 31 **Arulselvan P**, Fard MT, Tan WS, Gothai S, Fakurazi S, Norhaizan ME, Kumar SS. Role of Antioxidants and Natural Products in Inflammation. *Oxid Med Cell Longev* 2016; **2016**: 5276130 [PMID: 27803762]
 - 32 **Hui B**, Zhang L, Zhou Q, Hui L. Pristimerin Inhibits LPS-Triggered Neurotoxicity in BV-2 Microglia Cells Through Modulating IRAK1/TRAF6/TAK1-Mediated NF- κ B and AP-1 Signaling Pathways In Vitro. *Neurotox Res* 2018; **33**: 268-283 [PMID: 29119451 DOI: 10.1007/s12640-017-9837-3]

P- Reviewer: Zouiten-Mekki L **S- Editor:** Gong ZM
L- Editor: Filipodia **E- Editor:** Huang Y



Retrospective Cohort Study

Potential triggering factors of acute liver failure as a first manifestation of autoimmune hepatitis-a single center experience of 52 adult patients

Matthias Buechter, Paul Manka, Falko Markus Heinemann, Monika Lindemann, Hideo Andreas Baba, Martin Schlattjan, Ali Canbay, Guido Gerken, Alisan Kahraman

Matthias Buechter, Paul Manka, Guido Gerken, Alisan Kahraman, Department of Gastroenterology and Hepatology, University Clinic of Essen, Essen 45147, Germany

Paul Manka, Division of Transplantation Immunology and Mucosal Biology, King's College, London SE59RJ, United Kingdom

Falko Markus Heinemann, Monika Lindemann, Institute of Transfusion Medicine, University Clinic of Essen, Essen 45147, Germany

Hideo Andreas Baba, Martin Schlattjan, Institute of Pathology, University Clinic of Essen, Essen 45147, Germany

Ali Canbay, Department of Gastroenterology, Hepatology, and Infectious Diseases, Otto-von-Guericke University, Magdeburg 39120, Germany

ORCID number: Matthias Buechter (0000-0003-3394-5492); Paul Manka (0000-0001-8589-7280); Falko Markus Heinemann (0000-0002-9642-1154); Monika Lindemann (0000-0001-6708-4390); Hideo Andreas Baba (0000-0002-1750-5318); Martin Schlattjan (0000-0001-6639-5568); Ali Canbay (0000-0001-6069-7899); Guido Gerken (0000-0001-6734-5001); Alisan Kahraman (0000-0002-2823-6774).

Author contributions: Buechter M analyzed the data and wrote the manuscript; Manka P analyzed the data and performed the statistics; Heinemann FM and Lindemann M performed the HLA-typing; Baba HA and Schlattjan M evaluated the histological specimens; Canbay A and Gerken G treated the patients; Kahraman A treated the patients and designed the study.

Institutional review board statement: This study was reviewed and approved by the ethics committee of the University Clinic of Essen.

Informed consent statement: All patients gave their written informed consent prior to study inclusion.

Conflict-of-interest statement: All authors have nothing to declare.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Kahraman Alisan, MD, Associate Professor, Department of Gastroenterology and Hepatology, University Hospital of Essen, Hufelandstr 55, Essen 45122, Germany. alisan.kahraman@uk-essen.de

Telephone: +49-201-72384797

Fax: +49-201-7235655

Received: December 6, 2017

Peer-review started: December 6, 2017

First decision: January 18, 2018

Revised: February 21, 2018

Accepted: March 3, 2018

Article in press: March 3, 2018

Published online: April 7, 2018

Abstract

AIM

To investigate potential triggering factors leading to acute liver failure (ALF) as the initial presentation of autoimmune hepatitis (AIH).

METHODS

A total of 565 patients treated at our Department between 2005 and 2017 for histologically-proven AIH were retrospectively analyzed. However, 52 patients (9.2%) fulfilled the criteria for ALF defined by the "American Association for the Study of the Liver (AASLD)". According to this definition, patients with "acute-on-chronic" or "acute-on-cirrhosis" liver failure were excluded. Following parameters with focus on potential triggering factors were evaluated: Patients' demographics, causation of liver failure, laboratory data (liver enzymes, MELD-score, autoimmune markers, virus serology), liver histology, immunosuppressive regime, and finally, outcome of our patients.

RESULTS

The majority of patients with ALF were female (84.6%) and mean age was 43.6 ± 14.9 years. Interestingly, none of the patients with ALF was positive for anti-liver kidney microsomal antibody (LKM). We could identify potential triggering factors in 26/52 (50.0%) of previously healthy patients presenting ALF as their first manifestation of AIH. These were drug-induced ALF (57.7%), virus-induced ALF (30.8%), and preceding surgery in general anesthesia (11.5%), respectively. Unfortunately, 6 out of 52 patients (11.5%) did not survive ALF and 3 patients (5.7%) underwent liver transplantation (LT). Comparing data of survivors and patients with non-recovery following treatment, MELD-score ($P < 0.001$), age ($P < 0.05$), creatinine ($P < 0.01$), and finally, ALT-values ($P < 0.05$) reached statistical significance.

CONCLUSION

Drugs, viral infections, and previous surgery may trigger ALF as the initial presentation of AIH. Advanced age and high MELD-score were associated with lethal outcome.

Key words: Acute liver failure; Autoimmune hepatitis; Drug-induced liver injury; Triggering factors; MELD-score

© The Author(s) 2018. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Autoimmune hepatitis is considered to manifest as a chronic disease. In few cases, the clinician is challenged with patients revealing acute liver failure as their first manifestation of autoimmune hepatitis (AIH). The aim of our study was to investigate features of especially these patients with focus on potential triggering factors. We identified triggering factors in half of our patients (26 out of 52 patients with acute liver failure within a total cohort of 565 AIH patients). These were drugs, viral infections, and surgery. Advanced age and high MELD-score were associated with lethal outcome. Consequently, the clinician would be well-advised to document these underlying conditions.

triggering factors of acute liver failure as a first manifestation of autoimmune hepatitis-a single center experience of 52 adult patients. *World J Gastroenterol* 2018; 24(13): 1410-1418 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1410.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1410>

INTRODUCTION

Autoimmune hepatitis (AIH) is a complex disease characterized by immune-mediated destruction of hepatic parenchyma, female gender bias, presence of auto-antibodies, hypergammaglobulinaemia, association with other autoimmune conditions, and excellent response to immunosuppressive therapy^[1]. Since its first description by Waldenström in the early 1950's, AIH was considered to manifest as a chronic liver disease and its fulminant presentation was not commonly reported^[2-4]. Over the last decades, it has become apparent that AIH can occur with diverse clinical phenotypes and its classical perception of a chronic inflammatory liver disease that affects mainly young Caucasian women has been expanded^[5-8].

However, approximately 20%-30% of the patients reveal an acute presentation which may be induced by a triggering agent such as previous viral infections, toxic injury or treatment with immune-modifying drugs. Infectious triggers are commonly indicated as being involved in the induction of autoimmune diseases, with Epstein-Barr (EBV) or Cytomegalovirus (CMV) being implicated in several autoimmune liver disorders, such as type I autoimmune hepatitis or primary biliary cholangitis (PBC)^[9]. A remarkable proportion of patients with acute manifestation can develop acute liver failure (ALF), particularly in case of delayed diagnosis and treatment^[4,10,11].

ALF is characterized by a rapid onset of severe hepatocyte injury without prior liver disease that is associated with significant morbidity and mortality^[12,13]. The most widely accepted definition of ALF includes evidence of coagulation abnormality, usually an INR ≥ 1.5 , and any degree of mental alteration (hepatic encephalopathy) in a patient without pre-existing chronic liver disease with an illness of < 26 wk duration^[14].

Patients with AIH-induced ALF are frequently difficult to diagnose due to absence of serological markers including anti-nuclear (ANA), anti-smooth muscle (SMA), anti-liver kidney microsomal (LKM) antibodies, and normal immunoglobulin G (IgG)-levels in numerous cases^[15,16]. In this setting, determination of major histocompatibility complex HLA-loci (e.g., HLA-DR3 and -DR4), which are reported to have a strong association with AIH, might be helpful additional diagnostic tools^[1,8,17].

In the daily clinical setting, the hepatologist is frequently faced with patients having unknown elevations of their liver enzymes for years or even decades. Most of these asymptomatic patients present only marginal increased liver enzymes with normal liver

Buechter M, Manka P, Heinemann FM, Lindemann M, Baba HA, Schlattjan M, Canbay A, Gerken G, Kahraman A. Potential

function. Routine work-up of these cases leads finally to the diagnosis of underlying AIH and - following steroid therapy - transaminases often return to normal ranges. However, in few cases, one is challenged with patients previously being healthy without any signs of hepatopathy but rapidly demonstrating a life-threatening acute liver failure as their first manifestation of AIH with the necessity of urgent organ transplantation. Therefore, the aim of our study was to investigate demographic characteristics and clinical course of especially these patients presented with ALF as their initial presentation of AIH with special focus on potential triggering factors which may activate the "autoimmune machinery" leading to onset of the disease.

MATERIALS AND METHODS

Patients' characteristics, data collection and ethical considerations

In this retrospective study between 01/2005 and 04/2017, a total of 565 patients with histologically-proven AIH were analyzed, from whom 52 previously healthy patients suffered from ALF as their initial presentation of autoimmune hepatitis. According to the criteria defined by the "American Association for the Study of the Liver (AASLD)", ALF was diagnosed by elevation of liver enzymes in combination with hepatic encephalopathy (HE) and coagulopathy (INR > 1.5) in the absence of a pre-existing chronic liver disease^[14]. AIH was diagnosed according to the "Diagnostic Scoring System of the International Autoimmune Hepatitis Group" including analysis of auto-antibodies, IgG-levels, histological features, and exclusion of viral markers^[18,19]. Liver histology was available for the whole study population and was obtained either by percutaneous- or laparoscopy-guided biopsy (Figure 1A and B). Only adult patients (age ≥ 18 years) were included in the study. The University Clinic of Essen ethics committee approved the retrospective, anonymous analysis of the data and the study was conducted according to the principles expressed in the Declaration of Helsinki. All patients gave their written informed consent prior to study inclusion.

Laboratory parameters

At initial presentation, alanine-aminotransferase (ALT), total bilirubin, serum creatinine, INR, IgG, IgM, γ -globulins, antibody profile (ANA, AMA, ANCA, SMA, LKM, SLA), and finally, HLA-loci (HLA- A1, -B8, -DR3, and -DR4) were analyzed. Each patient was also tested for viral markers (anti-HAV IgM, HBs-Ag, anti-HBc IgM, HBeAg, anti-HBe, anti-HBs, anti-HCV, anti-HDV-EIA, anti-HEV IgM, and PCR's for HBV, HCV, HEV, HSV, CMV, EBV), transferrin-saturation, ceruloplasmin, copper in serum, soluble interleukin-II receptor, α 1-antitrypsin, and finally, GLDH. In suspected cases of Wilson's disease,

additional examinations were performed (Kayser-Fleischer ring, copper in urine, parameters of hemolysis, and also determination of copper in the liver biopsy).

RUCAM instrument

The appropriate diagnosis of drug-induced liver injury (DILI) vs DILI-AIH involves the collection of historical and laboratory data, including the latency in onset, the rate of resolution after discontinuing treatment, and exclusion of other reasonable causes of liver injury. The advantage of the RUCAM instrument is that it is systematic, thorough, and objective. We therefore used this instrument in our study population as - at present - it is considered the best method for assessing causality in DILI.

Liver histology

Liver biopsy was performed in all patients with evidence of typical histopathological features of AIH, namely presence of interface hepatitis, lymphoplasmacytic cell infiltration exceeding the borders of the portal tract, emperipolesis, and rosette formations, respectively. Differentiating between drug-induced ALF and AIH-induced ALF is difficult on the basis of histology alone. In both cases plasma cell rich inflammation with interface hepatitis can be present. In order to differentiate DILI from AIH clinical, historical, and laboratory data have to be considered. If EBV, CMV or HEV infection was suspected, viral detection by means of immunohistochemistry, in situ hybridization, and PCR was also performed.

Immunosuppressive therapy and definition of non-recovery

After diagnosis of AIH-induced ALF, each patient received standard steroid therapy (1 mg per kg body weight/d) intravenously with consecutive down-tapering to a maintenance dose of 7.5 mg daily. Non-recovery was defined as death or liver transplantation (LT) within 28 d despite steroid treatment and initiation of immunosuppressive therapy with azathioprine.

Statistical analysis

Statistical analysis was performed with GraphPad Prism, version 6.00 for MacOSX (GraphPad Software, San Diego, CA, United States). For descriptive statistics medians and IQR were determined. All variables were tested for normal distribution with the Kolmogorov-Smirnov test, the Shapiro-Wilk test, and calculation of skew and kurtosis. The Mann-Whitney *U* test was used to compare differences between independent groups. Categorical data were tested with the chi-square test and the Kruskal-Wallis test was used for multiple comparisons. *P* < 0.05 was considered statistically significant. The whiskers used in the graphs extend down to the 5th percentile and up to the 95th percentile.

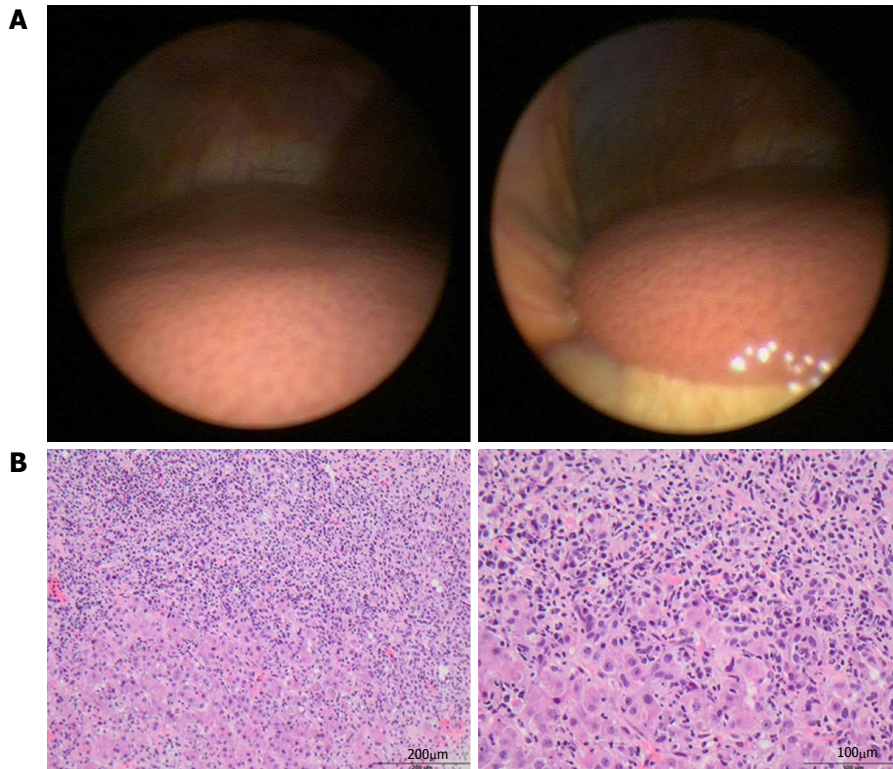


Figure 1 Mini-laparoscopy of a patient with acute liver failure due to newly diagnosed autoimmune hepatitis exemplarily showing the right liver lobe with diffuse capsular swelling, regenerative nodules, and rounded lower margin (upper panel) (A), and liver histology of the same patient with AIH-induced ALF (B). Left panel demonstrating severe inflammation with interface hepatitis (original magnification 200 ×) and the right panel with higher magnification (400 ×) revealing numerous plasma cells extending from the portal tract into the adjacent parenchyma (lower panel).

RESULTS

Patients' demographics, laboratory data, and immunosuppressive regime

Fifty-two out of 565 patients with AIH suffered from ALF as their initial presentation (9.2%) (Figure 1A and B) and were included in the study. Mean age of the study population was 43.6 ± 14.9 (19-76) years while the majority was of female gender (44/52, 84.6%). Laboratory parameters with median values on admission were as follows: ALT: 1391.0 (843.5-2154.5) U/L, total bilirubin: 14.3 (11.7-18.7) mg/dL, serum creatinine: 0.76 (0.55-0.95) mg/dL, INR: 1.78 (1.64-2.00), immunoglobulin G: 17.2 (13.1-22.8) g/L, and finally, γ -globulin-fraction: 24.5% (19.5%-29.3%). Calculated median labMELD-score was 24 (22-26) points. All patients with AIH-induced ALF received a pulse therapy with steroids starting with 1 mg/kg body weight intravenously. A total of 30 patients (57.7%) continued steroids in a daily dose of 7.5 mg to maintain remission. Azathioprine (in 27 patients, 51.9%) and also cyclosporine A (in 7 patients, 13.5%) were also used to maintain remission. Hepatic encephalopathy (HE) was classified using the West Haven criteria. We found HE grade I in 46 of our 52 patients (88.4%), HE grade II in 2 patients, HE grade III in 2 patients, and finally, HE grade IV in 2 further patients, respectively. Unfortunately, patients with HE

grade IV had poor prognosis and died of acute liver failure. We found no correlation between grade of HE and antibody or HLA-profiles. Moreover, we also did not find a correlation between the triggering factors with severity of HE (data not shown). Patients' demographics and laboratory data are summarized in Table 1.

HLA-typing and auto-antibody profiles

Thirty-six out of 52 patients were positive for ANA (69.2%), 6/52 for AMA (11.5%), 4/52 for SMA (7.7%), and 2/52 for SLA (3.8%), respectively. Interestingly, none of the adult patients with acute liver failure was either positive for ANCA or LKM. Data on HLA-loci were available for 34 patients (65.4%) showing the following results: HLA-A1 positivity in 15/34 (44.1%), HLA-B8 in 6/34 (17.6%), HLA-DR3 in 8/34 (23.5%), and HLA-DR4 in 11/34 patients (32.4%). HLA- and antibody profiles of our study population are demonstrated in Figure 2A and B.

Potential triggering factors for ALF in patients with first manifestation of AIH

We could identify potential triggering factors for ALF in patients with their first manifestation of AIH in 26/52 patients (50.0%). These triggers were predominantly drugs [15/26 (57.7%)], namely non-steroidal anti-inflammatory drugs (NSAID) ($n = 8$), antibiotics ($n = 5$),

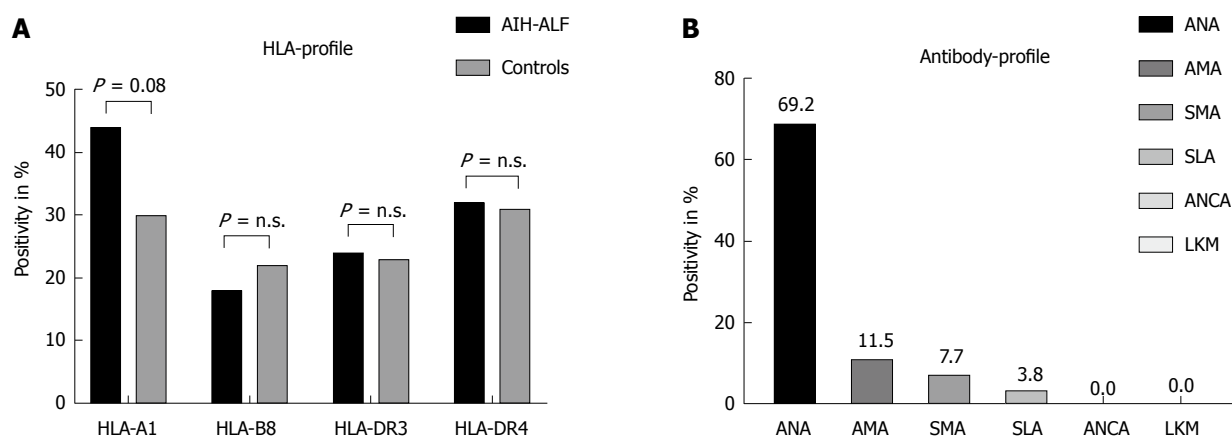


Figure 2 HLA-profile of the study population ($n = 34$) investigating HLA-A1, -B8, -DR3, and -DR4 status (A), and antibody-profile of the study population ($n = 52$) demonstrating positivity for ANA-, AMA-, SMA-, SLA-, ANCA-, and LKM-titers (B). ANA: Anti-nuclear; SMA: Anti-smooth muscle; LKM: Anti-liver kidney microsomal.

Table 1 Patients' demographics and laboratory with $n = 52$ demonstrating autoimmune hepatitis-induced acute liver failure (9.2%)

Study population ($n = 52$) with AIH-induced ALF	
Mean age (yr)	43.6 \pm 14.9 (19-76)
Male	8 (15.4%)
Female	44 (84.6%)
Hepatic encephalopathy	Grade I : 46/52 (88.4%) Grade II : 2/52 (3.8%) Grade III : 2/52 (3.8%) Grade IV : 2/52 (3.8%)
Immunosuppressive therapy	Steroid induction: 52/52 (100%) Steroid maintenance: 30/52 (57.7%) Steroid withdrawal: 20/52 (42.3%) Azathioprine: 27/52 (51.9%) Cyclosporine A: 7/52 (13.5%)
ALT (U/L)	1391.0 (843.5-2154.5)
Total bilirubin (mg/dL)	14.3 (11.7-18.7)
Creatinine (mg/dL)	0.76 (0.55-0.95)
INR	1.78 (1.64-2.00)
LabMELD-score	24 (22-26)
Immunoglobulin G (g/L)	17.2(13.1-22.8)
γ -globulin-fraction (%)	24.5 (19.5-29.3)

Data represents median and IQR. AIH: Autoimmune hepatitis; ALF: Acute liver failure; ALT: Alanine-aminotransferase.

ipilimumab ($n = 1$), and rivaroxaban ($n = 1$)] followed by previous acute viral infections [8/26 (30.8%), namely Epstein-Barr virus ($n = 4$), Cytomegalovirus ($n = 3$), and hepatitis E virus ($n = 1$)]. Finally, the remaining 3 patients underwent previous surgery in general anesthesia (11.5%) (Figure 3).

Higher age, creatinine, and MELD-score were associated with lethal outcome or need for liver transplantation, while elevated liver enzymes indicated recovery

Unfortunately, 6 out of 52 patients did not receive an organ offer and died due to ALF while 3 further patients underwent liver transplantation (non-recovery group). When comparing data of these patients with patients who responded to steroids and survived

ALF (recovery group), statistical analysis revealed significant differences in terms of age [median age: 40.0 (28.0-52.0) years for the recovery group vs median age: 49.0 (44.0-62.5) years, for the non-recovery group, $P = 0.031$], serum creatinine [median for the recovery group: 0.72 (0.51-0.92) mg/dL vs median for the non-recovery group: 0.98 (0.77-1.61) mg/dL, $P = 0.0069$], labMELD-score [median for the recovery group: 23 (22-25) vs median for the non-recovery group: 27 (25-30), $P = 0.0007$, and finally, median ALT-values for the recovery group 1512 (904-2276) U/L vs median for the non-recovery group: 711 (324-1391) U/L, $P = 0.0157$] (Table 2 and Figure 4A-D). None of the remaining AIH patients of our cohort ($n = 513$ patients) developed acute liver failure under therapy with corticosteroids and/or immunosuppressive therapy. However, no significance was found for markedly increased bilirubin levels comparing both groups.

DISCUSSION

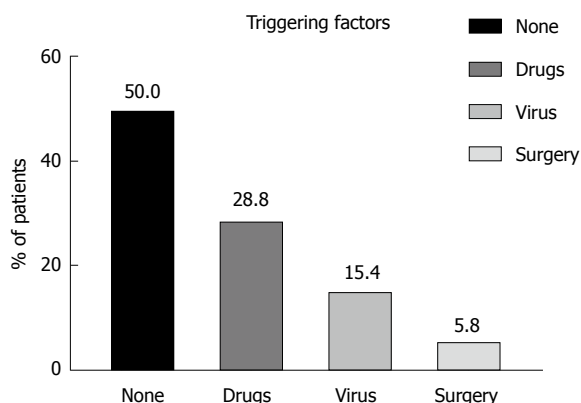
The etiology of autoimmune hepatitis (AIH) is uncertain but - in some cases - the disease can be triggered by external factors such as viruses or drugs. AIH usually develops in individuals with genetic background. Many drugs have been linked to AIH phenotypes, which sometimes persist after drug discontinuation, suggesting that they awaken latent autoimmunity. Growing information on the relationship of drugs and AIH is being available, being drugs and biologic agents more frequently involved in cases allowing to establish a causal relationship^[20-23]. According to current literature, the frequency of patients with drug-induced AIH (DILI-AIH) is reported to range from 9%-17% in overall patients diagnosed with AIH^[24,25].

Drug-induced liver injury (DILI) is the most common cause of acute liver failure (ALF) and responsible for approximately 50% of the cases in the United States and Western Europe. DILI may be dose-dependent and predictable (e.g., acetaminophen-induced hepatotoxicity)

Table 2 Patients' data stratified by recovery and non-recovery (*n* = 52)

	Recovery (<i>n</i> = 43)	Non-recovery (<i>n</i> = 9)	<i>P</i> value
Age (yr)	40.0 (28.0-52.0)	49.0 (44.0-62.5)	0.031
Male/female	7/36	1/8	NS
ALT (U/L)	1512 (904-2276)	711 (324-1391)	0.0157
Total bilirubin (mg/ dL)	14.0 (11.3-18.7)	16.1 (11.8-23.6)	NS
Creatinine (mg/ dL)	0.72 (0.51-0.92)	0.98 (0.77-1.61)	0.0069
INR	1.76 (1.63-1.98)	1.96 (1.75-2.79)	0.0644
LabMELD-score	23 (22-25)	27 (25-30)	0.0007

Data represents median and IQR. ALF: Acute liver failure.

**Figure 3** Potential triggering factors for acute liver failure in patients with their first manifestation of autoimmune hepatitis (*n* = 52).

or idiosyncratic, unpredictable, and probably independent of dose^[12,26]. Autoimmune hepatitis - on the contrary - is a relatively rare cause of ALF in developed countries with an incidence of approximately 5%^[27]. According to the "Acute Liver Failure Study Group (ALFSG)" registry, including 2436 patients between 1998 and 2016 in the United States, 163 (6.7%) were diagnosed with ALF due to AIH^[28]. In our cohort 9.2% of AIH patients presented ALF. DILI is reported to have a phenotype of autoimmunity similar to AIH and distinguishing these entities still remains a challenge^[29]. However, immune-mediated DILI nearly always resolves or becomes quiescent when drugs are withdrawn^[24,30]. In contrast, in patients with drug-induced AIH, it can be assumed that predisposition for AIH existed before, but the disease was quiescent and remained undiagnosed until this drug triggered the autoimmune process. Recently, Licata and colleagues reported 12 patients from a series of 136 subjects with DILI that were diagnosed as drug-induced AIH (9%)^[31]. Accordingly, Kuzu *et al.*^[32] described 82 DILI patients from whom five (6%) were diagnosed with DILI-AIH.

AIH - in its classical perception - commonly presents as a chronic hepatopathy. On the one hand, the majority of patients with AIH are diagnosed due to accidentally and repeatedly elevated liver enzymes in routine check-up examinations without having symptoms. On the other hand, AIH can lead to fulminant acute liver failure which is associated with high morbidity and

mortality. The most important genetic risk factor is human leukocyte antigen, especially HLA-DR, whereas the role of environmental factors is not completely understood. Immunologically, disruption of the immune tolerance to autologous liver antigens may be a trigger of AIH. According to current data, triggering agents which may lead to this disruption are mainly unknown, but may include viral infections, environmental toxins, drugs, and vaccinations. There is growing evidence for a loss of immune tolerance to self-antigens playing a part in the development of this condition^[33-38]. Genetic risk association studies have identified HLA loci for the development of disease and providing prognostic information^[38,39]. Interestingly, when compared to published allele frequencies in healthy controls, HLA genotypes of our cohort did not reach statistical significance while only HLA-A1 status revealed a positive trend^[40]. Moreover, none of our adult patients included in the analyses was diagnosed with LKM-positive AIH type 2. This observation matches with current literature: Kessler and colleagues, who analyzed 30 patients with fulminant hepatic failure as the initial presentation of acute AIH, found that only 3% were LKM-positive^[3]. Likewise, di Giorgio *et al.*^[41] described 46 children with fulminant hepatic failure of autoimmune etiology, none was LKM-positive.

In this study, we aimed to check potential triggering factors which may lead to ALF as the initial presentation of AIH. However, in 50% of the patients, triggering factors for ALF as the initial presentation of AIH were suspected. These were predominantly drugs (e.g., NSAID and antibiotics), followed by previous viral infections, and surgery in general anesthesia, respectively. Non-recovery - defined as death or liver transplantation within 28 d - was found among 9/52 patients (17.3%). Reviewing current literature, results of non-recovery in patients with ALF due to AIH differ significantly and range from 10%-60%^[3,41,42]. Risk factors for non-recovery among our patients were increase in age, MELD-score, and creatinine levels, while higher liver enzymes (ALT-values) were associated with improved spontaneous recovery. Our center previously demonstrated that high ammonia, low albumin, and low ALT-levels were associated with worse outcome in childhood acute liver failure^[43]. From our long-time clinical experience in patients with acute

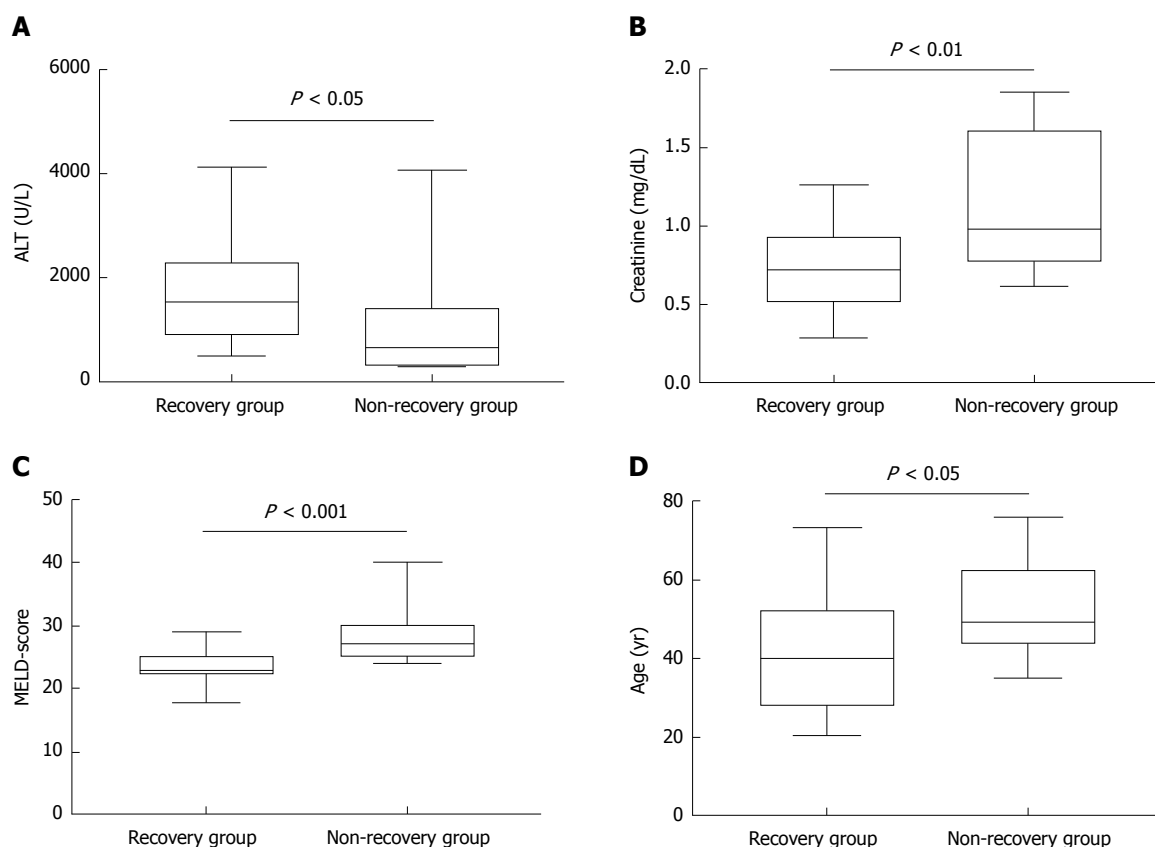


Figure 4 Higher age, creatinine, and MELD-score were associated with lethal outcome. A: Median alanine-aminotransferase (ALT)-values of patients with recovery as compared to the non-recovery group, $P < 0.05$; B: Median serum creatinine levels of patients with recovery and non-recovery, $P < 0.01$; C: Median labMELD-score of patients with recovery and non-recovery, $P < 0.001$; D: Median age of patients with recovery and non-recovery, $P < 0.05$.

liver failure, we observed that patients with high ALT-values recovered better than their counterparts with low ALT-values indicating that there is still functioning liver parenchyma despite the fact of acute liver injury. On the one hand, extreme inflammation may reflect less grade of already existing hepatic necrosis and higher proportion of vital hepatocytes while on the other hand, inflammation may increase the probability of response to immunosuppressive treatment.

In summary, approximately 9% of our patients were diagnosed with acute liver failure as their initial presentation of autoimmune hepatitis which may be potentially induced by drugs, viral infections, and surgery in general anesthesia. Consequently, the clinician would be well-advised to accurately document these underlying conditions. Increases of age, MELD-score, and creatinine levels may be risk factors for lethal outcome or need for urgent liver transplantation, while higher levels of transaminases come along with improved spontaneous recovery.

ARTICLE HIGHLIGHTS

Research background

Autoimmune hepatitis (AIH) is generally considered to manifest as a chronic liver disease. So far, only limited data are available investigating patients presenting a fulminant acute liver failure as a first manifestation of this

autoimmune disorder. The significance of our study was therefore to investigate the circumstances leading to acute liver failure and onset of autoimmune hepatitis.

Research motivation

In the daily clinical setting, the hepatologist is frequently faced with patients demonstrating only a mild elevation of their liver enzymes. Routine work-up of these cases leads finally to the diagnosis of underlying AIH. However, in few cases, one is challenged with patients without any signs of hepatopathy but rapidly developing a life-threatening acute liver failure (ALF) as their first manifestation of AIH. We here presented potential triggering factors which may activate the “autoimmune machinery” leading to ALF.

Research objectives

The main objective of the present study was to gather more information with focus on potential triggering factors leading to acute presentation of AIH with consecutive liver failure. The clinician would be well-advised to accurately document these underlying conditions.

Research methods

In our retrospective cohort study we investigated patients with histologically-proven AIH and further analyzed the patients who presented acute liver failure. Patients’ demographics, laboratory data, immunosuppressive regime, histology, and outcome were documented and studied.

Research results

We were able to identify potential triggering factors in 26/52 (50.0%) of our previously healthy patients presenting ALF as their first manifestation of AIH. These were drug-induced (e.g., non-steroidal anti-inflammatory drugs and antibiotics) ALF (57.7%), virus-induced (Epstein-Barr, Cytomegalovirus and

HEV) ALF (30.8%), and surgery in general anesthesia (11.5%), respectively.

Research conclusions

Approximately 9% of our patients were diagnosed with ALF as their initial presentation of AIH which may be potentially induced by drugs, viral infections, and surgery in general anesthesia. Consequently, the clinician would be well-advised to ask his patients for hepato-toxic drugs and accurately document these underlying conditions. Increases of age, MELD-score, and creatinine levels were associated with lethal outcome or need for urgent liver transplantation.

Research perspectives

With our study and findings we hope to further attract the physician's attention especially in cases of acute liver failure induced by autoimmune hepatitis. In some cases, these disorders may be triggered by drugs and hepato-tropic viruses. We hope that more studies investigating acute liver failure as a first manifestation of AIH will be available in future.

ACKNOWLEDGMENTS

We thank Professor Dr. Gregory Gores from the Division of Gastroenterology and Hepatology, Mayo Clinic Rochester (Minnesota, United States) for his valuable contribution and his permanent support. We also thank the anonymous Reviewers for his/her thoughtful and constructive examination of our manuscript.

REFERENCES

- 1 Wang Q, Yang F, Miao Q, Krawitt EL, Gershwin ME, Ma X. The clinical phenotypes of autoimmune hepatitis: A comprehensive review. *J Autoimmun* 2016; **66**: 98-107 [PMID: 26614611 DOI: 10.1016/j.jaut.2015.10.006]
- 2 WALDENSTROM J. Liver, blood proteins and nutritive protein. *Dtsch Z Verdau Stoffwechselkr* 1953; **9**: 113-119 [PMID: 13150939]
- 3 Kessler WR, Cummings OW, Eckert G, Chalasani N, Lumeng L, Kwo PY. Fulminant hepatic failure as the initial presentation of acute autoimmune hepatitis. *Clin Gastroenterol Hepatol* 2004; **2**: 625-631 [PMID: 15224287]
- 4 Mendizabal M, Marciano S, Videla MG, Anders M, Zerega A, Balderramo DC, Tisi Baña MR, Barrabino M, Gil O, Mastai R, Yantorno S, Gadano A, Silva MO. Fulminant presentation of autoimmune hepatitis: clinical features and early predictors of corticosteroid treatment failure. *Eur J Gastroenterol Hepatol* 2015; **27**: 644-648 [PMID: 25923939 DOI: 10.1097/MEG.0000000000000353]
- 5 Czaja AJ. Diagnosis and Management of Autoimmune Hepatitis: Current Status and Future Directions. *Gut Liver* 2016; **10**: 177-203 [PMID: 26934884 DOI: 10.5009/gnl15352]
- 6 European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Autoimmune hepatitis. *J Hepatol* 2015; **63**: 971-1004 [PMID: 26341719 DOI: 10.1016/j.jhep.2015.06.030]
- 7 Lammert C, Loy VM, Oshima K, Gawrieh S. Management of Difficult Cases of Autoimmune Hepatitis. *Curr Gastroenterol Rep* 2016; **18**: 9 [PMID: 26780632 DOI: 10.1007/s11894-015-0484-7]
- 8 Heneghan MA, Yeoman AD, Verma S, Smith AD, Longhi MS. Autoimmune hepatitis. *Lancet* 2013; **382**: 1433-1444 [PMID: 23768844 DOI: 10.1016/S0140-6736(12)62163-1]
- 9 Rigopoulou EI, Smyk DS, Matthews CE, Billinis C, Burroughs AK, Lenzi M, Bogdanos DP. Epstein-barr virus as a trigger of autoimmune liver diseases. *Adv Virol* 2012; **2012**: 987471 [PMID: 22693505 DOI: 10.1155/2012/987471]
- 10 Dhawan A. Acute liver failure in children and adolescents. *Clin Res Hepatol Gastroenterol* 2012; **36**: 278-283 [PMID: 22521555 DOI: 10.1016/j.clinre.2012.03.022]
- 11 Sonthalia N, Rath PM, Jain SS, Surude RG, Mohite AR, Pawar SV, Contractor Q. Natural History and Treatment Outcomes of Severe Autoimmune Hepatitis. *J Clin Gastroenterol* 2017; **51**: 548-556 [PMID: 28272079 DOI: 10.1097/MCG.0000000000000805]
- 12 Bernal W, Wendon J. Acute liver failure. *N Engl J Med* 2013; **369**: 2525-2534 [PMID: 24369077 DOI: 10.1056/NEJMra1208937]
- 13 Reddy KR, Ellerbe C, Schilsky M, Stravitz RT, Fontana RJ, Durkalski V, Lee WM; Acute Liver Failure Study Group. Determinants of outcome among patients with acute liver failure listed for liver transplantation in the United States. *Liver Transpl* 2016; **22**: 505-515 [PMID: 26421889 DOI: 10.1002/lt.24347]
- 14 Polson J, Lee WM; American Association for the Study of Liver Disease. AASLD position paper: the management of acute liver failure. *Hepatology* 2005; **41**: 1179-1197 [PMID: 15841455 DOI: 10.1002/hep.20703]
- 15 Czaja AJ. Autoantibodies as prognostic markers in autoimmune liver disease. *Dig Dis Sci* 2010; **55**: 2144-2161 [PMID: 20464491 DOI: 10.1007/s10620-010-1268-4]
- 16 Bernal W, Ma Y, Smith HM, Portmann B, Wendon J, Vergani D. The significance of autoantibodies and immunoglobulins in acute liver failure: a cohort study. *J Hepatol* 2007; **47**: 664-670 [PMID: 17602781 DOI: 10.1016/j.jhep.2007.05.011]
- 17 Zachou K, Muratori P, Koukoulis GK, Granito A, Gatselis N, Fabbri A, Dalekos GN, Muratori L. Review article: autoimmune hepatitis -- current management and challenges. *Aliment Pharmacol Ther* 2013; **38**: 887-913 [PMID: 24010812 DOI: 10.1111/apt.12470]
- 18 Czaja AJ. Performance parameters of the diagnostic scoring systems for autoimmune hepatitis. *Hepatology* 2008; **48**: 1540-1548 [PMID: 18924244 DOI: 10.1002/hep.22513]
- 19 Hennes EM, Zeniya M, Czaja AJ, Parés A, Dalekos GN, Krawitt EL, Bittencourt PL, Porta G, Boberg KM, Hofer H, Bianchi FB, Shibata M, Schramm C, Eisenmann de Torres B, Galle PR, McFarlane I, Dienes HP, Lohse AW; International Autoimmune Hepatitis Group. Simplified criteria for the diagnosis of autoimmune hepatitis. *Hepatology* 2008; **48**: 169-176 [PMID: 18537184 DOI: 10.1002/hep.22322]
- 20 Castiella A, Zapata E, Lucena MI, Andrade RJ. Drug-induced autoimmune liver disease: A diagnostic dilemma of an increasingly reported disease. *World J Hepatol* 2014; **6**: 160-168 [PMID: 24799984 DOI: 10.4254/wjh.v6.i4.160]
- 21 Watkins PB, Seeff LB. Drug-induced liver injury: summary of a single topic clinical research conference. *Hepatology* 2006; **43**: 618-631 [PMID: 16496329 DOI: 10.1002/hep.21095]
- 22 Alla V, Abraham J, Siddiqui J, Raina D, Wu GY, Chalasani NP, Bonkovsky HL. Autoimmune hepatitis triggered by statins. *J Clin Gastroenterol* 2006; **40**: 757-761 [PMID: 16940892]
- 23 Grasset L, Guy C, Ollagnier M. Cyclines and acne: pay attention to adverse drug reactions! A recent literature review. *Rev Med Interne* 2003; **24**: 305-316 [PMID: 12763176]
- 24 Björnsson E, Talwalkar J, Treeprasertsuk S, Kamath PS, Takahashi N, Sanderson S, Neuhauser M, Lindor K. Drug-induced autoimmune hepatitis: clinical characteristics and prognosis. *Hepatology* 2010; **51**: 2040-2048 [PMID: 20512992 DOI: 10.1002/hep.23588]
- 25 Castiella A, Lucena MI, Zapata EM, Otazua P, Andrade RJ. Drug-induced autoimmune-like hepatitis: a diagnostic challenge. *Dig Dis Sci* 2011; **56**: 2501-2502; author reply 2502-2503 [PMID: 21674172 DOI: 10.1007/s10620-011-1787-7]
- 26 Reuben A, Koch DG, Lee WM; Acute Liver Failure Study Group. Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. *Hepatology* 2010; **52**: 2065-2076 [PMID: 20949552 DOI: 10.1002/hep.23937]
- 27 Lee WM, Squires RH Jr, Nyberg SL, Doo E, Hoofnagle JH. Acute liver failure: Summary of a workshop. *Hepatology* 2008; **47**: 1401-1415 [PMID: 18318440 DOI: 10.1002/hep.22177]
- 28 Tujios SR, Lee WM. Acute liver failure induced by idiosyncratic reaction to drugs: Challenges in diagnosis and therapy. *Liver Int* 2018; **38**: 6-14 [PMID: 28771932 DOI: 10.1111/liv.13535]
- 29 de Boer YS, Kosinski AS, Urban TJ, Zhao Z, Long N, Chalasani N, Kleiner DE, Hoofnagle JH; Drug-Induced Liver Injury Network. Features of Autoimmune Hepatitis in Patients With Drug-induced Liver Injury. *Clin Gastroenterol Hepatol* 2017; **15**: 103-112.e2

- [PMID: 27311619 DOI: 10.1016/j.cgh.2016.05.043]
- 30 **Liu ZX**, Kaplowitz N. Immune-mediated drug-induced liver disease. *Clin Liver Dis* 2002; **6**: 755-774 [PMID: 12362579]
 - 31 **Licata A**, Maida M, Cabibi D, Butera G, Macaluso FS, Alessi N, Caruso C, Craxi A, Almasio PL. Clinical features and outcomes of patients with drug-induced autoimmune hepatitis: a retrospective cohort study. *Dig Liver Dis* 2014; **46**: 1116-1120 [PMID: 25224696 DOI: 10.1016/j.dld.2014.08.040]
 - 32 **Kuzu UB**, Öztaş E, Turhan N, Saygılı F, Suna N, Yıldız H, Kaplan M, Akpınar MY, Akdoğan M, Kaçar S, Kiliç ZM, Köksal AŞ, Ödemiş B, Kayaçetin E. Clinical and histological features of idiosyncratic liver injury: Dilemma in diagnosis of autoimmune hepatitis. *Hepatol Res* 2016; **46**: 277-291 [PMID: 25926402 DOI: 10.1111/hepr.12530]
 - 33 **Aizawa Y**, Hokari A. Autoimmune hepatitis: current challenges and future prospects. *Clin Exp Gastroenterol* 2017; **10**: 9-18 [PMID: 28176894 DOI: 10.2147/CEG.S101440]
 - 34 **Maggiore G**, Nastasio S, Sciveres M. Juvenile autoimmune hepatitis: Spectrum of the disease. *World J Hepatol* 2014; **6**: 464-476 [PMID: 25067998 DOI: 10.4254/wjh.v6.i7.464]
 - 35 **Pischke S**, Iking-Konert C. Hepatitis E infections in rheumatology. A previously underestimated infectious disease? *Z Rheumatol* 2015; **74**: 731-736 [PMID: 26450437 DOI: 10.1007/s00393-015-1631-0]
 - 36 **Lauletta G**, Russi S, Pavone F, Marzullo A, Tampoia M, Sansonno D, Dammacco F. Autoimmune Hepatitis: Factors Involved in Initiation and Methods of Diagnosis and Treatment. *Crit Rev Immunol* 2016; **36**: 407-428 [PMID: 28605347 DOI: 10.1615/CritRevImmunol.2017017868]
 - 37 **van Gemenen MA**, van Wijngaarden P, Doukas M, de Man RA. Vaccine-related autoimmune hepatitis: the same disease as idiopathic autoimmune hepatitis? Two clinical reports and review. *Scand J Gastroenterol* 2017; **52**: 18-22 [PMID: 27565372 DOI: 10.1080/00365521.2016.1224379]
 - 38 **Arndtz K**, Hirschfield GM. The Pathogenesis of Autoimmune Liver Disease. *Dig Dis* 2016; **34**: 327-333 [PMID: 27170385 DOI: 10.1159/000444471]
 - 39 **Webb GJ**, Hirschfield GM. Using GWAS to identify genetic predisposition in hepatic autoimmunity. *J Autoimmun* 2016; **66**: 25-39 [PMID: 26347073 DOI: 10.1016/j.jaut.2015.08.016]
 - 40 **Maiers M**, Gragert L, Madbouly A, Steiner D, Marsh SG, Gourraud PA, Oudshoorn M, van der Zanden H, Schmidt AH, Pingel J, Hofmann J, Müller C, Eberhard HP. 16(th) IHIW: global analysis of registry HLA haplotypes from 20 million individuals: report from the IHIW Registry Diversity Group. *Int J Immunogenet* 2013; **40**: 66-71 [PMID: 23280139 DOI: 10.1111/iji.12031]
 - 41 **Di Giorgio A**, Bravi M, Bonanomi E, Alessio G, Sonzogni A, Zen Y, Colledan M, D'Antiga L. Fulminant hepatic failure of autoimmune aetiology in children. *J Pediatr Gastroenterol Nutr* 2015; **60**: 159-164 [PMID: 25304891 DOI: 10.1097/MPG.0000000000000593]
 - 42 **Yeoman AD**, Westbrook RH, Zen Y, Bernal W, Al-Chalabi T, Wendon JA, O'Grady JG, Heneghan MA. Prognosis of acute severe autoimmune hepatitis (AS-AIH): the role of corticosteroids in modifying outcome. *J Hepatol* 2014; **61**: 876-882 [PMID: 24842305 DOI: 10.1016/j.jhep.2014.05.021]
 - 43 **Kathemann S**, Bechmann LP, Sowa JP, Manka P, Dechêne A, Gerner P, Lainka E, Hoyer PF, Feldstein AE, Canbay A. Etiology, outcome and prognostic factors of childhood acute liver failure in a German Single Center. *Ann Hepatol* 2015; **14**: 722-728 [PMID: 26256901]

P- Reviewer: Eshraghian A, Lei YC, McMillin MA
S- Editor: Wang XJ **L- Editor:** A **E- Editor:** Huang Y



Retrospective Cohort Study

Helicobacter pylori infection in subjects negative for high titer serum antibody

Osamu Toyoshima, Toshihiro Nishizawa, Masahide Arita, Yosuke Kataoka, Kosuke Sakitani, Shuntaro Yoshida, Hiroharu Yamashita, Keisuke Hata, Hidenobu Watanabe, Hidekazu Suzuki

Osamu Toyoshima, Toshihiro Nishizawa, Masahide Arita, Yosuke Kataoka, Kosuke Sakitani, Shuntaro Yoshida, Hiroharu Yamashita, Keisuke Hata, Department of Gastroenterology, Toyoshima Endoscopy Clinic, Tokyo 1570066, Japan

Hidenobu Watanabe, Department of Pathology, Pathology and Cytology Laboratory Japan, Tokyo 1660003, Japan

Hidekazu Suzuki, Medical Education Center, Keio University School of Medicine, Tokyo 1608582, Japan

ORCID number: Osamu Toyoshima (0000-0002-6953-6079); Toshihiro Nishizawa (0000-0003-4876-3384); Masahide Arita (0000-0003-1952-8086); Yosuke Kataoka (0000-0002-8374-6558); Kosuke Sakitani (0000-0002-4537-6023); Shuntaro Yoshida (0000-0002-9437-9132); Hiroharu Yamashita (0000-0002-9468-3716); Keisuke Hata (0000-0003-4064-8701); Hidenobu Watanabe (0000-0002-7871-4738); Hidekazu Suzuki (0000-0002-3855-3140).

Author contributions: All authors were involved in designing the study; Toyoshima O, Nishizawa T and Suzuki H prepared the manuscript; Watanabe H was involved with histological diagnoses; Toyoshima O contributed to statistical analyses.

Institutional review board statement: This retrospective study was approved by the Ethical Review Committee of Hattori Clinic on September 7, 2017.

Informed consent statement: Written informed consents were obtained from the participants.

Conflict-of-interest statement: During the last five years, Toyoshima O received personal fees from Otsuka Pharmaceutical Co., Ltd. and Takeda Pharmaceutical Co., Ltd. outside of the submitted work; Suzuki H received scholarship funds for the research from Astellas Pharma Inc., Astra-Zeneca K.K., Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Zeria Pharmaceutical Co., Ltd. and received service honoraria from Astellas Pharma, Inc., Astra-Zeneca K.K., Otsuka Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Zeria Pharmaceutical Co., Ltd.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Osamu Toyoshima, MD, Director, Department of Gastroenterology, Toyoshima Endoscopy Clinic, 6-17-5 Seijo, Setagaya-ku, Tokyo 1570066, Japan. t@ichou.com
Telephone: +81-3-54299555
Fax: +81-3-54299511

Received: February 25, 2018
Peer-review started: February 25, 2018
First decision: March 9, 2018
Revised: March 13, 2018
Accepted: March 18, 2018
Article in press: March 18, 2018
Published online: April 7, 2018

Abstract

AIM

To investigate the clinicopathological features of the patients testing negative for high titer serum anti-*Helicobacter pylori* (*H. pylori*) antibody.

METHODS

The antibody titers were measured using antigens

derived from Japanese individuals. ^{13}C -urea breath test-positive individuals were defined as having *H. pylori* infection. We investigated the demographic characteristics, laboratory data, endoscopic findings including Kyoto classification of gastritis, and histology in negative-high titer patients without *H. pylori* eradication therapy. Kyoto classification consisted of scores for gastric atrophy, intestinal metaplasia, enlarged folds, nodularity, and redness.

RESULTS

Of the 136 subjects enrolled, 23 (17%) had *H. pylori* infection. Kyoto classification had an excellent area under the receiver operating characteristics curve (0.886, 95% confidence interval: 0.803-0.968, $P = 3.7 \times 10^{-20}$) for predicting *H. pylori* infection with a cut-off value of 2. Further, Kyoto classification, *H. pylori* density, and neutrophil activity had high accuracies (89.7%, 96.3%, and 94.1%, respectively). Kyoto classification was independent of the demographic and laboratory parameters in multivariate analysis.

CONCLUSION

Endoscopic Kyoto classification of gastritis is a useful predictor of *H. pylori* infection in negative-high titer antibody patients.

Key words: Kyoto classification; Gastritis; *Helicobacter pylori*; Antibody; Endoscopy

© The Author(s) 2018. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Compared with negative-low titer (< 3 U/mL on E-plate Eiken kit), negative-high titer (3-9.9 U/mL) have been reported to be at higher risk for intestinal gastric cancer. *Helicobacter pylori* (*H. pylori*)-infected patients accounted for 94% of gastric cancer patients with an antibody titer of < 10 U/mL. Seventeen percent of subjects with negative-high titer serum anti-*H. pylori* antibody tested positive for *H. pylori* infection. Endoscopic Kyoto classification of gastritis was an excellent predictor of *H. pylori* infection with large area under the receiver operating characteristics curve (0.886), cut-off value of 2, and high accuracy (89.7%), indicating its high confidence.

Toyoshima O, Nishizawa T, Arita M, Kataoka Y, Sakitani K, Yoshida S, Yamashita H, Hata K, Watanabe H, Suzuki H. *Helicobacter pylori* infection in subjects negative for high titer serum antibody. *World J Gastroenterol* 2018; 24(13): 1419-1428 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1419.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1419>

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a group 1 carcinogen

for gastric cancer. Therefore, the International Agency for Research on Cancer has recommended screening for and eradication of *H. pylori* for preventing gastric cancer^[1]. The main non-invasive methods for diagnosing *H. pylori* infection are the serum immunoglobulin G antibody test, ^{13}C -urea breath test (UBT), and stool antigen test. Endoscopy, histology, culture, and rapid urease test have been used as the main invasive methods. The Maastricht V/Florence consensus report states that the urea breath test using ^{13}C -urea is the best test to diagnose *H. pylori* infection^[2,3]. However, some of the available serum antibody kits including E-plate Eiken are excellent kits, with sensitivity and specificity above 90%^[4,5]. Serology is hardly affected by the changes in the stomach that result in a low bacterial load, including gastrointestinal bleeding, atrophic gastritis, gastric mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma^[2,6]. Additionally, proton pump inhibitors and antibiotics have little influence on serological tests as well^[7]. A serological test, with levels of serum anti-*H. pylori* antibody and pepsinogen I and II, is useful for identifying patients at increased risk of gastric cancer^[2,8,9]. These are some of the merits of serological testing. However, subjects with an E-plate antibody titer of < 10 U/mL include patients with spontaneous disappearance of *H. pylori* from the gastric mucosa, who are known to have extremely severe gastritis and high risk for gastric cancer^[10].

In clinical practice, in addition to evaluating the results of *H. pylori* serology as a categorical variable (*i.e.*, positive or negative), it is also important to consider the titer of *H. pylori* antibodies because there is a relationship between the antibody titer and the risk of gastric cancer. We mainly use the E-plate Eiken kit as an anti-*H. pylori* antibody test in Japan. The cut-off titer of this kit for diagnosing *H. pylori* infection is ≥ 10 U/mL, while the lower sensitivity limit of this kit is 3 U/mL. Previous reports have defined the titer between 3 and 9.9 U/mL as negative-high titer, and the titer < 3 U/mL as a negative-low titer. Compared with the negative-low titer, the negative-high titer has been reported to carry a higher risk, especially for intestinal gastric cancer in subjects with gastric atrophy^[10-12].

There are some false negative results when screening for current *H. pylori* infection in patients with an E-plate antibody titer of < 10 U/mL. *H. pylori*-infected patients accounted for 94% of patients with gastric cancer with an E-plate antibody titer of < 10 U/mL. Additionally, in patients with gastric cancer with an E-plate antibody titer of < 10 U/mL, *H. pylori* infection was associated with higher titers of antibodies^[13].

Thus, seronegative-high titer antibody is associated with gastric cancer. However, the clinicopathological characteristics of negative-high titer patients, including the prevalence of *H. pylori* infection, have not been studied extensively. This study focused on serum negative-high titer antibody subjects without history

of *H. pylori* eradication therapy and investigated the features of *H. pylori*-infected patients in the category.

MATERIALS AND METHODS

Subjects

We conducted this retrospective case-control study in patients with negative-high titer serum anti-*H. pylori* antibodies, who underwent esophagogastroduodenoscopy (EGD) and histological evaluation based on the updated Sydney system at Toyoshima Endoscopy Clinic between September 2016 to May 2017. EGDs were performed for screening, surveillance for gastrointestinal diseases, and investigation of some symptoms or abnormal results of the other assessments. We did not include subjects with history of gastric cancer, gastrectomy, *H. pylori* eradication therapy, and severe concomitant illnesses, and those who did not consent to this study. The following demographic characteristics were collected from the medical records: age, sex, body mass index (BMI), first-degree family history of gastric cancer, smoking history, and habitual drinking^[14]. A score of at least 400 on the Brinkman index was defined as positive smoking history. Consumption of at least one drink of alcohol per day was defined as habitual.

This retrospective study was approved by the Ethical Review Committee of Hattori Clinic on September 7, 2017. Written informed consents were obtained from the participants. All clinical investigations were conducted according to the ethical guidelines of the Declaration of Helsinki.

Diagnosis of *H. pylori* and related findings

The *H. pylori* antibody titer was measured in the blood samples obtained at the time of the first visit or EGD. The antibody titer was measured using an enzyme immunoassay kit using antigens derived from Japanese individuals (E-plate Eiken *H. pylori* antibody II; Eiken Chemical, Tokyo, Japan). A negative-high titer was defined as 3-9.9 U/mL of anti-*H. pylori* antibodies.

UBT-positive individuals were defined as subjects with *H. pylori* infection^[2,15,16]. We performed UBT using a 100 mg ¹³C-urea tablet (Pylonic; Sumitomo Dainippon Pharma, Osaka, Japan) after at least 2 wk of cessation of proton pump inhibitors or antibiotics. The result was declared negative if it was lower than 3 per mil.

Kyoto classification of gastritis is based on the sum of scores of the following five endoscopic findings, which are scored from 0 to 8: atrophy, intestinal metaplasia (IM), enlarged folds, nodularity, and redness. A high score represents increased risk for gastric cancer^[13,17]. Gastric atrophy was classified according to the extent of mucosal atrophy as described by Kimura and Takemoto^[14,18,19]. C-II and C-III of Kimura-

Takemoto classification were scored as 1, and O-I to O-III as 2. IM is observed as grayish-whitish and slightly opalescent patches. IM within the antrum was scored as 1, and IM extending into the corpus as 2. The presence of folds enlarged over 5 mm or more was scored as 1. Nodularity is characterized by the appearance of multiple whitish elevated lesions mainly in the pyloric gland mucosa. The presence of nodularity was scored as 1. Diffuse redness refers to uniform redness involving the entire fundic gland mucosa. The presence of redness with regular arrangements of collecting venules was scored as 1, and that without regular arrangement of collecting venules as 2. We also considered the presence of gastric sticky mucus and gastroduodenal ulcer as positive findings of *H. pylori* infection. On the contrary, gastroesophageal reflux disease, hiatal hernia, and fundic gland polyp were considered as findings of absence of *H. pylori* infection. Sticky mucus refers to grayish or yellowish mucus that adheres to the mucosal surface prior to washing with water. Gastroduodenal ulcer scars were included in the positive group. Grade A or more severe of Los Angeles classification in gastroesophageal reflux disease was defined as positive. We defined hiatal hernia of 2 cm or more as positive. Figure 1A-F shows the representative endoscopic findings related to *H. pylori* infection in negative-high titer patients of this study.

EGDs were performed by 14 expert physicians using Olympus Evis Lucera Elite system with endoscope: GIF-HQ290 or GIF-H290Z (Olympus Corporation, Tokyo, Japan). We carried out EGDs under conscious sedation with midazolam and/or pethidine hydrochloride. The EGD images were retrospectively reviewed by the chief investigator (OT). Any disagreements were resolved by consulting a third reviewer (TN). Discrepancies in diagnoses between the two sets of physicians were resolved through discussions.

Pathological findings were evaluated using the updated Sydney system score, including *H. pylori* density, neutrophil activity, chronic inflammation, IM, and glandular atrophy, with hematoxylin and eosin stains^[20-22]. The biopsy samples were collected from the greater curvature of the corpus and antrum. We defined one or more score in either of the two points as present. The histological diagnosis was performed by an expert gastrointestinal pathologist, who was not an endoscopist, and was from another organization.

Statistical analysis

First, we evaluated the effects of age, sex, BMI, family history of gastric cancer, smoking, habitual drinking, serum anti-*H. pylori* antibody titer, endoscopic findings, and histological findings on *H. pylori* infection in univariate analysis using Fisher's exact test or Cochran-Armitage test for categorical variables and Mann-Whitney *U* test for quantitative variables. Next,

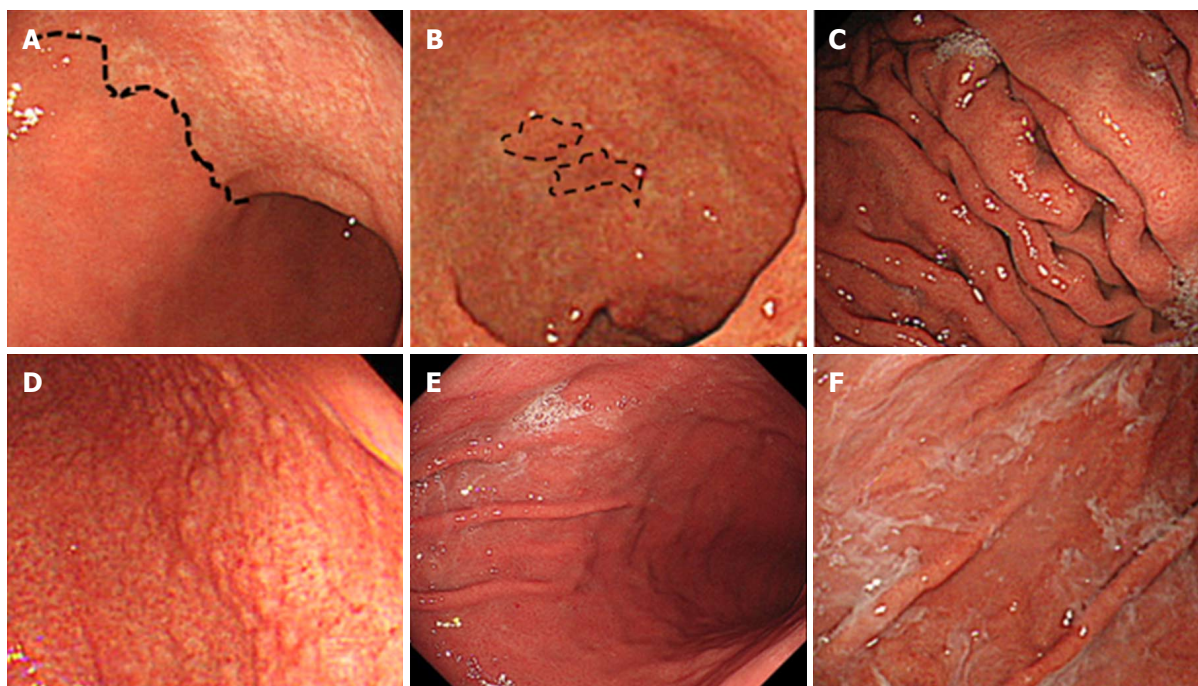


Figure 1 Endoscopic findings related to *Helicobacter pylori* infection. A: Atrophy is diagnosed based on the vascular pattern and rugal atrophy. The dotted line indicates an atrophic border in the anterior wall of the body (43-year-old woman; antibody titer: 4.3 U/mL; UBT: 55.3 per mil; Kyoto classification score: 2); B: Intestinal metaplasia is visible as grayish-whitish, slightly opalescent patches. The dotted line indicates the extent of the lesions in the lesser curvature of the antrum (81-year-old woman; antibody titer: 4.7 U/mL; UBT: 7.3 per mil; Kyoto classification score: 5); C: An enlarged fold is defined as that which is 5 mm or more in diameter. Enlarged folds are present in the greater curvature of the body (56-year-old man; antibody titer: 3.8 U/mL; UBT: 7.0 per mil; Kyoto classification score: 3); D: Nodularity is characterized by the appearance of multiple whitish elevated lesions mainly in the pyloric gland mucosa. Nodularity is present in the antrum (28-year-old man; antibody titer: 9.4 U/mL; UBT: 3.6 per mil; Kyoto classification score: 2); E: Redness refers to uniform redness involving the entire fundic gland mucosa. Redness is visible in the greater curvature of the body (44-year-old man; antibody titer: 8.7 U/mL; UBT: 26.5 per mil; Kyoto classification score: 3); F: Sticky mucus refers to grayish or yellowish mucus adhering to the mucosal surface. There is sticky mucus in the greater curvature of the body (70-year-old woman; antibody titer: 6.5 U/mL; UBT: 26.4 per mil; Kyoto classification score: 4). UBT: Urea breath test.

the values of the area under the receiver operating characteristic curve (AUC) for predicting *H. pylori* infection were compared with the value of 0.5 using the chi-squared test. The cut-off values for predicting *H. pylori* infection were estimated using the Youden index, which is the farthest point on the receiver operating characteristic curve from the positive diagonal^[23]. We compared AUC values with the use of a chi-square test. Then, the performances of the endoscopic and histological findings for *H. pylori* infection, including accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were investigated. The predictors associated with *H. pylori* infection were subsequently assessed using multiple logistic regression analysis to distinguish the independent factors from other demographic and laboratory variables. A two-sided *P* value less than 0.05 was considered as significant. The data were analyzed using Ekuseru-Toukei 2015 software (Social Survey Research Information, Tokyo, Japan).

RESULTS

The characteristics of the participants of the present study are shown in Table 1. A total of 136 subjects

were enrolled. The median age of the subjects was 45 (range: 17-82, interquartile range: 37-56) years, and 39% were males. The median titer of *H. pylori* antibody was 4.7 (interquartile range, 3.7-6.6) U/mL. Seventeen percent (*n* = 23) were diagnosed as *H. pylori*-infected based on UBT.

On comparing *H. pylori*-infected and -uninfected patients regarding the demographic characteristics and laboratory data, *H. pylori*-infected patients were older (53 years vs 42 years, *P* = 0.0057), and had higher BMI (22.7 kg/m² vs 21.2 kg/m², *P* = 0.028) and serum antibody titer (5.4 U/mL vs 4.7 U/mL, *P* = 0.048). No significant differences due to sex, family history of gastric cancer, habitual smoking, or habitual drinking were demonstrated. Regarding endoscopic findings, we found significant differences between them in Kyoto classification of gastritis score (*P* = 3.8×10^{-13}), gastric sticky mucus (*P* = 0.013), and fundic gland polyp (*P* = 0.0022). Histologically, *H. pylori* density (*P* = 2.8×10^{-18}), chronic inflammation (*P* = 4.5×10^{-10}), and neutrophil activity (*P* = 1.4×10^{-14}) were significantly different between the two groups (Table 1).

Then, we analyzed AUC for predicting *H. pylori* infection based on the variables that had significant differences between *H. pylori*-infected and -uninfected

Table 1 Characteristics of enrolled subjects

	Total	<i>H. pylori</i> infected ¹	<i>H. pylori</i> uninfected	<i>P</i> value ³
<i>n</i> (%)	136	23 (17)	113 (83)	
Demographic characteristics				
Age median (IQR), yr	45 (37-56)	53 (44-68)	42 (35-53)	0.0057
Male sex (%)	53 (39)	7 (30)	46 (41)	0.48
Body mass index median (IQR), kg/m ²	21.2 (19.6-23.7)	22.7 (20.4-25.6)	21.2 (19.5-23.3)	0.028
Family history of gastric cancer, present/absent	12/124	3/20	9/104	0.43
Smoking, present/absent	4/132	0/23	4/109	1.0
Drinking, present/absent	25/111	4/19	21/92	1.0
Laboratory data				
Anti- <i>H. pylori</i> antibody median (IQR), U/mL	4.7 (3.7-6.6)	5.4 (4.2-7.9)	4.7 (3.7-6.4)	0.048
¹³ C-urea breath test result median (IQR), per mil	0.3 (0.1-0.8)	19.3 (9.3-26.3)	0.3 (0.1-0.4)	3.6 × 10 ⁻¹⁴
Endoscopic findings				
Kyoto classification of gastritis ⁴ , 5/4/3/2/1/0	1/3/14/9/13/96	1/3/8/6/2/3	0/0/6/3/11/93	3.8 × 10 ⁻¹³
Atrophy, 2/1/0	15/20/101	10/4/2009	6/10/97	5.8 × 10 ⁻¹²
Intestinal metaplasia, 1/0	14/122	9/14	5/108	2.9 × 10 ⁻⁵
Enlarged folds, 1/0	5/131	4/19	1/112	0.0029
Nodularity, 1/0	2/134	2/21	0/113	0.028
Redness, 1/0	19/117	12/11	7/106	7.3 × 10 ⁻⁷
Gastric sticky mucus, present/absent	22/114	8/15	14/99	0.013
Gastric ulcer, present/absent	1/135	1/22	0/113	0.17
Duodenal ulcer, present/absent	3/133	1/22	2/111	0.43
Gastroesophageal reflux disease, present/absent	20/116	2/21	18/95	0.53
Hiatal hernia, present/absent	18/118	2/21	16/97	0.74
Fundic gland polyp, present/absent	41/95	1/22	40/73	0.0022
Histological findings ⁵				
<i>H. pylori</i> density, present/absent	18/118	18/5	0/113	2.8 × 10 ⁻¹⁸
Chronic inflammation, present/absent	46/90	21/2	25/88	4.5 × 10 ⁻¹⁰
Neutrophil activity, present/absent	15/121	15/8	0/113	1.4 × 10 ⁻¹⁴
Intestinal metaplasia, present/absent	4/132	1/22	3/110	0.53
Glandular atrophy, present/absent	4/132	1/22	3/110	0.53

¹¹³C-urea breath test-positive subjects were defined as *H. pylori*-infected patients; ³Fisher's exact test, Cochran-Armitage test, or Mann-Whitney *U* test was used as appropriate; ⁴Kyoto classification of gastritis was estimated by gastric atrophy, intestinal metaplasia, enlarged folds, nodularity, and redness^[13]; ⁵We defined one or more score classified by the updated Sydney system in either the great curvature of the corpus or the antrum as present. *H. pylori*: *Helicobacter pylori*; IQR: Interquartile range.

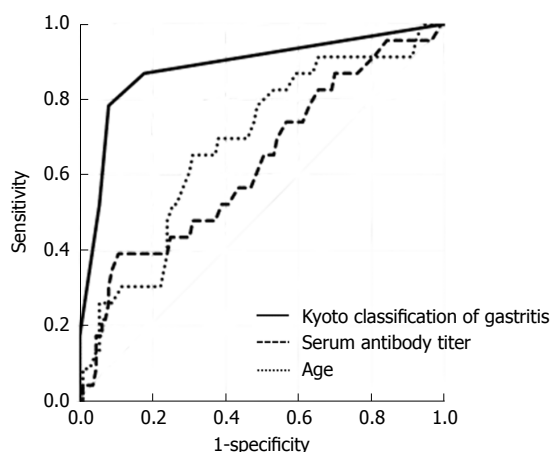


Figure 2 Receiver operating characteristics curves for predicting *Helicobacter pylori* infection. Receiver operating characteristics curves were based on endoscopic Kyoto classification of gastritis score, serum antibody titer, and age in 136 patients with negative-high titer antibody. Positive UBT was defined as *H. pylori* infection. UBT: Urea breath test.

patients (Table 2). AUC of *H. pylori* density (0.891, 95%CI: 0.805-0.977, $P = 5.6 \times 10^{-19}$) was the largest followed by those of Kyoto classification (0.886,

95%CI: 0.803-0.968, $P = 3.7 \times 10^{-20}$) and endoscopic atrophy (0.848, 95%CI: 0.760-0.936, $P = 7.7 \times 10^{-15}$). There was no significant difference between the three AUC values. The cut-off value of Kyoto classification of gastritis score for correlation with *H. pylori* infection was 2 and that of endoscopic atrophy was 1. The receiver operating characteristic curves based on Kyoto endoscopic classification, serum antibody titer, and age in 136 patients with negative-high titer antibody are shown in Figure 2.

The performances of endoscopic and histological findings for *H. pylori* infection are shown in Table 3. The highest accuracy was found in histological *H. pylori* density (96.3%), and its specificity and PPV were 100%. *H. pylori* density also had the second highest NPV (95.8%). The second highest accuracy was in neutrophil activity (94.1%), and its specificity and PPV were 100%. With regards to endoscopic findings, Kyoto classification of gastritis showed the highest accuracy (89.7%). The accuracies of redness, IM, atrophy (1 or more score as positive), enlarged folds, and nodularity followed that of Kyoto classification in order. The highest sensitivity (91.3%) and highest

Table 2 Area under the receiver operating characteristic curve for predicting *Helicobacter pylori* infection

	AUC	95%CI	P value
Age	0.684	0.564-0.804	0.0027
Body mass index	0.646	0.518-0.774	0.026
Serum antibody titer	0.631	0.500-0.763	0.051
Kyoto classification of gastritis	0.886	0.803-0.968	3.7×10^{-20}
Endoscopic atrophy	0.848	0.760-0.936	7.7×10^{-15}
Endoscopic intestinal metaplasia	0.674	0.570-0.777	0.0010
Enlarged fold	0.583	0.503-0.662	0.042
Nodularity	0.543	0.485-0.602	0.15
Redness	0.730	0.623-0.837	2.4×10^{-5}
Gastric sticky mucus	0.612	0.508-0.716	0.035
Fundic gland polyp	0.655	0.594-0.717	7.4×10^{-7}
<i>H. pylori</i> density	0.891	0.805-0.977	5.6×10^{-19}
Chronic inflammation	0.846	0.776-0.916	5.3×10^{-22}
Neutrophil activity	0.826	0.727-0.926	1.3×10^{-10}

Positive urea breath test was defined as *H. pylori* infection. The values of the AUC were compared with the value of 0.5 using the chi-square test. AUC: Area under the receiver operating characteristics curve; CI: Confidence interval; *H. pylori*: *Helicobacter pylori*.

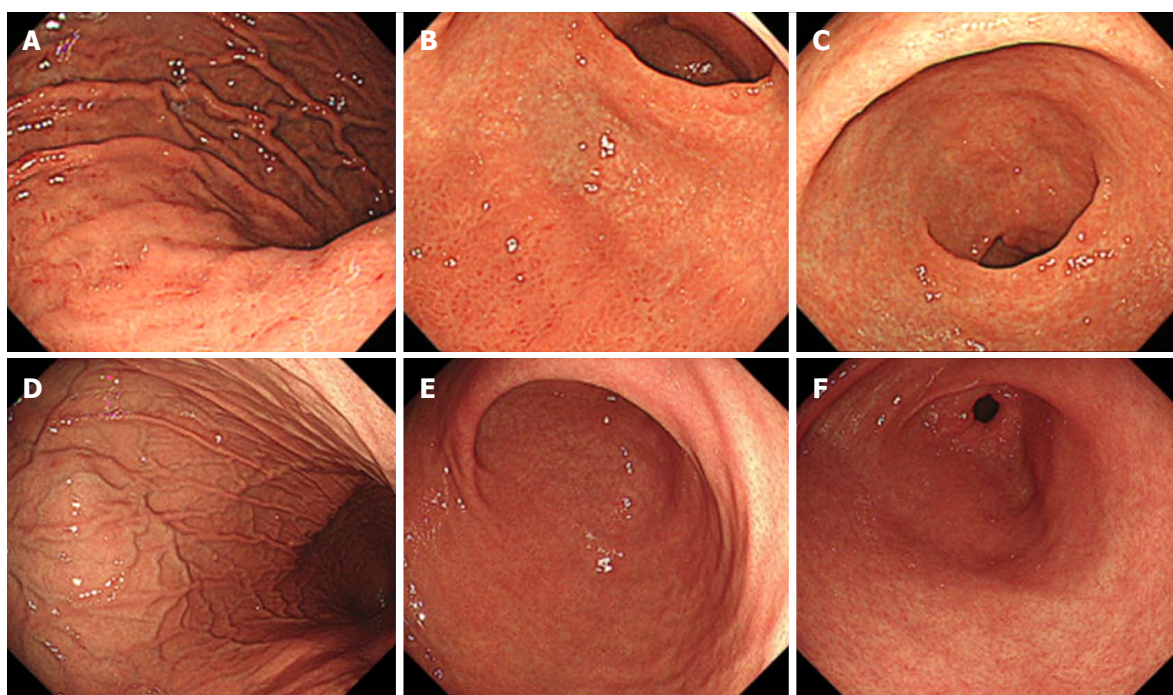


Figure 3 Representative endoscopic findings of negative-high titer antibody cases. A case with *Helicobacter pylori* infection; 81-year-old woman with antibody titer of 4.7 U/mL, UBT of 7.3 per mil, and Kyoto classification score of 5 (A-C). A: Greater curvature of the body of the stomach. Enlarged folds and redness are present; B: Lower body of the stomach. Endoscopic atrophic border lies in the anterior wall and greater curvature. Redness is present in the greater curvature; C: Antrum. Intestinal metaplasia is present in the lesser curvature. The mucosa is atrophic. A case without *H. pylori* infection; 31-year-old man with antibody titer of 5.7 U/mL, UBT of 1.2 per mil, and Kyoto classification score of 0 (D-F). D: The greater curvature of the body of the stomach. Regular arrangement of collecting venules and fundic gland polyps are present; E: Lower body of the stomach. Atrophy and redness are absent; F: Antrum. Intestinal metaplasia and atrophy are absent. UBT: Urea breath test.

NPV (97.8%) were shown with histological chronic inflammation.

Lastly, Kyoto classification was assessed using multivariate logistic regression analysis to identify any association with the variables such as age, BMI, and serum antibody titer. Kyoto classification was identified as an independent predictor of *H. pylori* infection ($P = 2.2 \times 10^{-6}$, Table 4).

Representative endoscopic findings of negative-high titer cases with or without *H. pylori* infection are demonstrated in Figure 3A-F.

DISCUSSION

We found that 17% of subjects with negative-high titer serum anti-*H. pylori* antibody were positive for *H.*

Table 3 Performance of endoscopic and histological findings for *Helicobacter pylori* infection

	Accuracy	Sensitivity	Specificity	PPV	NPV
Endoscopic findings					
Kyoto classification of gastritis ¹	89.7	78.3	92.0	66.7	95.4
Atrophy ²	85.3	82.6	85.8	54.3	96.0
Intestinal metaplasia	86.0	39.1	95.6	64.3	88.5
Enlarged folds	85.3	17.4	99.1	80.0	85.5
Nodularity	84.6	8.7	100	100	84.3
Redness	86.8	52.2	93.8	63.2	90.6
Gastric sticky mucus	78.7	34.8	87.6	36.4	86.8
Gastric ulcer	83.8	4.3	100	50.0	83.7
Duodenal ulcer	82.4	4.3	98.2	33.3	83.5
Gastroesophageal reflux disease	71.3	8.7	84.1	10.0	81.9
Hiatal hernia	72.8	8.7	85.8	11.1	82.2
Fundic gland polyp	54.4	4.3	64.6	2.4	76.8
Histological findings					
<i>H. pylori</i> density	96.3	78.3	100	100	95.8
Chronic inflammation	80.1	91.3	77.9	45.7	97.8
Neutrophil activity	94.1	65.2	100	100	93.4
Intestinal metaplasia	81.6	4.3	97.3	25.0	83.3
Glandular atrophy	81.6	4.3	97.3	25.0	83.3

¹A score of 2 or more was defined as positive; ²A score of 1 or more was defined as positive. The data are presented as %. Positive urea breath test was defined as *H. pylori* infection. PPV: Positive predictive value; NPV: Negative predictive value; *H. pylori*: *Helicobacter pylori*.

Table 4 Multivariate analysis for independent predictors of *Helicobacter pylori* infection

	Odds ratio	95%CI	P value
Age	0.98	0.93-1.03	0.49
Body mass index	1.06	0.90-1.24	0.50
Serum antibody titer	1.21	0.87-1.68	0.26
Kyoto classification of gastritis	4.23	2.33-7.67	2.2 × 10 ⁻⁶

pylori infection. Higher bacterial counts induce intense immune responses, resulting in subsequent higher antibody titers, while genetic differences between human hosts may affect the antibody levels in response to pathogens^[24]. Precise diagnosis in patients with seronegativity is necessary to reduce the false negative estimation of gastric cancer risk^[13]. We should identify *H. pylori*-infected cases in negative-high titer patients and carefully examine them.

Endoscopic Kyoto classification of gastritis proved to be an excellent predictor of *H. pylori* infection with large AUC (0.886), cut-off value of 2, high accuracy (89.7%), and was comparable to histological *H. pylori* density, indicating its high confidence. Kyoto classification also demonstrated to be independent of demographic and laboratory data. These results show that Kyoto classification is useful in the diagnosis of *H. pylori* infection among negative high-titer serum antibody patients. Endoscopic atrophy and nodularity have been attributed to *H. pylori* infection consistently, as was also seen with our results^[25-28].

Kyoto classification score is believed to provide an estimate of the risk of gastric cancer. Sugimoto *et al.*^[29] showed that the mean Kyoto classification score in gastric cancer group was 4.6 ± 1.2, which

was significantly higher than in gastritis-alone group (3.8 ± 1.1; *P* < 0.001). In subgroup analysis within the cancer group, the mean Kyoto classification score in the *H. pylori*-uneradicated subgroup was 4.8 ± 1.1, which was significantly higher than that in the eradicated subgroup (4.2 ± 1.2; *P* < 0.001). Our study showed that Kyoto classification score might be useful for not only estimating the risk of gastric cancer but also the prediction of *H. pylori* infection in negative-high titer patients.

Cases with negative-high titer antibodies with negative UBT could include not only subjects who have never been infected but also patients in whom infections resolved spontaneously^[10,30]. Patients with spontaneous resolution are known to be at very high risk for gastric cancer. In this study, nine patients with Kyoto classification score 2 or more had negative results with UBT. These cases might be after spontaneous disappearance of *H. pylori* infection. Such patients would need careful surveillance.

Histological *H. pylori* density was the strongest contributing factor to *H. pylori* infection with the largest AUC and highest accuracy (96.3%). Neutrophil activity had the second highest accuracy (94.1%). Several investigators have inferred significant associations of anti-*H. pylori* antibody titers with *H. pylori* density and neutrophil activity^[25,31-33]. Our findings are in accordance with their reports. Chronic inflammation had the highest sensitivity and NPV. Chronic inflammation has been reported to progress in parallel with increases in serum anti-*H. pylori* antibodies, and our results are consistent with this observation^[25,28,32].

H. pylori-infected patients were older than the uninfected patients among negative-high titer antibody

participants. Kiso *et al.*^[13] reported that in serum *H. pylori* antibody-negative subjects, those with *H. pylori* infection and gastric cancer were older than those with gastric cancer but without the infection. Our results were concordant with their results. In this study, the BMI of *H. pylori*-infected patients was higher than that of -uninfected patients. Our results are in agreement with the results of a report that concluded a positive association between being overweight and serum *H. pylori* antibody^[34].

There are some limitations to this study. We used UBT as the gold standard for *H. pylori* infection; however, its accuracy is not 100%. Better performance in serological screening depends on the use of the appropriate antigens and adjustment of cut-off values^[35]. As we used antibodies against the Japanese strain, further investigation of the other antibodies is needed. Non-*H. pylori* *Helicobacter* species, including *H. suis* and *H. felis*, could provoke serum anti-*H. pylori* antibody positivity^[36], and anti-*H. pylori* antibody correlates with the presence of cytotoxin associated gene A-positive strains^[37]; however, we did not assess them. Furthermore, we did not analyze the long-term outcomes in 17% of the patients with negative-high titer anti-*H. pylori* antibodies without history of eradication therapy who had *H. pylori* infection. Further studies should be performed to analyze the long-term outcomes and the association between the presence of CagA positive *H. pylori* infection and Kyoto classification.

In conclusion, 17% of the patients with negative-high titer serum anti-*H. pylori* antibodies without history of eradication therapy had *H. pylori* infection. Endoscopic Kyoto classification of gastritis with a score of 2 or more could predict *H. pylori* infection in negative high-titer patients. Further examination including UBT should be considered in these patients with Kyoto classification score 2 or more.

ARTICLE HIGHLIGHTS

Research background

Patients who test negative but in the negative-high titer range of serum anti-*Helicobacter pylori* (*H. pylori*) antibodies are at a high risk for gastric cancer, especially the intestinal type, and sometimes have *H. pylori* infection. Patients with negative-high titers with *H. pylori* infection have higher risk for gastric cancer than do those without *H. pylori* infection.

Research motivation

The clinicopathological features including *H. pylori* infection rate in the negative-high titer patients are unclear.

Research objectives

The objective of this research was to elucidate the clinicopathological features of the negative-high titer patients.

Research methods

The antibody titers were measured using antigens derived from Japanese individuals, E-plate Eiken. ¹³C-urea breath test (UBT)-positive individuals were defined as having *H. pylori* infection. We investigated the demographic

characteristics, laboratory data, endoscopic findings including Kyoto classification of gastritis, and histology in negative-high titer patients without history of *H. pylori* eradication therapy.

Research results

Of the 136 subjects enrolled, 23 (17%) had *H. pylori* infection. Kyoto classification had an excellent area under the receiver operating characteristics curve (0.886) for predicting *H. pylori* infection, with a cut-off value of 2. Further, Kyoto classification had high accuracy (89.7%). Kyoto classification was independent of the demographic and laboratory parameters in multivariate analysis.

Research conclusions

In this study, 17% of patients with negative-high titer had *H. pylori* infection. Endoscopic Kyoto classification of gastritis with a score of 2 or more could predict *H. pylori* infection in negative high-titer patients. Further investigations including UBT should be considered in these patients.

Research perspectives

Long-term prospective studies are expected to investigate the role of serum antibody titer and Kyoto classification of gastritis in predicting not only *H. pylori* infection but also the risk of gastric cancer.

ACKNOWLEDGEMENTS

We would also like to thank Kanazawa T, Matsumoto S, Isomura Y, Arano T, Kinoshita H, Ohki D, Fukagawa K, and Sekiba K for performing esophagogastroduodenoscopy and Sugita K, Sakurai C, and Yamakawa T for collecting data.

REFERENCES

- 1 **Cancer IAFRo.** *Helicobacter pylori* eradication as a strategy for preventing gastric cancer. IARC Working Group Reports. Volume 8
- 2 **Malfertheiner P,** Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM; European Helicobacter and Microbiota Study Group and Consensus panel. Management of *Helicobacter pylori* infection-the Maastricht V/Florence Consensus Report. *Gut* 2017; **66**: 6-30 [PMID: 27707777 DOI: 10.1136/gutjnl-2016-312288]
- 3 **Nishizawa T,** Suzuki H, Fujimoto A, Kinoshita H, Yoshida S, Isomura Y, Toyoshima A, Kanai T, Yahagi N, Toyoshima O. Effects of patient age and choice of antisecretory agent on success of eradication therapy for *Helicobacter pylori* infection. *J Clin Biochem Nutr* 2017; **60**: 208-210 [PMID: 28584402 DOI: 10.3164/jcbn.16-86]
- 4 **Buruco C,** Delchier JC, Courillon-Mallet A, de Korwin JD, Mégraud F, Zerbib F, Raymond J, Fauchère JL. Comparative evaluation of 29 commercial *Helicobacter pylori* serological kits. *Helicobacter* 2013; **18**: 169-179 [PMID: 23316886 DOI: 10.1111/hel.12030]
- 5 **Ueda J,** Okuda M, Nishiyama T, Lin Y, Fukuda Y, Kikuchi S. Diagnostic accuracy of the E-plate serum antibody test kit in detecting *Helicobacter pylori* infection among Japanese children. *J Epidemiol* 2014; **24**: 47-51 [PMID: 24240631]
- 6 **Tonkic A,** Tonkic M, Lehours P, Mégraud F. Epidemiology and diagnosis of *Helicobacter pylori* infection. *Helicobacter* 2012; **17** Suppl 1: 1-8 [PMID: 22958148 DOI: 10.1111/j.1523-5378.2012.00975.x]
- 7 **Parente F,** Sainaghi M, Sangaletti O, Imbesi V, Maconi G, Anderloni A, Bianchi Porro G. Different effects of short-term omeprazole, lansoprazole or pantoprazole on the accuracy of the

- 13C-urea breath test. *Aliment Pharmacol Ther* 2002; **16**: 553-557 [DOI: 10.1046/j.1365-2036.2002.01192.x]
- 8 **Sugano K**, Tack J, Kuipers EJ, Graham DY, El-Omar EM, Miura S, Haruma K, Asaka M, Uemura N, Malfertheiner P; faculty members of Kyoto Global Consensus Conference. Kyoto global consensus report on *Helicobacter pylori* gastritis. *Gut* 2015; **64**: 1353-1367 [PMID: 26187502 DOI: 10.1136/gutjnl-2015-309252]
 - 9 **Yamaji Y**, Mitsushima T, Ikuma H, Okamoto M, Yoshida H, Kawabe T, Shiratori Y, Saito K, Yokouchi K, Omata M. Inverse background of *Helicobacter pylori* antibody and pepsinogen in reflux oesophagitis compared with gastric cancer: analysis of 5732 Japanese subjects. *Gut* 2001; **49**: 335-340 [PMID: 11511553]
 - 10 **Kishikawa H**, Kimura K, Takarabe S, Kaida S, Nishida J. *Helicobacter pylori* Antibody Titer and Gastric Cancer Screening. *Dis Markers* 2015; **2015**: 156719 [PMID: 26494936 DOI: 10.1155/2015/156719]
 - 11 **Yamaji Y**, Mitsushima T, Ikuma H, Okamoto M, Yoshida H, Kawabe T, Shiratori Y, Saito K, Yokouchi K, Omata M. Weak response of *Helicobacter pylori* antibody is high risk for gastric cancer: a cross-sectional study of 10,234 endoscoped Japanese. *Scand J Gastroenterol* 2002; **37**: 148-153 [PMID: 11843049]
 - 12 **Tatemichi M**, Sasazuki S, Inoue M, Tsugane S; JPHC Study Group. Clinical significance of IgG antibody titer against *Helicobacter pylori*. *Helicobacter* 2009; **14**: 231-236 [PMID: 19702853 DOI: 10.1111/j.1523-5378.2009.00681.x]
 - 13 **Kiso M**, Yoshihara M, Ito M, Inoue K, Kato K, Nakajima S, Mabe K, Kobayashi M, Uemura N, Yada T, Oka M, Kawai T, Boda T, Kotachi T, Masuda K, Tanaka S, Chayama K. Characteristics of gastric cancer in negative test of serum anti-*Helicobacter pylori* antibody and pepsinogen test: a multicenter study. *Gastric Cancer* 2017; **20**: 764-771 [PMID: 28025702 DOI: 10.1007/s10120-016-0682-5]
 - 14 **Nishizawa T**, Suzuki H, Sakitani K, Yamashita H, Yoshida S, Hata K, Kanazawa T, Fujiwara N, Kanai T, Yahagi N, Toyoshima O. Family history is an independent risk factor for the progression of gastric atrophy among patients with *Helicobacter pylori* infection. *United European Gastroenterol J* 2017; **5**: 32-36 [PMID: 28405319 DOI: 10.1177/2050640616642341]
 - 15 **Gisbert JP**, Calvet X. *Helicobacter Pylori* "Test-and-Treat" Strategy for Management of Dyspepsia: A Comprehensive Review. *Clin Transl Gastroenterol* 2013; **4**: e32 [PMID: 23535826 DOI: 10.1038/ctg.2013.3]
 - 16 **Ferwana M**, Abdulmajed I, Alhajiahmed A, Madani W, Firwana B, Hasan R, Altayar O, Limburg PJ, Murad MH, Knawy B. Accuracy of urea breath test in *Helicobacter pylori* infection: meta-analysis. *World J Gastroenterol* 2015; **21**: 1305-1314 [PMID: 25632206 DOI: 10.3748/wjg.v21.i4.1305]
 - 17 **Shichijo S**, Hirata Y, Niihara R, Hayakawa Y, Yamada A, Koike K. Association between gastric cancer and the Kyoto classification of gastritis. *J Gastroenterol Hepatol* 2017; **32**: 1581-1586 [PMID: 28217843 DOI: 10.1111/jgh.13764]
 - 18 **Kimura K**, Takemoto T. An endoscopic recognition of the atrophic border and its significance in chronic gastritis. *Endoscopy* 1969; **3**: 87-97
 - 19 **Toyoshima O**, Yamaji Y, Yoshida S, Matsumoto S, Yamashita H, Kanazawa T, Hata K. Endoscopic gastric atrophy is strongly associated with gastric cancer development after *Helicobacter pylori* eradication. *Surg Endosc* 2017; **31**: 2140-2148 [PMID: 27604367 DOI: 10.1007/s00464-016-5211-4]
 - 20 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181 [PMID: 8827022]
 - 21 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789 [PMID: 11556297 DOI: 10.1056/NEJMoa001999]
 - 22 **Toyoshima O**, Tanikawa C, Yamamoto R, Watanabe H, Yamashita H, Sakitani K, Yoshida S, Kubo M, Matsuo K, Ito H, Koike K, Seto Y, Matsuda K. Decrease in PSCA expression caused by *Helicobacter pylori* infection may promote progression to severe gastritis. *Oncotarget* 2017; **9**: 3936-3945 [PMID: 29423095 DOI: 10.18632/oncotarget.23278]
 - 23 **Perkins NJ**, Schisterman EF. The inconsistency of "optimal" cutpoints obtained using two criteria based on the receiver operating characteristic curve. *Am J Epidemiol* 2006; **163**: 670-675 [PMID: 16410346 DOI: 10.1093/aje/kwj063]
 - 24 **Rubicz R**, Leach CT, Kraig E, Dhurandhar NV, Duggirala R, Blangero J, Yolken R, Göring HH. Genetic factors influence serological measures of common infections. *Hum Hered* 2011; **72**: 133-141 [PMID: 21996708 DOI: 10.1159/000331220]
 - 25 **Kamada T**, Sugiu K, Hata J, Kusunoki H, Hamada H, Kido S, Nagashima Y, Kawamura Y, Tanaka S, Chayama K, Haruma K. Evaluation of endoscopic and histological findings in *Helicobacter pylori*-positive Japanese young adults. *J Gastroenterol Hepatol* 2006; **21**: 258-261 [PMID: 16460483 DOI: 10.1111/j.1440-1746.2006.04128.x]
 - 26 **Adachi K**, Mishihiro T, Tanaka S, Kinoshita Y. Analysis of negative result in serum anti-*H. pylori* IgG antibody test in cases with gastric mucosal atrophy. *J Clin Biochem Nutr* 2016; **59**: 145-148 [PMID: 27698543 DOI: 10.3164/jcbn.16-13]
 - 27 **Watanabe M**, Kato J, Inoue I, Yoshimura N, Yoshida T, Mukoubayashi C, Deguchi H, Enomoto S, Ueda K, Maekita T, Iguchi M, Tamai H, Utsunomiya H, Yamamichi N, Fujishiro M, Iwane M, Tekeshita T, Mohara O, Ushijima T, Ichinose M. Development of gastric cancer in nonatrophic stomach with highly active inflammation identified by serum levels of pepsinogen and *Helicobacter pylori* antibody together with endoscopic rugal hyperplastic gastritis. *Int J Cancer* 2012; **131**: 2632-2642 [PMID: 22383377 DOI: 10.1002/ijc.27514]
 - 28 **Bruden DL**, Bruce MG, Miernyk KM, Morris J, Hurlburt D, Hennessy TW, Peters H, Sacco F, Parkinson AJ, McMahon BJ. Diagnostic accuracy of tests for *Helicobacter pylori* in an Alaska Native population. *World J Gastroenterol* 2011; **17**: 4682-4688 [PMID: 22180710 DOI: 10.3748/wjg.v17.i42.4682]
 - 29 **Sugimoto M**, Ban H, Ichikawa H, Sahara S, Otsuka T, Inatomi O, Bamba S, Furuta T, Andoh A. Efficacy of the Kyoto Classification of Gastritis in Identifying Patients at High Risk for Gastric Cancer. *Intern Med* 2017; **56**: 579-586 [PMID: 28321054 DOI: 10.2169/internalmedicine.56.7775]
 - 30 **Bergey B**, Marchildon P, Peacock J, Mégraud F. What is the role of serology in assessing *Helicobacter pylori* eradication? *Aliment Pharmacol Ther* 2003; **18**: 635-639 [DOI: 10.1046/j.1365-2036.2003.01716.x]
 - 31 **Kreuning J**, Lindeman J, Biemond I, Lamers CB. Relation between IgG and IgA antibody titres against *Helicobacter pylori* in serum and severity of gastritis in asymptomatic subjects. *J Clin Pathol* 1994; **47**: 227-231 [PMID: 8163693]
 - 32 **Sheu BS**, Shiesh SC, Yang HB, Su IJ, Chen CY, Lin XZ. Implications of *Helicobacter pylori* serological titer for the histological severity of antral gastritis. *Endoscopy* 1997; **29**: 27-30 [PMID: 9083733 DOI: 10.1055/s-2007-1004057]
 - 33 **Park CH**, Kim EH, Jung DH, Chung H, Park JC, Shin SK, Lee SK, Lee YC. The new modified ABCD method for gastric neoplasm screening. *Gastric Cancer* 2016; **19**: 128-135 [PMID: 25663259 DOI: 10.1007/s10120-015-0473-4]
 - 34 **Thjodleifsson B**, Olafsson I, Gislason D, Gislason T, Jögi R, Janson C. Infections and obesity: A multinational epidemiological study. *Scand J Infect Dis* 2008; **40**: 381-386 [PMID: 17943636 DOI: 10.1080/00365540701708293]
 - 35 **Hoang TT**, Wheelon TU, Bengtsson C, Phung DC, Sörberg M, Granström M. Enzyme-linked immunosorbent assay for *Helicobacter pylori* needs adjustment for the population

- investigated. *J Clin Microbiol* 2004; **42**: 627-630 [PMID: 14766827]
- 36 **Flahou B**, Haesebrouck F, Smet A, Yonezawa H, Osaki T, Kamiya S. Gastric and enterohepatic non-*Helicobacter pylori* *Helicobacters*. *Helicobacter* 2013; **18** Suppl 1: 66-72 [PMID: 24011248 DOI: 10.1111/hel.12072]
- 37 **Loffeld RJ**, Werdmuller BF, Kusters JG, Kuipers EJ. IgG antibody titer against *Helicobacter pylori* correlates with presence of cytotoxin associated gene A-positive *H. pylori* strains. *FEMS Immunol Med Microbiol* 2000; **28**: 139-141 [PMID: 10799804]

P- Reviewer: Engin AB, Papamichail K, Park WS
S- Editor: Wang XJ **L- Editor:** A **E- Editor:** Huang Y



Retrospective Study

Impact of postoperative TNM stages after neoadjuvant therapy on prognosis of adenocarcinoma of the gastro-oesophageal junction tumours

Michael Thomaschewski, Richard Hummel, Ekaterina Petrova, Juliana Knief, Ulrich Friedrich Wellner, Tobias Keck, Dirk Bausch

Michael Thomaschewski, Richard Hummel, Ekaterina Petrova, Ulrich Friedrich Wellner, Tobias Keck, Dirk Bausch, Department of Surgery, University Medical Center Schleswig-Holstein, Campus Lübeck, Lübeck 23538, Germany

Juliana Knief, Department of Pathology, University Medical Center Schleswig-Holstein, Campus Lübeck, Lübeck 23538, Germany

ORCID number: Michael Thomaschewski (0000-0002-5405-9716); Richard Hummel (0000-0001-5671-5222); Ekaterina Petrova (0000-0001-7740-6410); Juliana Knief (0000-0002-5036-3817); Ulrich Friedrich Wellner (0000-0002-8632-166X); Tobias Keck (0000-0001-7651-6183); Dirk Bausch (0000-0001-6511-1535).

Author contributions: Thomaschewski M, Hummel R and Bausch D drafted the original manuscript, contributed to design of the study, performance of statistical analyses and interpretation of the results; Petrova E collected the data; Wellner UF performed, reviewed and approved the statistical analyses; Keck T contributed to the design of the study and critically revised the manuscript for important intellectual content; all authors read and approved the final manuscript.

Supported by Land Schleswig-Holstein within the funding programme Open Access Publikationsfonds.

Institutional review board statement: The study was reviewed and approved by the institutional ethics committee (Ethik-Kommission Universität zu Lübeck/Aktenzeichen: 17-379A).

Conflict-of-interest statement: All authors declare no conflict-of-interest related to this article.

Data sharing statement: Consent for data sharing was not obtained and the data are not available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on

different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Michael Thomaschewski, MD, Doctor, Department of Surgery, University Medical Center Schleswig-Holstein, Campus Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany. michael.thomaschewski@uksh.de
Telephone: +49-451-50040220
Fax: +49-451-5002069

Received: January 22, 2018

Peer-review started: January 22, 2018

First decision: February 24, 2018

Revised: March 6, 2018

Accepted: March 10, 2018

Article in press: March 10, 2018

Published online: April 7, 2018

Abstract

AIM

To compare prognostic relevance of postoperative tumour/node/metastasis (TMN) stages between patients with and without neoadjuvant treatment.

METHODS

Data from patients with adenocarcinoma of the gastro-oesophageal junction (AEG) who had undergone surgical resection at a single German university centre were retrospectively analysed. Patients with or without neoadjuvant preoperative treatment were selected by exact matching based on preoperative staging. Standard assessment of preoperative (c)TNM stage was based on endoscopic ultrasound and computed tomography of the thorax and abdomen, according to the American Joint Committee on Cancer/Union

for International Cancer Control classification system. Patients with cT1cN0cM0 and cT2cN0cM0 stages were excluded from the study, as these patients are generally not recommended for pretreatment. Long-term survival among the various postoperative TNM stages was compared between the groups of patients with or without neoadjuvant treatment. For statistical assessments, a *P*-value of ≤ 0.05 was considered significant.

RESULTS

The study included a total of 174 patients. The group of patients who had received preoperative neoadjuvant treatment included more cases of AEG (Siewert) type 1 carcinoma ($P < 0.001$), and consequently oesophagectomy was performed more frequently among these patients ($P < 0.001$). The two groups (with or without preoperative neoadjuvant treatment) had comparable preoperative T stages, but the group of patients with preoperative neoadjuvant treatment presented a higher rate of preoperative N-positive disease ($P = 0.020$). Overall long-term survival was not different between the two groups of patients according to tumours of different AEG classifications, receipt of oesophagectomy or gastrectomy, nor between patients with similar postoperative TNM stage, resection margin and grading. However, an improvement of long-term survival was found for patients with nodal down-staging after neoadjuvant therapy ($P = 0.053$).

CONCLUSION

The prognostic relevance of postoperative TNM stages is similar for AEG in patients with or without neoadjuvant preoperative treatment, but treatment-related nodal down-staging prognosticates longer-term survival.

Key words: Adenocarcinoma of the gastro-oesophageal junction; American Joint Committee on Cancer/Union for International Cancer Control; TNM system; Neoadjuvant therapy; Oesophageal cancer

© **The Author(s) 2018.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Neoadjuvant therapy is the standard treatment for locally advanced adenocarcinoma of the gastro-oesophageal junction (AEG). Prognosis of AEG is based mainly on postoperative tumour/node/metastasis (TNM) stages, using the American Joint Committee on Cancer/Union for International Cancer Control classification system. Yet, whether prognostication based on postoperative TNM stage is affected by preoperative neoadjuvant therapy is unclear. Retrospective analysis of 174 patients showed that the prognostic relevance of postoperative TNM stage is independent of preoperative neoadjuvant therapy. However, nodal down-stage response following neoadjuvant therapy was found to result in improvement of survival.

Thomaschewski M, Hummel R, Petrova E, Knief J, Wellner UF, Keck T, Bausch D. Impact of postoperative TNM stages after neoadjuvant therapy on prognosis of adenocarcinoma of the gastro-oesophageal junction tumours. *World J Gastroenterol* 2018; 24(13): 1429-1439 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1429.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1429>

INTRODUCTION

Adenocarcinoma of the gastro-oesophageal junction (AEG) is one of the most common cancers worldwide, with a global incidence of 0.7 per 100000^[1,2]. It represents an aggressive disease with poor prognosis, and diagnosis is often delayed due to a lack of early disease-specific symptoms. Moreover, these tumours tend to spread to (local) lymph nodes even in early stages^[3,4].

Today, curative treatment options involve multidisciplinary approaches including endoscopy, surgery, chemotherapy and radiotherapy. These treatments have led to improvements in clinical management and patient outcome over the last years^[4,5]. In particular, effective neoadjuvant chemotherapy and/or radiotherapy approaches have been established for patients with locally advanced adenocarcinoma of the distal oesophagus and the gastro-oesophageal junction. When applied prior to surgery, these pretreatments provide a survival benefit, improve the potential for down-staging of the primary tumour and/or lymph node metastasis, and yield higher rates of complete tumour resection (R0) in contrast to a surgery-alone approach^[6-10]. However, whether a patient benefits from neoadjuvant therapy depends on tumour biology, individual patient-related risk factors and stage of disease^[6,8,9].

The American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) tumour/node/metastasis (TNM) system has been established as an international standard of classification of local, regional and distant extension/spread for many solid tumours, including AEG, and proven a powerful tool for prediction of prognosis of cancer patients^[10-14].

For the first time, the recently published 8th edition of AJCC staging of cancers of the oesophagus and oesophago-gastric junction introduced the post-neoadjuvant (yp)TNM stage groupings in addition to the clinical (c)TNM and pathological (p)TNM stagings^[12]. Whereas the separate definitions from the previous 7th AJCC/UICC edition for depth of wall infiltration by the primary tumour (the T staging), lymph node involvement (the N staging) and presence of distant metastases (the M staging) of AEG were not changed, stage grouping for neoadjuvant categories (*i.e.*, ypTNM) was newly classified with separate stage grouping for squamous cell carcinoma and adenocarcinoma, to account for the different prognostic implications

between ypTNM (postneoadjuvant) and pTNM cancer categories^[12,13].

Based on data derived from the Worldwide Esophageal Cancer Collaboration (WECC), involving 7723 patients from different countries and continents, survival for neoadjuvant groups (ypTNM patients) differed from that for equivalent-stage patients that underwent surgery alone (pTNM patients)^[13]. In detail, survival for node-negative (ypN0) patients and early-stage disease (ypTNM groups I and II) patients is significantly lower than for equivalently categorised patients that underwent surgery alone (pTNM)^[13,15]. However, two other retrospective analyses showed that the prognostic relevance of postoperative AJCC/UICC TNM staging is similar for patients with or without neoadjuvant treatment^[16,17].

In summary, the data available on the actual prognostic relevance of postoperative TNM stages of AEG patients who underwent neoadjuvant treatment are still limited and heterogeneous. The objective of this study, therefore, was to retrospectively analyse data from our University Cancer Center to compare the prognostic relevance of postoperative TMN stages between patients with and without preoperative neoadjuvant treatment, following surgery for tumours of the gastro-oesophageal junction.

MATERIALS AND METHODS

Patient selection and study parameters

Between 1996 and 2014, a total of 254 consecutive patients underwent curative surgery for AEG at the University Medical Center Schleswig-Holstein, Campus Lübeck. Data of all these patients were obtained from the institutional database and selected according to the following inclusion criteria: age > 18 years; histological confirmation of AEG (Siewert types I to III) on the basis of postoperative resection specimen analysis; curative intent of surgery/treatment; and, formal eligibility for neoadjuvant/perioperative treatment based on preoperative cTNM stages (according to AJCC Classification 8th edition^[12]; for details, please see the "Neoadjuvant/perioperative treatment" section below). Exclusion criteria were in-hospital death (as we aimed to analyse long-term outcome) and early-stage cancers (cT1cN0cM0 and cT2cN0cM0). After identification of eligible patients, we applied exact matching techniques to select the final retrospective study population of patients for the "neoadjuvant treatment" and "no neoadjuvant treatment" groups. Local ethics board approval was obtained (Ethik-Kommission Universität zu Lübeck/Aktenzeichen: 17-379A).

Study parameters included sex, age, AEG (Siewert) classification^[18], surgical procedure (see below), preoperative staging (including cT, cN and cM categories according to the AJCC Cancer Staging Manual 8th edition^[12]), postoperative staging [including T, N and M categories according to the AJCC/UICC Cancer Staging Manual 7th edition^[11], grade of differentiation (G) and

resection margin status (R)], long-term survival (defined as time in months from the day of hospital discharge) and pathologic down-staging/response in T and N stages after neoadjuvant therapy. For this study, we defined any pathologic down-staging/improvement in T and N stages after neoadjuvant therapy as 'down-staged', in contrast to 'unchanged' or 'up-staged' T and N stages.

Preoperative and postoperative tumour staging

Standard assessment of diagnosis and preoperative TNM stage (cTNM) was based on findings from endoscopy with biopsy, including endoscopic ultrasound and computed tomography of the thorax and abdomen. The postoperative staging was based on the resection specimen (according to the AJCC/UICC Cancer Staging Manual 7th edition^[11] and including G and R parameters). In this context, the resected specimens were re-evaluated by an independent pathologist for the purpose of this study.

Neoadjuvant / perioperative treatment

From the year 2005 onward, neoadjuvant chemotherapy has been used as standard treatment in the context of a multidisciplinary approach for locally advanced cancers. Our local standard protocol for neoadjuvant/perioperative treatment is based on the German National Guidelines for Diagnostics and Treatment of Adenocarcinomas of the Stomach and the Gastroesophageal Junction (http://www.awmf.org/uploads/tx_szleitlinien/032009l_S3_Magenkarzinom_Diagnostik_Therapie_Adenokarzinome_oesophagogastraler_Uebergang_2012abgelaufen.pdf). Generally, patients are deemed eligible for neoadjuvant/perioperative treatment if the tumour is locally advanced. In detail, we recommend neoadjuvant/perioperative treatment for patients with locally advanced tumour stages (cT2 node-positive disease as well as cT3/4), and patients with cT1 cN0 cM0 or cT2 cN0 cM0 are not recommended for pretreatment. Prior to 2005, patients received neoadjuvant/perioperative treatment on an individual basis based on recommendations of the local interdisciplinary tumour board. Neoadjuvant/perioperative therapy mainly consisted of cisplatin and fluorouracil (5-FU)-based regimens and included, over the time, different protocols such as cisplatin/5-FU, ECX, FLOT or ECF. For better presentation of results, neoadjuvant/perioperative treatment is referred to as 'neoadjuvant treatment' throughout the rest of the manuscript. In 26 cases, patients without neoadjuvant pretreatment had received adjuvant therapy (if recommended according to the local interdisciplinary tumor board). The decision was based on postoperative tumour stages and individual patient-specific risk factors.

Surgical procedures

The type of surgical resection for the AEG tumours was selected in accordance with tumour location and extent, and was chosen from among either oesophagectomy

Table 1 Demographics of the study population

	All, <i>n</i> = 174	No neoadjuvant tx	Neoadjuvant tx	<i>P</i> value
Male sex	85.1%	81.7	87.4	0.387
Age, median	61.5	64	58	0.043
Siewert stage				< 0.001
I	35.1%	16.9	47.6	
II	51.7%	67.6	40.8	
III	13.2%	15.5	11.7	
cT stage				0.9
T2	7.5%	8.5	6.8	
T3	74.7%	74.6	74.8	
T4	17.8%	16.9	18.4	
cN stage				0.02
Negative	17.2%	28.2	9.7	
Positive	82.8%	72.8	90.3	
Surgery				< 0.001
Oesophagectomy	54.0%	26.8	72.8	
Gastrectomy	46.0%	73.2	27.2	
pT stage				
T0	11%	-	20.0	
T1/2	26%	21.4	30.0	
T3/4	63%	78.6	50.0	
pN stage				
N0	37%	24.3	48.8	
N1	17%	18.6	15	
N2	21%	20	21.2	
N3	25%	37.1	15	
pM stage				
M0	94%	94.3	93.8	
M1	6%	5.7	6.2	

Data represent the entire study population ("All"), and the subgroups of patients with ("Neoadjuvant tx") or without ("No neoadjuvant tx") neoadjuvant pretreatment. Tx: Treatment.

techniques (open/hybrid/totally minimally invasive oesophagectomies), gastrectomy techniques (transhiatal extended gastrectomy) or combined oesophagectomy-and-total-gastrectomy techniques. For the purpose of this study, patients treated with the combined oesophagectomy-and-total-gastrectomy technique were included in the oesophagectomy group. The standard surgical procedure in our hospital included two-field lymphadenectomy for oesophagectomies and D2-lymphadenectomy for gastrectomies.

Follow-up

The Department of Surgery includes an Outpatient Cancer Clinic for follow up of cancer patients. Most of the cancer patients in our study are receiving their follow-up care in this outpatient clinic. However, for those patients who requested follow up with their general practitioner (e.g., based on the location of their residence), we obtained their follow-up information *via* telephone and entered the respective information into our database.

Statistical analysis

Statistical analyses were performed using SPSS software (version 22; IBM Corp., Armonk, NY, United States). For analysis of categorical variables [sex, age, AEG (Siewert) classification, surgical procedure and pre-

operative staging (cTNM)], Pearson's chisquare and Fisher's exact tests were used. Long-term survival was analysed using the Kaplan-Meier method. Log-rank test was used for statistical comparison. For all statistical analyses, a *P*-value of ≤ 0.05 was considered significant.

RESULTS

Demographics and overall survival

Following the exact matching patient selection, we identified 174 out of the 254 patients for study inclusion. Table 1 presents an overview of the two study groups: "neoadjuvant treatment (tx)" vs "no neoadjuvant tx". The patients who underwent neoadjuvant treatment were significantly younger than their nontreated counterparts (58 years vs 64 years, *P* = 0.043) and presented significantly more often with Siewert type 1 AEG tumours (*P* < 0.001) mandating oesophagectomy rather than gastrectomy (*P* < 0.001). While patients in both groups presented comparable preoperative T stages, patients in the neoadjuvant treatment group presented higher preoperative rates of N-positive disease (*P* = 0.02). Rates of N-positive disease were 90% for neoadjuvant tx and 73% for no neoadjuvant tx. Analysis of overall survival of the entire patient population based on postoperative T and N

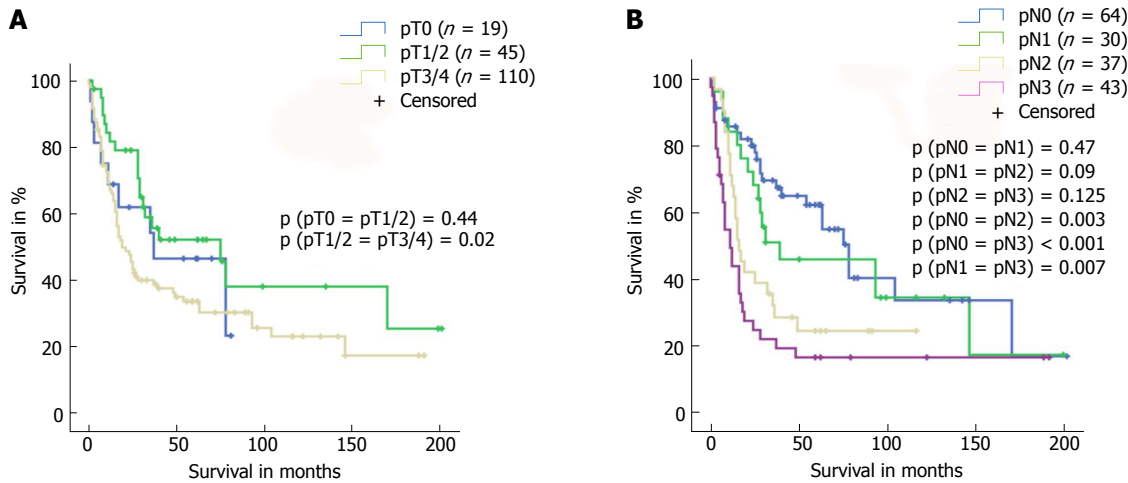


Figure 1 Overall survival. The graphs present an overview of the long-term survival of the entire study cohort based on tumour (A) and nodal (B) stages.

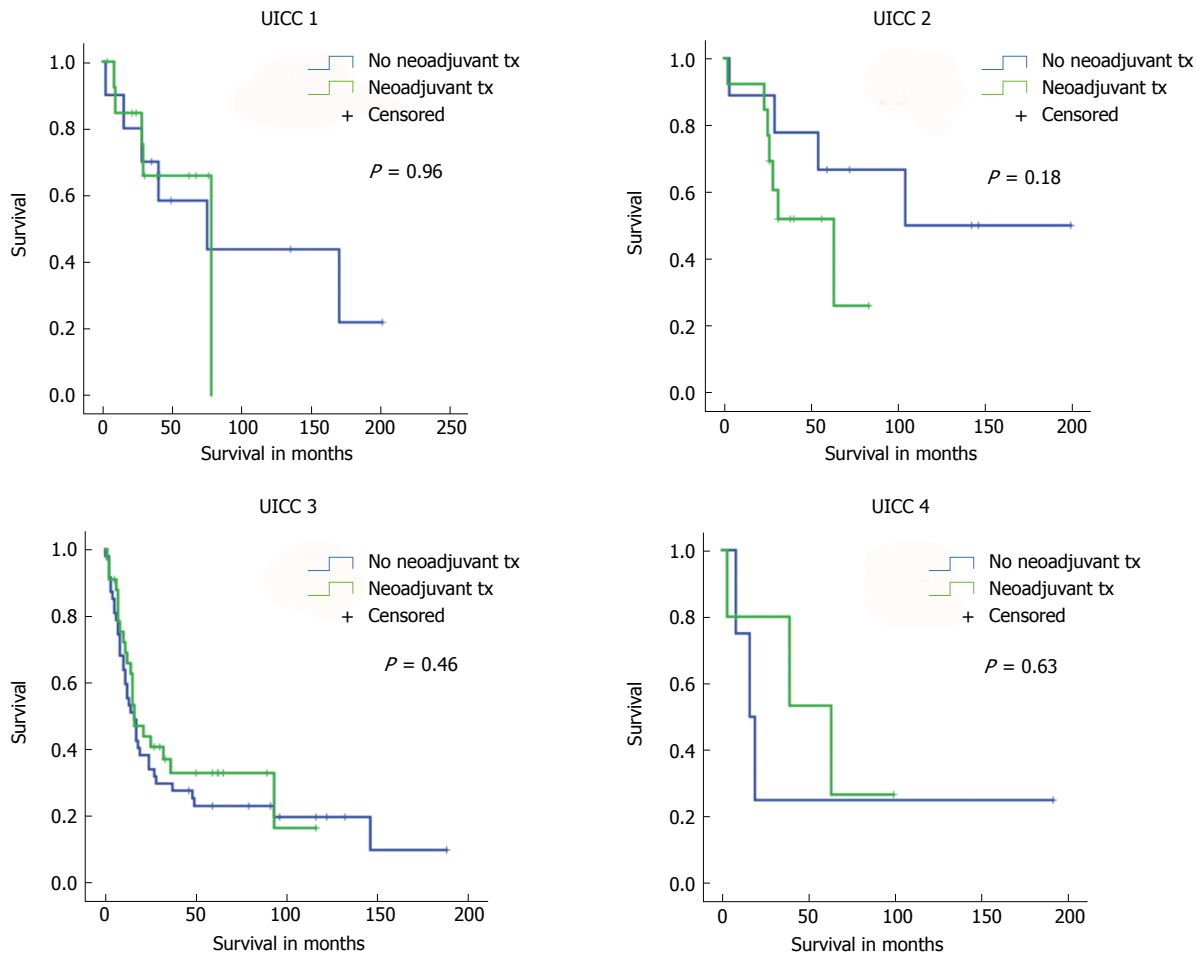


Figure 2 Impact of American Joint Committee on Cancer/Union for International Cancer Control stage on survival of patients with or without neoadjuvant pretreatment. The graphs show the long-term survival of patients with or without neoadjuvant pretreatment and with the same postoperative UICC stage. Tx: treatment; UICC: Union for International Cancer Control.

stages confirmed that long-term survival depended on disease stages (Figure 1).

Survival of patients with or without neoadjuvant treatment who had equivalent postoperative TNM stages
First, we compared long-term survival between groups

of patients with or without neoadjuvant treatment who had equivalent postoperative AJCC/UICC TNM stages (stages I -IV according to the 7th edition AJCC/UICC staging). We found no significant differences in long-term survival according to receipt of neoadjuvant treatment for the four AJCC/UICC stage subgroups (Figure 2).

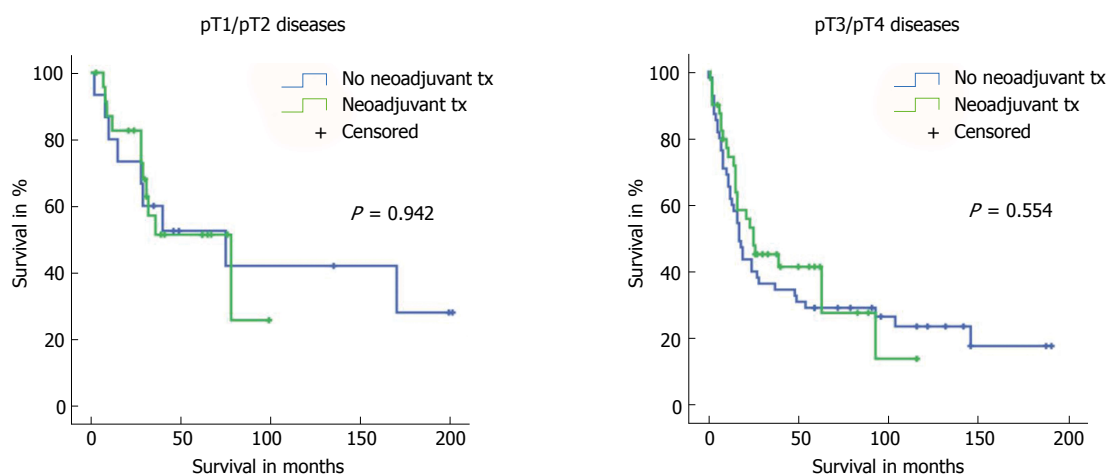


Figure 3 Impact of tumour stage on survival of patients with or without neoadjuvant pretreatment. The graphs present the long-term survival of patients with or without neoadjuvant pretreatment and with similar postoperative tumour stages (early stage cancers: pT1/2; advanced stage cancers: pT3/4). Tx: Treatment.

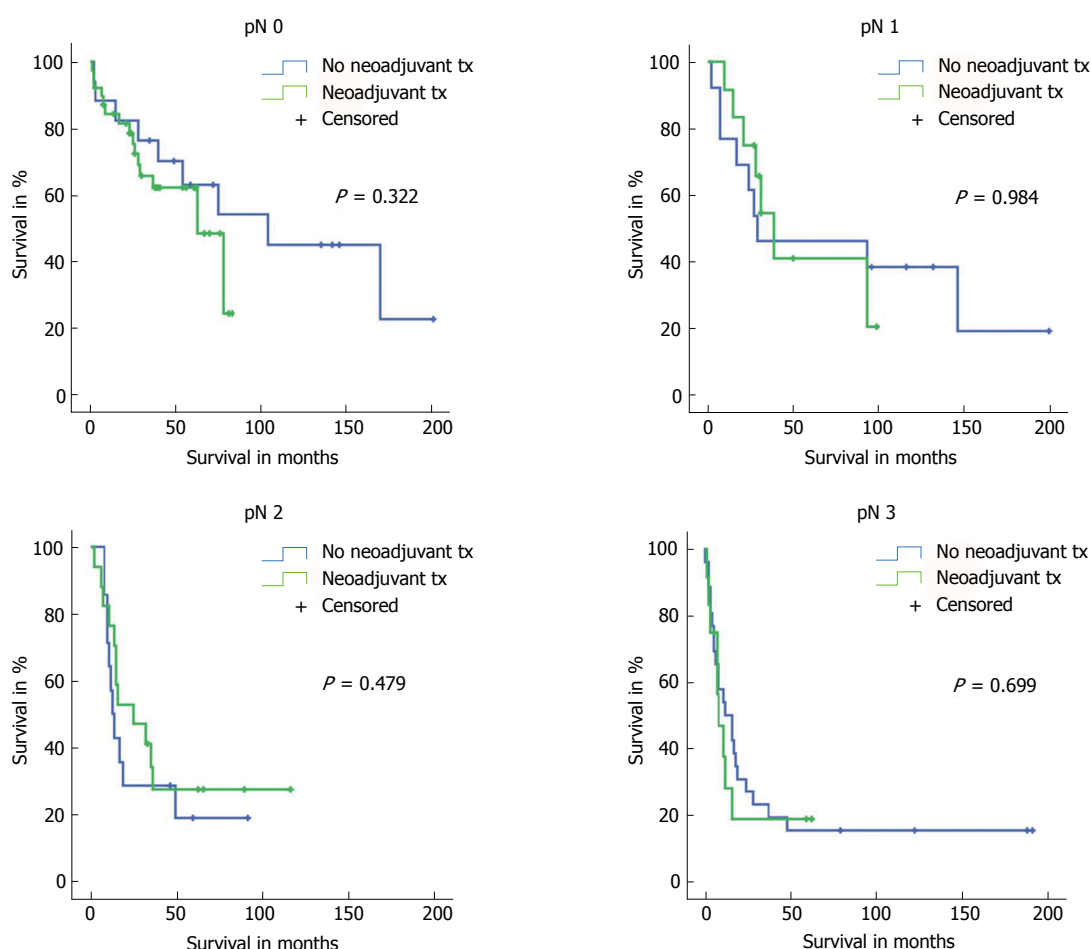


Figure 4 Impact of nodal stage on survival of patients with or without neoadjuvant pretreatment. The graphs show the long-term survival of patients with or without neoadjuvant pretreatment and with the same postoperative nodal stages (pN0/pN1/pN2/pN3). Tx: Treatment.

Furthermore, analysis of patients with either pT1/pT2 diseases or advanced pT3/pT4 diseases showed no significant difference in long-term survival related to receipt (or no receipt) of neoadjuvant pretreatment (Figure 3).

Subgroup analyses on all tumour (pT) stages se-

parately showed that only neoadjuvant-pretreated patients with pT1 stage had slightly better long-term survival ($P = 0.046$). However, the statistical comparison of these groups included only 8 vs 5 patients. With regards to postoperative N stages (pN), we did not find any differences in outcome between patients with or

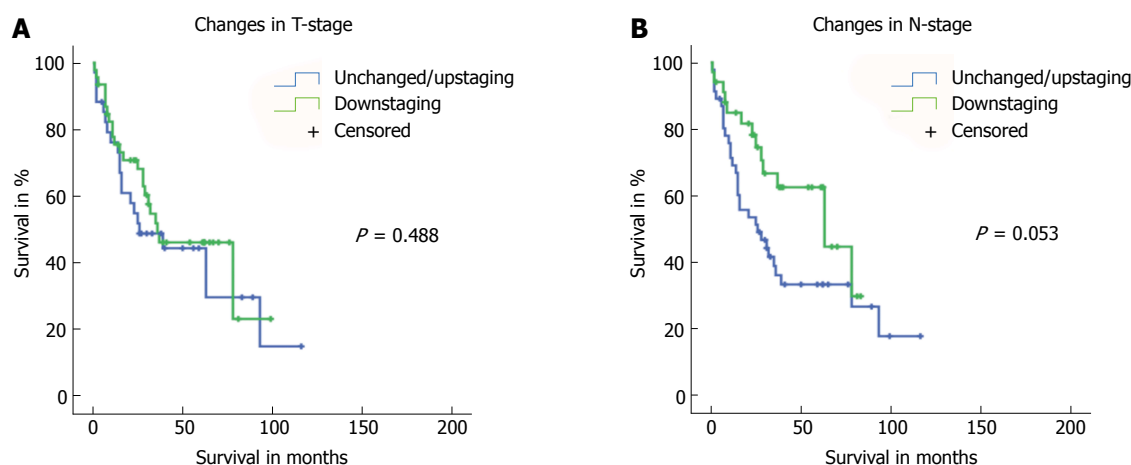


Figure 5 Impact of T and N down-staging on outcome after neoadjuvant treatment. A: The long-term survival of patients with either T “down-staging” or “unchanged/up-staging” after neoadjuvant treatment; B: The long-term survival of patients with either N “down-staging” or “unchanged/up-staging” after neoadjuvant treatment. N: Nodal; T: Tumour; Tx: Treatment.

without neoadjuvant therapy who represented the same postoperative pN stages (Figure 4).

Further subgroup analyses investigating effects of surgical procedure, postoperative G, positive/negative R and Siewert type I / II / III AEG tumours on outcome showed that long-term survival rates were comparable between patients with the same G stage or R stage regardless of neoadjuvant pretreatment (G2: $P = 0.580$; G3: $P = 0.417$; R0: $P = 0.389$; R1: $P = 0.825$). With regards to the Siewert classification, only patients with pT1 tumours in Siewert type 2 AEG showed a better survival after neoadjuvant therapy ($P = 0.017$; 7 patients with neoadjuvant tx vs 3 patients with no neoadjuvant tx); otherwise, the location of the tumour classified by Siewert classification did not impact outcome of patients with or without neoadjuvant pretreatment. Similarly, patients with pT1 tumours who received gastrectomy showed a significantly better survival rate after neoadjuvant therapy ($P = 0.020$; 3 patients with neoadjuvant tx vs 4 patients with no neoadjuvant tx); otherwise, surgical procedures (oesophagectomy vs gastrectomy) did not impact outcome.

Effect of T and N down-staging after neoadjuvant therapy on long-term survival

Finally, we analysed if T or N down-staging after neoadjuvant treatment impacted outcome by comparing preoperative and postoperative T and N stages only for those patients that underwent neoadjuvant pretreatment. We found that T down-staging after neoadjuvant therapy did not affect long-term survival ($P = 0.488$; Figure 5). Subgroup analysis on patients with either unchanged or up-staged disease showed a trend towards worse survival for patients with up-staged T stage ($P = 0.628$; Supplementary Figure 1). However, these results are limited by the very low number of patients included ($n = 30$ vs $n = 4$). In contrast, N down-staging after neoadjuvant treatment resulted in

borderline significant improvement in long-term survival ($P = 0.053$) (Figure 5).

DISCUSSION

The development of new therapeutic approaches and strategies for AEG within recent years has led to a multidisciplinary approach involving neoadjuvant/perioperative chemotherapy and/or radiotherapy. In contrast to the surgery-alone approaches, the multidisciplinary approaches have resulted in a relevant overall survival benefit to patients^[7,19-22] and have become part of standard treatment for AEG tumours. In addition to a survival benefit, neoadjuvant pretreatment further showed potential for down-staging of the primary tumour and/or lymph node metastasis, and finally in improving rates of complete tumour resection (R0)^[7,19-22]. While there is a broad consensus that neoadjuvant treatment affects outcome and prognosis of patients with AEG tumours, data are scarce on the exact prognostic relevance of postoperative AJCC/UICC TNM staging in the era of neoadjuvant treatment.

With this current study, we showed that there were no significant differences in the overall long-term survival of patients with or without neoadjuvant treatment, if they presented similar postoperative AJCC/UICC stages (stages I-IV, according to the 7th edition AJCC/UICC), T stages (early pT1/2 and advanced pT3/4 cancers) or N stages (pN0/pN1/pN2/pN3). Furthermore, we could show that surgical procedure, postoperative G, positive/negative R and location (Siewert classification of AEG the tumour) did not affect outcome between patients with or without neoadjuvant treatment, except in some cases of patients with pT1 tumours.

In summary, in our opinion, these results provide an interesting contribution towards answering the question of whether the AJCC TNM staging system can predict or estimate individual prognosis of patients with AEG tumours, regardless of whether they received

neoadjuvant pretreatment or not. Oesophageal cancer staging in the 7th edition AJCC/UICC TNM is based on pTNM of patients that had undergone surgery alone^[11,14]. Our data provide evidence that this system might also be applicable to patients who receive neoadjuvant treatment, and that prognosis of patients with similar T and N stages might indeed be comparable regardless of neoadjuvant treatment.

However, Rice *et al.*^[12,13] recently published the 8th edition of AJCC TNM, which includes, for the first time, neoadjuvant pretreatment stage groupings (*i.e.* ypTNM). A retrospective comparison of actual WECC survival data of patients with neoadjuvant treatment (ypTNM from the 8th edition) with data of patients who underwent surgery-alone (pTNM from the previous 7th edition) indicated that survival for the neoadjuvant-treated patients (ypTNM data) was lower than that found for patients of equivalent pathological staging that underwent surgery alone (pTNM data)^[13,15]. However, these data only partly contradict our results, as Rice *et al.*^[13,15] described a worse prognosis of neoadjuvant categories (ypTNM) for adenocarcinoma patients compared to corresponding pTNM data alone for early-stage disease (stages I and II); advanced stage adenocarcinoma patients (stages III-IV) showed no differences in survival^[13,15].

We did not find any significant difference in survival for either early or advanced stage disease, apart from limited pT1 cases as discussed below. Our data are further supported by a retrospective analysis published by Davies *et al.*^[16] that showed prognostic relevance of postoperative pTNM stage was similar between patients with or without neoadjuvant pretreatment. Similarly, in another series, Swisher *et al.*^[17] demonstrated that pTNM-specific survival was similar for patients with down-staged disease but not for those with unchanged disease. We must acknowledge in this context that our study did not include analysis of patients with complete tumour regression (ypT0N0), as this subgroup of patients did not exist among the patients without neoadjuvant treatment. This caveat might impact our results for early-stage cancer patients and might lead to differences in results compared to the data of Rice *et al.*^[13]. On the other hand, WECC data represents a fairly heterogeneous patient population as well as of different treatment standards in different countries and continents, which is reflected in the heterogeneous survival rate^[13]. In contrast, the patient population in our study might be more homogenous since all data were collected from a single cancer centre. Of note, in our study cohort, patients with ypT0N0 showed long-term survival similar to that of patients with pT1N0 (data not shown).

We found that T down-staging did not affect long-term outcome, whereas N down-staging appeared to improve survival (borderline significance; $P = 0.053$). This observation is supported by the fact that N involvement is one of the most important and strongest prognostic

factors of AEG tumours. Recent data, for example, show that lymph node involvement is more important than regional anatomic location for prognosis^[23,24]. The 7th edition AJCC TNM has already heralded the era of data-driven cancer staging and the incorporation of nonanatomic cancer characteristics^[25]. And, indeed, factors beyond those included in the AJCC TNM system (*e.g.*, down-staging of the primary tumour and/or lymph node metastasis after neoadjuvant treatment) have been shown to represent independent prognostic factors for overall survival^[7,16,17,19-22,26]. However, for prognostication, T or/and N down-staging were still not considered in the currently used 8th AJCC TNM edition^[12].

In order to improve prognostication, some authors have suggested modification of the pTNM staging system to incorporate the extent of pathologic response following neoadjuvant treatment, rather than developing separate ypTNM stages^[17]. This idea is supported by our data, which indicate that it might be necessary to include information on T or/and N down-staging in the AJCC TNM staging system in order to improve prognostic assessment of patients with AEG tumours.

There are, however, a number of aspects and limitations of the current study that must be considered for proper interpretation of the presented data. First, and most importantly, our study embodies all the known disadvantages of a retrospective study, including potential inhomogeneity of data acquisition and quality, single-centre data, changes of treatment protocols over time, *etc.* Second, we have to acknowledge that the patients without neoadjuvant treatment had been mainly recruited from the years 1996 to 2004, and patients with neoadjuvant treatment were from the year 2005 onward. This is based on the development and introduction of neoadjuvant treatment protocols into daily clinical practice since 2005. We are fully aware that inclusion of historical cohorts of patients might impact outcome of the respective groups^[27]; however, it will be very difficult to recruit a significant number of patients in the current era who qualify for but do not receive any neoadjuvant treatment, as this treatment is part of standard protocols nowadays in most parts of the world.

It is also important to note that our two study groups (neoadjuvant tx vs no neoadjuvant tx) are not completely homogenous. In fact, there are significant differences between the groups in regards to age, location (Siewert classification), N involvement and surgical technique. This fact is based on the use of the method of exact matching that allowed for us to include different numbers of patients into both groups as long as the selected parameters (preoperative TNM stages) were identical. However, the aim of this study was to analyse if similar postoperative T and N stages indicate similar prognosis in patients with or without neoadjuvant pretreatment; such a question might not be highly affected by this selection of patients. We

found in our analyses that patients with postoperative T1 stages showed differences in survival between groups in limited cases. This observation, in our opinion, needs very careful interpretation, as the number of patients with pT1 stage in our study cohort was extremely low ($n = 24$ patients in total). These findings warrant further confirmation, and clinical relevance remains unclear. Furthermore, in our study, 26 patients without neoadjuvant pretreatment received adjuvant therapy; yet, subgroup analysis excluding this patient cohort produced no difference in the results (data not shown).

A number of studies have found that prognosis and tumour biology differs between AEG tumours at different locations according to the Siewert classification (types I to III), supporting the concept that Siewert type III carcinoma represents true gastric adenocarcinoma, having a worse prognosis than Siewert types I and II carcinoma^[28,29]. Interestingly, the 7th edition AJCC/UICC TNM classification did not include Siewert classification for prognostication, and instead classified all tumours within 5 cm of the gastro-oesophageal junction as oesophageal carcinoma.

Based on the discrepancy of available data, we performed subgroup analyses with regards to outcome of tumours in different locations according to the Siewert classification. Our data showed that, in general, location of the tumour classified by Siewert classification did not impact outcome of patients with or without neoadjuvant pretreatment. Only patients with pT1 tumours in Siewert type 2 AEG tumours showed a better survival after neoadjuvant therapy ($P = 0.017$), but this analysis was based on only 7 vs 3 patients, casting suspicion on the final significance of these findings. We can only hypothesize that our study might be underpowered for answering the question of whether location of tumours impacts outcome. Further studies are needed to elucidate this specific and highly relevant question in more detail.

In summary, our retrospective analysis of patients with AEG tumours demonstrated that there were no significant differences in the overall long-term survival of patients with or without neoadjuvant treatment, if they presented similar postoperative AJCC/UICC stages (stages I–IV), T stages (early pT1/2 and advanced pT3/4 cancers) or N stages (pN0/pN1/pN2/pN3). Furthermore, we showed that N down-staging, especially, affected long-term survival of patients undergoing neoadjuvant treatment. Collectively, our data indicate that the pTNM staging system is reliable for assessment of individual prognosis for patients with AEG tumours, regardless of whether neoadjuvant treatment has been received or not. Furthermore, our data support the inclusion of T and/or N down-staging information, rather than separate pTNM and ypTNM stages, as independent risk factors for survival in the

next edition of the AJCC TNM staging system.

ARTICLE HIGHLIGHTS

Research background

Adenocarcinoma of the gastro-oesophageal junction (AEG) has a poor prognosis. Neoadjuvant chemotherapy and radiotherapy have significantly improved clinical management and outcome of patients, leading to a major evolution in treatment of oesophageal cancer. Neoadjuvant therapy provides a survival benefit to patients with AEG, through its elimination of micrometastatic disease and potential for down-staging of the primary tumour and/or lymph node metastasis, ultimately leading to higher rates of complete resections (R0). For prediction of prognosis of cancer patients, the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) system has been established. The 8th edition of AJCC staging of cancers of the oesophagus and oesophagogastric junction includes, for the first time, postneoadjuvant tumour/node/metastasis (ypTNM) stage groupings; the previous editions only referred to patients that underwent surgery alone. This raises the question of whether prognosis according to the postoperative pTNM/ypTNM stages is similar between patients that receive neoadjuvant pretreatment (ypTNM) or patients that undergo surgery alone (pTNM). According to the 8th edition AJCC, there are different prognostic implications between postneoadjuvant (ypTNM) and pathologic (pTNM) AEG categories. In detail, prognosis of node-negative (ypN0) and early-stage diseases (ypTNM groups I and II) is worse compared to patients with similar stages who underwent surgery alone. In contrast, for advanced stage AEG, there is no difference of prognosis among patients with identical pTNM/ypTNM stages. Other studies, however, have shown contradictory results. In these studies, the prognostic relevance of postoperative AJCC/UICC TNM staging did not differ between patients with or without neoadjuvant pretreatment.

Research motivation

Due to limited and heterogeneous data, the prognostic relevance of postoperative TNM staging in the era of neoadjuvant therapy of AEG remains unclear. However, due to the generally poor prognosis of AEG and the relevant risk of recurrence, an exact assessment of prognosis according to the TNM staging system is extremely important for the individual patient and for further treatment decision-making.

Research objectives

The main objective of this study was to compare the prognostic relevance of similar postoperative TNM stages between patients with or without neoadjuvant pretreatment. The results were expected to clarify the need of a separate postneoadjuvant stage grouping (ypTNM) for prognostication of AEG patients. Furthermore, in the era of neoadjuvant treatment, other prognostic factors may be relevant for prognostication of survival of patients with AEG.

Research methods

We conducted a retrospective study analysing 254 patients that underwent curative surgical treatment at our University Medical Center Schleswig-Holstein, Campus Lübeck. After excluding patients with preoperative tumour stages that preclude neoadjuvant pretreatment (cT1cN0cM0 and cT2cN0cM0), we performed exact matching to identify patients with or without neoadjuvant pretreatment who would be eligible for the study. Additionally, in-hospital deaths were excluded since we aimed to analyse long-term survival. Study parameters included sex, age, AEG (Siewert) classification, surgical procedure, preoperative staging (including cT, cN and cM categories according to the AJCC Cancer Staging Manual 8th edition), postoperative staging (including T, N and M categories according to the AJCC/UICC Cancer Staging Manual 7th edition, grade of differentiation (G) and resection margin status (R)), long-term survival (defined as time in months as from the day of hospital discharge) and pathologic down-staging/response in tumour (T) and nodal (N) stages after neoadjuvant therapy. Pearson's chi-square and Fisher's exact tests were used for statistical analyses of categorical variables (sex, age, AEG (Siewert) classification, surgical procedure and preoperative staging (cTNM)). Long-term survival was analysed using the Kaplan-Meier method. For statistical comparisons, log-rank test was used. A P -value of ≤ 0.05 was considered

significant for all statistical analyses.

Research results

After patient selection and exact matching, 174 of the 254 patients were included in the study. Regarding demographics of both groups (no neoadjuvant treatment vs neoadjuvant treatment), patients who received neoadjuvant treatment were significantly younger (58 years vs 64 years, $P = 0.043$) and presented Siewert type I AEG tumours significantly more often ($P < 0.001$), resulting in significantly more oesophagectomies than gastrectomies ($P < 0.001$) for surgical treatment in this group. Patients who received neoadjuvant treatment presented higher preoperative rates of lymph node-positive disease ($P = 0.020$). Regarding overall survival of the entire study cohort, survival worsened at advanced postoperative AJCC/UICC TNM stages. Comparing long-term survival between patients with or without neoadjuvant pretreatment with identical postoperative TNM stages, no difference could be found. In addition, no difference was found in long-term survival of patients with or without neoadjuvant pretreatment for identical pT, pN or pM stages, G or R. Investigation of other prognostic markers for patients who received neoadjuvant pretreatment involved analysis of the effect of T and N down-staging on long-term survival. Here, we found that T down-staging did not have an impact on long-term survival ($P = 0.488$), while N down-staging after neoadjuvant treatment provided a significant but borderline improvement in long-term survival ($P = 0.053$).

Research conclusions

Our retrospective study demonstrated that the prognostic relevance of equivalent postoperative AJCC/UICC TNM stages is similar between patients with or without neoadjuvant pretreatment. Our data provide evidence that the pTNM staging system can be applied for assessment of individual prognosis of patients with AEG, regardless of whether or not they received neoadjuvant treatment. Furthermore, our study showed that N down-staging following neoadjuvant treatment positively affects long-term outcome, emphasizing the need of novel markers for prognostication in the era of neoadjuvant therapy.

Research perspectives

Our data support the idea of modifying the pTNM staging system by incorporating the extent of pathologic response following neoadjuvant treatment, rather than developing separate ypTNM stages. Prognostic factors or markers that reflect tumour biology, rather than the anatomical extent of growth, are promising for the development of new assessments for prognostication of survival of patients with AEG.

REFERENCES

- 1 Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut* 2015; **64**: 381-387 [PMID: 25320104 DOI: 10.1136/gutjnl-2014-308124]
- 2 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]
- 3 Pennathur A, Farkas A, Krasinskas AM, Ferson PF, Gooding WE, Gibson MK, Schuchert MJ, Landreneau RJ, Luketich JD. Esophagectomy for T1 esophageal cancer: outcomes in 100 patients and implications for endoscopic therapy. *Ann Thorac Surg* 2009; **87**: 1048-54; discussion 1054-5 [PMID: 19324126 DOI: 10.1016/j.athoracsur.2008.12.060]
- 4 Pennathur A, Gibson MK, Jobe BA, Luketich JD. Oesophageal carcinoma. *Lancet* 2013; **381**: 400-412 [PMID: 23374478 DOI: 10.1016/S0140-6736(12)60643-6]
- 5 Enzinger PC, Mayer RJ. Esophageal cancer. *N Engl J Med* 2003; **349**: 2241-2252 [PMID: 14657432 DOI: 10.1056/NEJMra035010]
- 6 Li B, Li J, Xu WW, Guan XY, Qin YR, Zhang LY, Law S, Tsao SW, Cheung AL. Suppression of esophageal tumor growth and chemoresistance by directly targeting the PI3K/AKT pathway. *Oncotarget* 2014; **5**: 11576-11587 [PMID: 25344912 DOI: 10.18632/oncotarget.2596]
- 7 van Hagen P, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, Richel DJ, Nieuwenhuijzen GA, Hospers GA, Bonenkamp JJ, Cuesta MA, Blaisse RJ, Busch OR, ten Kate FJ, Creemers GJ, Punt CJ, Plukker JT, Verheul HM, Spillenaar Bilgen EJ, van Dekken H, van der Sagen MJ, Rozema T, Biermann K, Beukema JC, Piet AH, van Rij CM, Reinders JG, Tilanus HW, van der Gaast A; CROSS Group. Preoperative chemoradiotherapy for esophageal or junctional cancer. *N Engl J Med* 2012; **366**: 2074-2084 [PMID: 22646630 DOI: 10.1056/NEJMoa1112088]
- 8 Forde PM, Kelly RJ. Genomic alterations in advanced esophageal cancer may lead to subtype-specific therapies. *Oncologist* 2013; **18**: 823-832 [PMID: 23853247 DOI: 10.1634/theoncologist.2013-0130]
- 9 Findlay JM, Middleton MR, Tomlinson I. A systematic review and meta-analysis of somatic and germline DNA sequence biomarkers of esophageal cancer survival, therapy response and stage. *Ann Oncol* 2015; **26**: 624-644 [PMID: 25214541 DOI: 10.1093/annonc/mdl449]
- 10 Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol* 2010; **17**: 1471-1474 [PMID: 20180029 DOI: 10.1245/s10434-010-0985-4]
- 11 Rice TW, Blackstone EH, Rusch VW. 7th edition of the AJCC Cancer Staging Manual: esophagus and esophagogastric junction. *Ann Surg Oncol* 2010; **17**: 1721-1724 [PMID: 20369299 DOI: 10.1245/s10434-010-1024-1]
- 12 Rice TW, Patil DT, Blackstone EH. 8th edition AJCC/UICC staging of cancers of the esophagus and esophagogastric junction: application to clinical practice. *Ann Cardiothorac Surg* 2017; **6**: 119-130 [PMID: 28447000 DOI: 10.21037/acs.2017.03.14]
- 13 Rice TW, Lerut TE, Orringer MB, Chen LQ, Hofstetter WL, Smithers BM, Rusch VW, van Lanschot J, Chen KN, Davies AR, D'Journo XB, Kesler KA, Luketich JD, Ferguson MK, Räsänen JV, van Hillegersberg R, Fang W, Durand L, Allum WH, Cecconello I, Cerfolio RJ, Pera M, Griffin SM, Burger R, Liu JF, Allen MS, Law S, Watson TJ, Darling GE, Scott WJ, Duranceau A, Denlinger CE, Schipper PH, Ishwaran H, Apperson-Hansen C, DiPaola LM, Semple ME, Blackstone EH. Worldwide Esophageal Cancer Collaboration: neoadjuvant pathologic staging data. *Dis Esophagus* 2016; **29**: 715-723 [PMID: 27731548 DOI: 10.1111/dote.12513]
- 14 Rice TW, Rusch VW, Ishwaran H, Blackstone EH; Worldwide Esophageal Cancer Collaboration. Cancer of the esophagus and esophagogastric junction: data-driven staging for the seventh edition of the American Joint Committee on Cancer/International Union Against Cancer Cancer Staging Manuals. *Cancer* 2010; **116**: 3763-3773 [PMID: 20564099 DOI: 10.1002/encr.25146]
- 15 Rice TW, Ishwaran H, Kelsen DP, Hofstetter WL, Apperson-Hansen C, Blackstone EH; Worldwide Esophageal Cancer Collaboration Investigators. Recommendations for neoadjuvant pathologic staging (ypTNM) of cancer of the esophagus and esophagogastric junction for the 8th edition AJCC/UICC staging manuals. *Dis Esophagus* 2016; **29**: 906-912 [PMID: 27905170 DOI: 10.1111/dote.12538]
- 16 Davies AR, Gossage JA, Zylstra J, Mattsson F, Lagergren J, Maisey N, Smyth EC, Cunningham D, Allum WH, Mason RC. Tumor stage after neoadjuvant chemotherapy determines survival after surgery for adenocarcinoma of the esophagus and esophagogastric junction. *J Clin Oncol* 2014; **32**: 2983-2990 [PMID: 25071104 DOI: 10.1200/JCO.2014.55.9070]
- 17 Swisher SG, Hofstetter W, Wu TT, Correa AM, Ajani JA, Komaki RR, Chirieac L, Hunt KK, Liao Z, Phan A, Rice DC, Vaporciyan AA, Walsh GL, Roth JA. Proposed revision of the esophageal cancer staging system to accommodate pathologic response (pP) following preoperative chemoradiation (CRT). *Ann Surg* 2005; **241**: 810-7; discussion 817-20 [PMID: 15849517]
- 18 Siewert JR, Stein HJ. Classification of adenocarcinoma of the oesophagogastric junction. *Br J Surg* 1998; **85**: 1457-1459 [PMID: 9823902 DOI: 10.1046/j.1365-2168.1998.00940.x]
- 19 Kelsen DP, Ginsberg R, Pajak TF, Sheahan DG, Gunderson L, Mortimer J, Estes N, Haller DG, Ajani J, Kocha W, Minsky

- BD, Roth JA. Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. *N Engl J Med* 1998; **339**: 1979-1984 [PMID: 9869669 DOI: 10.1056/NEJM199812313392704]
- 20 **Urba SG**, Orringer MB, Turrisi A, Iannettoni M, Forastiere A, Strawderman M. Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional esophageal carcinoma. *J Clin Oncol* 2001; **19**: 305-313 [PMID: 11208820 DOI: 10.1200/JCO.2001.19.2.305]
- 21 **Arnott SJ**, Duncan W, Gignoux M, Hansen HS, Launois B, Nygaard K, Parmar MK, Rousell A, Spilopoulos G, Stewart G, Tierney JF, Wang M, Rhugang Z; Oesophageal Cancer Collaborative Group. Preoperative radiotherapy for esophageal carcinoma. *Cochrane Database Syst Rev* 2005; **19**: CD001799 [PMID: 16235286 DOI: 10.1002/14651858.CD001799.pub2]
- 22 **Cunningham D**, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Loftis FJ, Falk SJ, Iveson TJ, Smith DB, Langle RE, Verma M, Weeden S, Chua YJ, MAGIC Trial Participants. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20 [PMID: 16822992 DOI: 10.1056/NEJMoa055531]
- 23 **Mariette C**, Piessen G, Briez N, Triboulet JP. The number of metastatic lymph nodes and the ratio between metastatic and examined lymph nodes are independent prognostic factors in esophageal cancer regardless of neoadjuvant chemoradiation or lymphadenectomy extent. *Ann Surg* 2008; **247**: 365-371 [PMID: 18216546 DOI: 10.1097/SLA.0b013e31815aaadf]
- 24 **DaVee T**, Ajani JA, Lee JH. Is endoscopic ultrasound examination necessary in the management of esophageal cancer? *World J Gastroenterol* 2017; **23**: 751-762 [PMID: 28223720 DOI: 10.3748/wjg.v23.i5.751]
- 25 **Rusch VW**, Rice TW, Crowley J, Blackstone EH, Rami-Porta R, Goldstraw P. The seventh edition of the American Joint Committee on Cancer/International Union Against Cancer Staging Manuals: the new era of data-driven revisions. *J Thorac Cardiovasc Surg* 2010; **139**: 819-821 [PMID: 20304130 DOI: 10.1016/j.jtcvs.2010.02.013]
- 26 **Ronellenfitch U**, Schwarzbach M, Hofheinz R, Kienle P, Kieser M, Slanger TE, Jensen K; GE Adenocarcinoma Meta - analysis Group. Perioperative chemo(radio)therapy versus primary surgery for resectable adenocarcinoma of the stomach, gastroesophageal junction, and lower esophagus. *Cochrane Database Syst Rev* 2013; **31**: CD008107 [PMID: 23728671 DOI: 10.1002/14651858.CD008107.pub2]
- 27 **Cooke DT**, Calhoun RF, Kuderer V, David EA. A Defined Esophagectomy Perioperative Clinical Care Process Can Improve Outcomes and Costs. *Am Surg* 2017; **83**: 103-111 [PMID: 28234134]
- 28 **Curtis NJ**, Noble F, Bailey IS, Kelly JJ, Byrne JP, Underwood TJ. The relevance of the Siewert classification in the era of multimodal therapy for adenocarcinoma of the gastro-oesophageal junction. *J Surg Oncol* 2014; **109**: 202-207 [PMID: 24243140 DOI: 10.1002/jso.23484]
- 29 **Kulig P**, Sierzega M, Pach R, Kolodziejczyk P, Kulig J; Polish Gastric Cancer Study Group. Differences in prognosis of Siewert II and III oesophagogastric junction cancers are determined by the baseline tumour staging but not its anatomical location. *Eur J Surg Oncol* 2016; **42**: 1215-1221 [PMID: 27241921 DOI: 10.1016/j.ejso.2016.04.061]

P- Reviewer: Herbella F, Nishida T, Tsoulfas G **S- Editor:** Gong ZM
L- Editor: A **E- Editor:** Huang Y



Retrospective Study

Mild drinking habit is a risk factor for hepatocarcinogenesis in non-alcoholic fatty liver disease with advanced fibrosis

Takefumi Kimura, Naoki Tanaka, Naoyuki Fujimori, Ayumi Sugiura, Tomoo Yamazaki, Satoru Joshita, Michiharu Komatsu, Takeji Umemura, Akihiro Matsumoto, Eiji Tanaka

Takefumi Kimura, Naoyuki Fujimori, Ayumi Sugiura, Tomoo Yamazaki, Satoru Joshita, Michiharu Komatsu, Takeji Umemura, Akihiro Matsumoto, Eiji Tanaka, Department of Internal Medicine, Division of Gastroenterology, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

Naoki Tanaka, Department of Metabolic Regulation, Shinshu University Graduate School of Medicine, Matsumoto 390-8621, Japan

Naoki Tanaka, Research Center for Agricultural Food Industry, Shinshu University, Matsumoto 390-8621, Japan

ORCID number: Takefumi Kimura (0000-0002-1481-1029); Naoki Tanaka (0000-0002-0606-2101); Naoyuki Fujimori (0000-0001-8744-8139); Ayumi Sugiura (0000-0001-5427-7628); Tomoo Yamazaki (0000-0001-6958-1366); Satoru Joshita (0000-0002-6364-9654); Michiharu Komatsu (0000-0002-7860-2816); Takeji Umemura (0000-0001-7985-919X); Akihiro Matsumoto (0000-0001-6453-8529); Eiji Tanaka (0000-0002-0724-2104).

Author contributions: Kimura T and Tanaka N designed the research; Fujimori N, Sugiura A, Yamazaki T, Joshita S, Komatsu M, Umemura T, and Matsumoto A treated the patients and collected materials and clinical data; Kimura T analyzed the data; Kimura T and Tanaka N wrote the paper; Tanaka E supervised the research.

Institutional review board statement: The study was reviewed and approved by the Committee for Medical Ethics of Shinshu University School of Medicine Institutional Review Board.

Informed consent statement: Informed written consent was obtained from all patients.

Conflict-of-interest statement: The authors declare that no conflict of interest exists.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative

Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Invited manuscript

Correspondence to: Naoki Tanaka, MD, PhD, Associate Professor, Doctor, Department of Metabolic Regulation, Shinshu University Graduate School of Medicine and Research Center for Agricultural Food Industry, Shinshu University, 3-1-1 Asahi, Matsumoto 390-8621, Japan. naopi@shinshu-u.ac.jp
Telephone: +81-263-372634
Fax: +81-263-329412

Received: January 31, 2018

Peer-review started: January 31, 2018

First decision: February 26, 2015

Revised: March 3, 2015

Accepted: March 10, 2018

Article in press: March 10, 2018

Published online: April 7, 2018

Abstract

AIM

The impact of mild drinking habit (less than 20 g/d of ethanol) on the clinical course of non-alcoholic fatty liver disease (NAFLD) has not been determined. We examined the influence of a mild drinking habit on liver carcinogenesis from NAFLD.

METHODS

A total of 301 patients who had been diagnosed as having NAFLD by liver biopsy between 2003 and 2016 [median age: 56 years, 45% male, 56% with non-alcoholic steatohepatitis, 26% with advanced fibrosis (F3-4)] were divided into the mild drinking group with

ethanol consumption of less than 20 g/d (mild drinking group, $n = 93$) and the non-drinking group ($n = 208$). Clinicopathological features at the time of liver biopsy and factors related to hepatocellular carcinoma (HCC) occurrence were compared between the groups.

RESULTS

We observed significant differences in male prevalence ($P = 0.01$), platelet count ($P = 0.04$), and gamma-glutamyl transpeptidase ($P = 0.02$) between the test groups. Over 6 years of observation, the HCC appearance rate was significantly higher in the mild drinking group (6.5% *vs* 1.4%, $P = 0.02$). Multivariate survival analysis using Cox's regression model revealed that hepatic advanced fibrosis (F3-4) ($P < 0.01$, risk ratio: 11.60), diabetes mellitus ($P < 0.01$, risk ratio: 89.50), and serum triglyceride ($P = 0.04$, risk ratio: 0.98) were factors significantly related to HCC in all NAFLD patients, while the effect of a drinking habit was marginal ($P = 0.07$, risk ratio: 4.43). In patients with advanced fibrosis (F3-4), however, a drinking habit ($P = 0.04$, risk ratio: 4.83), alpha-fetoprotein ($P = 0.01$, risk ratio: 1.23), and diabetes mellitus ($P = 0.03$, risk ratio: 12.00) were identified as significant contributors to HCC occurrence.

CONCLUSION

A mild drinking habit appears to be a risk factor for hepatocarcinogenesis in NAFLD patients, especially those with advanced fibrosis.

Key words: Non-alcoholic fatty liver disease; Ethanol; Hepatocellular carcinoma; Risk factor

© The Author(s) 2018. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: This study focused on the impact of a mild drinking habit on liver carcinogenesis in 301 biopsy-proven non-alcoholic fatty liver disease (NAFLD) patients. Multivariate analysis revealed that mild drinking of < 20 g/d might increase the risk of hepatocellular carcinoma in NAFLD patients, particularly those with advanced fibrosis (F3-4). NAFLD patients with severe fibrosis should abstain from even small amounts of regular alcohol consumption.

Kimura T, Tanaka N, Fujimori N, Sugiura A, Yamazaki T, Joshita S, Komatsu M, Umemura T, Matsumoto A, Tanaka E. Mild drinking habit is a risk factor for hepatocarcinogenesis in non-alcoholic fatty liver disease with advanced fibrosis. *World J Gastroenterol* 2018; 24(13): 1440-1450 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1440.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1440>

INTRODUCTION

Over the past several decades, it has become clear

that non-alcoholic fatty liver disease (NAFLD) and its advanced form non-alcoholic steatohepatitis (NASH) are major chronic liver diseases worldwide^[1,2]. The prevalence rate of NAFLD has doubled during the last 20 years, while those of other chronic liver conditions have remained stable or even decreased^[3]. Recent evidence has confirmed that NAFLD and NASH incidence is increasing in Japan as well^[4].

The etiology of NAFLD/NASH has not been fully elucidated. The most widely accepted theory involves insulin resistance as an important mechanism leading to liver steatosis and perhaps steatohepatitis^[5]. Others have proposed that "multiple hits" of additional oxidative stress and lipotoxicity are necessary for the necro-inflammatory component of steatohepatitis and carcinogenesis^[6]. Liver iron, leptin, anti-oxidant deficiency, and intestinal microbiota have also been suggested as potential factors in the progression from steatosis to steatohepatitis^[6-9]. However, research on NAFLD/NASH pathogenesis and carcinogenesis is ongoing.

Although it is widely accepted that more than 60 g/d of ethanol consumption may lead to alcoholic liver disease and steatosis, steatohepatitis, and hepatic fibrosis^[10], there is uncertainty on the influence of a mild drinking habit (< 20 g/d of ethanol) on human health. For example, mild habitual drinking improved insulin resistance and hepatic steatosis but either worsened or improved hepatic fibrosis^[11-14]. Since NAFLD/NASH is defined as fatty liver disease with average ethanol intake of less than 20 g daily^[10], some NAFLD patients may habitually consume small amounts of ethanol while others abstain completely. There are no reports to date investigating the influence of a mild drinking habit on NAFLD/NASH patients despite a growing number of reports on hepatocellular carcinoma (HCC). To investigate the influence of a mild drinking habit on liver carcinogenesis from NAFLD, we compared clinicopathological features and outcomes between NAFLD patients with a mild drinking habit and the non-drinking NAFLD patients.

MATERIALS AND METHODS

Ethics

This study was carried out in accordance with the World Medical Association Helsinki Declaration and was approved by the ethics committee of Shinshu University School of Medicine (approval ID: 2802).

Patients

We enrolled 301 patients who were diagnosed as having NAFLD by liver biopsy between 2003 and 2016 [median age: 56 years, 45% male, 56% with NASH, 26% with advanced fibrosis (F3-4)] at Shinshu University Hospital in Matsumoto, Nagano, Japan. These patients originally referred to our department from local hospitals in Nagano prefecture to confirm the diagnosis by liver biopsy.

The diagnosis of NAFLD was based on the criteria of: (1) the presence of hepato-renal contrast and increased hepatic echogenicity on abdominal ultrasonography (US), (2) an average daily consumption of < 20 g of ethanol, and (3) the absence of other causes of liver dysfunction, such as viral hepatitis, drug-induced liver injury, autoimmune liver diseases, primary sclerosing cholangitis, Wilson's disease, hereditary hemochromatosis, and citrin deficiency^[15,16]. The diagnosis of NAFLD was confirmed based on histological findings of biopsied specimens. Pathology details are described below.

All patients were followed by US or computed tomography with measurements of serum alpha-fetoprotein every 6 mo. HCC was identified radiologically in all affected patients ($n = 9$). The radiological diagnosis of HCC was based on the American Association for the Study of Liver Diseases practice guidelines on the management of HCC as either: (1) the presence of a hepatic lesion > 2 cm in diameter with typical vascular pattern for HCC on one dynamic imaging technique or alpha-fetoprotein > 200 ng/mL; or (2) the presence of a lesion 1-2 cm in diameter with typical vascular pattern for HCC on two dynamic imaging techniques^[17]. Follow-up time was defined as the number of days from biopsy to HCC diagnosis or from biopsy to the last follow-up visit when protocol surveillance confirmed no HCC. Patient drinking habits were confirmed as remaining unchanged during follow-up.

Clinical data collection

All laboratory data in a fasting state on the day of liver biopsy were obtained from our medical database. Past and current drinking habit data were collected by self-reported questionnaires and interviews with doctors performing the liver biopsy. We divided the subjects into two groups: the mild drinking group with ethanol consumption of less than 20 g/d (mild drinking group, $n = 93$) and the non-drinking group ($n = 208$). Patients were considered to be hypertensive if their systolic/diastolic pressure was > 140/90 mmHg or if they were taking anti-hypertensive drugs^[18]. Patients were judged as having hyperlipidemia if their fasting serum levels of cholesterol or triglyceride were ≥ 220 mg/dL or ≥ 150 mg/dL, respectively, or if they were taking lipid-lowering drugs^[19]. Patients were considered to be diabetic if they had a fasting glucose level of ≥ 126 mg/dL or hemoglobin A1c (HbA1c) was $\geq 6.5\%$, or if they were taking insulin or oral hypoglycemic agents^[19].

Histological findings

Liver specimens of at least 1.5 cm in length were obtained from segment 5 or 8 using a 14-gauge needle, as described previously, and immediately fixed in 10% neutral formalin^[20]. Sections of 4 μ m in thickness were stained by means of the hematoxylin and eosin and Azan-Mallory methods. The histological activity of NAFLD was assessed by an independent expert pathologist in a

blinded manner according to the NAFLD scoring system proposed by Kleiner *et al.*^[21]. Steatosis grade was scored as 0 to 3 by the fat degeneration rate of hepatocytes (< 5%, 5%-33%, 33%-66%, and > 66%, respectively). Lobular inflammation grade was also scored as 0 to 3 by overall assessment of all inflammatory foci (none, < 2 foci/200 \times field, 2-4 foci/200 \times field, and > 4 foci/200 \times field, respectively). Ballooning grade was determined by the number of degenerating hepatocytes as 0 to 2, corresponding to none, few, and many, respectively. NAFLD activity score was the total of steatosis, lobular inflammation, and ballooning scores. Fibrosis was staged as 0 to 4 depending on the degree of fibrosis (F0, none; F1, perisinusoidal or periportal; F2, perisinusoidal and portal/periportal; F3, bridging fibrosis; and F4, cirrhosis)^[21].

Statistical analysis

Clinical and histological data were expressed as a number (percentage) or median (range). Chi-square and Mann-Whitney *U* tests were used for comparisons between the groups. Kaplan-Meier analysis was performed to estimate HCC cumulative incidence from the time of liver biopsy, and plots of cumulative events vs years of follow-up were constructed. Receiver operating characteristic curves were plotted, and optimal cut-off points were determined as the values showing maximum sensitivity plus specificity. In order to assess which factors were associated with the development of HCC after liver biopsy, univariate and multivariate Cox's proportional hazard regression analysis was employed. Variables revealed as significant by univariate analysis were further tested by multivariate analysis. $P < 0.05$ was considered to be statistically significant. Data were analyzed using a statistical software package (SPSS for Windows, SPSS Inc., Chicago, IL, United States).

RESULTS

Overall HCC occurrence rate

HCC appeared in 9 subjects (3%) within a median of 6 years of follow-up from liver biopsy. Kaplan-Meier analysis revealed the HCC occurrence rate in our cohort to be 0.9/2.6/6.0% in 3/5/10 years, respectively (Figure 1).

Comparison of clinicopathological features at the time of biopsy between the mild drinking and non-drinking groups

Comparisons of clinicopathological features at the time of biopsy between the mild drinking and non-drinking groups revealed significant differences for male prevalence ($P = 0.01$), platelet count ($P = 0.04$), and gamma-glutamyl transpeptidase ($P = 0.02$) (Table 1). No differences were observed between the groups for co-existing disease rate, serum albumin, bilirubin, or alpha-fetoprotein, HbA1c, or pathological features, such as grades for steatosis, lobular inflammation, ballooning, or NAFLD activity score (Table 1). The

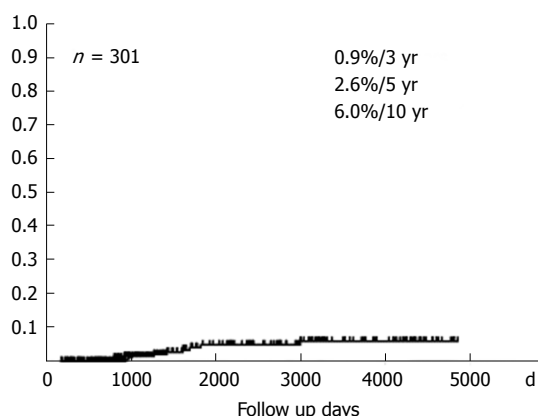


Figure 1 Cumulative incidence rate of hepatocellular carcinoma by Kaplan Meier analysis. The horizontal and vertical axes show days from liver biopsy and cumulative incidence rate of hepatocellular carcinoma, respectively.

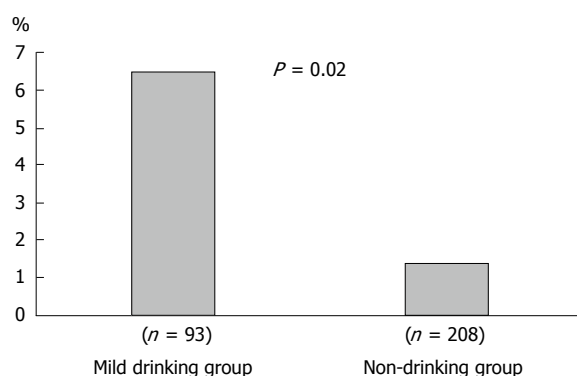


Figure 2 Comparison of incidence rates of hepatocellular carcinoma between mild drinking and non-drinking groups. The vertical axis shows incidence rate (percentage) of hepatocellular carcinoma during follow-up time.

prevalence of liver cirrhosis (F4) was higher in the mild drinking group compared to non-drinking groups (9 vs 8 cases: 10% vs 4%, $P = 0.04$) (Table 1), while the rate of hepatic advanced fibrosis (F3-4) was not different between the groups. Interestingly, the HCC appearance rate was higher in the mild drinking group (6 vs 3 cases: 6.5% vs 1.4%, $P = 0.02$) (Figure 2).

Comparison of clinicopathological features at the time of biopsy between the HCC and non-HCC groups

In comparisons of clinicopathological features at the time of biopsy between HCC and non-HCC patients (Table 2), those with HCC had significantly higher age ($P < 0.01$), higher prevalence of a drinking habit ($P = 0.02$), diabetes mellitus ($P < 0.01$), and hypertension ($P < 0.01$), higher HbA1c ($P = 0.01$), type IV collagen 7S ($P = 0.03$), and alpha-fetoprotein ($P < 0.01$), and lower albumin ($P < 0.01$), cholinesterase ($P < 0.01$), total cholesterol ($P = 0.02$), triglyceride ($P = 0.02$), and platelet count ($P < 0.01$) (Table 2). In pathological findings, the HCC group had a lower steatosis score ($P = 0.01$) and significantly higher fibrosis stage ($P < 0.01$) (Table 2).

Kaplan-Meier analysis was carried out for the HCC and non-HCC groups (Figure 3). Factors associated with higher HCC occurrence included age ≥ 63 years at the time of biopsy ($P = 0.04$, Figure 3A), a mild drinking habit ($P < 0.01$, Figure 3B), diabetes mellitus ($P < 0.01$, Figure 3C), hypertension ($P < 0.01$, Figure 3D), albumin ≤ 4.0 g/dL ($P < 0.01$, Figure 3E), HbA1c $\geq 6.6\%$ ($P = 0.01$, Figure 3G), triglyceride < 133 mg/dL ($P = 0.02$, Figure 3I), platelet count $< 13.3 \times 10^4/\mu\text{L}$ ($P < 0.01$, Figure 3J), type IV collagen 7S ≥ 5.0 ng/mL ($P = 0.01$, Figure 3K), alpha-fetoprotein ≥ 6.0 ng/mL ($P < 0.01$, Figure 3L), steatosis grade 1 ($P = 0.01$, Figure 3M), and F3-4 ($P < 0.01$, Figure 3N).

Multivariate survival analysis using Cox's regression model revealed that hepatic advanced fibrosis (F3-4) ($P < 0.01$, risk ratio: 11.60), diabetes mellitus ($P < 0.01$, risk ratio: 89.50), and serum triglyceride ($P = 0.04$, risk ratio: 0.98) were factors significantly related to HCC, while a mild drinking habit appeared to be marginally related ($P = 0.07$, risk ratio: 4.43) (Table 3).

The result of all HCC patients having advanced hepatic fibrosis (F3-4) at the time of biopsy corroborated the close association between HCC and hepatic fibrosis. To elucidate the additional impact of mild drinking on HCC development in the HCC high-risk group, we evaluated the clinicopathological features of the HCC and non-HCC groups in NAFLD patients with advanced fibrosis (Table 4). Compared with the non-HCC group ($n = 68$), the HCC group ($n = 9$) had a significantly higher rate of a drinking habit ($P = 0.03$), diabetes mellitus ($P = 0.02$), and hypertension ($P = 0.01$), higher alpha-fetoprotein ($P = 0.04$), and lower cholinesterase ($P = 0.02$), triglyceride ($P = 0.02$), and platelet count ($P < 0.01$) (Table 4). There were no differences in pathological findings between the groups (Table 4).

In NAFLD cases with advanced fibrosis, multivariate survival analysis using Cox's regression model revealed that a mild drinking habit ($P = 0.04$, risk ratio: 4.83), alpha-fetoprotein ($P = 0.01$, risk ratio: 1.23), and diabetes mellitus ($P = 0.03$, risk ratio: 12.00) were factors significantly associated with HCC (Table 5). Accordingly, a mild drinking habit appeared to be a risk factor for hepatocarcinogenesis in NAFLD patients with advanced fibrosis.

DISCUSSION

Although continuous and excessive ethanol consumption is harmful to the liver, a mild drinking habit reportedly improves insulin sensitivity and decreases cardiovascular mortality in the general population^[22]. One question arises on whether mild drinking is similarly beneficial for NAFLD patients, but there are few studies on NAFLD regarding the impact of light ethanol consumption. This study demonstrated that a mild drinking habit may be associated with HCC occurrence in NAFLD patients with advanced fibrosis. We therefore propose the abstinence

Table 1 Comparison of clinicopathological features at the time of biopsy between mild drinking and non-drinking groups

	Mild drinking group (<i>n</i> = 93)	Non-drinking group (<i>n</i> = 208)	<i>P</i> value
Age (yr)	55 (19-77)	56 (10-84)	0.50
Male	52 (56)	84 (40)	0.01
Body mass index (kg/m ²)	26.5 (18.3-40.0)	26.2 (17.8-41.0)	0.53
Co-existing disease			
Diabetes mellitus	32 (34)	78 (38)	0.57
Hypertension	41 (44)	81 (39)	0.44
Hyperlipidemia	55 (59)	135 (66)	0.29
Laboratory data			
Albumin (mg/dL)	4.5 (3.2-5.4)	4.5 (3.0-5.2)	0.48
Total bilirubin (mg/dL)	0.91 (0.40-2.20)	0.86 (0.38-2.64)	0.12
Aspartate aminotransferase (IU/L)	44 (20-175)	47 (13-263)	0.80
Alanine aminotransferase (IU/L)	68 (22-237)	67 (13-522)	0.74
Gamma-glutamyl transpeptidase (IU/L)	69 (19-400)	54 (7-544)	0.02
Cholinesterase (IU/L)	363 (171-586)	384 (189-591)	0.05
Fasting blood sugar (mg/dL)	106 (84-215)	108 (77-221)	0.63
HOMA-IR	3.4 (1.0-42.6)	3.3 (0.3-24.5)	0.58
HbA1c (%)	5.9 (5.1-9.8)	6.0 (4.9-12.3)	0.21
Total cholesterol (mg/dL)	205 (138-336)	208 (70-295)	0.68
Triglyceride (mg/dL)	115 (42-404)	133 (32-801)	0.14
Platelet (×10 ³ /μL)	21.1 (5.3-45.4)	22.1 (7.2-40.7)	0.04
Hyaluronic acid (ng/mL)	43 (12-320)	49 (9-1611)	0.57
Type IV collagen 7s (ng/mL)	4.3 (2-20)	4.5 (2-11)	0.93
Alpha-fetoprotein (ng/mL)	3.3 (0.7-13.5)	3.0 (0.7-20.3)	0.41
Pathology			
Steatosis			0.86
1	33 (36)	64 (31)	
2	36 (39)	90 (43)	
3	24 (26)	54 (26)	
Lobular inflammation			0.14
0	5 (5)	8 (4)	
1	47 (51)	83 (40)	
2	37 (40)	95 (46)	
3	4 (4)	22 (11)	
Ballooning			0.82
0	18 (19)	41 (20)	
1	50 (54)	118 (57)	
2	25 (27)	49 (24)	
NAFLD activity score			0.79
1	4 (4)	5 (2)	
2	8 (9)	16 (8)	
3	16 (17)	34 (16)	
4	17 (18)	32 (15)	
5	25 (27)	62 (30)	
6	14 (15)	36 (17)	
7	9 (10)	18 (9)	
8	0 (0)	5 (2)	
Fibrosis			0.39
0	17 (18)	40 (19)	
1	39 (42)	93 (45)	
2	10 (11)	25 (12)	
3	18 (19)	42 (20)	
4	9 (10)	8 (4)	
Fibrosis 3-4 (Advanced fibrosis)	27 (29)	50 (24)	0.36
Fibrosis 4 (Cirrhosis)	9 (10)	8 (4)	0.04

Data are expressed as median (range) or *n* (%). HbA1c: Hemoglobin A1c; HOMA-IR: Homeostasis model assessment for insulin resistance.

of ethanol, even in small amounts, in such individuals.

Mild to moderate alcohol consumption has been shown to decrease insulin resistance and improve components of metabolic syndrome^[23]. Dunn *et al*^[12] and Kwon *et al*^[13] reported a positive association between moderate alcohol intake and decreased steatosis/ballooning and fibrosis grades in NAFLD patients, which might explain the protective effects

of moderate alcohol intake on preventing histological injury. In our cohort, however, the mild and non-drinking groups did not differ with regard to steatosis/ballooning grade and the rate of liver cirrhosis was higher in the mild drinking group. The reason for these discrepancies is unknown, along with why there were no significant reductions in body mass index or homeostasis model assessment for insulin resistance score between our

Table 2 Comparison of clinicopathological features at the time of biopsy between hepatocellular carcinoma and non-hepatocellular carcinoma groups

	HCC group (<i>n</i> = 9)	Non-HCC group (<i>n</i> = 292)	<i>P</i> value
Age (yr)	65 (56-84)	55 (10-81)	< 0.01
Male	3 (33)	133 (46)	0.52
Body mass index (kg/m ²)	26.0 (18.3-28.7)	26.2 (17.8-41.0)	0.23
Drinking habit	6 (67)	87 (30)	0.02
Co-existing disease			
Diabetes mellitus	8 (89)	102 (35)	< 0.01
Hypertension	9 (100)	113 (39)	< 0.01
Hyperlipidemia	3 (33)	187 (65)	0.06
Laboratory data			
Albumin (mg/dL)	4.0 (3.7-4.6)	4.5 (3.0-5.4)	< 0.01
Total bilirubin (mg/dL)	0.91 (0.44-1.61)	0.88 (0.38-2.64)	0.75
Aspartate aminotransferase (IU/L)	52 (32-95)	46 (13-263)	0.71
Alanine aminotransferase (IU/L)	53 (16-132)	68 (13-522)	0.11
Gamma-glutamyl transpeptidase (IU/L)	65 (28-192)	56 (7-544)	0.48
Cholinesterase (IU/L)	249 (190-434)	380 (171-591)	< 0.01
Fasting blood sugar (mg/dL)	133 (84-172)	106 (77-221)	0.13
HOMA-IR	5.8 (1.2-9.8)	3.3 (0.3-42.6)	0.18
HbA1c (%)	6.6 (6.0-7.0)	5.9 (4.9-12.3)	0.01
Total cholesterol (mg/dL)	176 (153-264)	208 (70-336)	0.02
Triglyceride (mg/dL)	85 (64-140)	130 (32-801)	0.02
Platelet (×10 ⁴ /μL)	11.0 (6.4-18.6)	22.0 (5.3-45.4)	< 0.01
Hyaluronic acid (ng/mL)	42 (17-263)	42 (9-1180)	0.96
Type IV collagen 7s (ng/mL)	5.6 (4.7-8.4)	4.4 (2.0-20.0)	0.03
Alpha-fetoprotein (ng/mL)	6.0 (3.7-20.3)	3.0 (0.7-13.2)	< 0.01
Pathology			
Steatosis			0.01
1	7 (78)	90 (31)	
2	2 (22)	124 (43)	
3	0 (0)	78 (27)	
Lobular inflammation			0.21
0	0 (0)	13 (4)	
1	2 (22)	128 (44)	
2	7 (78)	125 (43)	
3	0 (0)	26 (9)	
Ballooning			0.76
0	1 (11)	58 (20)	
1	6 (67)	162 (56)	
2	2 (22)	72 (25)	
NAFLD activity score			0.53
1	0 (0)	9 (3)	
2	0 (0)	24 (8)	
3	3 (33)	47 (16)	
4	2 (22)	47 (16)	
5	4 (44)	83 (28)	
6	0 (0)	50 (17)	
7	0 (0)	27 (9)	
8	0 (0)	5 (2)	
Fibrosis			< 0.01
0	0 (0)	57 (20)	
1	0 (0)	132 (45)	
2	0 (0)	35 (12)	
3	4 (44)	56 (19)	
4	5 (56)	12 (4)	

Data are expressed as median (range) or *n* (%). HCC: Hepatocellular carcinoma; HOMA-IR: Homeostasis model assessment for insulin resistance; HbA1c: Hemoglobin A1c; NAFLD: Non-alcoholic fatty liver disease.

test groups. We presume that ethnic differences may account for differences in ethanol consumption effects on steatosis, ballooning, and fibrosis.

In 2010, Ascha *et al.*^[24] described that a mild drinking habit was associated with an increased risk of carcinogenesis in a NASH-associated cirrhosis cohort.

Here, we focused on the impact of a mild drinking habit on liver carcinogenesis originating from NAFLD. All HCC-developing patients (*n* = 9) had advanced fibrosis (F3-4). Among all NAFLD patients, multivariate analysis revealed that fibrosis, diabetes mellitus, and serum triglyceride were factors significantly related to

Table 3 Factors related to hepatic carcinogenesis by multivariate survival analysis using Cox's regression model for all patients

	<i>P</i> value	Relative risk	95%CI
Fibrosis	< 0.01	11.6	2.36-56.9
Diabetes mellitus	< 0.01	89.5	6.01-1331.2
Triglyceride	0.04	0.98	0.95-0.99
Drinking habit	0.07	4.43	0.88-22.4

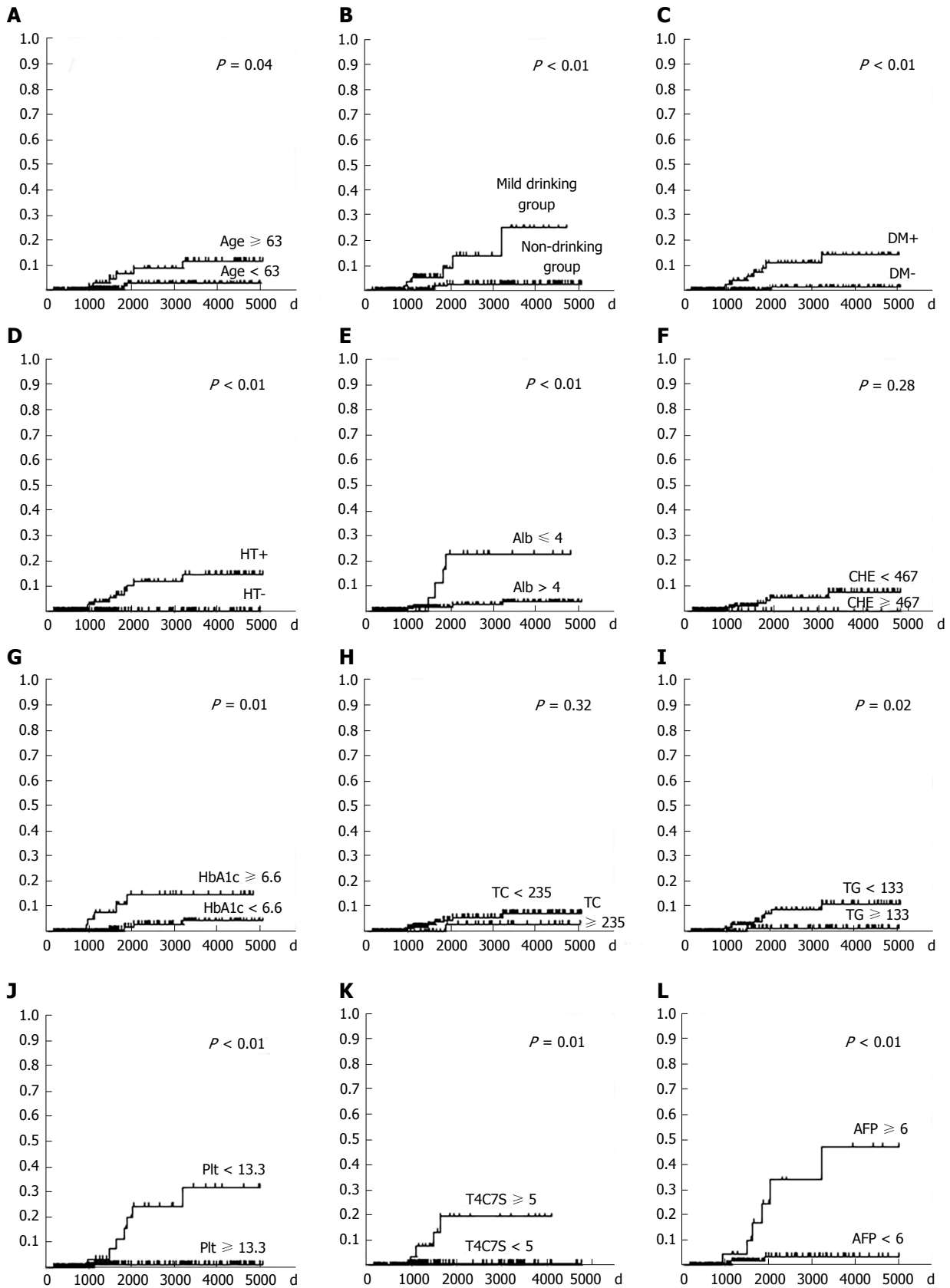
Table 4 Comparison of clinicopathological features at the time of biopsy between hepatocellular carcinoma and non- hepatocellular carcinoma groups in non-alcoholic fatty liver disease patients with advanced fibrosis (F3-4)

	HCC group (<i>n</i> = 9)	Non-HCC group (<i>n</i> = 68)	<i>P</i> value
Age (yr)	65 (56-84)	64 (30-81)	0.32
Male	3 (33)	14 (21)	0.39
Body mass index (kg/m ²)	26.0 (18.3-28.7)	27.7 (20.1-41.0)	0.05
Drinking habit	6 (67)	21 (31)	0.03
Co-existing disease			
Diabetes mellitus	8 (89)	32 (47)	0.02
Hypertension	9 (100)	38 (56)	0.01
Hyperlipidemia	3 (33)	35 (52)	0.31
Laboratory data			
Albumin (mg/dL)	4.0 (3.7-4.6)	4.3 (3.2-5.0)	0.07
Total bilirubin (mg/dL)	0.91 (0.44-1.61)	0.91 (0.48-2.15)	0.99
Aspartate aminotransferase (IU/L)	52 (32-95)	63 (20-200)	0.13
Alanine aminotransferase (IU/L)	53 (16-132)	71 (19-298)	0.09
Gamma-glutamyl transpeptidase (IU/L)	65 (28-192)	65 (25-205)	0.75
Cholinesterase (IU/L)	249 (190-434)	345 (171-467)	0.02
Fasting blood sugar (mg/dL)	133 (84-172)	110 (82-215)	0.30
HOMA-IR	5.8 (1.2-9.8)	4.3 (1.1-15.6)	0.54
HbA1c (%)	6.6 (6.0-7.0)	6.1 (5.0-10.9)	0.14
Total cholesterol (mg/dL)	176 (153-264)	201 (131-294)	0.10
Triglyceride (mg/dL)	85 (64-140)	119 (42-351)	0.03
Platelet (×10 ³ /μL)	11.0 (6.4-18.6)	16.6 (5.3-32.6)	< 0.01
Hyaluronic acid (ng/mL)	42 (17-263)	59 (11-1180)	0.45
Type IV collagen 7s (ng/mL)	5.6 (4.7-8.4)	6.8 (3.5-20.0)	0.61
Alpha-fetoprotein (ng/mL)	6.0 (3.7-20.3)	5.0 (1.1-11.3)	0.04
Pathology			
Steatosis			0.07
1	7 (78)	26 (38)	
2	2 (22)	32 (47)	
3	0 (0)	10 (15)	
Lobular inflammation			0.45
0	0 (0)	1 (2)	
1	2 (22)	17 (25)	
2	7 (78)	37 (54)	
3	0 (0)	13 (19)	
Ballooning			0.76
0	1 (11)	1 (2)	
1	6 (67)	34 (50)	
2	2 (22)	33 (49)	
NAFLD activity score			0.25
1	0 (0)	1 (2)	
2	0 (0)	1 (2)	
3	3 (33)	5 (7)	
4	2 (22)	10 (15)	
5	4 (44)	26 (38)	
6	0 (0)	16 (24)	
7	0 (0)	8 (12)	
8	0 (0)	1 (2)	

Data are expressed as median (range) or *n* (%). HCC: Hepatocellular carcinoma; HOMA-IR: Homeostasis model assessment for insulin resistance; HbA1c: Hemoglobin A1c; NAFLD: Non-alcoholic fatty liver disease.

HCC, while a mild drinking habit appeared to be only marginally related to carcinogenesis. On the other hand, in NAFLD cases with F3-4, multivariate survival analysis

showed a mild drinking habit, alpha-fetoprotein, and diabetes mellitus to be factors significantly associated with HCC. Our results indicated that mild drinking may



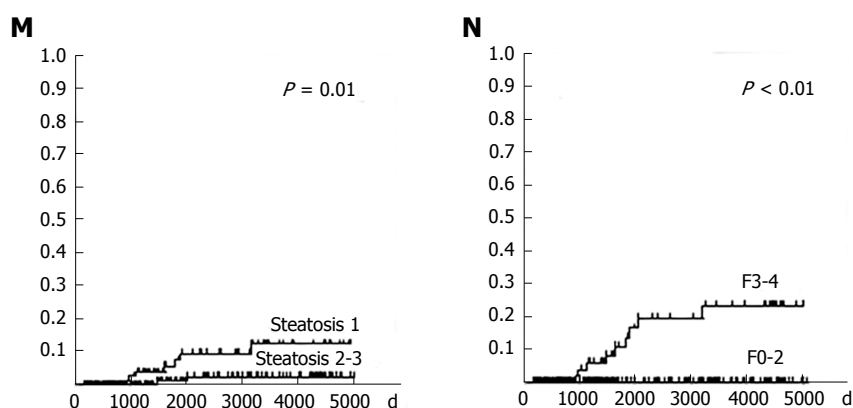


Figure 3 Cumulative incidence rate of hepatocellular carcinoma based on data at the time of liver biopsy. A: Age; B: Drinking habit; C: Diabetes mellitus; D: Hypertension; E: Albumin; F: Cholinesterase; G: HbA1c; H: Total cholesterol; I: Triglyceride; J: Platelet; K: Type IV collagen 7S; L: Alpha-fetoprotein; M: Steatosis; N: Fibrosis. The horizontal and vertical axes show days from liver biopsy and cumulative incidence rate of hepatocellular carcinoma, respectively. DM: Diabetes mellitus; HT: Hypertension; Alb: Albumin; CHE: Cholinesterase; TC: Total cholesterol; TG: Triglyceride; Plt: Platelet; T4C7S: Type IV collagen 7S; AFP: Alpha-fetoprotein; F: Fibrosis.

Table 5 Factors related to hepatic carcinogenesis by multivariate survival analysis using Cox's regression model for patients with advanced fibrosis (F3-4)

	P value	Relative risk	95%CI
Drinking habit	0.04	4.83	1.01-23.00
Alpha-fetoprotein	0.01	1.23	1.04-1.44
Diabetes mellitus	0.03	12.00	1.20-119.66

increase the risk of HCC in NAFLD patients with not only cirrhosis (F4), but also advanced fibrosis.

The International Agency for Cancer Research (WHO) has certified that alcohol intake is carcinogenic for humans^[25]. Indeed, alcohol consumption has been associated with increased risks of head and neck, oral cavity, pharynx, larynx, esophagus, bowel, breast, and liver cancers^[25]. Ethanol is metabolized into acetaldehyde by alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1) in the liver, which is then oxidized to acetate by aldehyde dehydrogenase (ALDH)^[26]. Although the underlying causes of cancers related to ethanol consumption are not yet clear, various factors have been proposed as key contributors of hepatocarcinogenesis, including the direct genotoxicity of ethanol and its metabolite acetaldehyde, malnutrition, chronic inflammation, oxidative stress, interactions with retinoids, methylation level alterations, immunological surveillance, and angiogenesis^[27]. Ethanol also reduces the levels of glutathione S-transferase, a detoxifier of oxidative stress, and increases the expression of CYP2E1, a generator of oxidative stress^[28-32]. The net increases in oxidative stress by long-term ethanol consumption may lead to hepatocarcinogenesis in the presence of steatosis, while it is undetermined which factor is most affecting this oncogenic process^[33-36]. The impact of ethanol per hepatocyte might be greater in cirrhotic patients because of the decreases in the number and function of hepatocytes. Actually, Vidal *et al.*^[37] reported that ALDH activity was significantly reduced in patients

with advanced liver fibrosis compared with those having mild fibrosis. Therefore, we presume that increased acetaldehyde and resultant DNA damage may induce pro-carcinogenic gene mutations and/or epigenetic changes, even with mild drinking, in NAFLD patients with advanced fibrosis.

Based on the results of the present study, mild ethanol consumption should be abandoned for NAFLD patients, especially those with advanced fibrosis, due to the possible risk of liver tumorigenesis. The main limitation of this study was its retrospective design; there remains a need for future large-scale longitudinal studies that evaluate the outcomes of NAFLD patients with mild ethanol intake. Prospective studies investigating the effect of ethanol cessation in NAFLD patients with a mild drinking habit are also required to confirm the impact of mild drinking on the clinical course of NAFLD.

In conclusion, in NAFLD patients, especially those with advanced fibrosis, a mild drinking habit is a risk factor for hepatocarcinogenesis that should be discouraged.

ARTICLE HIGHLIGHTS

Research background

The prevalence rate of non-alcoholic fatty liver disease (NAFLD) has doubled during the last 20 years.

Research motivation

The impact of mild drinking habit (less than 20 g/d of ethanol) on the clinical

course of NAFLD has not been determined. We examined the influence of a mild drinking habit on liver carcinogenesis from NAFLD.

Research objectives

A total of 301 patients who had been diagnosed as having NAFLD by liver biopsy between 2003 and 2016 (median age: 56 years, 45% male, 56% with non-alcoholic steatohepatitis, 26% with advanced fibrosis (F3-4)) were divided into the mild drinking group with ethanol consumption of less than 20 g/d (mild drinking group, $n = 93$) and the non-drinking group ($n = 208$).

Research methods

Clinicopathological features at the time of liver biopsy and factors related to hepatocellular carcinoma (HCC) occurrence were compared between the groups.

Research results

We observed significant differences in male prevalence ($P = 0.01$), platelet count ($P = 0.04$), and gamma-glutamyl transpeptidase ($P = 0.02$) between the test groups. Over 6 years of observation, the HCC appearance rate was significantly higher in the mild drinking group (6.5% vs 1.4%, $P = 0.02$). Multivariate survival analysis using Cox's regression model revealed that hepatic advanced fibrosis (F3-4) ($P < 0.01$, risk ratio: 11.60), diabetes mellitus ($P < 0.01$, risk ratio: 89.50), and serum triglyceride ($P = 0.04$, risk ratio: 0.98) were factors significantly related to HCC in all NAFLD patients, while the effect of a drinking habit was marginal ($P = 0.07$, risk ratio: 4.43). In patients with advanced fibrosis (F3-4), however, a drinking habit ($P = 0.04$, risk ratio: 4.83), alpha-fetoprotein ($P = 0.01$, risk ratio: 1.23), and diabetes mellitus ($P = 0.03$, risk ratio: 12.00) were identified as significant contributors to HCC occurrence.

Research conclusions

A mild drinking habit appears to be a risk factor for hepatocarcinogenesis in NAFLD patients, especially those with advanced fibrosis.

Research perspectives

Prospective studies investigating the effect of ethanol cessation in NAFLD patients with a mild drinking habit are also required to confirm the impact of mild drinking on the clinical course of NAFLD.

REFERENCES

- 1 NCD Risk Factor Collaboration (NCD-RisC). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. *Lancet* 2016; **387**: 1377-1396 [PMID: 27115820 DOI: 10.1016/S0140-6736(16)30054-X]
- 2 Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]
- 3 Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]
- 4 Eguchi Y, Hyogo H, Ono M, Mizuta T, Ono N, Fujimoto K, Chayama K, Saibara T; JSG-NAFLD. Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. *J Gastroenterol* 2012; **47**: 586-595 [PMID: 22328022 DOI: 10.1007/s00535-012-0533-z]
- 5 Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183-1192 [PMID: 11266382 DOI: 10.1053/gast.2001.23256]
- 6 Duvnjak M, Lerotić I, Barsić N, Tomasić V, Virović Jukić L, Velagić V. Pathogenesis and management issues for non-alcoholic fatty liver disease. *World J Gastroenterol* 2007; **13**: 4539-4550 [PMID: 17729403]
- 7 Tanaka N, Aoyama T, Kimura S, Gonzalez FJ. Targeting nuclear receptors for the treatment of fatty liver disease. *Pharmacol Ther* 2017; **179**: 142-157 [PMID: 28546081 DOI: 10.1016/j.pharmthera.2017.05.011]
- 8 Tanaka N, Takahashi S, Hu X, Lu Y, Fujimori N, Golla S, Fang ZZ, Aoyama T, Krausz KW, Gonzalez FJ. Growth arrest and DNA damage-inducible 45a protects against nonalcoholic steatohepatitis induced by methionine- and choline-deficient diet. *Biochim Biophys Acta* 2017; **1863**: 3170-3182 [PMID: 28844958 DOI: 10.1016/j.bbadis.2017.08.017]
- 9 Kitabatake H, Tanaka N, Fujimori N, Komatsu M, Okubo A, Kakegawa K, Kimura T, Sugiura A, Yamazaki T, Shibata S, Ichikawa Y, Joshita S, Umemura T, Matsumoto A, Koinuma M, Sano K, Aoyama T, Tanaka E. Association between endotoxemia and histological features of nonalcoholic fatty liver disease. *World J Gastroenterol* 2017; **23**: 712-722 [PMID: 28216979 DOI: 10.3748/wjg.v23.i4.712]
- 10 Kimura T, Kobayashi A, Tanaka N, Sano K, Komatsu M, Fujimori N, Yamazaki T, Shibata S, Ichikawa Y, Joshita S, Umemura T, Matsumoto A, Horiuchi A, Mori H, Wada S, Kiyosawa K, Miyagawa SI, Tanaka E. Clinicopathological characteristics of non-B non-C hepatocellular carcinoma without past hepatitis B virus infection. *Hepatol Res* 2017; **47**: 405-418 [PMID: 27288988 DOI: 10.1111/hepr.12762]
- 11 Bell RA, Mayer-Davis EJ, Martin MA, D'Agostino RB Jr, Haffner SM. Associations between alcohol consumption and insulin sensitivity and cardiovascular disease risk factors: the Insulin Resistance and Atherosclerosis Study. *Diabetes Care* 2000; **23**: 1630-1636 [PMID: 11092284]
- 12 Dunn W, Sanyal AJ, Brunt EM, Unalp-Arida A, Donohue M, McCullough AJ, Schwimmer JB. Modest alcohol consumption is associated with decreased prevalence of steatohepatitis in patients with non-alcoholic fatty liver disease (NAFLD). *J Hepatol* 2012; **57**: 384-391 [PMID: 22521357 DOI: 10.1016/j.jhep.2012.03.024]
- 13 Kwon HK, Greenon JK, Conjeevaram HS. Effect of lifetime alcohol consumption on the histological severity of non-alcoholic fatty liver disease. *Liver Int* 2014; **34**: 129-135 [PMID: 23809459 DOI: 10.1111/liv.12230]
- 14 Ajmera VH, Terrault NA, Harrison SA. Is moderate alcohol use in nonalcoholic fatty liver disease good or bad? A critical review. *Hepatology* 2017; **65**: 2090-2099 [PMID: 28100008 DOI: 10.1002/hep.29055]
- 15 Fujimori N, Tanaka N, Shibata S, Sano K, Yamazaki T, Sekiguchi T, Kitabatake H, Ichikawa Y, Kimura T, Komatsu M, Umemura T, Matsumoto A, Tanaka E. Controlled attenuation parameter is correlated with actual hepatic fat content in patients with non-alcoholic fatty liver disease with none-to-mild obesity and liver fibrosis. *Hepatol Res* 2016; **46**: 1019-1027 [PMID: 27183219 DOI: 10.1111/hepr.12649]
- 16 Komatsu M, Kimura T, Yazaki M, Tanaka N, Yang Y, Nakajima T, Horiuchi A, Fang ZZ, Joshita S, Matsumoto A, Umemura T, Tanaka E, Gonzalez FJ, Ikeda S, Aoyama T. Steatogenesis in adult-onset type II citrullinemia is associated with down-regulation of PPARα. *Biochim Biophys Acta* 2015; **1852**: 473-481 [PMID: 25533124 DOI: 10.1016/j.bbadis.2014.12.011]
- 17 Bruix J, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]
- 18 Kimura T, Shinji A, Tanaka N, Koinuma M, Yamaura M, Nagaya T, Joshita S, Komatsu M, Umemura T, Horiuchi A, Wada S, Tanaka E. Association between lower air pressure and the onset of ischemic colitis: a case-control study. *Eur J Gastroenterol Hepatol* 2017; **29**: 1071-1078 [PMID: 28562393 DOI: 10.1097/MEG.0000000000000913]
- 19 Kimura T, Shinji A, Horiuchi A, Tanaka N, Nagaya T, Shigeno T, Nakamura N, Komatsu M, Umemura T, Arakura N, Matsumoto A,

- Tanaka E. Clinical characteristics of young-onset ischemic colitis. *Dig Dis Sci* 2012; **57**: 1652-1659 [PMID: 22383082 DOI: 10.1007/s10620-012-2088-5]
- 20 Umemura T, Joshita S, Sekiguchi T, Usami Y, Shibata S, Kimura T, Komatsu M, Matsumoto A, Ota M, Tanaka E. Serum Wisteria floribunda Agglutinin-Positive Mac-2-Binding Protein Level Predicts Liver Fibrosis and Prognosis in Primary Biliary Cirrhosis. *Am J Gastroenterol* 2015; **110**: 857-864 [PMID: 25916223 DOI: 10.1038/ajg.2015.118]
- 21 Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]
- 22 Thun MJ, Peto R, Lopez AD, Monaco JH, Henley SJ, Heath CW Jr, Doll R. Alcohol consumption and mortality among middle-aged and elderly U.S. adults. *N Engl J Med* 1997; **337**: 1705-1714 [PMID: 9392695 DOI: 10.1056/NEJM199712113372401]
- 23 Alkerwi A, Boutsen M, Vaillant M, Barre J, Lair ML, Albert A, Guillaume M, Dramaix M. Alcohol consumption and the prevalence of metabolic syndrome: a meta-analysis of observational studies. *Atherosclerosis* 2009; **204**: 624-635 [PMID: 19084839 DOI: 10.1016/j.atherosclerosis.2008.10.036]
- 24 Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 1972-1978 [PMID: 20209604 DOI: 10.1002/hep.23527]
- 25 Boffetta P, Hashibe M. Alcohol and cancer. *Lancet Oncol* 2006; **7**: 149-156 [PMID: 16455479 DOI: 10.1016/S1470-2045(06)70577-0]
- 26 Zakhari S. Overview: how is alcohol metabolized by the body? *Alcohol Res Health* 2006; **29**: 245-254 [PMID: 17718403]
- 27 Testino G, Leone S, Borro P. Alcohol and hepatocellular carcinoma: a review and a point of view. *World J Gastroenterol* 2014; **20**: 15943-15954 [PMID: 25473148 DOI: 10.3748/wjg.v20.i43.15943]
- 28 Tsutsumi T, Suzuki T, Moriya K, Shintani Y, Fujie H, Miyoshi H, Matsuura Y, Koike K, Miyamura T. Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. *Hepatology* 2003; **38**: 820-828 [PMID: 14512869 DOI: 10.1053/jhep.2003.50399]
- 29 Lu Y, Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. *Free Radic Biol Med* 2008; **44**: 723-738 [PMID: 18078827 DOI: 10.1016/j.freeradbiomed.2007.11.004]
- 30 Kanbe H, Kamijo Y, Nakajima T, Tanaka N, Sugiyama E, Wang L, Fang ZZ, Hara A, Gonzalez FJ, Aoyama T. Chronic ethanol consumption decreases serum sulfatide levels by suppressing hepatic cerebroside sulfotransferase expression in mice. *Arch Toxicol* 2014; **88**: 367-379 [PMID: 24065054 DOI: 10.1007/s00204-013-1132-3]
- 31 Okiyama W, Tanaka N, Nakajima T, Tanaka E, Kiyosawa K, Gonzalez FJ, Aoyama T. Polyene phosphatidylcholine prevents alcoholic liver disease in PPARalpha-null mice through attenuation of increases in oxidative stress. *J Hepatol* 2009; **50**: 1236-1246 [PMID: 19398233 DOI: 10.1016/j.jhep.2009.01.025]
- 32 Nakajima T, Kamijo Y, Tanaka N, Sugiyama E, Tanaka E, Kiyosawa K, Fukushima Y, Peters JM, Gonzalez FJ, Aoyama T. Peroxisome proliferator-activated receptor alpha protects against alcohol-induced liver damage. *Hepatology* 2004; **40**: 972-980 [PMID: 15382117 DOI: 10.1002/hep.20399]
- 33 Mantena SK, King AL, Andringa KK, Eccleston HB, Bailey SM. Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol- and obesity-induced fatty liver diseases. *Free Radic Biol Med* 2008; **44**: 1259-1272 [PMID: 18242193 DOI: 10.1016/j.freeradbiomed.2007.12.029]
- 34 Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Todoroki T, Kimura S, Koike K. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001; **61**: 4365-4370 [PMID: 11389061]
- 35 Ramadori P, Cubero FJ, Liedtke C, Trautwein C, Nevzorova YA. Alcohol and Hepatocellular Carcinoma: Adding Fuel to the Flame. *Cancers (Basel)* 2017; **9**: [PMID: 28946672 DOI: 10.3390/cancers9100130]
- 36 Ambade A, Satishchandran A, Gyongyosi B, Lowe P, Szabo G. Adult mouse model of early hepatocellular carcinoma promoted by alcoholic liver disease. *World J Gastroenterol* 2016; **22**: 4091-4108 [PMID: 27122661 DOI: 10.3748/wjg.v22.i16.4091]
- 37 Vidal F, Toda R, Gutiérrez C, Broch M, Fernández-Muixí F, Lorenzo A, Richart C. Influence of chronic alcohol abuse and liver disease on hepatic aldehyde dehydrogenase activity. *Alcohol* 1998; **15**: 3-8 [PMID: 9426831]

P- Reviewer: Peltec A, Sharafi H, Sanal MG S- Editor: Wang XJ
L- Editor: A E- Editor: Huang Y



Retrospective Study

Prognostic significance of combined preoperative fibrinogen and CA199 in gallbladder cancer patients

Wei-Yu Xu, Hao-Hai Zhang, Xiao-Bo Yang, Yi Bai, Jian-Zhen Lin, Jun-Yu Long, Jian-Ping Xiong, Jun-Wei Zhang, Xin-Ting Sang, Hai-Tao Zhao

Wei-Yu Xu, Hao-Hai Zhang, Xiao-Bo Yang, Yi Bai, Jian-Zhen Lin, Jun-Yu Long, Jian-Ping Xiong, Jun-Wei Zhang, Xin-Ting Sang, Hai-Tao Zhao, Department of Liver Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

ORCID number: Wei-Yu Xu (0000-0002-2101-4829); Hao-Hai Zhang (0000-0002-5292-6505); Xiao-Bo Yang (0000-0003-1929-8866); Yi Bai (0000-0002-1179-3734); Jian-Zhen Lin (0000-0002-4767-8834); Jun-Yu Long (0000-0001-5745-7165); Jian-Ping Xiong (0000-0002-6163-2621); Jun-Wei Zhang (0000-0002-2833-0090); Xin-Ting Sang (0000-0003-1952-0527); Hai-Tao Zhao (0000-0002-3444-8044).

Author contributions: Xu WY, Zhang HH and Yang XB contributed equally to this work. Xu WY conceived, designed and wrote the manuscript that led to the submission; Xu WY, Yang XB, Bai Y and Zhang JW collected the clinical data and followed up the patients; Lin JZ, Long JY and Xiong JP helped to analyze the data, Bai Y revised the manuscript, Sang XT and Zhao HT provided financial support for this work; Sang XT and Zhao HT are co-corresponding authors, and contributed equally to this work; all authors read and approved the final manuscript.

Supported by National Key Project Research and Development Projects, No. S2016G9012; International Science and Technology Cooperation Projects, No. 2015DFA30650; and The Capital Special Research Project for Clinical Application, No. Z151100004015170.

Institutional review board statement: The publication of this manuscript has been reviewed and approved by the PUMCH institutional review board.

Informed consent statement: All patients and their families signed informed consent statements before surgery, and the type of surgical procedure was performed according to the approved guidelines.

Conflict-of-interest statement: We declare that the authors have no conflict of interest.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Hai-Tao Zhao, MD, Professor, Department of Liver Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Shuaifuyuan, Wangfujing, Beijing 100730, China. zhaoht@pumch.cn
Telephone: +86-10-69156042
Fax: +86-10-69156042

Received: January 26, 2018
Peer-review started: January 26, 2018
First decision: February 10, 2018
Revised: March 7, 2018
Accepted: March 10, 2018
Article in press: March 10, 2018
Published online: April 7, 2018

Abstract

AIM

To investigate the prognostic value of the combination of preoperative plasma fibrinogen and CA199 in patients with gallbladder carcinoma (GBC).

METHODS

The clinicopathological data of 154 GBC patients were retrospectively reviewed after surgery. A receiver

operating characteristic (ROC) curve was plotted to verify the optimum cut-off values for plasma fibrinogen and CA199. Univariate and multivariate survival analyses were performed to identify the factors associated with GBC prognosis. Based on the HRs calculated *via* multivariate survival analyses, patients with elevated plasma fibrinogen and CA199 levels were allocated a score of 2.1; those with an elevated plasma fibrinogen level only were allocated a score of 1, those with an elevated CA199 level only were allocated a score of 1.1, and those with neither of these abnormalities were allocated a score of 0.

RESULTS

ROC curve analysis showed that the optimum cut-off values for preoperative plasma fibrinogen and CA199 were 3.47 g/L and 25.45 U/mL, respectively. Multivariate analysis indicated that elevated preoperative plasma fibrinogen and CA199 levels were significantly correlated with worse overall survival (OS) (HR = 1.711, 95%CI: 1.114-2.627, $P = 0.014$, and HR = 1.842, 95%CI: 1.111-3.056, $P = 0.018$). When we combined these two parameters, the area under the ROC curve increased from 0.735 (for preoperative plasma fibrinogen only) and 0.729 (for preoperative CA199 only) to 0.765. When this combined variable was added to the multivariate analysis, the combination of plasma fibrinogen and CA199 ($P < 0.001$), resection margin ($P < 0.001$) and TNM stage ($P = 0.010$) were independent prognostic factors for GBC.

CONCLUSION

The combination of plasma fibrinogen and CA199 may serve as a more efficient independent prognostic biomarker for postoperative GBC patients than either parameter alone.

Key words: Prognostic factor; Plasma fibrinogen; CA199; Survival; Gallbladder cancer

© The Author(s) 2018. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Elevated plasma fibrinogen and CA199 levels are associated with poor prognosis in patients with gallbladder carcinoma (GBC). The prognostic value of the combination of plasma fibrinogen and CA199 for GBC has not been reported. The most important finding in this study was that the combination of preoperative plasma fibrinogen and CA199 levels was a better independent prognostic indicator for GBC than either parameter alone.

Xu WY, Zhang HH, Yang XB, Bai Y, Lin JZ, Long JY, Xiong JP, Zhang JW, Sang XT, Zhao HT. Prognostic significance of combined preoperative fibrinogen and CA199 in gallbladder cancer patients. *World J Gastroenterol* 2018; 24(13): 1451-1463 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1451.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1451>

INTRODUCTION

Primary gallbladder carcinoma (GBC) is relatively rare worldwide, but is the most common malignancy of the biliary tract system^[1]. GBC is the seventh most common gastrointestinal cancer^[2] and is attributable to approximately 1% of all cancer cases in China^[3]. The incidence of this malignancy was recently reported to be approximately 2.5 per 100000 persons^[4]. The prognosis of GBC is still typically poor due to nonspecific symptoms, late diagnosis, lack of treatment options, and the absence of effective prognostic markers. According to epidemiological studies, the overall survival (OS) of GBC patients is 6 mo, with a 5-year survival rate of less than 10%^[1,5-7]. Therefore, investigations on the prognostic factors of GBC are especially important.

The association between hemostasis and cancer, and the influence of hemostatic factors on cancer development, growth, and metastasis are evident^[8,9]. Fibrinogen is a 340-kDa plasma glycoprotein that is upregulated during systemic inflammation and tissue injury. Fibrinogen is synthesized in the liver and transformed into fibrin through the activity of activated thrombin, which is a key coagulation factor in platelet aggregation, clot formation, wound healing, and coagulation^[10-12]. A number of studies have shown that plasma fibrinogen levels are upregulated in various cancer types, such as respiratory system tumors^[13,14], digestive system tumors^[15-18], gynecological tumors^[19-22], head and neck cancer^[23,24] and genitourinary tumors^[25,26], and may indicate cancer progression, metastasis and recurrence^[17,22,27-29]. However, to our knowledge, studies on the prognostic value of plasma fibrinogen levels in GBC are very rare^[30].

In addition, CA199 has been traditionally used for the diagnosis and prognosis of GBC^[31]; however, the reported results on its prognostic value in GBC patients are inconsistent and controversial^[30,32,33]. Therefore, we hypothesized that the combination of plasma fibrinogen and CA199 levels may avoid inconsistent results and increase the prognostic accuracy for GBC.

Hence, the aim of the current study was to investigate the prognostic value of the combination of plasma fibrinogen and CA199 levels in patients with GBC. Additionally, we aimed to determine whether the combination of plasma fibrinogen and CA199 levels can serve as a more efficient predictive factor than either parameter alone in patients with GBC.

MATERIALS AND METHODS

Study population

From January 2005 to May 2017, a retrospective analysis of 154 GBC patients was conducted following surgery in the Department of Liver Surgery at the Peking Union Medical College Hospital of the Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC). The patients included in the analysis

met the following criteria: (1) GBC diagnosis confirmed by histopathology and cancer stage determined in accordance with the American Joint Committee on Cancer staging system, 8th Edition (AJCC-8), and the histopathologic postoperative pathologic tumor-node-metastasis (pTNM) categorization system (International Union against Cancer Staging Manual, 7th Edition; UICC-7); and (2) no adjuvant chemotherapy and/or radiotherapy before gallbladder resection surgery. Patients with the following characteristics were excluded: (1) other tumors; (2) inflammatory conditions, including infections, collagen diseases, anemia, other diseases concerning the hematological system, and absolute cardiovascular and cerebrovascular disorders; (3) liver disease; (4) oral administration of anticoagulants or acetylsalicylic acids within 3 mo before surgery; (5) lack of adequate clinical data or loss to follow-up. This study was approved by the Medical Ethics Committee of the Peking Union Medical College Hospital of the CAMS & PUMC, and all participants signed written informed consent forms.

Data collection

Patient characteristics were obtained *via* a retrospective medical record review using a standardized data collection form. Based on the medical records, the following data were collected for each patient: age, gender, plasma fibrinogen concentration, CA199 level, tumor size (defined as the longest diameter of the general postoperative pathological specimens), gallstone history, jaundice, comorbidity (diabetes), resection margin, tumor differentiation (categorized as poorly differentiated, moderately differentiated and well differentiated), T stage, N stage, M stage, pTNM stage (as defined by AJCC-8), pathological type and other miscellaneous characteristics.

Plasma fibrinogen concentration and CA199 level

The plasma fibrinogen concentration and CA199 level were measured within 3 d before surgery as part of a routine workup in these patients. The fibrinogen concentration was measured based on the Clauss method as previously described^[34], and the CA199 level was determined *via* an electrochemiluminescence immunoassay at the Department of Liver Surgery of the Peking Union Medical Hospital affiliated to Peking Union Medical College, Beijing, China. According to the assay protocols, the normal reference values were as follows: serum fibrinogen concentration ≤ 4.0 g/L and CA199 level ≤ 39 U/mL.

Clinical treatment and follow-up assessments

All patients were treated by modified radical cholecystectomy or radical cholecystectomy and received systemic therapy in the adjuvant setting. All patients were followed *via* telephone interviews. The patients were carefully followed at 3-mo intervals for the first 2 years after surgery, at 6-mo intervals during the third year, and at 1-year intervals thereafter. The date of

surgery marked the beginning of the follow-up period, which ended at the last follow-up visit (December 2017) or death.

Statistical analysis

Continuous variables are expressed as means \pm standard deviation for normally distributed variables (Kolmogorov-Smirnov test, $P > 0.05$) or as medians (range) for non-normally distributed variables, and categorical variables were expressed as frequencies and percentages. OS was defined as the time from surgery to death from any cause or the last follow-up. A receiver operating characteristic (ROC) curve for OS prediction was constructed to estimate the optimal cut-off value for plasma fibrinogen, which allowed us to treat this parameter as a binary variable. The optimal cut-off value was determined as the point on the ROC curve that maximizes the Youden index. The area under the ROC curve (AUC) was used to calculate discrimination ability. The associations between clinicopathological variables and pretreatment plasma fibrinogen levels were assessed using either the chi-square test or the trend version of the chi-square test, as appropriate. Survival curves were generated using the Kaplan-Meier method, and the log-rank test was used to evaluate survival differences between groups. A univariate analysis using the log-rank test was performed to screen variables that could potentially predict prognosis. The statistically significantly predictive variables were then included in a multivariate Cox regression model to determine the independent prognostic risk factors. Statistical analysis of the data was performed using Statistical Package for the Social Sciences (SPSS®, version 24.0; IBM Corp., Armonk, NY, United States). Statistical significance was defined as a two-sided $P < 0.05$.

RESULTS

Patient characteristics

The detailed baseline clinicopathological characteristics of the 154 GBC patients are displayed in Table 1. There were 91 (59.1%) women and 63 (40.9%) men, and 98 (63.6%) of the patients were > 60 years old. The median age at diagnosis was 64 years (range: 29-85 years). There were 75 (48.7%) patients with a history of gallstones before surgery. Thirty-eight (24.7%) patients had diabetes before surgery. The entire cohort comprised 150 (97.4%) adenocarcinoma carcinoma patients, 3 (1.9%) adenosquamous carcinoma patients and 1 (0.6%) papillary carcinoma patient. The majority of patients had moderately or well differentiated cancer [94 (61.0%) patients with moderately or well differentiated cancer, 60 (39.0%) patients with poorly differentiated cancer]. Fifty-eight (37.7%) patients had a positive resection margin. Tumor invasion depths of Tis-T1a, T1b-T2b, T3, and T4 were observed in 10 (6.5%), 29 (18.8%), 103 (66.9%), and 12 (7.8%) patients, respectively. In terms of lymph node metastasis, 98 (63.6%) patients were N0, 47 (30.5%)

Table 1 Baseline characteristics of 154 patients who underwent potential curative cholecystectomy *n* (%)

Characteristic	Patients (<i>n</i> = 154)
Age (yr)	64 (29-85)
≤ 60	56 (36.4)
> 60	98 (63.6)
Sex	
Male	63 (40.9)
Female	91 (59.1)
Cholecystolithiasis	
Absent	79 (51.3)
Present	75 (48.7)
Diabetes	
Absent	116 (75.3)
Present	38 (24.7)
Jaundice	
Absent	129 (83.8)
Present	25 (8.9)
Blood groups	
A	43 (27.9)
B	56 (36.4)
AB	9 (5.8)
O	46 (29.9)
Pathological types	
Adenosquamous carcinoma	3 (1.9)
Adenocarcinoma	150 (97.4)
Papilocarcinoma	1 (0.6)
Degree of differentiation	
Poor	60 (39.0)
Moderate-well	94 (61.0)
Resection margin status	
Negative	96 (62.3)
Positive	58 (37.7)
Maximum tumor diameter (cm)	3 (0.2-13)
≤ 2.45	68 (44.2)
> 2.45	86 (55.8)
T stage	
Tis-T1a	10 (6.5)
T1b-T2b	29 (18.8)
T3	103 (66.9)
T4	12 (7.8)
N stage	
0	98 (63.6)
1	47 (30.5)
2	9 (5.8)
Distant metastasis	
Absent	142 (92.2)
Present	12 (7.8)
TNM stage	
0- I stage	16 (10.4)
II A- II B stage	16 (10.4)
III A- III B stage	92 (59.7)
IV A- IV B stage	30 (19.5)
CA199 (U/mL)	69.3 (0.6-10524)
≤ 25.45	57 (37.0)
> 25.45	97 (63.0)
Fibrinogen concentration (g/L)	3.54 (1.71-7.47)
≤ 3.47	75 (48.7)
> 3.47	79 (51.3)

patients were N1 (1-3 positive lymph nodes), and 9 (5.8%) patients were N2 (≥ 4 positive lymph nodes). The majority [142 (92.2%)] of patients did not have distant metastasis. Of the 154 patients, 16 (10.4%) had stage 0- I disease, 16 (10.4%) had stage II A- II B, 92 (59.7%) had stage III A- III B, and 30 (19.5%) had stage IV A- IV B.

Association between plasma fibrinogen levels and patient clinicopathological characteristics

The median plasma fibrinogen concentration in all patients was 3.54 g/L (range: 1.71-7.47 g/L). The optimum cut-off value for plasma fibrinogen according to the ROC curve was 3.47 g/L, with a sensitivity of 0.709 and a specificity of 0.721 (Figure 1A); the AUC was 0.735 (95%CI: 0.654-0.816). The entire cohort was stratified into 2 groups for further analysis: group A, with a plasma fibrinogen concentration > 3.47 g/L, included 79 patients (51.3%); group B, with a plasma fibrinogen concentration ≤ 3.47 g/L, included 75 patients (48.7%) (Table 1). As shown in Table 2, an elevated plasma fibrinogen level was significantly correlated with resection margin ($P = 0.003$), degree of differentiation ($P = 0.048$), jaundice ($P = 0.003$), T stage ($P < 0.001$), CA199 level ($P = 0.003$) and TNM stage ($P = 0.011$), but was not significantly correlated with gender, age, gallstone history, comorbidity (diabetes), pathological type, N stage, distant metastasis, ABO blood group, or tumor size ($P > 0.05$).

Association between CA199 levels and patient clinicopathological characteristics

The median CA199 level in all patients was 69.3 U/mL (range: 0.6-10524 U/mL). The optimum cut-off value for CA199 according to the ROC curve was 25.45 U/mL, with a sensitivity of 0.791 and a specificity of 0.574 (Figure 1B); the AUC was 0.729 (95%CI: 0.650-0.808). The entire cohort was stratified into 2 groups for further analysis: group A, with a CA199 level > 25.45 U/mL, included 97 patients (63.0%); group B, with a CA199 level ≤ 25.45 U/mL, included 57 patients (37.0%) (Table 1). As shown in Table 3, an elevated CA199 level was significantly correlated with resection margin ($P = 0.001$), jaundice ($P = 0.022$), T stage ($P < 0.001$), plasma fibrinogen concentration ($P = 0.003$) and TNM stage ($P < 0.001$), but was not significantly correlated with other factors ($P > 0.05$).

Analysis of factors influencing prognosis

The median follow-up time was 17 mo. One hundred and three patients died during the follow-up period, with an estimated median OS duration of 14.5 mo (range: 0.5-153.0 mo). The 1-year and 2-year survival rates were 55.8% and 35.7%, respectively.

A Cox univariate analysis of OS showed that resection margin (HR: 3.683, 95%CI: 2.468-5.496, $P < 0.001$), distant metastasis (HR = 2.550, 95%CI: 1.388-4.684, $P = 0.003$), jaundice (HR = 2.598, 95%CI: 1.644-4.106, $P < 0.001$), CA199 level (HR = 3.570, 95%CI: 2.213-5.760, $P < 0.001$), lymph node metastasis ($P < 0.001$), degree of differentiation (HR = 1.527, 95%CI: 1.031-2.261, $P = 0.035$), T stage ($P < 0.001$), TNM stage ($P < 0.001$), and plasma fibrinogen level (HR = 2.795, 95%CI: 1.853-4.214, $P < 0.001$) were significantly associated with unfavorable OS (Table 4). The OS curve stratified by plasma fibrinogen level

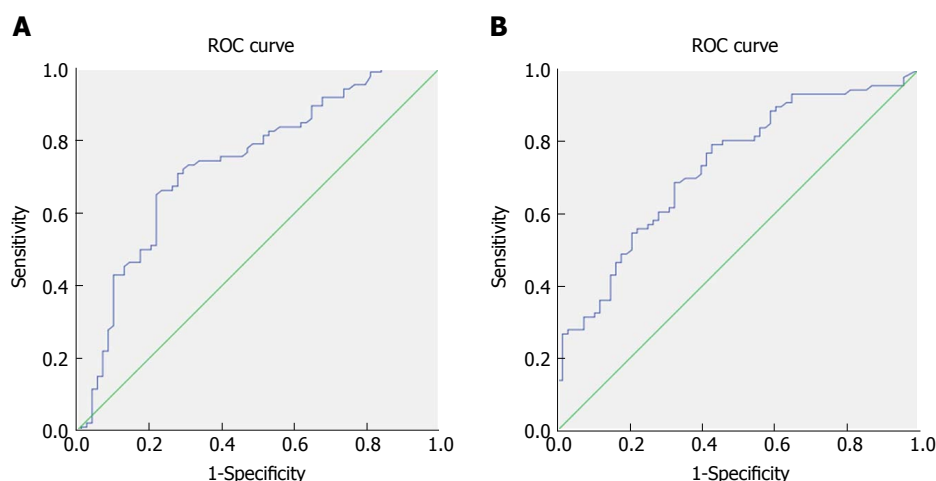


Figure 1 Receiver operating characteristic curve analysis based on fibrinogen for overall survival. A: The area under the ROC curve (AUC) indicates the diagnostic power of preoperative plasma fibrinogen concentration. In this model, the optimum cut-off point for fibrinogen concentration was 3.47 g/L, AUC was 0.735 (95%CI: 0.654-0.816), with a sensitivity of 0.709 and a specificity of 0.721 by the Youden index. B: AUC indicates the diagnostic power of preoperative CA199 level. In this model, the optimum cut-off point for CA199 level was 25.45 U/mL, AUC was 0.729 (95%CI: 0.650-0.808), with a sensitivity of 0.791 and a specificity of 0.574 by the Youden index. AUC: Area under curve. ROC: Receiver operating characteristic curve.

showed that GBC patients with a plasma fibrinogen level ≤ 3.47 g/L had longer OS durations than those with a plasma fibrinogen level > 3.47 g/L (Figure 2A). In addition, the OS curve stratified by CA199 showed that GBC patients with a CA199 level ≤ 25.45 U/mL had longer OS durations than those with a CA199 level > 25.45 U/mL (Figure 2B). Next, we selected the risk factors identified by the univariate analysis described above for multivariate Cox regression analysis of survival. Resection margin (HR = 1.971, 95%CI: 1.288-3.017, $P = 0.002$), TNM stage ($P = 0.003$), CA199 level (HR = 1.842, 95%CI: 1.111-3.056, $P = 0.018$) and plasma fibrinogen level (HR = 1.711, 95%CI: 1.114-2.627, $P = 0.014$) were identified as independent prognostic factors for GBC patient survival (Table 5).

Prognostic significance of the combination of plasma fibrinogen and CA199 in predicting the long-term survival of GBC patients

As shown by the above results of multivariate analysis, plasma fibrinogen and CA199 were independent prognostic biomarkers in GBC patients, but whether the combination of plasma fibrinogen and CA199 had the same efficacy remained unclear. As the HR for CA199/the HR for plasma fibrinogen = 1.842/1.711 approximately 1.10, patients with elevated plasma fibrinogen and CA199 levels were allocated a score of 2.1, those with an elevated plasma fibrinogen level only were allocated a score of 1, those with an elevated CA199 level only were allocated a score of 1.1, and those with neither of these abnormalities were allocated a score of 0. We then used the Kaplan-Meier method and a Cox regression model to investigate the prognostic significance of the combination of plasma fibrinogen and CA199 in these GBC patients.

Both the univariate and multivariate Cox regression

analyses of survival revealed that the combination of plasma fibrinogen and CA199 was an independent prognostic factor for survival in GBC patients following surgery (Table 6). The results of the OS curve are presented in Figure 3. Finally, a ROC curve was generated to assess the prognostic accuracy of the combination of plasma fibrinogen and CA199. The results showed that for OS, the AUC of the combination of plasma fibrinogen and CA199 was 0.765 (95%CI: 0.688-0.841) (Figure 4), which was higher than that of plasma fibrinogen (0.735, 95%CI: 0.654-0.816) (Figure 1A) and that of CA199 (0.729, 95%CI: 0.650-0.808) (Figure 1B). These results indicated that the combination of plasma fibrinogen and CA199 may serve as a significant prognostic biomarker that is superior to either plasma fibrinogen or CA199 alone.

DISCUSSION

The incidence of GBC appears to be increasing worldwide, creating an enormous public health and economic burden. Due to a lack of effective prognostic biomarkers, the prognosis of GBC is typically poor. In the present study, we investigated the correlations between biomarkers, clinicopathological characteristics, and survival in patients with GBC undergoing surgical resection. Our results showed that plasma fibrinogen, CA199, resection margin and TNM stage were independent prognostic factors associated with OS in patients with GBC. Elevated plasma fibrinogen and CA199 levels were significantly correlated with worse OS. Moreover, to the best of our knowledge, the current study indicated for the first time that the combination of plasma fibrinogen and CA199 was more efficient than plasma fibrinogen or CA199 alone in predicting the prognosis of GBC patients who have undergone surgical resection.

Table 2 Correlation between fibrinogen concentration and clinicopathological characteristics in gallbladder carcinoma patients *n* (%)

Characteristics	Fibrinogen concentration		<i>P</i> value
	≤ 3.47 g/L (<i>n</i> = 75)	> 3.47 g/L (<i>n</i> = 79)	
Age (yr)			
≤ 60	31 (20.1)	25 (16.2)	0.243
> 60	44 (28.6)	54 (35.1)	
Sex			
Male	33 (21.4)	30 (19.5)	0.513
Female	42 (27.3)	49 (31.8)	
Cholecystolithiasis			
Absent	38 (24.7)	41 (26.6)	0.878
Present	37 (24.0)	38 (24.7)	
Diabetes			
Absent	57 (37.0)	59 (38.3)	0.850
Present	18 (11.7)	20 (13.0)	
Jaundice			
Absent	68 (44.2)	61 (39.6)	0.029
Present	7 (4.5)	18 (11.7)	
Blood groups			
A	19 (12.3)	24 (15.6)	0.145
B	33 (21.4)	23 (14.9)	
AB	2 (1.3)	7 (4.5)	
O	21 (13.6)	25 (16.2)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.142
Adenocarcinoma	75 (48.7)	75 (48.7)	
Papilocarcinoma	0 (0)	1 (0.6)	
Degree of differentiation			
Poor	23 (14.9)	37 (24.0)	0.048
Moderate-well	52 (33.8)	42 (27.3)	
Resection margin status			
Negative	56 (36.4)	40 (26.4)	0.003
Positive	19 (12.3)	39 (25.3)	
Maximum tumor diameter (cm)			
≤ 2.45	34 (22.1)	34 (22.1)	0.871
> 2.45	41 (26.6)	45 (29.2)	
T stage			
Tis-T1a	8 (5.2)	2 (1.3)	< 0.001
T1b-T2b	22 (14.3)	7 (4.5)	
T3	43 (27.9)	60 (39.0)	
T4	2 (1.3)	10 (6.5)	
N stage			
N0	50 (32.5)	48 (31.2)	0.748
N1	21 (13.6)	26 (16.9)	
N2	4 (2.6)	5 (3.2)	
Distant metastasis			
Absent	69 (44.8)	73 (47.4)	0.925
Present	6 (3.9)	6 (3.9)	
TNM stage			
0- I stage	12 (7.8)	4 (2.6)	0.011
II A- II B stage	12 (7.8)	4 (2.6)	
III A- III B stage	39 (25.3)	53 (34.4)	
IV A-IV B stage	12 (7.8)	18 (11.7)	
CA199 (U/mL)			
≤ 25.45	37 (24.0)	20 (13.0)	0.003
> 25.45	38 (24.7)	59 (38.3)	

Due to the low incidence of GBC, few studies have examined the correlations between inflammation-related factors and GBC prognosis. The inflammation-related factors explored in previous studies include platelet count (PLT)^[35], platelet to lymphocyte ratio (PLR)^[32,33], neutrophil to lymphocyte ratio (NLR)^[36,37] and plasma fibrinogen level^[30].

Wang *et al*^[35] showed that a PLT > 178 × 10⁹/L was significantly correlated with worse prognosis of

GBC (HR = 1.541, 95%CI: 1.038-2.287, *P* = 0.032) and identified 178 × 10⁹/L as the optimal cut-off value (AUC = 0.798, 95%CI: 0.737-0.858, sensitivity: 0.746, specificity: 0.722). The prognostic accuracy of PLT for GBC was higher than that of plasma fibrinogen in our study (AUC = 0.735, 95%CI: 0.654-0.816, sensitivity: 0.709, specificity: 0.721). However, Pang *et al*^[32] and Zhang *et al*^[33] indicated that PLT was not associated with the prognosis of GBC (HR = 1.013,

Table 3 Correlation between CA199 level and clinicopathological characteristics in gallbladder carcinoma patients *n* (%)

Characteristics	CA199 level		<i>P</i> value
	≤ 25.45 U/mL (<i>n</i> = 57)	> 25.45 U/mL (<i>n</i> = 97)	
Age (yr)			
≤ 60	24 (15.6)	32 (20.8)	0.299
> 60	33 (21.4)	65 (42.2)	
Sex			
Male	23 (14.9)	40 (26.0)	0.914
Female	34 (21.1)	57 (37.0)	
Cholecystolithiasis			
Absent	32 (20.8)	47 (30.5)	0.406
Present	25 (16.2)	50 (32.5)	
Diabetes			
Absent	45 (29.2)	71 (46.1)	0.447
Present	12 (7.8)	26 (16.9)	
Jaundice			
Absent	53 (34.4)	76 (49.4)	0.022
Present	4 (2.6)	21 (13.6)	
Blood groups			
A	19 (12.3)	24 (15.6)	0.303
B	21 (13.6)	35 (22.7)	
AB	1 (0.6)	8 (5.2)	
O	16 (10.4)	30 (19.5)	
Pathological types			
Adenosquamous carcinoma	0 (0)	3 (1.9)	0.299
Adenocarcinoma	57 (37.0)	93 (60.4)	
Papillocarcinoma	0 (0)	1 (0.6)	
Degree of differentiation			
Poor	33 (21.4)	61 (39.6)	0.069
Moderate-well	24 (15.6)	36 (23.4)	
Resection margin status			
Negative	45 (29.2)	51 (33.1)	0.001
Positive	12 (7.8)	46 (29.9)	
Maximum tumor diameter (cm)			
≤ 2.45	30 (19.5)	38 (24.7)	0.131
> 2.45	27 (17.5)	59 (38.3)	
T stage			
Tis-T1a	7 (4.5)	3 (1.9)	< 0.001
T1b-T2b	18 (11.7)	11 (7.1)	
T3	32 (20.8)	71 (46.1)	
T4	0 (0.0)	12 (7.8)	
N stage			
N0	43 (27.9)	55 (35.7)	0.056
N1	11 (7.1)	36 (23.4)	
N2	3 (1.9)	6 (3.9)	
Distant metastasis			
Absent	53 (34.4)	89 (57.8)	0.783
Present	4 (2.6)	8 (5.2)	
TNM stage			
0- I stage	11 (7.1)	5 (3.2)	< 0.001
II A- II B stage	12 (7.8)	4 (2.6)	
III A- III B stage	27 (17.5)	65 (42.2)	
IV A- IV B stage	7 (4.5)	23 (14.9)	
Fibrinogen concentration (g/L)			
≤ 3.47	37 (24.0)	38 (24.7)	0.003
> 3.47	20 (13.0)	59 (38.3)	

95%CI: 0.647-1.594, $P = 0.956$, and HR = 1.172, 95%CI: 0.794-1.731, $P = 0.423$). These results may be related to the different cut-off values chosen ($300 \times 10^9/L$ and $200 \times 10^9/L$) and different sample sizes (316 patients and 145 patients). Therefore, the prognostic significance of PLT in GBC patients requires further validation.

Zhang *et al.*^[33] and Zhang *et al.*^[36] demonstrated that NLR was significantly associated with an un-

favorable prognosis of GBC (HR = 2.059, 95%CI: 1.253-3.384, $P = 0.004$, and HR = 1.65, $P < 0.001$), but the prognostic accuracy of NLR for GBC according to Lingqiang Zhang *et al.*^[36] (AUC = 0.637, 95%CI: 0.556-0.718, sensitivity: 0.713, specificity: 0.565) was not higher than that of plasma fibrinogen in our study (AUC = 0.735, 95%CI: 0.654-0.816, sensitivity: 0.709, specificity: 0.721).

Pang *et al.*^[32] showed that PLR was a negative

Table 4 Univariate analysis of overall survival in gallbladder cancer patients

Characteristics	HR (95%CI)	P value
Age (yr)	1.473 (0.973-2.230)	0.067
≤ 60		
> 60		
Sex	0.995 (0.670-1.477)	0.981
Male		
Female		
Cholecystolithiasis	1.198 (0.814-1.764)	0.360
Absent		
Present		
Diabetes	1.028 (0.651-1.623)	0.906
Absent		
Present		
Jaundice	2.598 (1.644-4.106)	< 0.001
Absent		
Present		
Blood groups	-	0.113
A		
B		
AB		
O		
Pathological types	-	0.165
Adenosquamous carcinoma		
Adenocarcinoma		
Papilocarcinoma		
Degree of differentiation	1.527 (1.031-2.261)	0.035
Poor		
Moderate-well		
Resection margin status	3.683 (2.468-5.496)	< 0.001
Negative		
Positive		
Maximum tumor diameter (cm)	1.101 (0.744-1.630)	0.631
≤ 2.45		
> 2.45		
T stage	-	< 0.001
Tis-T1a		
T1b-T2b		
T3		
T4		
N stage	-	< 0.001
N0		
N1		
N2		
Distant metastasis	2.550 (1.388-4.684)	< 0.003
Absent		
Present		
TNM stage	-	< 0.001
0- I stage		
II A- II B stage		
III A- III B stage		
IV A- IV B stage		
CA199 (U/mL)	3.570 (2.213-5.760)	< 0.001
≤ 25.45		
> 25.45		
Fibrinogen concentration (g/L)	2.790 (1.853-4.214)	< 0.001
≤ 3.47		
> 3.47		

prognostic factor for GBC patients (HR = 2.02, 95%CI: 1.24-3.28, $P < 0.001$), although the prognostic accuracy of PLR for GBC (AUC = 0.620, 95%CI: 0.542-0.698, $P = 0.040$, sensitivity: 0.736, specificity: 0.532) was not superior to that of plasma fibrinogen in our study (AUC = 0.735, 95%CI: 0.654-0.816, sensitivity: 0.709, specificity: 0.721). However, Zhang *et al*^[33] showed that PLR was not associated with

the prognosis of patients with GBC. Therefore, the prognostic significance of PLR in GBC patients requires further validation.

To date, the only study to explore the prognostic significance of plasma fibrinogen in GBC patients was conducted by Shu *et al*^[30]. Consistent with the results of our study, their results indicated that an elevated preoperative plasma fibrinogen level, poorer margin

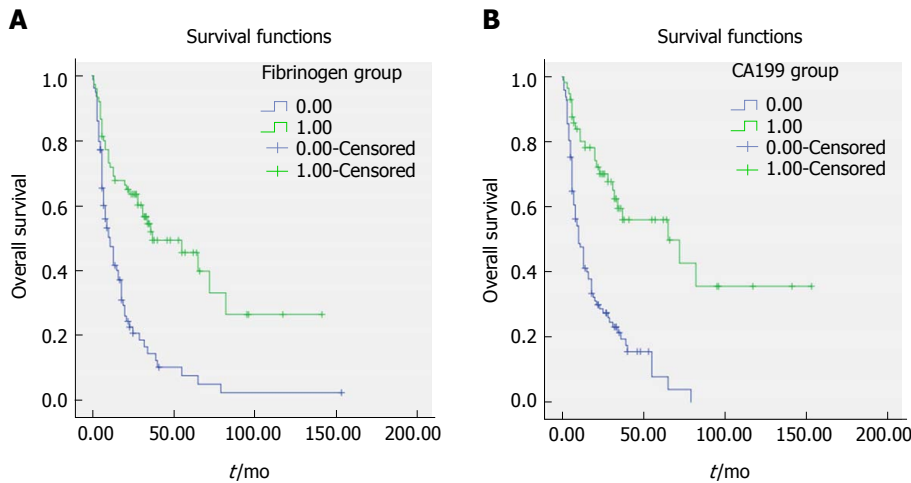


Figure 2 Survival curve according to the preoperative fibrinogen concentration (A) and CA199 level (B). A: Data compares fibrinogen concentration > 3.47 g/L vs ≤ 3.47 g/L group ($P < 0.05$). The number 1 for ≤ 3.47 g/L group, number 2 for > 3.47 g/L group. B: Data compares CA199 level > 25.45 U/mL vs ≤ 25.45 U/mL ($P < 0.05$). The number 1 for ≤ 25.45 U/mL group, number 2 for > 25.45 U/mL group.

Table 5 Multivariate analysis of overall survival in gallbladder cancer patients

Characteristics	HR (95%CI)	Wald	P value
Resection margin status	1.971 (1.288-3.017)		0.002
Negative			
Positive			
TNM stage		11.299	0.003
II A-II B stage/0-1 stage	1.336 (0.317-5.627)	0.156	0.693
III A-III B stage/0-1 stage	3.831 (1.167-12.571)	4.907	0.027
IV A-IV B stage/0-1 stage	5.204 (1.497-18.093)	6.730	0.009
Fibrinogen concentration (g/L)	1.711 (1.114-2.627)		0.014
≤ 3.47			
> 3.47			
CA199 (U/mL)	1.842 (1.111-3.056)		0.018
≤ 25.45			
> 25.45			

Table 6 Univariate and multivariate analysis of overall survival in gallbladder cancer patients according to the combination of fibrinogen and CA199

Characteristics	HR (95%CI)	Wald	P value
Univariate analysis			
Combined fibrinogen and CA199	-		< 0.001
0			
1			
1.1			
2.1			
Multivariate analysis			
Resection margin status	1.973 (1.289-3.020)		0.002
Negative			
Positive			
TNM stage	11.299		0.011
IIA-IIB stage/0-1 stage	1.342 (0.318-5.659)	0.160	0.689
III A-III B stage/0-1 stage	3.812 (1.158-12.545)	4.848	0.028
IV A-IV B stage/0-1 stage	5.189 (1.491-18.055)	6.699	0.010
Combined fibrinogen and CA199	14.218		0.003
1/0	1.784 (0.775-4.104)	1.854	0.173
1.1/0	1.895 (0.943-3.806)	3.222	0.073
2.1/0	3.195 (1.676-6.090)	12.454	0.000

The number 0 for fibrinogen concentration > 3.47g/L with CA199 level > 25.45 U/mL group, number 1 for fibrinogen concentration > 3.47 g/L with CA199 level ≤ 25.45 U/mL group, number 1.1 for fibrinogen concentration ≤ 3.47g/L with CA199 level > 25.45 U/mL group, and number 2.1 for fibrinogen concentration ≤ 3.47 g/L with CA199 level ≤ 25.45 U/mL group.

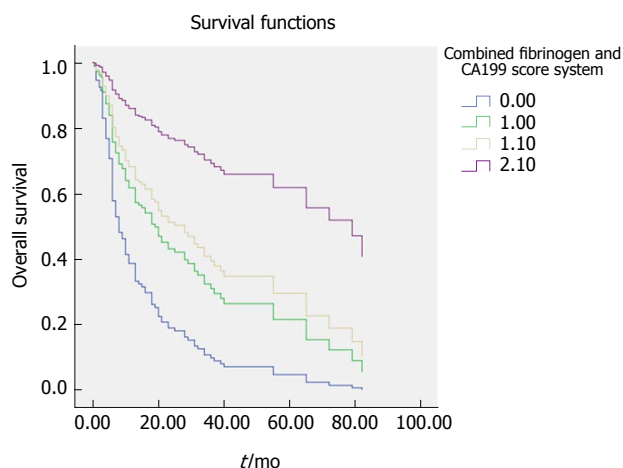


Figure 3 Survival curve according to the combined fibrinogen and CA199 scoring system. Data compares the fibrinogen concentration > 3.47 g/L with CA199 level > 25.45 U/mL group, fibrinogen concentration > 3.47 g/L with CA199 level ≤ 25.45 U/mL group, fibrinogen concentration ≤ 3.47 g/L with CA199 level > 25.45 U/mL group and fibrinogen concentration ≤ 3.47 g/L with CA199 level ≤ 25.45 U/mL group. The number 0 for fibrinogen concentration > 3.47 g/L with CA199 level > 25.45 U/mL group, number 1 for fibrinogen concentration > 3.47 g/L with CA199 level ≤ 25.45 U/mL group, number 1.1 for fibrinogen concentration ≤ 3.47 g/L with CA199 level > 25.45 U/mL group, number 2.1 for fibrinogen concentration ≤ 3.47 g/L with CA199 level ≤ 25.45 U/mL group.

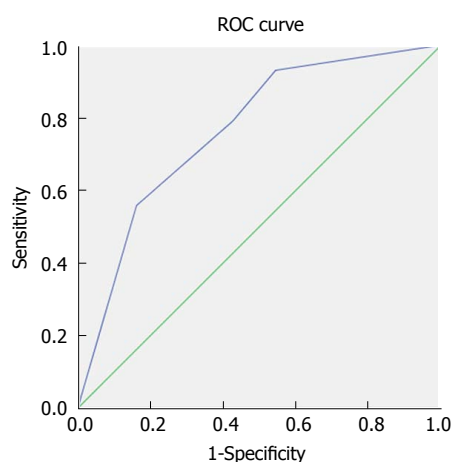


Figure 4 Receiver operating characteristic curve analysis based on the combination of fibrinogen and CA199 for overall survival. AUC indicates the diagnostic power of the combination of fibrinogen and CA199. In this model, AUC was 0.765 (95%CI: 0.688-0.841). AUC: Area under curve.

status and higher TNM stage were independently associated with worse OS. They also showed that lymphatic metastasis was a negative prognostic factor for GBC, but in our study, lymphatic metastasis was not identified as a prognostic factor. This difference may be related to the TNM stage classification standard used in the present study. The newly published AJCC-8 indicated that the number of positive lymph nodes rather than the location of lymph node metastasis is closely related to the prognosis of GBC. The new N stage defined 1-3 positive lymph nodes

as N1, 4 or more positive lymph nodes as N2, and no positive lymph nodes as N0.

The prognostic significance of plasma fibrinogen in the study conducted by Shu *et al.*^[30] (AUC = 0.751, 95%CI: 0.653-0.848) was slightly superior to that observed in our study (AUC = 0.735, 95%CI: 0.654-0.816, sensitivity: 0.709, specificity: 0.721) and had a positive predictive value of 92.73%. This difference may be related to the different optimal cut-off values between the two studies (4.02 g/L and 3.47 g/L) and the different methods used to determine the optimal cut-off values. In their study, the dichotomous variable that was used to determine the optimal cut-off value on the ROC curve was TNM stage, whereas the dichotomous variable used in our study was OS. Our method is accepted by most researchers. Nonetheless, the results of their study support the conclusion reached in our study that the prognostic accuracy of the combination of plasma fibrinogen and CA199 (AUC = 0.765, 95%CI: 0.688-0.841) is higher than that of plasma fibrinogen alone (AUC = 0.751, 95%CI: 0.653-0.848).

Many recent studies have demonstrated that an elevated plasma fibrinogen level, as a marker of coagulation and fibrinolytic activation, is a strong predictor of poor prognosis for various malignant tumors^[13,15,19,23,25]. Additionally, it was found that fibrinogen synthesis is significantly upregulated by inflammation^[38]. However, the molecular mechanisms underlying the association between plasma fibrinogen and cancer prognosis is still uncertain. In an *in vitro* study, Shu *et al.*^[30] found that plasma fibrinogen at a high concentration induced epithelial-mesenchymal transition (EMT), thus increasing the migration, invasion, and metastatic capacity of co-cultured GBC cells by increasing the expression of vimentin (a mesenchymal marker) and reducing the expression of E-cadherin (an epithelial marker). EMT is known to confer migration, invasion, and metastatic capacity and multidrug resistance to cells^[38,39]. However, this explanation needs to be verified through basic research in the future.

One of the methodological innovations of our study is that we assigned different scores to patients according to the HR for elevated plasma fibrinogen levels and elevated CA199 levels, instead of assigning all patients a score of 1 as in the previous scoring system. This new scoring method further distinguished the difference in prognostic efficiency between plasma fibrinogen and CA199, rather than simply assigning a score of 1 to each parameter, considering that the HRs for the two parameters are not the same (CA199: 1.842, plasma fibrinogen: 1.711). From the OS curve (Figure 3) and ROC curve (Figure 4), we observed that the new scoring system effectively improved the prognostic accuracy of the biomarkers.

This study is the first to investigate the prognostic significance of the combination of plasma fibrinogen

and CA199 in GBC patients. Inevitably, our study has some limitations that should be acknowledged. First, this study was performed using a retrospective design. Second, although this new scoring method distinguished the prognostic value of different biomarkers and improved prognostic accuracy, the validity and predictive value of this scoring method still require further verification. Third, the data were obtained from a single institution and the sample size was relatively small, which may have influenced the final conclusions. Fourth, to obtain more data, we did not distinguish between patients who underwent radical surgery and those who received palliative cholecystectomy or extended resection. Therefore, our results should be validated by prospective, multicenter studies with a large sample size.

In conclusion, elevated preoperative levels of both plasma fibrinogen and CA199 are independent prognostic factors for GBC. Additionally, the combination of plasma fibrinogen and CA199 showed superior prognostic accuracy compared with either parameter alone. Therefore, the combination of plasma fibrinogen and CA199 can facilitate the identification of GBC patients with poorer survival prognosis before surgery. We hypothesize that the combination of plasma fibrinogen and CA199 could be used as an inexpensive, simple, reliable and reproducible method to determine GBC prognosis in clinical practice.

ARTICLE HIGHLIGHTS

Research background

Gallbladder cancer is a rare hepatobiliary tumor with a relatively low incidence. Due to the lack of significant specific symptoms at the early stage, the prognosis of gallbladder cancer is poor and can be fatal. Therefore, determining a convenient and cost-effective prognostic biomarker is urgently required for patients with gallbladder cancer. Elevated fibrinogen has been demonstrated to be associated with poor prognosis in multiple malignancies, while CA199 is considered a widely accepted diagnostic and prognostic marker of gallbladder cancer. There have been very few studies on the role of fibrinogen in the prognosis of patients with gallbladder cancer. To date, studies on the combined use of fibrinogen and CA199 to predict the prognosis of patients with gallbladder cancer have not been conducted. The combined use of fibrinogen and CA199 avoids inconsistencies caused by the use of a single indicator and enhances predictive efficacy.

Research motivation

The main aim of this study was to validate and identify a convenient and inexpensive combination of biomarkers with a higher prognostic value for patients with gallbladder cancer. From our research, we found that the combination of preoperative plasma fibrinogen and CA199 was a more efficient prognostic factor than either parameter alone in patients with gallbladder cancer. Due to this finding, we can screen potential high-risk candidates for gallbladder cancer and provide prognostic guidance for surgical patients with gallbladder cancer. In addition, this study also provides clinical evidence and preconditions for the future study of how fibrinogen promotes the proliferation and metastasis of malignant tumor cells.

Research objectives

The main objective of this study was to identify a convenient and more efficient prognostic biomarker for gallbladder cancer patients. From this study, we found that both elevated fibrinogen and elevated CA199 were independent risk factors

for gallbladder cancer patients. Furthermore, the combination of preoperative plasma fibrinogen and CA199 was a more efficient prognostic factor than either parameter alone in patients with gallbladder cancer. These findings not only provide a further example which proves the relationship between hemostasis and tumor, but also provide powerful clinical evidence for related basic research in the future.

Research methods

We used an Excel table to organize research-related clinical data, and imported these variables into SPSS 24.0 statistical software. We then assigned the different types of variables appropriately. We determined the optimal cut-off values for fibrinogen and CA199 by plotting ROC curves, and then determined the association of fibrinogen and CA199 with other clinicopathological variables using the $R \times C$ table. Finally, univariate and multivariate analyses were performed to determine the independent prognostic factors in patients with gallbladder cancer.

Given the different HRs of the two parameters (CA199: 1.842, plasma fibrinogen: 1.711), one methodological innovation of this study was that we assigned different scores to elevated plasma fibrinogen levels and elevated CA199 levels, instead of assigning the same score as in the previous scoring system. This scoring approach further differentiates the difference in the prognostic efficiency between plasma fibrinogen and CA199, rather than simply assigning each parameter with 1 point. Based on the overall survival curve (Figure 3) and the ROC curve (Figure 4) in the text, we observed that the new scoring system effectively improved the prognostic accuracy of the biomarkers.

Research results

Our study demonstrated that the best cut-off values for pretreatment fibrinogen and CA199 were 3.47 g/L and 25.45 U/mL, respectively, in patients with gallbladder cancer. After single factor and multivariate analysis, it was shown that elevated pretreatment fibrinogen, elevated pretreatment CA199, resection margin and TNM stage were independent risk factors for gallbladder cancer patients. When the elevated pretreatment fibrinogen and elevated pretreatment CA199 were combined with different assigned scores according to their different HRs, the prognostic accuracy and power was significantly improved (the AUROC increased to 0.765, a relatively high value). These research findings confirm the relationship between hemostatic factors and cancer, in this case gallbladder cancer. How does the hemostatic factor fibrinogen influence the development, growth, and metastasis of gallbladder cancer cells? The underlying mechanism is still unknown, and further studies are required to identify and confirm the mechanism involved.

Research conclusions

In the present study, we found that the combination of preoperative plasma fibrinogen and CA199 is a more efficient prognostic factor than either parameter alone in patients with gallbladder cancer. We proposed that the combination of hemostatic factor and specific oncology markers can better predict the prognosis of gallbladder cancer, as hemostatic and oncology markers can compensate for each other's inconsistency in predicting tumor prognosis and thus enhance overall prognostic efficacy. Fibrinogen is associated with the development, growth, and metastasis of cancer cells, and CA199 is a product of tumor cell growth and metabolism. However, from our study findings, we observed that they had different prognostic efficacy (as they had different HRs) in gallbladder cancer patients; therefore, we assigned different scores to them which differed from the previous traditional scoring system. Using this new method, the prognostic efficacy of these two prognostic biomarkers combined was significantly improved, and this combination was used to screen potential high-risk gallbladder cancer candidates, identify appropriate surgical patients, and adopt the best follow-up strategy.

Research perspectives

In this study, we found that the combination of a hemostatic factor and oncology factor could compensate for each other's inconsistency in predicting tumor prognosis and improve the overall prognostic efficacy. The combination of these factors was more efficient than either parameter alone in predicting the prognosis of gallbladder cancer patients. These factors are inexpensive, easy-to-use and highly accurate for determining the prognosis of gallbladder cancer

patients. Further large-scale, well-designed and prospective studies to verify the findings and conclusions of this investigation are required.

REFERENCES

- Hundal R, Shaffer EA. Gallbladder cancer: epidemiology and outcome. *Clin Epidemiol* 2014; **6**: 99-109 [PMID: 24634588 DOI: 10.2147/CLEP.S37357]
- Wu XS, Shi LB, Li ML, Ding Q, Weng H, Wu WG, Cao Y, Bao RF, Shu YJ, Ding QC, Mu JS, Gu J, Dong P, Liu YB. Evaluation of two inflammation-based prognostic scores in patients with resectable gallbladder carcinoma. *Ann Surg Oncol* 2014; **21**: 449-457 [PMID: 24081806 DOI: 10.1245/s10434-013-3292-z]
- Liu YB, He XW, Wang JW, Li JT, Li KQ, Liu FB, Xue JF, Zhu JH, Li B, Peng SY. [Establishment of liver metastasis model of human gallbladder cancer and isolation of the subpopulation with high metastatic potential]. *Zhonghua Yi Xue Za Zhi* 2006; **86**: 2117-2121 [PMID: 17064616]
- Boutros C, Gary M, Baldwin K, Somasundar P. Gallbladder cancer: past, present and an uncertain future. *Surg Oncol* 2012; **21**: e183-e191 [PMID: 23025910 DOI: 10.1016/j.suronc.2012.08.002]
- Srivastava K, Srivastava A, Mittal B. Potential biomarkers in gallbladder cancer: present status and future directions. *Biomarkers* 2013; **18**: 1-9 [PMID: 22931385 DOI: 10.3109/1354750X.2012.717105]
- Choi SB, Han HJ, Kim CY, Kim WB, Song TJ, Suh SO, Kim YC, Choi SY. Fourteen year surgical experience of gallbladder cancer: validity of curative resection affecting survival. *Hepatogastroenterology* 2012; **59**: 36-41 [PMID: 22251521 DOI: 10.5754/hge10297]
- Wang RT, Xu XS, Liu J, Liu C. Gallbladder carcinoma: analysis of prognostic factors in 132 cases. *Asian Pac J Cancer Prev* 2012; **13**: 2511-2514 [PMID: 22938413]
- Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; **357**: 539-545 [PMID: 11229684 DOI: 10.1016/S0140-6736(00)04046-0]
- Wang X, Wang E, Kavanagh JJ, Freedman RS. Ovarian cancer, the coagulation pathway, and inflammation. *J Transl Med* 2005; **3**: 25 [PMID: 15969748 DOI: 10.1186/1479-5876-3-25]
- Lawrence SO, Simpson-Haidaris PJ. Regulated de novo biosynthesis of fibrinogen in extrahepatic epithelial cells in response to inflammation. *Thromb Haemost* 2004; **92**: 234-243 [PMID: 15269818 DOI: 10.1160/TH04-01-0024]
- Collen D, Tytgat GN, Claeys H, Piessens R. Metabolism and distribution of fibrinogen. I. Fibrinogen turnover in physiological conditions in humans. *Br J Haematol* 1972; **22**: 681-700 [PMID: 5064500]
- Koenig W. Fibrin (ogen) in cardiovascular disease: an update. *Thromb Haemost* 2003; **89**: 601-609 [PMID: 12669113]
- Kim KH, Park TY, Lee JY, Lee SM, Yim JJ, Yoo CG, Kim YW, Han SK, Yang SC. Prognostic significance of initial platelet counts and fibrinogen level in advanced non-small cell lung cancer. *J Korean Med Sci* 2014; **29**: 507-511 [PMID: 24753697 DOI: 10.3346/jkms.2014.29.4.507]
- Ghanim B, Hoda MA, Klikovits T, Winter MP, Alimohammadi A, Grusch M, Dome B, Arns M, Schenk P, Jakopovic M, Samarzija M, Brcic L, Filipits M, Laszlo V, Klepetko W, Berger W, Hegedus B. Circulating fibrinogen is a prognostic and predictive biomarker in malignant pleural mesothelioma. *Br J Cancer* 2014; **110**: 984-990 [PMID: 24434429 DOI: 10.1038/bjc.2013.815]
- Zhang SS, Lei YY, Cai XL, Yang H, Xia X, Luo KJ, Su CH, Zou JY, Zeng B, Hu Y, Luo HH. Preoperative serum fibrinogen is an independent prognostic factor in operable esophageal cancer. *Oncotarget* 2016; **7**: 25461-25469 [PMID: 27009857 DOI: 10.18632/oncotarget.8171]
- Wang GY, Jiang N, Yi HM, Wang GS, Zhang JW, Li H, Zhang J, Zhang Q, Yang Y, Chen GH. Pretransplant Elevated Plasma Fibrinogen Level is a Novel Prognostic Predictor for Hepatocellular Carcinoma Recurrence and Patient Survival Following Liver Transplantation. *Ann Transplant* 2016; **21**: 125-130 [PMID: 26903139]
- Yamamoto M, Kurokawa Y, Miyazaki Y, Makino T, Takahashi T, Yamasaki M, Nakajima K, Takiguchi S, Mori M, Doki Y. Usefulness of Preoperative Plasma Fibrinogen Versus Other Prognostic Markers for Predicting Gastric Cancer Recurrence. *World J Surg* 2016; **40**: 1904-1909 [PMID: 26969673 DOI: 10.1007/s00268-016-3474-5]
- Hong T, Shen D, Chen X, Wu X, Hua D. Preoperative plasma fibrinogen, but not D-dimer might represent a prognostic factor in non-metastatic colorectal cancer: A prospective cohort study. *Cancer Biomark* 2017; **19**: 103-111 [PMID: 28269756 DOI: 10.3233/CBM-160510]
- Zhao K, Deng H, Qin Y, Liao W, Liang W. Prognostic significance of pretreatment plasma fibrinogen and platelet levels in patients with early-stage cervical cancer. *Gynecol Obstet Invest* 2015; **79**: 25-33 [PMID: 25278089 DOI: 10.1159/000365477]
- Seebacher V, Polterauer S, Grimm C, Husslein H, Leipold H, Hefler-Frischmuth K, Tempfer C, Reinthaller A, Hefler L. The prognostic value of plasma fibrinogen levels in patients with endometrial cancer: a multi-centre trial. *Br J Cancer* 2010; **102**: 952-956 [PMID: 20160724 DOI: 10.1038/sj.bjc.6605547]
- Bekos C, Grimm C, Brodowicz T, Petru E, Heffler L, Reimer D, Koch H, Reinthaller A, Polterauer S, Polterauer M. Prognostic role of plasma fibrinogen in patients with uterine leiomyosarcoma - a multicenter study. *Sci Rep* 2017; **7**: 14474 [PMID: 29101329 DOI: 10.1038/s41598-017-13934-8]
- Mei Y, Zhao S, Lu X, Liu H, Li X, Ma R. Clinical and Prognostic Significance of Preoperative Plasma Fibrinogen Levels in Patients with Operable Breast Cancer. *PLoS One* 2016; **11**: e0146233 [PMID: 26799214 DOI: 10.1371/journal.pone.0146233]
- Selzer E, Grah A, Heiduschka G, Kornek G, Thurnher D. Pretherapeutic fibrinogen levels are of prognostic significance in locally advanced head and neck cancer. *Wien Klin Wochenschr* 2016; **128**: 320-328 [PMID: 26919854 DOI: 10.1007/s00508-016-0963-3]
- Holzinger D, Danilovic I, Seemann R, Kornek G, Engelmann J, Pillerstorf R, Holawe S, Psyri A, Erovic BM, Farwell G, Perisanidis C. Prognostic Impact of Pretreatment Plasma Fibrinogen in Patients with Locally Advanced Oral and Oropharyngeal Cancer. *PLoS One* 2016; **11**: e0158697 [PMID: 27362659 DOI: 10.1371/journal.pone.0158697]
- Zhang B, Song Y, Jin J, Zhou LQ, He ZS, Shen C, He Q, Li J, Liu LB, Wang C, Chen XY, Fan Y, Hu S, Zhang L, Yu W, Han WK. Preoperative Plasma Fibrinogen Level Represents an Independent Prognostic Factor in a Chinese Cohort of Patients with Upper Tract Urothelial Carcinoma. *PLoS One* 2016; **11**: e0150193 [PMID: 26930207 DOI: 10.1371/journal.pone.0150193]
- Ma C, Zhou Y, Zhou S, Zhao K, Lu B, Sun E. Preoperative peripheral plasma fibrinogen level is an independent prognostic marker in penile cancer. *Oncotarget* 2017; **8**: 12355-12363 [PMID: 27738342 DOI: 10.18632/oncotarget.12563]
- Troppan KT, Melchardt T, Wenzl K, Schlick K, Deutsch A, Bullock MD, Reitz D, Beham-Schmid C, Weiss L, Neureiter D, Tränkenschuh W, Greil R, Neumeister P, Egle A, Pichler M. The clinical significance of fibrinogen plasma levels in patients with diffuse large B cell lymphoma. *J Clin Pathol* 2016; **69**: 326-330 [PMID: 26644520 DOI: 10.1136/jclinpath-2015-203356]
- Zhang D, Zhou X, Bao W, Chen Y, Cheng L, Qiu G, Sheng L, Ji Y, Du X. Plasma fibrinogen levels are correlated with postoperative distant metastasis and prognosis in esophageal squamous cell carcinoma. *Oncotarget* 2015; **6**: 38410-38420 [PMID: 26334098 DOI: 10.18632/oncotarget.4800]
- Zhu LR, Li J, Chen P, Jiang Q, Tang XP. Clinical significance of plasma fibrinogen and D-dimer in predicting the chemotherapy efficacy and prognosis for small cell lung cancer patients. *Clin Transl Oncol* 2016; **18**: 178-188 [PMID: 26184726 DOI: 10.1007/s12094-015-1350-7]
- Shu YJ, Weng H, Bao RF, Wu XS, Ding Q, Cao Y, Wang XA, Zhang F, Xiang SS, Li HF, Li ML, Mu JS, Wu WG, Liu YB. Clinical and prognostic significance of preoperative plasma hyperfibrinogenemia in gallbladder cancer patients following surgical resection: a retrospective and in vitro study. *BMC Cancer*

- 2014; **14**: 566 [PMID: 25096189 DOI: 10.1186/1471-2407-14-566]
- 31 **Wang YF**, Feng FL, Zhao XH, Ye ZX, Zeng HP, Li Z, Jiang XQ, Peng ZH. Combined detection tumor markers for diagnosis and prognosis of gallbladder cancer. *World J Gastroenterol* 2014; **20**: 4085-4092 [PMID: 24744600 DOI: 10.3748/wjg.v20.i14.4085]
- 32 **Pang Q**, Zhang LQ, Wang RT, Bi JB, Zhang JY, Qu K, Liu SS, Song SD, Xu XS, Wang ZX, Liu C. Platelet to lymphocyte ratio as a novel prognostic tool for gallbladder carcinoma. *World J Gastroenterol* 2015; **21**: 6675-6683 [PMID: 26074706 DOI: 10.3748/wjg.v21.i21.6675]
- 33 **Zhang Y**, Jiang C, Li J, Sun J, Qu X. Prognostic significance of preoperative neutrophil/lymphocyte ratio and platelet/lymphocyte ratio in patients with gallbladder carcinoma. *Clin Transl Oncol* 2015; **17**: 810-818 [PMID: 26077119 DOI: 10.1007/s12094-015-1310-2]
- 34 **CI A**. [Rapid physiological coagulation method in determination of fibrinogen]. *Acta Haematol* 1957; **17**: 237-246 [PMID: 13434757 DOI: 10.1159/000205234]
- 35 **Wang RT**, Zhang LQ, Mu YP, Li JB, Xu XS, Pang Q, Sun LK, Zhang X, Dong SB, Wang L, Liu C. Prognostic significance of preoperative platelet count in patients with gallbladder cancer. *World J Gastroenterol* 2015; **21**: 5303-5310 [PMID: 25954104 DOI: 10.3748/wjg.v21.i17.5303]
- 36 **Zhang L**, Wang R, Chen W, Xu X, Dong S, Fan H, Liu C. Prognostic significance of neutrophil to lymphocyte ratio in patients with gallbladder carcinoma. *HPB (Oxford)* 2016; **18**: 600-607 [PMID: 27346141 DOI: 10.1016/j.hpb.2016.03.608]
- 37 **Whelton SP**, Narla V, Blaha MJ, Nasir K, Blumenthal RS, Jenny NS, Al-Mallah MH, Michos ED. Association between resting heart rate and inflammatory biomarkers (high-sensitivity C-reactive protein, interleukin-6, and fibrinogen) (from the Multi-Ethnic Study of Atherosclerosis). *Am J Cardiol* 2014; **113**: 644-649 [PMID: 24393259 DOI: 10.1016/j.amjcard.2013.11.009]
- 38 **Romano S**, Staibano S, Greco A, Brunetti A, Nappo G, Ilardi G, Martinelli R, Sorrentino A, Di Pace A, Mascolo M, Bisogni R, Scalvenzi M, Alfano B, Romano MF. FK506 binding protein 51 positively regulates melanoma stemness and metastatic potential. *Cell Death Dis* 2013; **4**: e578 [PMID: 23559012 DOI: 10.1038/cddis.2013.109]
- 39 **He L**, Zhou X, Qu C, Hu L, Tang Y, Zhang Q, Liang M, Hong J. Musashi2 predicts poor prognosis and invasion in hepatocellular carcinoma by driving epithelial-mesenchymal transition. *J Cell Mol Med* 2014; **18**: 49-58 [PMID: 24305552 DOI: 10.1111/jcmm.12158]

P- Reviewer: Armellini E, Osuga T, Tokunaga Y **S- Editor:** Gong ZM
L- Editor: Webster JR **E- Editor:** Huang Y



Retrospective Study

Fecal microbial dysbiosis in Chinese patients with inflammatory bowel disease

Hai-Qin Ma, Ting-Ting Yu, Xiao-Jing Zhao, Yi Zhang, Hong-Jie Zhang

Hai-Qin Ma, Ting-Ting Yu, Xiao-Jing Zhao, Yi Zhang, Hong-Jie Zhang, Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

ORCID number: Hai-Qin Ma (0000-0002-2900-5994); Ting-Ting Yu (0000-0003-3433-9013); Xiao-Jing Zhao (0000-0001-5156-3864); Yi Zhang (0000-0002-3072-6043); Hong-Jie Zhang (0000-0003-4497-0503).

Author contributions: Ma HQ and Zhang HJ conceived the study; Ma HQ and Yu TT performed the research; Zhao XJ and Zhang Y analyzed the data; Ma HQ wrote this manuscript; Zhang HJ supervised the report.

Supported by the National Natural Science Foundation of China, No. 81470827.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors declare no conflicts of interest related to this article.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Hong-Jie Zhang, MD, PhD, Professor,

Department of Gastroenterology, First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu Province, China. hjzhang06@163.com
Telephone: +86-25-83718836-6920
Fax: +86-25-83674636

Received: January 10, 2018

Peer-review started: January 10, 2018

First decision: February 5, 2018

Revised: March 5, 2018

Accepted: March 7, 2018

Article in press: March 7, 2018

Published online: April 7, 2018

Abstract**AIM**

To analyze the alterations of fecal microbiota in Chinese patients with inflammatory bowel disease (IBD).

METHODS

Fecal samples from 15 patients with Crohn's disease (CD) (11 active CD, 4 inactive CD), 14 patients with active ulcerative colitis (UC) and 13 healthy individuals were collected and subjected to 16S ribosomal DNA (rDNA) gene sequencing. The V4 hypervariable regions of 16S rDNA gene were amplified from all samples and sequenced by the Illumina MiSeq platform. Quality control and operational taxonomic units classification of reads were calculated with QIIME software. Alpha diversity and beta diversity were displayed with R software.

RESULTS

Community richness (chao) and microbial structure in both CD and UC were significantly different from those in normal controls. At the phyla level, analysis of the microbial compositions revealed a significantly greater abundance of *Proteobacteria* in IBD as compared to

that in controls. At the genera level, 8 genera in CD and 23 genera in UC (in particular, the *Escherichia* genus) showed significantly greater abundance as compared to that in normal controls. The relative abundance of *Bacteroidetes* in the active CD group was markedly lower than that in the inactive CD group. The abundance of *Proteobacteria* in patients with active CD was nominally higher than that in patients with inactive CD; however, the difference was not statistically significant after correction. Furthermore, the relative abundance of *Bacteroidetes* showed a negative correlation with the CD activity index scores.

CONCLUSION

Our study profiles specific characteristics and microbial dysbiosis in the gut of Chinese patients with IBD. *Bacteroidetes* may have a negative impact on inflammatory development.

Key words: Crohn's disease; Ulcerative colitis; Chinese; Microbial dysbiosis; 16S ribosomal DNA

© The Author(s) 2018. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Intestinal microbiota plays an important role in the pathogenesis of inflammatory bowel disease. However, there are few data on global alteration of microbiota in Chinese patients. In this study, fecal samples were subjected to 16S ribosomal DNA sequencing. Community richness and microbial structure in inflammatory bowel disease were significantly different from those in normal controls. The relative abundance of *Bacteroidetes* in the active Crohn's disease group was significantly lower than that in the inactive Crohn's disease group, and it showed a negative correlation with Crohn's disease activity index, which indicates that *Bacteroidetes* may have a negative impact on inflammatory development.

Ma HQ, Yu TT, Zhao XJ, Zhang Y, Zhang HJ. Fecal microbial dysbiosis in Chinese patients with inflammatory bowel disease. *World J Gastroenterol* 2018; 24(13): 1464-1477 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1464.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1464>

INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by chronic relapsing inflammation of the gastrointestinal tract and includes two main clinical phenotypes: Crohn's disease (CD) and ulcerative colitis (UC). The etiopathogenesis of IBD is not completely understood. Several disease susceptibility genes, such as *NOD2*, *ATG16L1* and *IRGM*, have been implicated in its pathogenesis^[1]. However, the rapid increase in the incidence of IBD cannot be explained by genetic factors

alone; an accumulating body of evidence indicates that environmental factors play a key role in the development of IBD by triggering intestinal microbiota dysbiosis^[2].

Currently available data from experimental models and clinical studies suggest that intestinal microbiota plays an important role in the pathogenesis of IBD^[3]. The alterations in intestinal microbiota related to IBD include decrease in *Bacteroides*, *Firmicutes*, *Clostridia*, *Ruminococcaceae*, *Bifidobacterium*, *Lactobacillus*, and *Faecalibacterium prausnitzii*, but increase in *Gamma Proteobacteria* and presence of *Fusobacterium* and *Escherichia coli*, especially *adherent-invasive E. coli* (AIEC). In addition, IBD is also associated with alterations in the microbial metabolic functions, including decrease of short-chain fatty acids (SCFAs) and amino acid biosynthesis, and increase of auxotrophy, amino acid and sulfate transport, oxidative stress, and type II secretion system^[4-7].

With respect to changes (increase or decrease) in intestinal microbiota in IBD patients, some conflicting findings have been reported for several bacteria, including *Bifidobacterium*, *Clostridiales*, *Clostridium difficile*, *Campylobacter*, *Helicobacter* and *Faecalibacterium prausnitzii*^[8]. For example, the levels of *F. prausnitzii* in IBD patients were found to be reduced in several studies^[9-11]. However, one study of *de novo* pediatric IBD revealed an increase in *F. prausnitzii* in CD, but not in UC^[12]. Another study of twins showed an increase in *F. prausnitzii* in patients with colonic CD, but a decrease of *F. prausnitzii* in patients with ileal CD^[13].

The intestinal microbiota of Western IBD patients has been extensively studied. However, the intestinal microbial profiles of Chinese IBD patients are not well characterized^[14]. In the present study, we profiled and compared the fecal microbial community of IBD patients at different disease stages and healthy controls by using 16S rDNA amplicon-based analysis.

MATERIALS AND METHODS

Study population

Twenty-nine IBD patients (11 active CD, 4 inactive CD and 14 active UC patients) who regularly visited the First Affiliated Hospital of Nanjing Medical University (Jiangsu, China) from 2014 to 2016 were recruited to the study. The diagnosis of IBD was based on standard clinical, endoscopic, radiological and histological criteria^[15]. The control group consisted of sex- and age-matched healthy subjects. Patients with IBD who met any of the following criteria were excluded: (1) use of antibiotics, probiotics or prebiotics in the 3-mo period immediately preceding the sampling time point; (2) current infectious diarrhea; and (3) malignancy. UC activity was evaluated using the Mayo score^[16]; active UC was defined as UC disease activity index > 2. Activity of CD was scored by Crohn's disease activity

index (CDAI)^[17]; active CD was defined as a CDAI > 150. Written informed consent was obtained from all subjects prior to their enrollment and the study was approved by the Ethics Committee at the First Affiliated Hospital of Nanjing Medical University, Jiangsu, China.

Fecal sample collection and extraction of genomic DNA

Fecal samples were collected from all subjects and subsequently stored at -80 °C within 2 h to prevent exposure of anaerobic bacteria to oxygen and to avoid bacterial overgrowth prior to DNA extraction. Genomic DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Feces (200 mg) was added to a 2-mL screw cap vial containing 300 mg of 0.1-mm glass beads (Sigma, St. Louis, MO, United States) which was maintained on ice. The samples were added of 1.4 mL ASL buffer and then subjected to bead beating (45 s, speed 6.5) twice using a FastPrep-24 machine (MP Biomedicals, Solon, OH, United States) before the initial incubation for heat and chemical lysis at 95 °C for 5 min. Subsequent DNA extraction was performed following the QIAamp kit protocol for pathogen detection.

Sequencing

16S rDNA genes of V4 regions were amplified using specific primer with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, United States). The same volume of 1 × loading buffer (containing SYB green) was mixed with PCR products and electrophoresis on 2% agarose gel was carried out for detection. Samples with the bright main band between 400-450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. The mixture of PCR products was subsequently purified with Qiagen Gel Extraction Kit. Sequencing libraries were generated using TruSeq®DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, United States) following manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific, Waltham, MA, United States) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina MiSeq platform and 250 bp paired-end reads were generated.

Data analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>)^[18], which was designed to merge paired-end reads when at least some of the reads overlapped the read generated from the opposite end of the same DNA fragment, and the splicing sequences

were called raw tags. Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags^[19] according to the QIIME (V1.7.0, <http://qiime.org/index.html>)^[20] quality controlled process. The tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html)^[21] to detect chimera sequences, and then the chimera sequences were removed^[22]. Finally, the effective tags were obtained. Analysis of sequences was performed with Uparse software (Uparse v7.0.1001, <http://drive5.com/uparse/>)^[23]. Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). Representative sequence for each OTU was screened for further annotation. For each representative sequence, the GreenGene Database (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>)^[24] was used based on the RDP classifier (version 2.2, <http://sourceforge.net/projects/rdp-classifier/>)^[25] algorithm to annotate taxonomic information.

In order to study the phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignment was conducted using the MUSCLE software (version 3.8.31, <http://www.drive5.com/muscle/>)^[26]. OTUs' abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on this output normalized data. Alpha diversity and beta diversity were calculated with QIIME (version 1.7.0) and displayed with R software (version 2.15.3).

Statistical analysis was performed using Statistical Package for Social Sciences version 19.0 (SPSS Inc., Chicago, IL, United States). The microbiota data and community estimates were analyzed by Kruskal-Wallis one-way analysis of variance to compare median values of microbiota data between CD, UC and controls. Spearman correlation analysis was used to analyze the correlation between intestinal bacterial abundance and intestinal inflammatory status. *P* values were corrected for multiple comparisons using false discovery rate (FDR); *P* < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics and sequencing data

Fecal samples from patients with active CD (*n* = 11), inactive CD (*n* = 4), active UC (*n* = 14), and 13 healthy individuals were analyzed in the current study. The median disease duration in patients with CD and UC was 10 mo (range: 3-48 mo) and 30 mo (range: 2-93 mo), respectively. Detailed clinical characteristics of the study subjects are presented in Table 1.

Paired-end reads were generated with the Illumina MiSeq platform. The reads with sequencing adapters,

Table 1 Clinical characteristics of enrolled patients

	CD	UC	Control
<i>n</i>	15	14	13
Age, mean \pm SD, yr	37.7 \pm 13.0	37.5 \pm 17.1	39.8 \pm 14.3
Sex, male/female	11/4	7/7	10/3
Disease duration in months, median (range)	10 (3-48)	30 (2-93)	-
Smoking habits	4 (26.7)	1 (7.1)	2 (15.4)
Abdominal surgery	4 (26.7)	0	0
Montreal A (age of onset)			
A1 (< 17)	1 (6.7)	-	-
A2 (17-40)	7 (46.7)	-	-
A3 (> 40)	7 (46.7)	-	-
Montreal L (location)			
L1 (ileal)	8 (53.3)	-	-
L2 (colonic)	1 (6.7)	-	-
L3 (ileocolonic)	6 (40)	-	-
L4 (upper gastrointestinal tract)	0	-	-
Montreal B (behavior)			
B1 (nonstricturing, nonpenetrating)	8 (53.3)	-	-
B2 (stricturing)	6 (40)	-	-
B3 (penetrating)	1 (6.7)	-	-
p (perianal disease)	4 (26.7)	-	-
Montreal			
E1 ulcerative proctitis	-	4 (28.6)	-
E2 left sided ulcerative colitis	-	5 (35.7)	-
E3 extensive ulcerative colitis	-	5 (35.7)	-
CDAI score			
< 150	4 (26.7)	-	-
150-220	5 (33.3)	-	-
221-450	6 (40)	-	-
> 450	0	-	-
Mayo score			
0-2	-	0	-
3-5	-	7 (50.0)	-
6-10	-	5 (35.7)	-
11-12	-	2 (14.3)	-
Therapy			
5-ASA	14 (93.3)	14 (100)	-
Azathioprine	2 (13.3)	0	-
Steroids	1 (6.7)	6 (42.9)	-
Infliximab	0	0	-

Data are presented as *n* (%). 5-ASA: 5-aminosalicylic acid; CD: Crohn's disease; CDAI: CD activity index; SD: Standard deviation; UC: Ulcerative colitis.

N base, poly base, and low quality were filtered out with default parameters. High quality paired-end reads were combined to tags based on overlaps. A total of 1747775 tags were obtained with an average of 41613 tags per sample; the average length was 252 bp. Filtered tags were clustered into OTUs at 97% similarity and a total of 878 OTUs were generated from 42 samples (see Supplementary File 1).

Characteristics of the microbial community in IBD patients and controls

When comparing bacterial alpha diversity, including community richness (observed species, chao, and ace) and diversity (Shannon and Simpson) between CD, UC and control groups, we found overall differences with respect to each diversity index (Figure 1). Significant differences ($P < 0.05$) with respect to community richness (chao) were observed both between CD and controls and between UC and controls. The observed species and ace indices of CD patients were lower than

those of controls; however, the differences were not statistically significant ($P > 0.05$). Moreover, the pattern of richness was found to be similar in CD and UC. When considering the species diversity of microbiota (Shannon and Simpson), the differences between each group were not statistically significant.

We subsequently surveyed the alpha diversity in IBD patients at different disease stages (see Supplementary Figure 1). Generally, the richness indices in IBD patients showed a decreasing trend (controls > inactive CD > active CD), but the between-group differences were not statistically significant. However, the diversity indices in IBD patients were not significantly different from those in controls.

Microbial community structures in IBD are distinct from those in normal controls

We used principal component analysis (PCA) to investigate the community structure of microbiota in CD, UC and controls. We found that samples tended to cluster together

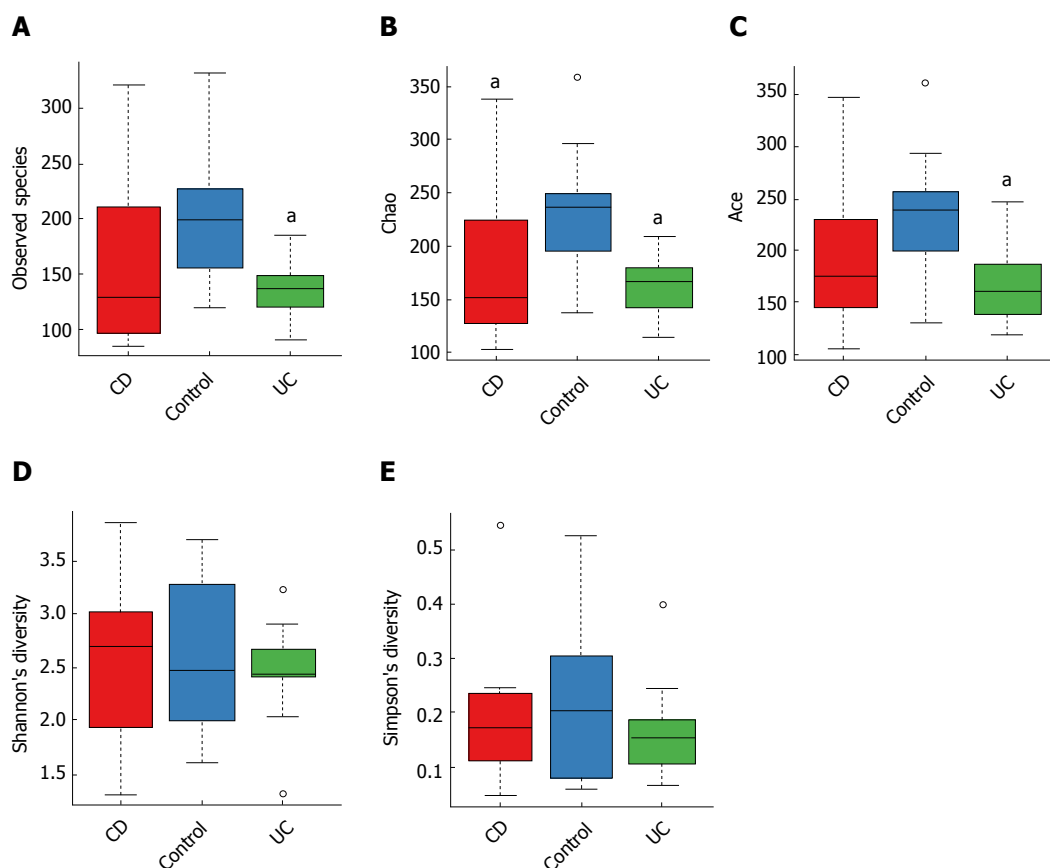


Figure 1 Alpha diversity indices boxplot, including community richness (observed species, chao, ace) and diversity (Shannon, Simpson) varied among each group. A: Observed species; B: Chao; C: Ace; D: Shannon; E: Simpson. ^a $P < 0.05$ vs control. CD: Crohn's disease; UC: Ulcerative colitis.

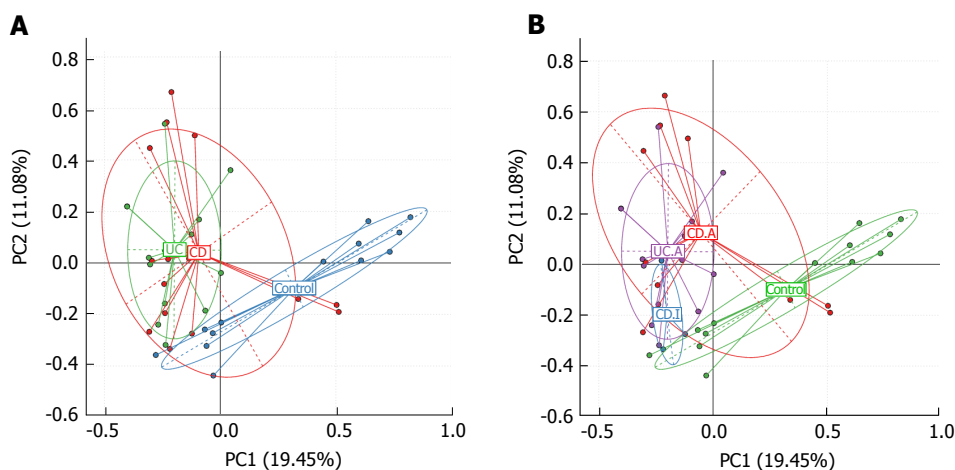


Figure 2 Principal component analysis based on the overall structure of the fecal microbiota in the entire study population. Each data point represents an individual sample. A: Disease phenotype group; B: Stages of disease group. CD: Crohn's disease; CD.A: Active CD; CD.I: Inactive CD; UC: Ulcerative colitis; UC.A: Active UC.

based on disease; however, to a certain extent, there was an overlap between all groups. IBD samples were mostly distinct from those of normal controls, which indicated differences with respect to community structure of the microbiota between IBD and controls (Anosim: CD vs control, $P = 0.02$; UC vs control, $P = 0.001$). However, samples of CD and UC were located closely, which

suggested a similar bacterial community structure in the context of both CD and UC (Anosim: $P = 0.133$) (Figure 2A).

Next, we visualized the PCA to compare the microbial structure in patients at different disease stages (Figure 2B). The results showed that samples could be well separated between active CD and controls

Table 2 Significant differences in microbial distribution of taxa (phylum and genus) in patients with inflammatory bowel disease

	CD	UC	CD/UC	CD.A/CD.I	CD.A/UC.A
<i>Firmicutes</i>					
<i>Abiotrophia</i> ¹	↑c				
<i>Butyricicoccus</i>	↓c		c ³		
<i>RFN20</i> ¹	↑c		c ²		
<i>Pseudoramibacter_Eubacterium</i> ¹	↑b		c ²		
<i>Holdemania</i> ¹		↓c			c ²
<i>02d06</i>		↓c	c ²		c ²
<i>Lachnobacterium</i>		↓c			
<i>Megamonas</i>		↓c			
<i>Mitsuokella</i>	↓c	↓c			
<i>Granulicatella</i>		↑b			
<i>Peptostreptococcus</i>		↑b			
<i>Schwartzia</i> ¹		↑b			
<i>Moryella</i> ¹			c ³		
<i>Staphylococcus</i> ¹			c ³		c ³
<i>Epulopiscium</i>					c ²
<i>Sarcina</i>					c ²
<i>Bacteroidetes</i>				b ³	
<i>Alistipes</i>		↓c			
<i>Butyricimonas</i>		↓c			
<i>Capnocytophaga</i> ¹		↑c	c ³		c ³
<i>Prevotella</i>		↓c			
<i>Proteobacteria</i>	↑b	↑b			
<i>Escherichia</i>	↑c	↑b			
<i>Haemophilus</i>	↓c		b ³		b ³
<i>Desulfovibrio</i>		↓c	b ²		c ²
<i>Oxalobacte</i> ¹		↓c			
<i>Janthinobacterium</i> ¹		↑b	b ³		
<i>Campylobacter</i>		↑b			
<i>Cardiobacterium</i> ¹			c ³		
<i>Lautropia</i> ¹			c ³		
<i>Lupinus</i> ¹			c ³		
<i>Shewanella</i> ¹			b ³		
<i>Actinobacteria</i>					
<i>Actinomyces</i>		↑c			
<i>Eggerthella</i> ¹		↑b			
<i>Corynebacterium</i> ¹		↑b	c ³		b ³
<i>Slackia</i> ¹			b ²		c ²
<i>Synergistetes</i>					
<i>Pyramidobacter</i> ¹		↓c			
<i>Synergistes</i> ¹		↓c			
<i>TG5</i> ¹			c ³		
<i>Spirochaetes</i>		↑c			c ³
<i>Lentisphaerae</i>		↓c			c ²
<i>Victivallis</i> ¹	↓c	↓c			

↑ and ↓ relative to controls; ¹Relative abundance of genera < 0.01%; ²Increase in value; ³Decrease in value. ^a*P* < 0.05; ^b*P* < 0.01; ^c*P* < 0.001. CD: Crohn's disease; CD.A: Active CD; CD.I: Inactive CD; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; UC.A: Active UC.

(Anosim: *P* = 0.016) as well as between active CD and active UC (Anosim: *P* = 0.01). However, there were no distinct microbiota structural patterns apparent between active CD and inactive CD groups, although the samples seemed to be clearly separated (Anosim: *P* = 0.719). There was also no separation between inactive CD and controls (Anosim: *P* = 0.564) based on the PCA. Our results indicated that the bacterial community structure in active CD was different from that in active UC; however, there was no difference with respect to the alterations of bacterial community structure in fecal samples of the total UC and CD patients.

Overall taxonomic analysis of IBD patients and controls

Taxonomic composition distribution histograms of each

sample were summarized at the phyla level (Figure 3A). The dominant sequences belonged to four bacterial phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Fusobacteria*), which accounted for over 97% of taxonomy generally (Figure 3B). Among all the relatively abundant dominant strains in IBD and normal controls, *Bacteroidetes* was, as a rule, the most abundant bacterial phylum.

Phylum-level analysis (Figure 3B, Table 2) revealed a nominal decrease in the relative abundance of *Bacteroidetes* in both CD and UC patients (CD vs control, 47.49% vs 66.85%, *P* = 0.015; UC vs control, 48.94% vs 66.85%, *P* = 0.019); however, these differences were not significant after adopting the FDR. On the contrary, *Proteobacteria* was significantly

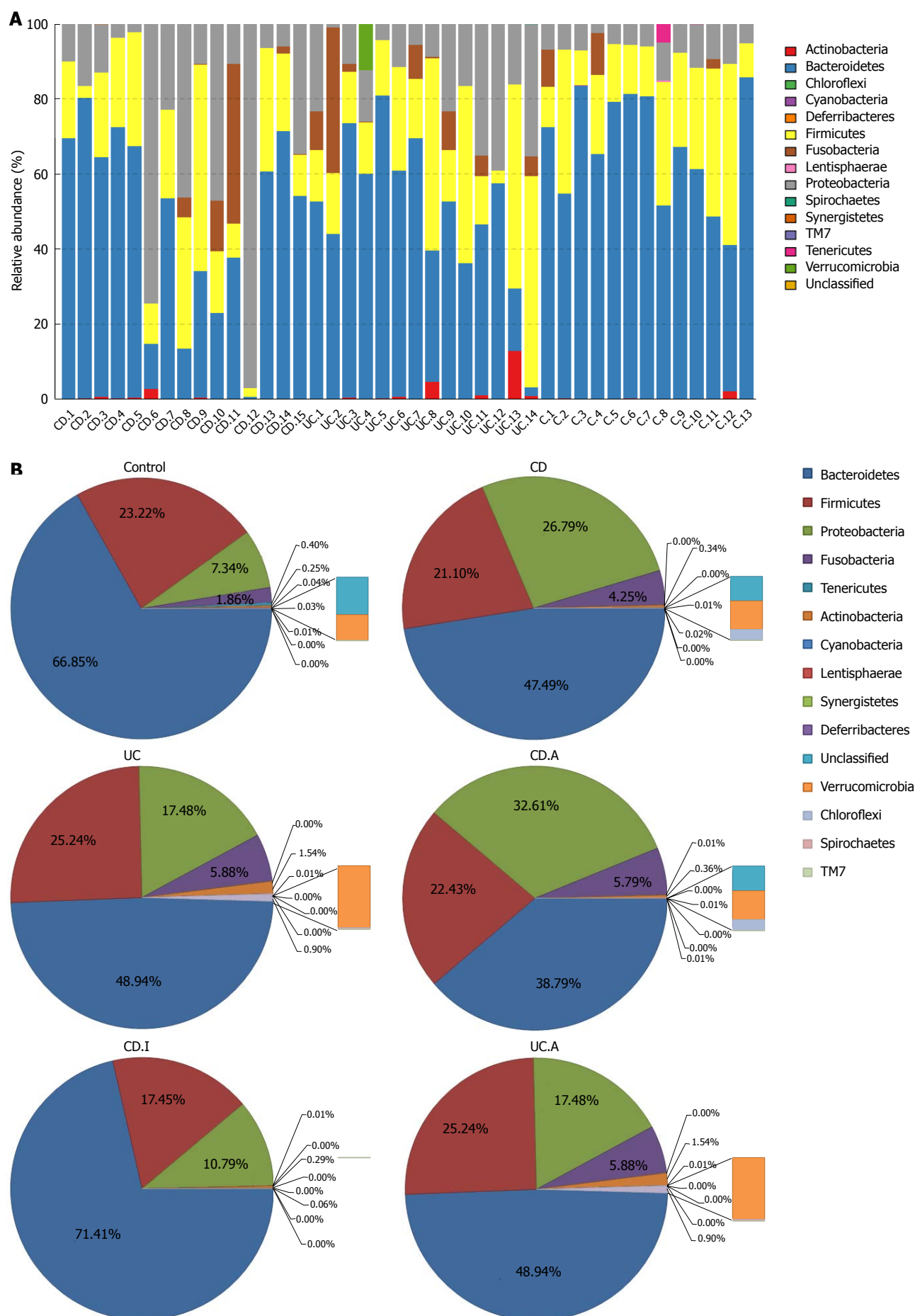


Figure 3 Taxonomic composition distribution in samples of phylum level. A: Individually; B: Integrally. CD: Crohn's disease; CD.A: Active CD; CD.I: Inactive CD; UC: Ulcerative colitis; UC.A: Active UC.

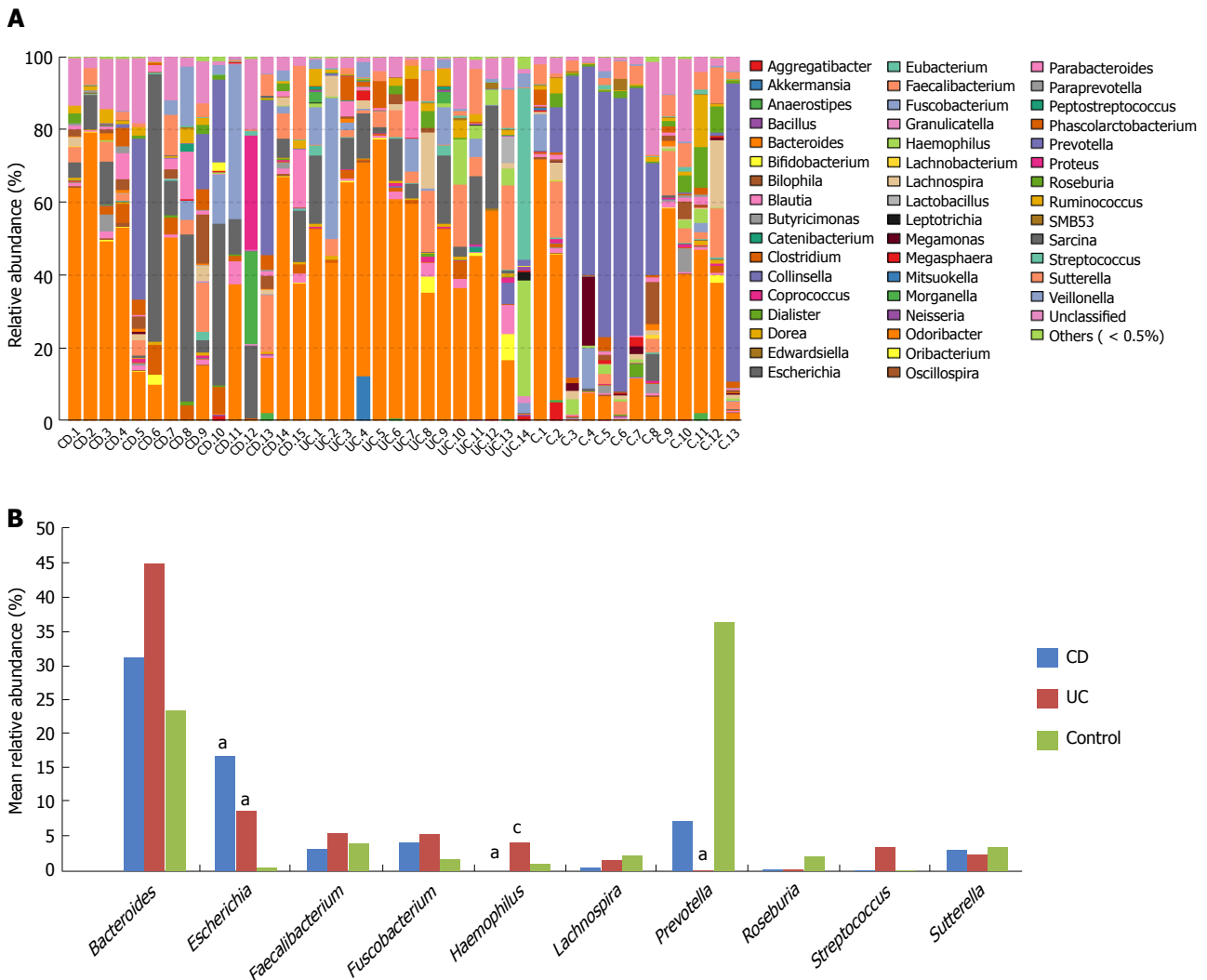


Figure 4 A: The taxonomic composition distribution in samples of genus level; B: Genera shown represent the 10 most abundant genera of CD, UC and control. ^a $P < 0.05$ vs control, ^b $P < 0.05$ vs CD. CD: Crohn's disease; UC: Ulcerative colitis.

increased in both CD and UC, as compared to that in controls (CD vs control, 26.79% vs 7.34%, $P = 0.002$; UC vs control, 17.48% vs 7.34%, $P = 0.005$). In addition, no *Spirochaetes* phylum was detected in CD and controls but it was observed in UC (0.015%). Similarly, *Lentisphaerae* phylum was found in the control group (accounting for 0.031%), but almost none was found in patients with IBD.

At the genus level, the relative abundance of all genera varied between different samples (Figure 4A). The top 10 abundant genera in UC, CD and controls were *Bacteroides*, *Escherichia*, *Faecalibacterium*, *Fusobacterium*, *Haemophilus*, *Lachnospira*, *Prevotella*, *Roseburia*, *Streptococcus*, and *Sutterella* (Figure 4B). Among these, the relative abundance of *Escherichia* in CD and UC was significantly higher than that in controls. In addition, abundance of *Haemophilus* in CD and *Prevotella* in UC patients were both markedly lower than that in normal controls. Moreover, the abundance of *Haemophilus* in CD was dramatically lower than that in UC. Besides the top 10 abundant

genera, the relative abundance of remaining genera was comparable between IBD patients and normal controls (Table 2). The abundance of 12 genera, *Butyricicoccus*, *Mitsuokella*, *02d06*, *Actinomyces*, *Alistipes*, *Butyricimonas*, *Campylobacter*, *Desulfovibrio*, *Granulicatella*, *Lachnobacterium*, *Megamonas* and *Peptostreptococcus*, was significantly different after correction among each group within the community; the sequence percentages for each of these 12 genera were more than 0.01%.

Taxonomic comparisons in IBD patients at different disease stages

On analysis of the alterations at the phyla level between active CD and inactive CD, we found that the dominant bacterial phyla were the same as described earlier (accounting for over 99% of taxonomy), with the exception that *Fusobacteria* was replaced by *Actinobacteria* in inactive CD (Figure 3B, Table 2). However, the abundance of *Bacteroidetes* was dramatically decreased in active CD group, as compared to that in the inactive CD group (CD.A

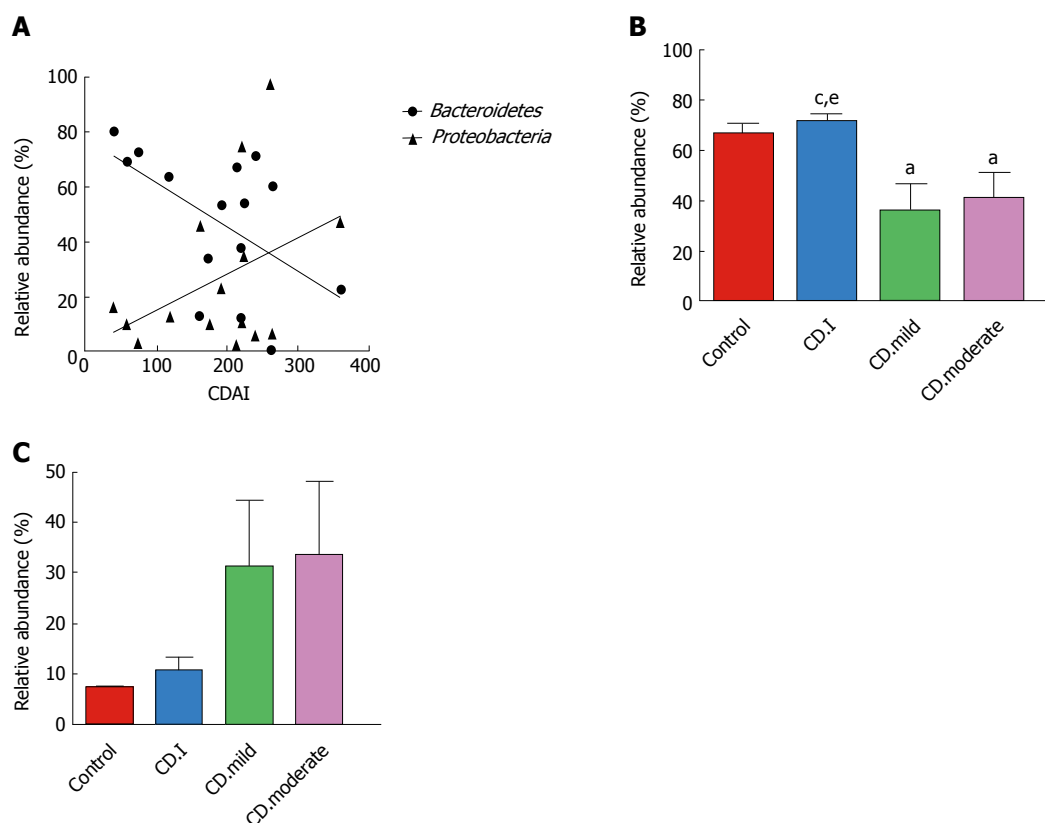


Figure 5 Correlation of the relative abundance of *Bacteroidetes* and *Proteobacteria* with Crohn's disease activity index scores (A). *Bacteroidetes* ($r = -0.538$, $P = 0.039$); *Proteobacteria* ($r = 0.250$, $P = 0.369$); B: Microbial composition of *Bacteroidetes* in patients with inactive/mild/moderate CD and in control; C: Microbial composition of *Proteobacteria* in patients with inactive/mild/moderate CD and in controls. ^a $P < 0.05$ vs control; ^c $P < 0.05$ vs CD.mild; ^e $P < 0.05$ vs CD.moderate. CDAI: CD activity index; CD.I: Inactive CD; CD.mild: Mild CD; CD.moderate: Moderate CD.

vs CD.I, 38.79% vs 71.41%, $P = 0.001$). The abundance of *Proteobacteria* was just nominally increased in active CD, as compared to that in inactive CD ($P = 0.023$), which did not hold significance after correction. Similarly, no differences were detected with respect to the remaining dominant bacteria between active CD and inactive CD. Microbiota in active CD and active UC were found to be similar at the phyla level.

We then investigated the genera with percentages of sequences $> 0.01\%$ of community in different phases of IBD and found that the abundance of *Bacteroides* and *Prevotella* in active CD were only nominally different from that in inactive CD. However, *Desulfovibrio*, *O2d06*, *Epulopiscium*, and *Sarcina* detected in active CD were markedly higher than that in active UC, while *Haemophilus* was markedly lower than that in active UC (Table 2).

Association between the inflammatory index of CD patients and microbiome

We assessed the correlation between the relative abundance of *Bacteroidetes* and CDAI scores of each CD patient; surprisingly, we found a negative correlation between the two ($r = -0.538$, $P = 0.039$) (Figure 5A). On the contrary, there was a trend of positive correlation between the abundance of *Proteobacteria* and CDAI (r

$= 0.250$, $P = 0.369$); however, the correlation was not statistically significant.

Next, we analyzed the correlation between microbial composition and disease severity. Patients with mild and moderate CD had notably decreased levels of *Bacteroidetes* as compared to that in patients with inactive CD; however, no significant difference in this respect was noted between patients with mild and moderate CD (Figure 5B). Interestingly, *Proteobacteria* exhibited a noteworthy trend (controls $<$ inactive CD $<$ mild CD $<$ moderate CD); however, the trend did not attain statistical significance (Figure 5C).

Effect of age and sex on intestinal microbial compositions

Although IBD mostly occurs in young adults (20- to 30-years-old), it can happen at any age. In the present research, no correlation was observed between microbial composition and age (see Supplementary Figure 2). Considering that most participants in our study (with the exception of one patient aged 14 years with UC) were adults, we divided the participants into two groups: age < 40 years and age > 40 years. However, no significant difference in microbial compositions was observed between the two groups (see Supplementary Table 1). On subgroup analysis based on sex, no notable

differences were observed between male and female patients in either subject subgroup (see Supplementary Table 2).

DISCUSSION

IBD is one of the most frequently studied human diseases linked to the gut microbiota. Distinctive microbial composition and its interaction with the host immunological response are believed to play a critical role in the pathogenesis of IBD^[27,28]; however, several aspects of the relationship are not well-characterized. In this study, we demonstrated differences with respect to fecal microbiota between Chinese IBD patients and healthy controls based on 16S rDNA sequencing analysis.

The dominant dysbiosis pattern unraveled by the present study was the decrease in community abundance of fecal microbiota both in CD and UC patients; while microbial diversity in CD patients was lower than that in controls, the difference was not statistically significant. Previous studies have shown reduced diversity of fecal microbiota in both Western^[29,30] and Chinese patients with IBD^[14], as compared to that in healthy controls. These inconsistencies are likely attributable to differences with respect to study design, stage of disease, or technique employed to survey the gut microbiota. The reasons for the changes of diversity in these conditions are still not known. Indeed, despite general trends such as a reduction in diversity, the response to IBD may, to some extent, be subject-specific.

We analyzed the bacterial community structure of microbiota in IBD patients and healthy individuals. The results showed distinct differences both in CD and UC, as compared to controls; however, the microbiota were similar within CD and UC groups or within active CD and inactive CD groups, which were not structurally distinguishable according to PCA. These data were also consistent with the previous studies conducted in Chinese and Western populations^[14,31]. However, Forbes *et al.*^[32] found a difference in the structure of microbiota between CD and UC. This result differed from those of other studies, as this study involved analysis of intestinal mucosa, while other studies were based on fecal analysis.

Detailed compositional alterations in fecal microbiota in IBD patients were detected at distinct taxonomic levels. The principle finding in our study was that the phylum *Proteobacteria* was significantly increased in IBD patients, which was in agreement with a consistent finding across published literature^[33,34]. The genus *Escherichia*, especially *Escherichia coli* (data not shown), was also found to be notably higher in IBD patients, as compared to that in normal controls. *Escherichia coli*, particularly AIEC, as an important pathobiont that may play a role in IBD development, has been isolated from ileal CD biopsy specimens^[35]. The initial lesions in the

colon mucosa can be aggravated by alpha-hemolysin secreted by *Escherichia coli*, which can damage host cell membranes and epithelial barrier^[36].

Moreover, both *E. coli* and *Campylobacter* (affiliated with *Proteobacteria*) are known to release cytolethal distending toxins, which leads to cell cycle arrest, chromatin fragmentation and apoptosis, all of which are involved in the pathogenesis of IBD^[37].

In the present study, patients with IBD exhibited relatively less number of *Bacteroidetes* compared to that in controls. The lower proportion of *Bacteroidetes* was mainly attributable to notably reduced abundance of *Prevotella* genus. The results were largely similar to those of another study which employed 16S rDNA sequencing analysis^[38]. Actually, alterations in *Bacteroidetes* in CD still remain controversial. Rehman *et al.*^[39] reported increased *Bacteroidetes* in CD patients and even demonstrated a notable increase in transcriptional activity, as compared to that in controls. Further studies are needed to clarify this issue. To minimize potential confounding factors, future studies should define gut dysbiosis in detail. Moreover, prospective cohort studies on newly diagnosed treatment-naïve patients will provide more definitive evidence in this respect.

In the present study, we documented increased abundance of *Haemophilus* and decreased *Desulfovibrio* (affiliated with *Proteobacteria*) in patients with UC. These findings were not observed in a previous study on fecal microbiota dysbiosis conducted by Chen *et al.*^[14] in Chinese patients with IBD. Recently, *Haemophilus* has been reported to contribute to oral dysbiosis in patients with IBD^[40] and *Haemophilus* spp., like the *Enterobacteriaceae*, are well adapted to survive under conditions of increased oxidative stress^[41]. To our knowledge, Rowan *et al.*^[42] demonstrated an increase of *Desulfovibrio* (sulfate-reducing bacteria) in patients with UC. *In vitro* studies have shown that 5-aminosalicylic acid (5-ASA) inhibits fecal sulfide production and fecal samples from patients not treated with this drug revealed higher levels of sulfide^[43]. It is conceivable that all participants in the present study were treated with 5-ASA, which may have contributed to the opposite phenomenon.

In addition, the study found an abundance of *Butyricoccus*, *Mitsuokella*, *O2d06*, *Lachnobacterium* and *Megamonas* (all affiliated with *Clostridia* class, *Firmicutes* phylum), which are obligate anaerobes. These were found significantly decreased in IBD patients in the current study. Dysanaerobiosis in patients with UC was observed recently^[44] and there seems to be a shift from anaerobiosis in healthy state to dysanaerobiosis in IBD, with an elevated oxygen level in the gut^[45]. Furthermore, studies conducted on experimental colitis models showed decrease in obligate anaerobes of *Firmicutes* and increase in facultative anaerobes of *Proteobacteria*, which indicates a role of oxygen in gut dysbiosis^[46]. In fact, both *Butyricoccus* (affiliated

with *Ruminococcaceae* family) and *Lachnobacterium* (affiliated with *Lachnospiraceae* family) produce SCFAs, which are known as the primary energy source for colonic epithelial cells^[47] and were shown to induce the expansion of colonic regulatory T cells^[48]. These alterations in microbial composition suggested that reduction in beneficial microbiota (*Clostridia* class and SCFA-producing bacteria) is more associated with IBD patients compared to the increment of pathobionts (*Escherichia* and *Campylobacter*).

When analyzing the fecal microbiota at different disease stages of IBD, only the abundance of *Bacteroidetes* was dramatically decreased in active CD, as compared to that in inactive CD. About the relationship between microbiome and disease activity, we also found a negative correlation between the relative abundance of *Bacteroidetes* and CDAI in the present study. The relative abundance of *Bacteroidetes* in active CD patients was lower than that in inactive CD or controls, but the relative abundance of *Bacteroidetes* was similar between mild and moderate CD. All these findings suggest that *Bacteroidetes* may have a negative impact on inflammatory development.

Potential links between age or sex and microbial compositions have been suggested recently^[49]. Gut microbiota vary in different age groups: infants, adults or the elderly. The microbiota in infants is often affected by the birth route, feeding patterns and illness history^[50]. Not until adulthood does the microbiota become stable, complex and shows improved resilience against perturbations^[51]. Then, the stability decreases in the elderly (≥ 65 years of age)^[52]. However, we did not find the effect of age and sex on microbiota in the current study. So, a different role for the microbiota in disease initiation and progression should be researched.

Our study faces several limitations. First of all, due to the small sample number and relatively high variability of microbial composition in each group, some of the relative abundances of specific bacteria between groups could not reach statistical significance after adopting the FDR. Secondly, 16S rDNA sequencing mainly focuses on the taxonomic profiling rather than providing greater insight into the function of the intestinal microbiota in disease^[53,54]. Thirdly, the nature and extent of difference between the fecal microbiota and mucosa-associated microbiota in IBD remains unclear. Controversy still exists between them because of different techniques used in separate studies^[55]. Several studies indicated that the fecal microbiota and mucosa-associated microbiota were similar^[13,56,57]. However, some studies have found a significant difference between them^[14,58,59]. It seems that the fecal microbiota represents a combination of a separate nonadherent luminal population and shed mucosal bacteria^[59]. Further study with a large population is required to confirm our data and mucosa-associated microbiota needs to be researched in Chinese patients with IBD.

In conclusion, we presented a comprehensive analysis of fecal microbiota in Chinese patients with IBD. Significant differences in microbial composition of patients with IBD and controls were observed. Additionally, the negative correlation between *Bacteroidetes* and CDAI suggested that *Bacteroidetes* might have a negative impact on development of inflammation.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is generally defined by two nonspecific inflammatory disorders, Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by chronic persistent inflammation of the intestinal mucosa lining the intestinal tract. Recently, distinctive microbial composition and its interaction with the host immunological response are believed to play critical roles in the pathogenesis of IBD. Although the intestinal microbial composition of Western IBD patients has been extensively studied, there are conflicting reports about changes of the bacterial abundance. What's more, the intestinal microbial profiles of Chinese IBD patients are not well characterized. In the present study, we use 16S rDNA amplicon-based analysis to analyze the alterations of fecal microbiota in Chinese patients with IBD.

Research motivation

Although the microbial community is gaining increasing attention for its influence on IBD, there is a lack of data on global alteration of microbiota in Chinese patients and the relationship is poorly understood. This study would characterize the important differences of fecal microbiota between Chinese IBD patients and healthy controls based on a 16S rDNA sequencing analysis, hoping to explore which kinds of the microbiota could be involved in the pathogenesis of IBD or providing important references for diagnosis or treatment of IBD.

Research objectives

The research aimed to investigate the differences in quantity, diversity and similarity of the fecal bacterial population taken from Chinese IBD patients at different stages of disease and healthy individuals.

Research methods

Twenty-nine IBD patients (11 active CD, 4 inactive CD and 14 active UC patients) from the First Affiliated Hospital of Nanjing Medical University (Jiangsu, China) and 13 sex and age well-matched healthy individuals were enrolled in the study. 16S rDNA amplicon-based sequencing was used to analyze the fecal microbiota of each sample.

Research results

In this study, community richness (chao) and microbial structure in IBD were significantly different from those in normal controls. The relative abundance of *Bacteroidetes* in the active CD group was significantly lower than that in the inactive CD group, and it showed a negative correlation with Crohn's disease activity index (CDAI). At the phyla level, the abundance of *Proteobacteria* was significantly higher in IBD than in controls. At the genera level, 8 genera in CD and 23 genera in UC (in particular, the *Escherichia* genus) showed significantly greater abundance as compared to that in normal controls.

Research conclusions

Our study presented a comprehensive analysis of fecal microbiota in the gut of Chinese patients with IBD. Significant differences in microbial composition of patients with IBD and controls were observed. Additionally, the negative correlation between *Bacteroidetes* and CDAI suggested that *Bacteroidetes* might have a negative impact on development of inflammation.

Research perspectives

Fecal microbial examination is noninvasive and easily collected compared

with the mucosal biopsy, which may increase the risk of unexpected bleeding. However, the mucosa-associated microbiota is believed to directly affect epithelial and mucosal function. In the future, both the fecal and mucosa-associated microbiota should be investigated together to better understand the role of the intestinal microbiota in health and disease.

ACKNOWLEDGMENTS

The authors appreciate technical and statistical supports of BGI Tech Solutions Co., Ltd (Shenzhen, China) and would like to express thanks.

REFERENCES

- Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]
- Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]
- Knights D**, Lassen KG, Xavier RJ. Advances in inflammatory bowel disease pathogenesis: linking host genetics and the microbiome. *Gut* 2013; **62**: 1505-1510 [PMID: 24037875 DOI: 10.1136/gutjnl-2012-303954]
- Kostic AD**, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology* 2014; **146**: 1489-1499 [PMID: 24560869 DOI: 10.1053/j.gastro.2014.02.009]
- Krause DO**, Little AC, Dowd SE, Bernstein CN. Complete genome sequence of adherent invasive *Escherichia coli* UM146 isolated from Ileal Crohn's disease biopsy tissue. *J Bacteriol* 2011; **193**: 583 [PMID: 21075930 DOI: 10.1128/JB.01290-10]
- Kang S**, Denman SE, Morrison M, Yu Z, Dore J, Leclerc M, McSweeney CS. Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflamm Bowel Dis* 2010; **16**: 2034-2042 [PMID: 20848492 DOI: 10.1002/ibd.21319]
- Morgan XC**, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB, Bousvaros A, Korzenik J, Sands BE, Xavier RJ, Huttenhower C. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* 2012; **13**: R79 [PMID: 23013615 DOI: 10.1186/gb-2012-13-9-r79]
- Li J**, Butcher J, Mack D, Stintzi A. Functional impacts of the intestinal microbiome in the pathogenesis of inflammatory bowel disease. *Inflamm Bowel Dis* 2015; **21**: 139-153 [PMID: 25248007 DOI: 10.1097/MIB.0000000000000215]
- Willing B**, Halfvarson J, Dicksved J, Rosenquist M, Järnerot G, Engstrand L, Tysk C, Jansson JK. Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 653-660 [PMID: 19023901 DOI: 10.1002/ibd.20783]
- Machiels K**, Joossens M, Sabino J, De Preter V, Arijis I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K, Ferrante M, Verhaegen J, Rutgeerts P, Vermeire S. A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 2014; **63**: 1275-1283 [PMID: 24021287 DOI: 10.1136/gutjnl-2013-304833]
- Sokol H**, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangeotte C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottière HM, Doré J, Marteau P, Seksik P, Langella P. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 2008; **105**: 16731-16736 [PMID: 18936492 DOI: 10.1073/pnas.0804812105]
- Hansen R**, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, Mukhopadhyay I, Bisset WM, Barclay AR, Bishop J, Flynn DM, McGrogan P, Loganathan S, Mahdi G, Flint HJ, El-Omar EM, Hold GL. Microbiota of de-novo pediatric IBD: increased *Faecalibacterium prausnitzii* and reduced bacterial diversity in Crohn's but not in ulcerative colitis. *Am J Gastroenterol* 2012; **107**: 1913-1922 [PMID: 23044767 DOI: 10.1038/ajg.2012.335]
- Willing BP**, Dicksved J, Halfvarson J, Andersson AF, Lucio M, Zheng Z, Järnerot G, Tysk C, Jansson JK, Engstrand L. A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. *Gastroenterology* 2010; **139**: 1844-1854.e1 [PMID: 20816835 DOI: 10.1053/j.gastro.2010.08.049]
- Chen L**, Wang W, Zhou R, Ng SC, Li J, Huang M, Zhou F, Wang X, Shen B, A Kamm M, Wu K, Xia B. Characteristics of fecal and mucosa-associated microbiota in Chinese patients with inflammatory bowel disease. *Medicine (Baltimore)* 2014; **93**: e51 [PMID: 25121355 DOI: 10.1097/MD.0000000000000051]
- Ouyang Q**, Tandon R, Goh KL, Pan GZ, Fock KM, Fiocchi C, Lam SK, Xiao SD. Management consensus of inflammatory bowel disease for the Asia-Pacific region. *J Gastroenterol Hepatol* 2006; **21**: 1772-1782 [PMID: 17074013 DOI: 10.1111/j.1440-1746.2006.04674.x]
- D'Haens G**, Sandborn WJ, Feagan BG, Geboes K, Hanauer SB, Irvine EJ, Lémann M, Marteau P, Rutgeerts P, Schölmerich J, Sutherland LR. A review of activity indices and efficacy end points for clinical trials of medical therapy in adults with ulcerative colitis. *Gastroenterology* 2007; **132**: 763-786 [PMID: 17258735 DOI: 10.1053/j.gastro.2006.12.038]
- Best WR**, Beckett JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 1976; **70**: 439-444 [PMID: 1248701]
- Magoč T**, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 2011; **27**: 2957-2963 [PMID: 21903629 DOI: 10.1093/bioinformatics/btr507]
- Bokulich NA**, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* 2013; **10**: 57-59 [PMID: 23202435 DOI: 10.1038/nmeth.2276]
- Caporaso JG**, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; **7**: 335-336 [PMID: 20383131 DOI: 10.1038/nmeth.f.303]
- Edgar RC**, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 2011; **27**: 2194-2200 [PMID: 21700674 DOI: 10.1093/bioinformatics/btr381]
- Haas BJ**, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, Ciulla D, Tabbaa D, Highlander SK, Sodergren E, Methé B, DeSantis TZ. Human Microbiome Consortium, Petrosino JF, Knight R, Birren BW. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* 2011; **21**: 494-504 [PMID: 21212162 DOI: 10.1101/gr.112730.110]
- Edgar RC**. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013; **10**: 996-998 [PMID: 23955772 DOI: 10.1038/nmeth.2604]
- DeSantis TZ**, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 2006; **72**: 5069-5072 [PMID: 16820507 DOI: 10.1128/aem.03006-05]

- 25 **Wang Q**, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007; **73**: 5261-5267 [PMID: 17586664 DOI: 10.1128/aem.00062-07]
- 26 **Edgar RC**. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004; **32**: 1792-1797 [PMID: 15034147 DOI: 10.1093/nar/gkh340]
- 27 **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594 [PMID: 18242222 DOI: 10.1053/j.gastro.2007.11.059]
- 28 **Swidsinski A**, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, Lochs H. Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 44-54 [PMID: 11781279]
- 29 **Manichanh C**, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, Roca J, Dore J. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 2006; **55**: 205-211 [PMID: 16188921 DOI: 10.1136/gut.2005.073817]
- 30 **Ott SJ**, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Fölsch UR, Timmis KN, Schreiber S. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* 2004; **53**: 685-693 [PMID: 15082587]
- 31 **Andoh A**, Imaeda H, Aomatsu T, Inatomi O, Bamba S, Sasaki M, Saito Y, Tsujikawa T, Fujiyama Y. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol* 2011; **46**: 479-486 [PMID: 21253779 DOI: 10.1007/s00535-010-0368-4]
- 32 **Forbes JD**, Van Domselaar G, Bernstein CN. Microbiome Survey of the Inflamed and Noninflamed Gut at Different Compartments Within the Gastrointestinal Tract of Inflammatory Bowel Disease Patients. *Inflamm Bowel Dis* 2016; **22**: 817-825 [PMID: 26937623 DOI: 10.1097/MIB.0000000000000684]
- 33 **Man SM**, Kaakoush NO, Mitchell HM. The role of bacteria and pattern-recognition receptors in Crohn's disease. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 152-168 [PMID: 21304476 DOI: 10.1038/nrgastro.2011.3]
- 34 **Sokol H**, Lepage P, Seksik P, Doré J, Marteau P. Temperature gradient gel electrophoresis of fecal 16S rRNA reveals active *Escherichia coli* in the microbiota of patients with ulcerative colitis. *J Clin Microbiol* 2006; **44**: 3172-3177 [PMID: 16954244 DOI: 10.1128/jcm.02600-05]
- 35 **Darfeuille-Michaud A**, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N, Bringer MA, Swidsinski A, Beaugerie L, Colombel JF. High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. *Gastroenterology* 2004; **127**: 412-421 [PMID: 15300573]
- 36 **Bücker R**, Schulz E, Günzel D, Bojarski C, Lee IF, John LJ, Wiegand S, Janßen T, Wieler LH, Dobrindt U, Beutin L, Ewers C, Fromm M, Siegmund B, Troeger H, Schulzke JD. α -Haemolysin of *Escherichia coli* in IBD: a potentiator of inflammatory activity in the colon. *Gut* 2014; **63**: 1893-1901 [PMID: 24534723 DOI: 10.1136/gutjnl-2013-306099]
- 37 **Smith JL**, Bayles DO. The contribution of cytolethal distending toxin to bacterial pathogenesis. *Crit Rev Microbiol* 2006; **32**: 227-248 [PMID: 17123907 DOI: 10.1080/10408410601023557]
- 38 **Lepage P**, Häslér R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, Ott S, Kupcinskis L, Doré J, Raedler A, Schreiber S. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* 2011; **141**: 227-236 [PMID: 21621540 DOI: 10.1053/j.gastro.2011.04.011]
- 39 **Rehman A**, Lepage P, Nolte A, Hellmig S, Schreiber S, Ott SJ. Transcriptional activity of the dominant gut mucosal microbiota in chronic inflammatory bowel disease patients. *J Med Microbiol* 2010; **59**: 1114-1122 [PMID: 20522625 DOI: 10.1099/jmm.0.021170-0]
- 40 **Said HS**, Suda W, Nakagome S, Chinen H, Oshima K, Kim S, Kimura R, Irahia A, Ishida H, Fujita J, Mano S, Morita H, Dohi T, Oota H, Hattori M. Dysbiosis of salivary microbiota in inflammatory bowel disease and its association with oral immunological biomarkers. *DNA Res* 2014; **21**: 15-25 [PMID: 24013298 DOI: 10.1093/dnares/dst037]
- 41 **Harrison A**, Bakaletz LO, Munson RS Jr. *Haemophilus influenzae* and oxidative stress. *Front Cell Infect Microbiol* 2012; **2**: 40 [PMID: 22919631 DOI: 10.3389/fcimb.2012.00040]
- 42 **Rowan F**, Docherty NG, Murphy M, Murphy B, Calvin Coffey J, O'Connell PR. *Desulfovibrio* bacterial species are increased in ulcerative colitis. *Dis Colon Rectum* 2010; **53**: 1530-1536 [PMID: 20940602 DOI: 10.1007/DCR.0b013e3181f1e620]
- 43 **Pitcher MC**, Beatty ER, Cummings JH. The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. *Gut* 2000; **46**: 64-72 [PMID: 10601057]
- 44 **Walujkar SA**, Dhotre DP, Marathe NP, Lawate PS, Bharadwaj RS, Shouche YS. Characterization of bacterial community shift in human Ulcerative Colitis patients revealed by Illumina based 16S rRNA gene amplicon sequencing. *Gut Pathog* 2014; **6**: 22 [PMID: 25018784 DOI: 10.1186/1757-4749-6-22]
- 45 **Rigottier-Gois L**. Dysbiosis in inflammatory bowel diseases: the oxygen hypothesis. *ISME J* 2013; **7**: 1256-1261 [PMID: 23677008 DOI: 10.1038/ismej.2013.80]
- 46 **Podolsky DK**. Inflammatory bowel disease (1) *N Engl J Med* 1991; **325**: 928-937 [PMID: 1881418 DOI: 10.1056/nejm199109263251306]
- 47 **Ahmad MS**, Krishnan S, Ramakrishna BS, Mathan M, Pulimood AB, Murthy SN. Butyrate and glucose metabolism by colonocytes in experimental colitis in mice. *Gut* 2000; **46**: 493-499 [PMID: 10716678]
- 48 **Smith PM**, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* 2013; **341**: 569-573 [PMID: 23828891 DOI: 10.1126/science.1241165]
- 49 **Blaser MJ**, Falkow S. What are the consequences of the disappearing human microbiota? *Nat Rev Microbiol* 2009; **7**: 887-894 [PMID: 19898491 DOI: 10.1038/nrmicro2245]
- 50 **Dominguez-Bello MG**, Blaser MJ, Ley RE, Knight R. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology* 2011; **140**: 1713-1719 [PMID: 21530737 DOI: 10.1053/j.gastro.2011.02.011]
- 51 **Lozupone CA**, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature* 2012; **489**: 220-230 [PMID: 22972295 DOI: 10.1038/nature11550]
- 52 **Claesson MJ**, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, van Sinderen D, O'Connor M, Harnedy N, O'Connor K, Henry C, O'Mahony D, Fitzgerald AP, Shanahan F, Twomey C, Hill C, Ross RP, O'Toole PW. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci U S A* 2011; **108** Suppl 1: 4586-4591 [PMID: 20571116 DOI: 10.1073/pnas.1000097107]
- 53 **Meyer F**, Trimble WL, Chang EB, Handley KM. Functional predictions from inference and observation in sequence-based inflammatory bowel disease research. *Genome Biol* 2012; **13**: 169 [PMID: 23013527 DOI: 10.1186/gb4042]
- 54 **Presley LL**, Ye J, Li X, Leblanc J, Zhang Z, Ruegger PM, Allard J, McGovern D, Ippoliti A, Roth B, Cui X, Jeske DR, Elashoff D, Goodglick L, Braun J, Borneman J. Host-microbe relationships in inflammatory bowel disease detected by bacterial and metaproteomic analysis of the mucosal-luminal interface. *Inflamm Bowel Dis* 2012; **18**: 409-417 [PMID: 21698720 DOI: 10.1002/ibd.21793]
- 55 **De Cruz P**, Prideaux L, Wagger J, Ng SC, McSweeney C, Kirkwood C, Morrison M, Kamm MA. Characterization of the gastrointestinal microbiota in health and inflammatory bowel disease. *Inflamm Bowel Dis* 2012; **18**: 372-390 [PMID: 21604329 DOI: 10.1002/ibd.21751]
- 56 **van der Waaij LA**, Harmsen HJ, Madjipour M, Kroese FG, Zwieters M, van Dullemen HM, de Boer NK, Welling GW, Jansen PL.

Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells. *Inflamm Bowel Dis* 2005; **11**: 865-871 [PMID: 16189415]

- 57 **Bibiloni R**, Tandon P, Vargas-Voracka F, Barreto-Zuniga R, Lupian-Sanchez A, Rico-Hinojosa MA, Guban J, Fedorak R, Tannock GW. Differential clustering of bowel biopsy-associated bacterial profiles of specimens collected in Mexico and Canada: what do these profiles represent? *J Med Microbiol* 2008; **57**: 111-117 [PMID:

18065676 DOI: 10.1099/jmm.0.47321-0]

- 58 **Durbán A**, Abellán JJ, Jiménez-Hernández N, Ponce M, Ponce J, Sala T, D'Auria G, Latorre A, Moya A. Assessing gut microbial diversity from feces and rectal mucosa. *Microb Ecol* 2011; **61**: 123-133 [PMID: 20734040 DOI: 10.1007/s00248-010-9738-y]
- 59 **Eckburg PB**, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. *Science* 2005; **308**: 1635-1638 [PMID: 15831718 DOI: 10.1126/science.1110591]

P-Reviewer: Naito Y, Zouiten-Mekki L **S-Editor:** Gong ZM

L-Editor: Filipodia **E-Editor:** Huang Y



Prospective Study

Hepatocellular carcinoma or interferon-based therapy history attenuates sofosbuvir/ribavirin for Japanese genotype 2 hepatitis C virus

Masayoshi Yada, Masayuki Miyazaki, Kosuke Tanaka, Akihide Masumoto, Kenta Motomura

Masayoshi Yada, Masayuki Miyazaki, Kosuke Tanaka, Akihide Masumoto, Kenta Motomura, Department of Hepatology, Iizuka Hospital, Iizuka, Fukuoka 820-8505, Japan

ORCID number: Masayoshi Yada (0000-0002-1129-5380); Masayuki Miyazaki (0000-0002-4192-8150); Kosuke Tanaka (0000-0003-1472-0597); Akihide Masumoto (0000-0003-4929-6271); Kenta Motomura (0000-0002-7515-099X).

Author contributions: Yada M and Motomura K wrote the paper; Yada M, Miyazaki M, Tanaka K, and Motomura K analyzed the data; Masumoto A supervised writing of the paper; all authors contributed to the manuscript.

Institutional review board statement: The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the Ethics Committee of Iizuka Hospital (Approve No. 26282).

Informed consent statement: Written informed consent was obtained from all patients.

Conflict-of-interest statement: The authors have no disclosures to report.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

Manuscript source: Unsolicited manuscript

Correspondence to: Masayoshi Yada, MD, PhD, Doctor, Department of Hepatology, Iizuka Hospital, 3-83 Yoshio-machi, Iizuka, Fukuoka 820-8505, Japan. myadah1@aih-net.com

Telephone: +81-948-223800
Fax: +81-948-295744

Received: February 5, 2018
Peer-review started: February 5, 2018
First decision: February 26, 2018
Revised: February 26, 2018
Accepted: March 7, 2018
Article in press: March 7, 2018
Published online: April 7, 2018

Abstract

AIM

To investigate the real-world efficacy and safety of sofosbuvir/ribavirin (SOF/RBV) therapy for Japanese patients with genotype 2 hepatitis C virus (GT2-HCV).

METHODS

A total of 182 patients with GT2-HCV infection who received SOF/RBV therapy for 12 wk at our hospital were enrolled. The patients comprised 122 men and 60 women (age range: 17-84 years; mean age \pm SD: 60.1 \pm 12.1 years). Relationships between virological response and clinical data were examined by logistic regression analyses.

RESULTS

The proportions of patients with liver cirrhosis and history of hepatocellular carcinoma (HCC) were 29.0% and 17.3%, respectively. The proportion of patients with prior interferon (IFN)-based therapy was 25.6%. SOF/RBV therapy rapidly decreased HCV RNA levels. Several patients required RBV dose reduction because of anemia or fatigue. Four patients discontinued the therapy. The rates of sustained virological response at 12 wk after the end of treatment were 87.9% (intention

to treat: 160/182) and 94.1% (per protocol: 159/169). Multivariate analyses showed that history of HCC or IFN-based therapy independently reduced the efficacy of SOF/RBV therapy.

CONCLUSION

SOF/RBV therapy for GT2-HCV is safe, highly tolerated, and effective. History of HCC or IFN-based therapy independently reduces the efficacy of this treatment.

Key words: Sofosbuvir; Ribavirin; Genotype 2; Hepatitis C virus; Interferon-based therapy; Hepatocellular carcinoma

© **The Author(s) 2018.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The real-world efficacy of sofosbuvir/ribavirin therapy for genotype 2 hepatitis C virus infection in Japan is high. Sofosbuvir/ribavirin therapy is safe and highly tolerated. History of hepatocellular carcinoma or interferon-based therapy independently reduces the efficacy of sofosbuvir/ribavirin therapy. Progressive liver fibrosis may attenuate the antiviral effect of sofosbuvir/ribavirin therapy.

Yada M, Miyazaki M, Tanaka K, Masumoto A, Motomura K. Hepatocellular carcinoma or interferon-based therapy history attenuates sofosbuvir/ribavirin for Japanese genotype 2 hepatitis C virus. *World J Gastroenterol* 2018; 24(13): 1478-1485 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1478.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1478>

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis, liver cirrhosis (LC), and hepatocellular carcinoma (HCC). In Global hepatitis report 2017^[1], World Health Organization (WHO) described that 71 million persons were living with chronic HCV infection in 2015 (the global prevalence of HCV infection was 1.0%). Approximately 399000 people died each year from HCV, mostly from LC and HCC. In 2011, Japanese estimated number of persons with chronic HCV infection was 1.25 million containing 64% with genotype 1B and 35% with genotype 2, respectively^[2]. WHO targets 80% of persons with HCV will be treated by 2030^[1]. Combination therapy with peg-interferon (PEG-IFN) and ribavirin (RBV) was the first-line therapy for genotype 2 HCV (GT2-HCV) infection, but only 80% of patients achieved elimination of HCV with this treatment^[3]. The introduction of direct-acting antiviral agents has drastically improved the efficacy of treatments for chronic HCV infection. In Japan, telaprevir (TVR), a first-generation NS3/4A protease inhibitor for GT2-HCV, was approved for clinical use

in 2013. Patients received TVR (750 mg, every 8 h) for 12 wk and PEG-IFN/RBV for 24 wk. The sustained virological response (SVR) rate in patients receiving triple therapy (TVR/PEG-IFN/RBV) was reported to be 85%^[4].

Subsequently, combination therapy with NS5B RNA-dependent RNA polymerase inhibitor sofosbuvir (SOF) and RBV for patients with GT2-HCV infection was approved for clinical use in June 2015. This therapy showed improved efficacy and was well tolerated in a phase 3 trial^[5]. We conducted a prospective study to investigate the efficacy and safety of SOF/RBV therapy for Japanese patients with GT2-HCV infection in a real-world clinical setting.

MATERIALS AND METHODS

Patients

A total of 182 patients with chronic GT2-HCV infection who received SOF/RBV therapy at Iizuka Hospital from September 2015 to January 2017 were enrolled. The patients comprised 122 men and 60 women with an age range of 17-86 years (mean \pm SD: 60.1 \pm 14.1 years). HCV genotype was determined by sequencing the NS5B region of the HCV genome. The results revealed 109 patients with genotype 2A and 70 patients with genotype 2B. Genotype was not determined in 3 patients. Presence of resistance-associated substitution (RAS) was analyzed by sequence determination around S282 in the NS5B region of the HCV genome. Two single nucleotide polymorphisms (SNPs), rs8099917 in the *interleukin-28B* (*IL28B*) locus associated with interferon (IFN) therapy^[6] and rs1127354 in the *inosine triphosphate pyrophosphatase* (*ITPA*) locus associated with RBV-induced hemolytic anemia^[7-9], were analyzed. fibrosis (FIB)-4 index was calculated as a noninvasive marker of liver fibrosis: FIB-4 index = age (years) \times aspartate aminotransferase (IU/L)/[platelet count \times 10⁹/L \times (alanine aminotransferase IU/L)^{1/2}]. FIB-4 index of > 3.25 was defined as progressive fibrosis, based on a previous report^[10].

Treatment regimen

SOF (Sovaldi[®], Gilead Sciences Inc., Durham, NC, United States) was administered orally at a dose of 400 mg once daily, and RBV (Rebetol[®], MSD, Tokyo, Japan or Copegus[®], Chugai, Shizuoka, Japan) was administered orally at a dose of 200-1000 mg for 12 wk. The starting RBV dose was determined by body weight (600 mg for < 60 kg; 800 mg for 60-80 kg; 1000 mg for > 80 kg) (Figure 1).

Evaluation of virological response

HCV RNA levels were measured by a COBAS TaqMan test (Roche Diagnostics, Tokyo, Japan) with a lower limit of quantitation of 1.2 logIU/mL. HCV RNA was measured at screening, at day 1 and weeks 4, 8, and 12 of treatment, and at 12 wk after the end of

Table 1 Pretreatment characteristics of the patients (*n* = 182)

Pretreatment characteristics	Values
Age ¹ , yr	60.1 ± 14.1
Age ≥ 70 yr	27.40%
Sex, M : F	122:60
Body height ¹ , cm	163.0 ± 8.9
Body weight ¹ , kg	62.3 ± 12.1
Liver cirrhosis	26.60%
History of HCC	15.90%
FIB-4 index ²	2.63 (0.45-19.03)
FIB-4 index > 3.25	40.70%
Wisteria floribunda agglutinin+-Mac-2 binding protein ²	1.94 (0.20-18.51)
Hyaluronic acid ² , ng/mL	97.4 (10.0-2750.0)
History of IFN-based therapy	23.60%
<i>IL28B</i> -SNP rs8099917 TT/non TT	136/38/8
<i>ITPA</i> -SNP rs1127354 CC	129/45/8
HCV genotype 2A/2B/ND	109/70/3
HCV RNA ² , logIU/mL	6.1 (1.2-7.6)
HCV RNA > 6 logIU/mL	58.80%
White blood cell count ² , /mm ³	4575 (1700-12010)
Hemoglobin ² , g/dL	13.7 (10.1-17.6)
Platelet count ² , /mm ³	165 (38-389) × 10 ³
Aspartate aminotransferase ² , IU/L	41 (14-336)
Alanine aminotransferase ² , IU/L	40 (5-391)
Albumin ² , g/dL	4.0 (2.6-5.0)
Total bilirubin ² , mg/dL	0.8 (0.3-3.0)
Blood urea nitrogen ² , mg/dL	13 (5-28)
Creatinine ² , mg/dL	0.71 (0.32-1.23)
Estimated glomerular filtration rate ² , mL/min/1.73 m ²	78.9 (42.6-164.2)

¹Mean ± SD; ²Median (range). M: Male; F: Female; HCC: Hepatocellular carcinoma; FIB: Fibrosis; *IL28B*: *Interleukin-28B*; SNP: Single nucleotide polymorphisms; *ITPA*: *Inosine triphosphate pyrophosphatase*; HCV: Hepatitis C virus.

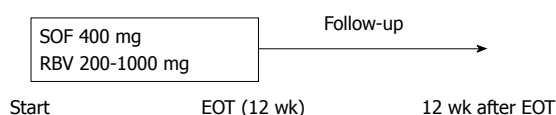


Figure 1 Treatment regimen of sofosbuvir and ribavirin. SOF: Sofosbuvir; RBV: Ribavirin; EOT: End of treatment.

treatment. The primary endpoint was the rate of SVR at 12 wk after the end of treatment (SVR12), defined as undetectable serum HCV RNA at this time point. Relapse and breakthrough were defined based on the guidelines of the American Association for the Study of Liver Diseases^[11].

Safety assessments

Safety evaluations included reported adverse events and serious adverse events, clinical laboratory tests, and physical examinations.

Statistical analysis

Statistical analyses were performed using JMP software version 8.0.2 (SAS Institute Inc., Cary, NC, United States). Hemoglobin levels and estimated glomerular filtration rate were compared by the Tukey honestly significant difference test. Categorical data were

compared by Chi-squared test. To identify independent factors predicting non-SVR, sex, age, liver cirrhosis, and variables with values of *P* < 0.05 in univariate analyses were entered into a multiple logistic regression analysis. Values of *P* < 0.05 were considered statistically significant.

RESULTS

Patient characteristics

The pretreatment characteristics of all patients enrolled in the study are presented in Table 1. There were 48 patients (26.4%) with LC, 43 (23.7%) with history of IFN-based therapy, and 28 (15.4%) with history of HCC. Presence of the SNPs in *IL28B* (rs8099917) and *ITPA* (rs1127354) was examined in 174 of 182 patients. HCV RAS of S282 in the NS5B domain was not detected in the 138 patients examined by the RAS test.

Treatment outcomes

Of the 182 patients, 178 (97.8%) completed the treatment. The causes of treatment discontinuation were fatigue (*n* = 1; 0.5%) and self-withdrawal (*n* = 3; 1.6%). The rates of SVR12 were 87.9% [intention to treat (ITT): 160/182] and 94.1% [per protocol (PP): 159/169]. SVR12 was not evaluated in 12 patients (6.7%) because of dropout from the study (Figure 2).

Hemoglobin levels during the treatment period were significantly reduced until 4 wk after the start of administration (Figure 3A). CC of the *ITPA* allele strongly contributed to anemia (Figure 3B). Twenty-nine (15.9%) of 182 patients needed RBV dose reduction because of anemia, but no patients discontinued the treatment for this reason. Although SOF and RBV cannot be used in patients with decreased renal function and we were concerned about SOF/RBV therapy decreasing the renal function of our patients, the estimated glomerular filtration rate did not change significantly during the treatment period (Figure 3C).

Factors contributing to SVR in the PP analysis

In the PP analysis, 13 of 182 patients who discontinued treatment and/or were not evaluated for SVR12 were excluded (Figure 2A). One hundred twenty three of 169 patients (72.8%) achieved rapid virological response (RVR), defined as undetectable serum HCV RNA after 4 wk of treatment. In univariate analyses, history of IFN-based therapy, LC, and history of HCC reduced the virological response, while age (≥ 70 years), sex, FIB-4 index, *IL28B* SNP, *ITPA* SNP, HCV genotype, pretreatment viral load, RBV dose reduction, and RVR had no effect on the treatment efficacy (Figures 4 and 5). The SNPs of *IL28B* and *ITPA* were determined for 161 patients in the PP analysis. The HCV viral genotype was determined in 166 patients. Multivariate analysis showed that history of HCC or IFN-based therapy was independently related to non-

Table 2 Factors contributing to sustained virological response in the per protocol analysis

	Univariate			Multivariate		
	Odds ratio	95%CI	P value	Odds ratio	95%CI	P value
Age, ≥ 70 yr	2.61	0.70, 9.82	0.1494			
Sex, male	4.76	0.85, 88.89	0.0781			
History of IFN-based therapy	5.12	1.39, 20.98	0.0147 ^a	7.05	1.65, 36.08	0.0084 ^a
Liver cirrhosis	6.72	1.78, 32.29	0.0048 ^a	2.13	0.31, 14.34	0.4315
FIB-4 index, > 3.25	2.11	0.58, 8.54	0.2546			
History of HCC	8.87	2.36, 37.04	0.0015 ^a	7.67	1.30, 60.30	0.0233 ^a
<i>IL28B</i> -SNP, non TT	1.21	0.17, 5.55	0.8214			
<i>ITPA</i> -SNP, non CC	0.45	0.02, 2.63	0.4177			
HCV genotype, 2A	2.78	0.67, 18.84	0.1687			
Viral load, ≥ 6 logIU/mL	1.53	0.41, 7.31	0.5478			
RBV dose reduction	2.29	0.47, 8.89	0.2757			
Rapid virological response	0.86	0.22, 4.15	0.8401			

^a $P < 0.05$, significant difference. IFN: Interferon; FIB: Fibrosis; HCC: Hepatocellular carcinoma; *IL28B*: Interleukin-28B; SNP: Single nucleotide polymorphisms; *ITPA*: Inosine triphosphate pyrophosphatase; HCV: Hepatitis C virus; RBV: Ribavirin.

Table 3 Assessment of patients with history of hepatitis C virus

	SVR ($n = 23$)	Non SVR ($n = 6$)	P value
Alpha fetoprotein	9.6 (1.7-348.9)	5.3 (2.6-65.7)	0.2815
Latest treatment for HCC			0.2558
Surgical resection or Radiofrequency ablation	73.9% (17)	66.6% (6)	
Transcatheter arterial chemoembolization	26.1% (6)	16.7% (1)	
Radiation for bone metastasis	0% (0)	16.7% (1)	
Recurrent HCC within 1 yr from the end of SOF/RBV	65.2% (15)	83.3% (5)	0.6328

SVR: Sustained virological response; HCC: Hepatocellular carcinoma; SOF/RBV: Sofosbuvir/ribavirin.

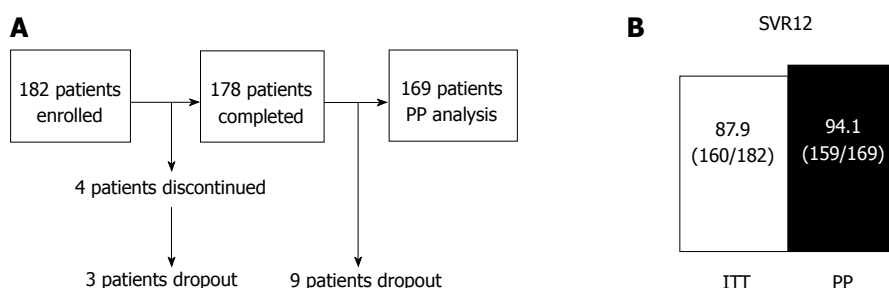


Figure 2 Flow sheet of this study (A) and virological response rates for combination therapy with sofosbuvir and ribavirin (B). The rates of sustained virological response at 12 wk after the end of treatment are shown for intention to treat and per protocol analyses. PP: Per protocol; ITT: Intention to treat; SVR12: Sustained virological response at 12 wk after the end of treatment.

SVR (Table 2).

Assessment of patients with history of HCC

We assessed 29 patients with history of HCC. We ascertained that their HCC was not detected by dynamic computed tomography or dynamic magnetic resonance imaging before initiation of SOF/RBV therapy. We compared alpha fetoprotein (AFP), latest therapy for HCC, and HCC recurrence within 1 year after the end of SOF/RBV therapy according to SVR or non-SVR (Table 3). The AFP level did not differ significantly between the two groups. Patients achieving SVR tended to be treated by surgical resection or radiofrequency ablation,

and also tended to have no HCC recurrence.

DISCUSSION

Combination therapy with SOF/RBV was the first IFN-free therapy for GT2-HCV infection approved for clinical use in Japan. In a phase 3 trial, 97% of patients achieved SVR12 and no patients discontinued the treatment^[5]. Thus, we expected high efficacy and tolerability, and conducted a prospective study in a real-world clinical practice setting to investigate the efficacy and safety of this combination therapy for Japanese patients with GT2-HCV infection.

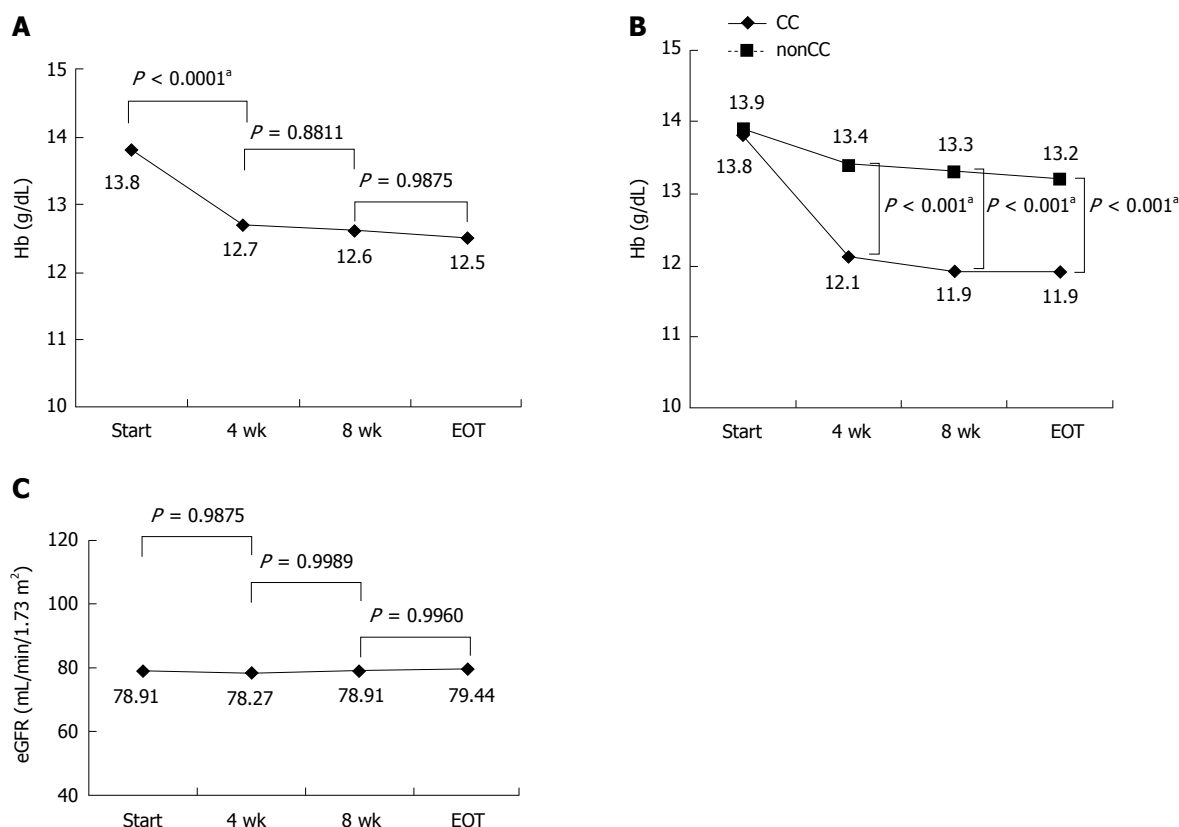


Figure 3 Hemoglobin levels in all patients during combination therapy with sofosbuvir and ribavirin (A), hemoglobin levels in 175 patients categorized by *inosine triphosphate pyrophosphatase* single nucleotide polymorphism rs1127354 (CC or non CC) (B), and estimated glomerular filtration rate levels in all patients during sofosbuvir/ribavirin therapy (C). Hb: Hemoglobin; EOT: End of treatment; eGFR: Estimated glomerular filtration rate. ^a $P < 0.05$, significant difference.

Hemoglobin levels rapidly reduced until 4 wk after the start of treatment. *ITPA* CC allele significantly contributed to anemia in this therapy, similar to the case for combination therapy of PEG-IFN and RBV^[7-9]. Therefore, patients with the *ITPA* CC allele should be monitored frequently for their hemoglobin levels until 4 wk after the start of treatment. Despite our concern that SOF and RBV may decrease renal function of the patients, renal function degeneration was not observed in any of the patients. Many elderly patients (27.4% were aged ≥ 70 years) and cirrhotic patients (26.6% had liver cirrhosis) were enrolled in this study. Thus, the present findings demonstrate that SOF/RBV therapy is safe and highly tolerated regardless of age and LC.

The ITT rate in this study was low compared with the rates in phase 3 clinical trials of SOF/RBV for Japanese^[5] and European^[12] patients with GT2-HCV infection. The reason for the difference was that SVR12 could not be evaluated in 12 patients (6.7%) because of dropout from the study. It is important to monitor carcinogenesis after virus elimination, and it is therefore necessary to provide patients with instructions that promote periodic examinations. Meanwhile, the PP rate was almost equal to the rates in the phase 3 clinical trial of SOF/RBV for Japanese patients with GT2-HCV infection^[5] and in real-world cohorts in North America and Europe^[13].

This finding shows that SOF/RBV therapy for Japanese patients with GT2-HCV infection is highly effective in real-world clinical practice.

In a meta-analysis, Rangnekar *et al.*^[14] reported that *IL28B* SNP was predictor of SVR in Caucasian patients with GT2-HCV infection receiving PEG-IFN/RBV for 24 wk. In contrast, *IL28B* SNP was not associated with SVR in Asian patients with GT2-HCV infection. This study also showed Japanese *IL28B* SNP had no relation to efficacy of SOF/RBV for GT2-HCV infection. Morisco *et al.*^[15] reported RVR was the only independent predictive factor of SVR in triple therapy (TVR/PEG-IFN/RBV) regardless of LC. In the present study, RVR did not affect efficacy of SOF/RBV for GT2-HCV infection. The multivariate regression analysis showed that history of HCC or IFN-based therapy was independently related to non-SVR. LC also reduced the efficacy in univariate analyses. It was previously reported that the efficacy of IFN-based therapy was inferior in LC patients^[16,17]. Furthermore, it was reported that patients with progressive liver fibrosis have a high probability of hepatocellular carcinogenesis^[18-21]. Prenner *et al.*^[22] reported that presence of active HCC at the start of direct acting antivirals, including SOF/RBV therapy, decreased the SVR rate. In our study, the proportion of non-SVR patients treated by surgical resection or radiofrequency ablation tended to be lower than the

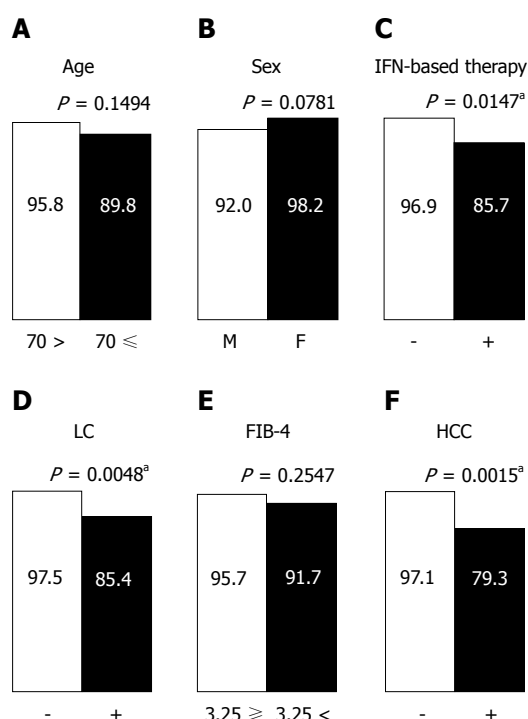


Figure 4 Virological response in patients with sofosbuvir and ribavirin (SOF/RBV) combination therapy categorized by patient characteristics. A: Age (< 70 or ≥ 70 yr); B: Sex (male or female); C: History of interferon-based therapy (- or +); D: Liver cirrhosis (- or +); E: Fibrosis-4 index (≤ 3.25 or > 3.25); F: History of hepatocellular carcinoma (- or +). ^a*P* < 0.05: Significant difference. M: Male; F: Female; IFN: Interferon; LC: Liver cirrhosis; FIB: Fibrosis; HCC: Hepatocellular carcinoma.

proportion of non-SVR patients treated by transcatheter arterial chemoembolization or radiation for bone metastasis. Meanwhile, the rate of HCC recurrence within 1 year tended to be higher in non-SVR patients than that in SVR patients. These findings show a high probability that active HCC was not detected by dynamic computed tomography or dynamic magnetic resonance imaging at the start of SOF/RBV therapy. Prenner *et al.*^[22] discussed that a putative biological explanation could be inadequate drug delivery by decreased blood flow or local fibrosis arising from HCC treatment. The present results suggested that liver fibrosis attenuated the antiviral effect of SOF/RBV in patients with history of IFN-based therapy or HCC. Although the FIB-4 index, a fibrosis marker^[10], had no effect on efficacy in the present study, the reason was unclear. History of IFN-based therapy and LC had no significant effect on SOF/RBV therapy in the phase 3 clinical trial for European patients with GT2-HCV infection^[12]. However, it was reported that LC and lower serum albumin decreased the SVR rate in the real-world cohorts in North America and Europe^[13]. Treatment-experienced patients tended to have ineffective outcomes in that study. These findings were similar to those in the present study. The real-world studies included larger populations of LC (26.6% in the present study and 26.8% in the North American and European study) than the phase 3 trials (11% in the Japanese trial^[5] and 15% in the European

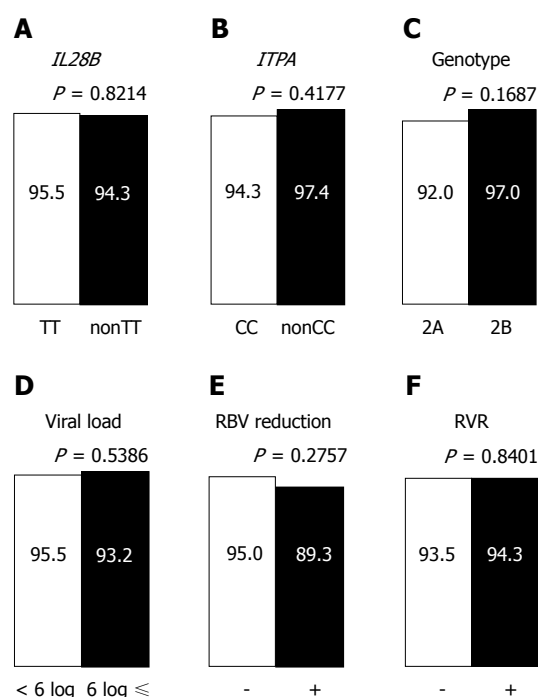


Figure 5 Virological response in patients with sofosbuvir and ribavirin combination therapy categorized by single nucleotide polymorphisms related to anti-hepatitis C virus therapy, pretreatment viral status, ribavirin dose, and rapid virological response. A: *IL28B* single nucleotide polymorphisms rs8099917 (TT or non TT); B: *ITPA* single nucleotide polymorphisms rs1127354 (CC or non CC); C: Hepatitis C virus genotype (2A or 2B); D: Pretreatment viral load (< 6 logIU/mL or ≥ 6 logIU/mL); E: Ribavirin dose reduction (- or +); F: Rapid virological response (- or +). ^a*P* < 0.05: Significant difference. *IL28B*: Interleukin-28B; *ITPA*: Inosine triphosphate pyrophosphatase; RBV: Ribavirin; RVR: Rapid virological response.

trial^[12]. Our study also enrolled patients with history of HCC (15.9%) and elderly patients aged ≥ 70 years (27.4%). It is suggested that the real-world studies enrolled more patients with severe fibrosis than the phase 3 clinical trials. As viral breakthrough did not occur in any of the non-SVR patients and all of these patients had relapses, it is suggested that SOF/RBV therapy was not ineffective for these patients. Extension of the treatment period for patients with history of IFN-based therapy, HCC, or LC should be considered to increase the efficacy of the therapy. As active HCC not detected by imaging was probably related to non-SVR, monitoring is required for carcinogenesis and recurrence of HCC after the end of SOF/RBV therapy, especially in non-SVR patients.

In conclusion, SOF/RBV therapy for Japanese patients with GT2-HCV infection is safe, highly tolerated, and effective. History of HCC or IFN-based therapy independently reduces the efficacy of the treatment.

ARTICLE HIGHLIGHTS

Research background

Combination therapy with peg-interferon (PEG-IFN) and ribavirin (RBV) was the first-line therapy for genotype 2 hepatitis C virus (HCV) infection, but only 80% of patients achieved elimination of HCV with this treatment. The introduction of direct-acting antiviral agents has drastically improved the efficacy of treatments

for chronic HCV infection. Combination therapy with NS5B RNA-dependent RNA polymerase inhibitor sofosbuvir (SOF) and RBV for patients with genotype 2 hepatitis C virus (GT2-HCV) infection was approved for clinical use in June 2015.

Research motivation

This therapy showed improved efficacy and was well tolerated in a phase 3 trial. However, predictive factor of sustained virological response (SVR) is not unclear.

Research objectives

We conducted a prospective study to investigate the efficacy and safety of sofosbuvir/ribavirin (SOF/RBV) therapy for Japanese patients with GT2-HCV infection in a real-world clinical setting.

Research methods

A total of 182 patients with GT2-HCV infection who received SOF/RBV therapy for 12 wk at our hospital were enrolled. The patients comprised 122 men and 60 women (age range: 17-84 years; mean age \pm SD: 60.1 \pm 12.1 years). One hundred sixty nine of 182 patients completed 12 wk treatment and were examined their virological response. To investigate predictive factors of SVR, we examined the relationships between virological response and clinical data by logistic regression analyses.

Research results

The rates of SVR at 12 wk after the end of treatment were 87.9% (intention to treat: 160/182) and 94.1% (per protocol: 159/169). Multivariate analyses showed that history of hepatocellular carcinoma (HCC) or IFN-based therapy independently reduced the efficacy of SOF/RBV therapy.

Research conclusions

This study showed Japanese *IL28B* single nucleotide polymorphisms had no relation to efficacy of SOF/RBV for GT2-HCV infection. Morisco *et al.*^[15] reported rapid virological response (RVR) was the only independent predictive factor of SVR in triple therapy (Telaprevir/PEG-IFN/RBV) regardless of cirrhosis. In the present study, RVR did not affect efficacy of SOF/RBV for GT2-HCV infection. The multivariate regression analysis showed that history of HCC or IFN-based therapy was independently related to non-SVR. In our study, the proportion of non-SVR patients treated by surgical resection or radiofrequency ablation tended to be lower than the proportion of non-SVR patients treated by transcatheter arterial chemoembolization or radiation for bone metastasis. Meanwhile, the rate of HCC recurrence within 1 year tended to be higher in non-SVR patients than that in SVR patients.

Research perspectives

Prenner *et al* reported that presence of active HCC at the start of direct acting antivirals, including SOF/RBV therapy, decreased the SVR rate. As active HCC not detected by imaging was probably related to non-SVR, monitoring is required for carcinogenesis and recurrence of HCC after the end of SOF/RBV therapy, especially in non-SVR patients.

ACKNOWLEDGMENTS

We are grateful to Ishibashi Y for assistance with manuscript preparation.

REFERENCES

- 1 World Health Organization. Global hepatitis report, 2017. 2017 Available from: URL: <http://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/>
- 2 Liakina V, Hamid S, Tanaka J, Olafsson S, Sharara AI, Alavian SM, Gheorghe L, El Hassan ES, Abaalkhail F, Abbas Z, Abdou A, Abourached A, Al Braiki F, Al Hosani F, Al Jaber K, Al Khatry M, Al Mulla MA, Al Quraishi H, Al Rifai A, Al Serkal Y, Alam A, Alashgar HI, Alawadhi S, Al-Dabal L, Aldins P, Alfaleh FZ, Alghamdi AS, Al-Hakeem R, Aljumah AA, Almessaabi A, Alqutub AN, Alswat KA, Altraif I, Alzaabi M, Andrea N, Assiri AM, Babatin MA, Baqir A, Barakat MT, Bergmann OM, Bizri AR, Blach S, Chaudhry A, Choi MS, Diab T, Djauzi S, El Khoury S, Estes C, Fakhry S, Farooqi JI, Fridjonsdottir H, Gani RA, Ghafoor Khan A, Goldis A, Gottfredsson M, Gregorcic S, Hajarizadeh B, Han KH, Hasan I, Hashim A, Horvath G, Hunyady B, Husni R, Jafri W, Jeruma A, Jonasson JG, Karlsdottir B, Kim DY, Kim YS, Koutoubi Z, Lesmana LA, Lim YS, Löve A, Maimets M, Makara M, Malekzadeh R, Maticic M, Memon MS, Merat S, Mokhtab JE, Mourad FH, Muljono DH, Nawaz A, Nugrahini N, Prihotomo S, Qureshi H, Rassam P, Razavi H, Razavi-Shearer D, Razavi-Shearer K, Rozentale B, Sadik M, Saeed K, Salamat A, Salupere R, Sanai FM, Sanityoso Sulaiman A, Sayegh RA, Schmelzer JD, Sibley A, Siddiq M, Siddiqui AM, Sigmundsdottir G, Sigurdardottir B, Speiciene D, Sulaiman A, Sultan MA, Taha M, Tarifi H, Tayyab G, Tolmane I, Ud Din M, Umar M, Valantinas J, Videcnik-Zorman J, Yaghi C, Yuniastuti E, Yusuf MA, Zuberi BF, Gunter J. Historical epidemiology of hepatitis C virus (HCV) in select countries - volume 3. *J Viral Hepat* 2015; **22** Suppl 4: 4-20 [PMID: 26513445 DOI: 10.1111/jvh.12475]
- 3 Sato K, Hashizume H, Yamazaki Y, Horiguchi N, Kakizaki S, Takagi H, Mori M; Gunma Liver Study Group. Response-guided peginterferon-alpha-2b plus ribavirin therapy for chronic hepatitis C patients with genotype 2 and high viral loads. *Hepatol Res* 2012; **42**: 854-863 [PMID: 22487210 DOI: 10.1111/j.1872-034X.2012.00997.x]
- 4 Kumada H, Sato K, Takehara T, Nakamuta M, Ishigami M, Chayama K, Toyota J, Suzuki F, Nakayasu Y, Ochi M, Yamada I, Okanoue T. Efficacy of telaprevir-based therapy for difficult-to-treat patients with genotype 2 chronic hepatitis C in Japan. *Hepatol Res* 2015; **45**: 745-754 [PMID: 25196718 DOI: 10.1111/hepr.12416]
- 5 Omata M, Nishiguchi S, Ueno Y, Mochizuki H, Izumi N, Ikeda F, Toyoda H, Yokosuka O, Nirei K, Genda T, Umemura T, Takehara T, Sakamoto N, Nishigaki Y, Nakane K, Toda N, Ide T, Yanase M, Hino K, Gao B, Garrison KL, Dvory-Sobol H, Ishizaki A, Omote M, Brainard D, Knox S, Symonds WT, McHutchison JG, Yatsushashi H, Mizokami M. Sofosbuvir plus ribavirin in Japanese patients with chronic genotype 2 HCV infection: an open-label, phase 3 trial. *J Viral Hepat* 2014; **21**: 762-768 [PMID: 25196837 DOI: 10.1111/jvh.12312]
- 6 Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41**: 1105-1109 [PMID: 19749757 DOI: 10.1038/ng.449]
- 7 Fellay J, Thompson AJ, Ge D, Gumbs CE, Urban TJ, Shianna KV, Little LD, Qiu P, Bertelsen AH, Watson M, Warner A, Muir AJ, Brass C, Albrecht J, Sulkowski M, McHutchison JG, Goldstein DB. ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature* 2010; **464**: 405-408 [PMID: 20173735 DOI: 10.1038/nature08825]
- 8 Ochi H, Maekawa T, Abe H, Hayashida Y, Nakano R, Kubo M, Tsunoda T, Hayes CN, Kumada H, Nakamura Y, Chayama K. ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy--a genome-wide study of Japanese HCV virus patients. *Gastroenterology* 2010; **139**: 1190-1197 [PMID: 20637204 DOI: 10.1053/j.gastro.2010.06.071]
- 9 Thompson AJ, Fellay J, Patel K, Tillmann HL, Naggie S, Ge D, Urban TJ, Shianna KV, Muir AJ, Fried MW, Afdhal NH, Goldstein DB, McHutchison JG. Variants in the ITPA gene protect against ribavirin-induced hemolytic anemia and decrease the need for ribavirin dose reduction. *Gastroenterology* 2010; **139**: 1181-1189 [PMID: 20547162 DOI: 10.1053/j.gastro.2010.06.016]
- 10 Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, S Sulkowski M, Torriani FJ, Dieterich DT, Thomas

- DL, Messinger D, Nelson M; APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317-1325 [PMID: 16729309 DOI: 10.1002/hep.21178]
- 11 **Ghany MG**, Strader DB, Thomas DL, Seeff LB; American Association for the Study of Liver Diseases. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335-1374 [PMID: 19330875 DOI: 10.1002/hep.22759]
 - 12 **Zeuzem S**, Dusheiko GM, Salupere R, Mangia A, Flisiak R, Hyland RH, Illeperuma A, Svarovskaia E, Brainard DM, Symonds WT, Subramanian GM, McHutchison JG, Weiland O, Reesink HW, Ferenci P, Hézode C, Esteban R; VALENCE Investigators. Sofosbuvir and ribavirin in HCV genotypes 2 and 3. *N Engl J Med* 2014; **370**: 1993-2001 [PMID: 24795201 DOI: 10.1056/NEJMoa1316145]
 - 13 **Welzel TM**, Nelson DR, Morelli G, Di Bisceglie A, Reddy RK, Kuo A, Lim JK, Darling J, Pockros P, Galati JS, Frazier LM, Alqahtani S, Sulkowski MS, Vainorius M, Akushevich L, Fried MW, Zeuzem S; HCV-TARGET Study Group. Effectiveness and safety of sofosbuvir plus ribavirin for the treatment of HCV genotype 2 infection: results of the real-world, clinical practice HCV-TARGET study. *Gut* 2017; **66**: 1844-1852 [PMID: 27418632 DOI: 10.1136/gutjnl-2016-311609]
 - 14 **Rangnekar AS**, Fontana RJ. IL-28B polymorphisms and the response to antiviral therapy in HCV genotype 2 and 3 varies by ethnicity: a meta-analysis. *J Viral Hepat* 2013; **20**: 377-384 [PMID: 23647954 DOI: 10.1111/jvh.12039]
 - 15 **Morisco F**, Masarone M, Rosato V, Camera S, Granata R, Tartaglione MT, Coppola C, Coppola N, Salomone-Megna A, Gentile I, De Luna A, Federico A, Precone D, Claar E, Abenavoli L, Persico M. Impact of Telaprevir in HCV Patients with Cirrhosis and RVR: Real-Life Data from Boceprevir or Telaprevir based "Triple Therapy" Experience in Southern Italy. *Rev Recent Clin Trials* 2016; **11**: 306-316 [PMID: 26672601]
 - 16 **Kaserer K**, Fiedler R, Steindl P, Müller CH, Wrba F, Ferenci P. Liver biopsy is a useful predictor of response to interferon therapy in chronic hepatitis C. *Histopathology* 1998; **32**: 454-461 [PMID: 9639122]
 - 17 **Akuta N**, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. *J Med Virol* 2007; **79**: 1686-1695 [PMID: 17854035 DOI: 10.1002/jmv.20979]
 - 18 **Zaman SN**, Melia WM, Johnson RD, Portmann BC, Johnson PJ, Williams R. Risk factors in development of hepatocellular carcinoma in cirrhosis: prospective study of 613 patients. *Lancet* 1985; **1**: 1357-1360 [PMID: 2861313]
 - 19 **Shiffman ML**. Natural history and risk factors for progression of hepatitis C virus disease and development of hepatocellular cancer before liver transplantation. *Liver Transpl* 2003; **9**: S14-S20 [PMID: 14586890 DOI: 10.1053/jlts.2003.50254]
 - 20 **Ikeda M**, Fujiyama S, Tanaka M, Sata M, Ide T, Yatsushashi H, Watanabe H. Risk factors for development of hepatocellular carcinoma in patients with chronic hepatitis C after sustained response to interferon. *J Gastroenterol* 2005; **40**: 148-156 [PMID: 15770398 DOI: 10.1007/s00535-004-1519-2]
 - 21 **Tokita H**, Fukui H, Tanaka A, Kamitsukasa H, Yagura M, Harada H, Okamoto H. Risk factors for the development of hepatocellular carcinoma among patients with chronic hepatitis C who achieved a sustained virological response to interferon therapy. *J Gastroenterol Hepatol* 2005; **20**: 752-758 [PMID: 15853990 DOI: 10.1111/j.1440-1746.2005.03800.x]
 - 22 **Prenner SB**, VanWagner LB, Flamm SL, Salem R, Lewandowski RJ, Kulik L. Hepatocellular carcinoma decreases the chance of successful hepatitis C virus therapy with direct-acting antivirals. *J Hepatol* 2017; **66**: 1173-1181 [PMID: 28161470 DOI: 10.1016/j.jhep.2017.01.020]

P- Reviewer: Abenavoli L, Dogan UB, El-Shabrawi MHF, Esmat S, Waheed Y **S- Editor:** Wang XJ **L- Editor:** A **E- Editor:** Huang Y



Gilbert syndrome combined with prolonged jaundice caused by contrast agent: Case report

Jian-Dan Qian, Feng-Qin Hou, Tai-Ling Wang, Chen Shao, Gui-Qiang Wang

Jian-Dan Qian, Feng-Qin Hou, Gui-Qiang Wang, Department of Infectious Diseases and the Center for Liver Diseases, Peking University First Hospital, Beijing 100034, China

Tai-Ling Wang, Department of Pathology, China-Japan Friendship Hospital, Beijing 100029, China

Chen Shao, Department of Pathology, Beijing YouAn Hospital Capital Medical University, Beijing 100069, China

Gui-Qiang Wang, The Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Gui-Qiang Wang, Peking University International Hospital, Beijing 102206, China

ORCID number: Jian-Dan Qian (0000-0002-0112-7500); Feng-Qin Hou (0000-0002-4771-0478); Tai-Ling Wang (0000-0003-2006-0978); Chen Shao (0000-0002-7914-5493); Gui-Qiang Wang (0000-0003-0515-7974).

Author contributions: Qian JD designed and wrote the report; Hou FQ reviewed the manuscript for its intellectual content and revised the entire work; Wang TL and Shao C performed the histological assessments and evaluations; Wang GQ reviewed the manuscript for its intellectual content.

Supported by National Natural Science Foundation of China, No. 81470849.

Informed consent statement: The patient involved in this study gave written informed consent authorizing the use and disclosure of his protected health information.

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to disclose.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

licenses/by-nc/4.0/

Manuscript source: Unsolicited manuscript

Correspondence to: Gui-Qiang Wang, MD, PhD, Professor, Department of Infectious Diseases and the Center for Liver Diseases, Peking University First Hospital, 8 Xishiku Dajie, Xicheng District, Beijing 100034, China. 04486@pkufh.com
Fax: +86-10-66551680

Received: January 19, 2018

Peer-review started: January 20, 2018

First decision: February 3, 2018

Revised: February 7, 2018

Accepted: February 26, 2018

Article in press: February 26, 2018

Published online: April 7, 2018

Abstract

This case highlights a patient with Gilbert syndrome who underwent endoscopic retrograde cholangiopancreatography (ERCP) with removal of bile duct stones, who then experienced an unexplained increase in bilirubin, with total bilirubin (TBIL) levels increasing from 159.5 $\mu\text{mol/L}$ to 396.2 $\mu\text{mol/L}$ and to a maximum of 502.8 $\mu\text{mol/L}$ after 9 d. Following the decrease in the TBIL level, enhanced magnetic resonance cholangiopancreatography (MRCP) was performed to exclude any possible remaining choledocholithiasis. Nevertheless, the serum bilirubin level increased again, with TBIL levels rising from 455.7 $\mu\text{mol/L}$ to 594.8 $\mu\text{mol/L}$ and a maximum level of 660.3 $\mu\text{mol/L}$ with no remaining bile duct stones. A liver biopsy showed severe bile duct cholestasis with no inflammation. Based on the exclusion of other potential causes of hyperbilirubinemia and the fact that both instances of increased bilirubin occurred after ERCP and MRCP, the contrast agents iopromide and gadoterate meglumine were suspected to be the causes of the hyperbilirubinemia. As of the writing of this report, the patient's bilirubin levels have spontaneously returned to baseline levels. In summary,

ERCP and MRCP utilizing the contrast agents iopromide and gadoterate meglumine may possibly induce prolonged hyperbilirubinemia.

Key words: Contrast agent; Iopromide; Gadoterate meglumine; Gilbert syndrome; Jaundice

© **The Author(s) 2018.** Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Over the past years, only few cases of prolonged postendoscopic retrograde cholangiopancreatography jaundice caused by toxicity of the contrast agent iobitridol have been reported in the world. Up to now, no case of postenhanced magnetic resonance cholangiopancreatography-related jaundice has been reported. Persistent jaundice affects the patient's quality of life, even seriously to life-threatening. Because of the high rarity, treatment experience is not sufficient and more cases need to be accumulated for further analysis.

Qian JD, Hou FQ, Wang TL, Shao C, Wang GQ. Gilbert syndrome combined with prolonged jaundice caused by contrast agent: Case report. *World J Gastroenterol* 2018; 24(13): 1486-1490 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i13/1486.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i13.1486>

INTRODUCTION

Gilbert syndrome is a liver disorder caused by a genetic mutation of the bilirubin UDP-glucuronosyltransferase (UGT1A1) gene, which results in elevated levels of unconjugated bilirubin. The elevation of serum bilirubin is usually mild, typically less than 102.6 $\mu\text{mol/L}$. Most cases of Gilbert syndrome are detected due to mild jaundice after pubescence or a mild elevation of unconjugated bilirubin found by chance during a blood examination^[1].

Both endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP) can be used for the diagnosis of cholelithiasis. The major complications related to ERCP involve acute pancreatitis, bleeding, sepsis and perforation. Prolonged postERCP jaundice has been described as a rare postERCP complication due to the known toxicity of the contrast agent iobitridol^[2]. However, no cases of postenhanced MRCP-related jaundice have been reported. We report here a case of postERCP and postenhanced-MRCP prolonged hyperbilirubinemia due to toxicity from the contrast agents iopromide and gadoterate meglumine.

CASE REPORT

A 35-year-old man presented with intermittent upper abdominal pain associated with eating greasy food for more than 4 mo. His eyes became yellow, and

he reported dark urine and generalized itching for more than 1 mo. The liver test results at the onset of the disease were as follows: total bilirubin (TBIL) 268.7 $\mu\text{mol/L}$, direct bilirubin (DBIL) 114.4 $\mu\text{mol/L}$, alanine aminotransferase (ALT) 155 IU/L, aspartate aminotransferase (AST) 71 IU/L, alkaline phosphatase (ALP) 172 IU/L, and gamma-glutamyl transpeptidase (GGT) 424 IU/L. Abdominal computed tomography showed the presence of cholecystolithiasis with features of cholecystitis and dilation of the common bile duct (CBD) and intrahepatic ducts, due to a distal CBD obstruction. MRCP showed a low signal of the distal CBD and bile duct expansion.

The patient had undergone ERCP with sphincterotomy and extraction of a CBD stone 2 wk before presenting to our hospital, and the contrast agent used was iopromide (370 mg/mL). After ERCP, the patient's ALT and GGT levels had significantly decreased and quickly returned to normal; although, the bilirubin levels had increased markedly, with the TBIL level increasing from 159.5 $\mu\text{mol/L}$ to 396.2 $\mu\text{mol/L}$ and the DBIL level increasing from 94.2 $\mu\text{mol/L}$ to 186.9 $\mu\text{mol/L}$, and with the ALP level increasing markedly from 135.6 IU/L to 192 IU/L (Figures 1 and 2). The amylase and lipase levels had been normal. Ursodeoxycholic acid (UDCA) had been prescribed at a dose of 250 mg three times daily, but the bilirubin levels continued to increase, resulting in levels of TBIL up to 442.5 $\mu\text{mol/L}$ and DBIL up to 334.9 $\mu\text{mol/L}$. Prednisolone (30 mg daily) had been added, although the hyperbilirubinemia did not improve, at which point the patient presented to our hospital for further diagnosis and treatment.

On the day the patient presented to our hospital, his TBIL level was 502.8 $\mu\text{mol/L}$ and his DBIL level was 295.51 $\mu\text{mol/L}$. Prednisolone was continued at a dose of 30 mg daily. The fourth day after he presented to our hospital, his bilirubin level decreased slightly, with the TBIL level decreasing from 502.8 $\mu\text{mol/L}$ to 455.7 $\mu\text{mol/L}$ and the DBIL level decreasing from 295.51 $\mu\text{mol/L}$ to 277.44 $\mu\text{mol/L}$. Once his bilirubin levels decreased, he underwent enhanced-MRCP with gadoterate meglumine as the contrast agent to exclude the possibility of any remaining choledocholithiasis. However, after the enhanced-MRCP, the patient's bilirubin levels increased again, with the TBIL level increasing from 455.7 $\mu\text{mol/L}$ to 594.8 $\mu\text{mol/L}$ and finally to a maximum of 660.3 $\mu\text{mol/L}$; meanwhile, the patient's DBIL level increased from 277.44 $\mu\text{mol/L}$ to 359.63 $\mu\text{mol/L}$ and then to a maximum of 396.46 $\mu\text{mol/L}$ (Figure 1). The enhanced-MRCP did not show any positive findings.

The markers of infection for hepatitis A, B and C, and other viruses (cytomegalovirus, Epstein-Barr virus) were all negative. Immunological tests for antinuclear antibody, smooth muscle antibody, antimitochondrial antibodies, anti-M2 and antibody to liver kidney microsomal antigen were also negative, with the exception of an increase in IgG-4 values (110 mg/dL). The patient's ferritin level, complete blood count and

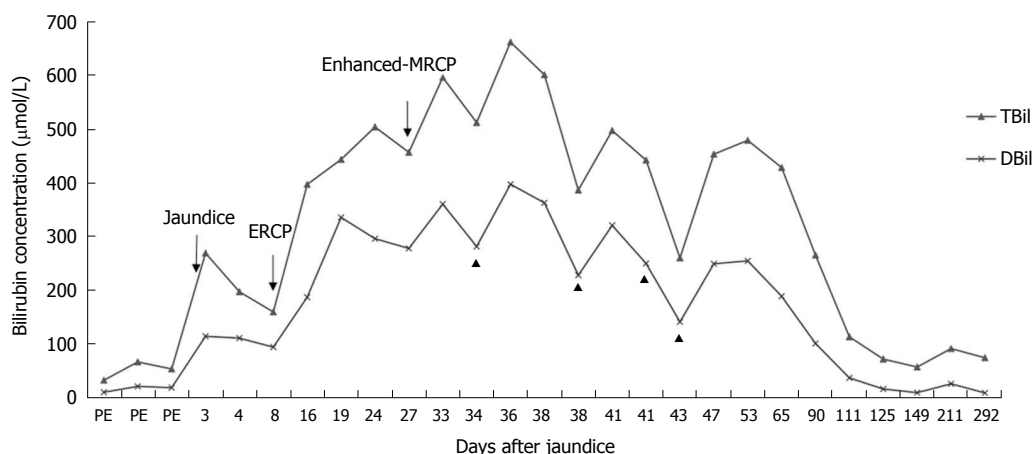


Figure 1 Bilirubin course from onset until the end of follow up. DBil: Direct bilirubin; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; TBil: Total bilirubin; ▲: After bilirubin adsorption treatment.

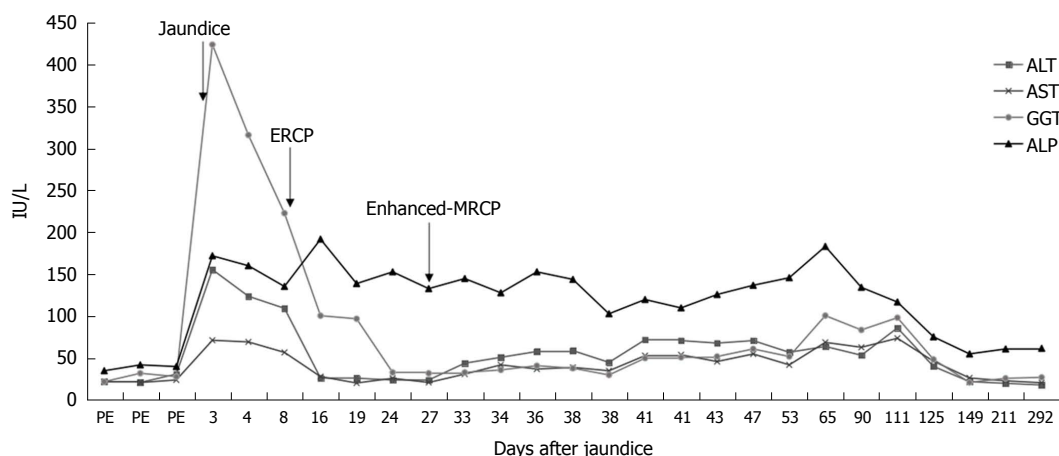


Figure 2 Liver enzyme concentrations from onset until the end of follow up. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase.

inflammatory markers were normal. Gilbert gene sequencing was performed, and the analysis revealed the A(TA)⁷TAA heterozygote [wild type: A(TA)⁶TAA] in the promoter region (UGT1A1*28) and an UGT1A1.80:-364C>T, UGT1A1.112:-1352A>C heterozygote. A liver biopsy showed marked bilirubinostasis in zones 3 and 2, canaliculi with no evidence of portal tract inflammation or interphase hepatitis, no lesions or paucity of bile ducts, and no bile infarcts or leaks (Figures 3 and 4).

Since the age of 17, the patient had experienced intermittent yellow discoloration of the eyes with no other symptoms. Laboratory examinations revealed normal hemoglobin, reticulocyte, aminotransferases and cholestatic enzymes, while the serum TBIL level fluctuated by 32.8 $\mu\text{mol/L}$ to 66.7 $\mu\text{mol/L}$, and the ratio of DBIL to TBIL was 32%. The diagnosis of congenital nonhemolytic bilirubin metabolic disorder was confirmed. The patient's family had no history of similar disorders, but his son had physiologic jaundice at birth. Before onset of the disease, the patient did not take any medications and had no history of alcohol abuse.

Combined with his history of nonhemolytic bilirubin metabolic disorder and genetic test results, the diagnosis of Gilbert syndrome was clear. Because the other potential causes of hyperbilirubinemia were ruled out and the two instances of increased bilirubin occurred postERCP and postMRCP, the use of the contrast agents iopromide and gadoterate meglumine were suspected to be the cause of the hyperbilirubinemia.

The patient was prescribed phenobarbital (60 mg three times daily) and UDCA (500 mg two times daily); prednisolone (30 mg daily) was continued, while cholestyramine (5 g three times daily) was used for symptomatic relief. In addition, with the worsening of cholestasis, bilirubin serum adsorption treatments were performed a total of four times; after each treatment, the serum bilirubin concentration decreased by 11%-23% and clinical symptoms were relieved, but the bilirubin level slightly increased again 1 to 2 d after the bilirubin serum adsorption treatment. Because the bilirubin level declined slowly, the patient was discharged after a 1-mo stay in our hospital.

In the outpatient setting, the oral prednisolone was

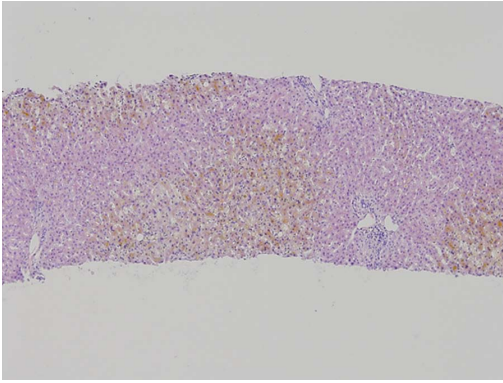


Figure 3 Cholestasis of liver tissue (hematoxylin-eosin stain, original magnification $\times 4$).

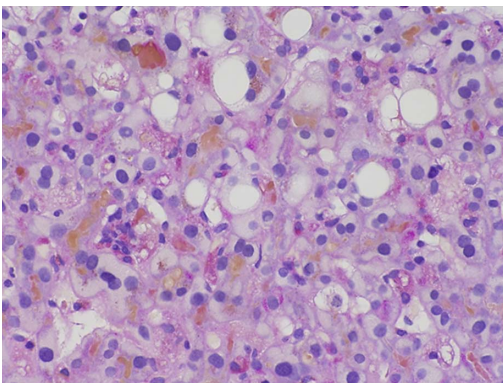


Figure 4 Liver lobular capillary bile duct cholestasis, slight inflammation, undamaged hepatocytes, and some ground-glass cytoplasm degeneration (D-PAS stain, original magnification $\times 60$).

tapered regularly, and phenobarbital and UDCA were discontinued. At his 11-mo follow-up visit, the serum bilirubin level had slowly decreased, and as of the writing of this report, the patient's bilirubin levels had spontaneously returned to baseline levels (Figure 1).

DISCUSSION

Due to the presence of Gilbert syndrome, the patient showed prolonged jaundice postERCP, and enhanced-MRCP accentuated this effect. A liver biopsy revealed marked intrahepatic cholestasis, which may be attributed to large bile obstruction, benign recurrent intrahepatic cholestasis (BRIC) or drug-induced liver toxicity. Enhanced-MRCP had already excluded bile obstruction by stones. BRIC is a rare genetic disorder characterized by intermittent episodes of jaundice and pruritus; each episode can last for several weeks to months. A completely asymptomatic phase occurs in between attacks that can last from months to years. Liver biochemistry is characterized by conjugated hyperbilirubinemia and increased ALP levels. Serum GGT, AST and ALT levels are either normal or mildly elevated. Liver biopsy shows intrahepatic and canalicular cholestasis predominantly involving zone 3^[3].

The patient's liver biopsy features were consistent with BRIC; however, genetic sequencing indicated no mutation in ATP8B1. In addition, the patient had no history of recurrent conjugated hyperbilirubinemia; therefore, the diagnosis of BRIC was not established. Because the two instances of increased bilirubin occurred after ERCP and MRCP, the use of the contrast agents iopromide and gadoterate meglumine was suspected to be the cause of the hyperbilirubinemia.

Prolonged cholestasis is a very rare complication of ERCP, and few cases of this complication are reported in the English literature^[2,4-6]. The exact mechanism of this complication, however, remains unknown. Some articles consider that it may be associated with iodine contrast agents (diatrizoate or iobitridol)^[2,4], while others posit that it may be caused by cefuroxime, which is one of the cephalosporins used after ERCP to prevent infections^[6]. Our patient did not receive cephalosporins postERCP; therefore, the prolonged jaundice was associated with the contrast agents. Additionally, the cause-result time connection between the use of the contrast agents and bilirubin increases supported this possibility.

Gadolinium-based contrast agents (GBCAs) play an important role in enhanced-MRCP examinations. The common side effects of GBCAs include nausea, chest tightness, rash and vascular edema. Gadolinium is mainly renally excreted, although it may also be deposited in the liver, brain, muscle and other organs^[7]. No cases of gadolinium-induced cholestasis have been reported in the literature. Although our patient's bilirubin level increased again after enhanced-MRCP, after his first instance of elevated bilirubin had decreased, we speculated that the gadolinium may have been involved in the cholestasis. The gadolinium used in our patient is ionic, which can lead to high osmotic pressure and dehydration of tissue cells, suggesting that the mechanism of gadolinium-induced hyperbilirubinemia may be due to the high permeability, which further aggravated the damage to the bile duct cells and affected the secretion and excretion of bile.

PostERCP jaundice has been reported to resolve after 2-4 mo, although it took approximately 1 year for this patient's bilirubin level to return to baseline levels, which may be related to the second incident of damage due to gadolinium.

The general treatment for cholestasis caused by contrast agents is UDCA, and cholestyramine can be used to relieve symptoms. If a trial of these two agents proves unsuccessful, corticosteroids may be added^[8] with or without plasmapheresis^[9]. In our case, UDCA (500 mg two times daily), cholestyramine (5 g three times daily) and prednisolone (30 mg daily) failed to relieve the patient's symptoms until bilirubin serum adsorption was performed, which efficiently stabilized the bilirubin level. Therefore, in the case of a poor response to traditional drug treatment methods, providers may try plasma exchange or bilirubin

adsorption, which can prevent damage to liver cells due to long-term elevated bilirubin levels.

Our patient's case illustrates a rare drug-induced liver injury due to contrast agents, which presented as prolonged cholestasis following "successful" therapeutic ERCP for an obstructing distal CBD stone and an enhanced-MRCP that excluded residual stones. Clinicians must be aware that ERCP and MRCP with the contrast agents iopromide and gadoterate meglumine may have the possibility of inducing prolonged hyperbilirubinemia.

ARTICLE HIGHLIGHTS

Case characteristics

A middle-aged male patient presented with abdominal pain, jaundice and dark urine.

Clinical diagnosis

The only physical sign of this case was a mild abdominal tenderness.

Differential diagnosis

Viral hepatitis, other viruses infection (cytomegalovirus, Epstein-Barr virus), autoimmune liver disease, IgG-4-related cholangitis, and benign recurrent intrahepatic cholestasis.

Laboratory diagnosis

The liver test results showed total bilirubin 268.7 $\mu\text{mol/L}$, direct bilirubin 114.4 $\mu\text{mol/L}$, alanine aminotransferase 155 IU/L, aspartate aminotransferase 71 IU/L, alkaline phosphatase 172 IU/L, and gamma-glutamyl transpeptidase 424 IU/L.

Imaging diagnosis

Abdominal computed tomography showed the presence of cholecystolithiasis, with features of cholecystitis and dilation of the common bile duct (CBD) and intrahepatic ducts due to a distal CBD obstruction.

Pathological diagnosis

A liver biopsy showed marked bilirubinostasis in zones 3 and 2, canaliculi with no evidence of portal tract inflammation or interphase hepatitis, no lesions or paucity of bile ducts, and no bile infarcts or leaks.

Treatment

The patient was prescribed phenobarbital, ursodeoxycholic acid, prednisolone and cholestyramine. In addition, with the worsening of cholestasis, bilirubin serum adsorption treatments were performed a total of four times.

Related reports

Prolonged cholestasis is a very rare complication of endoscopic retrograde cholangiopancreatography (ERCP), and few cases of this complication are reported in the English literature. The exact mechanism of this complication,

however, remains unknown. No cases of post enhanced magnetic resonance cholangiopancreatography (MRCP)-related jaundice have been reported.

Term explanation

ERCP: Endoscopic retrograde cholangiopancreatography, which can be used for the diagnosis of cholelithiasis.

Experiences and lessons

Our patient's case illustrates a rare drug-induced liver injury due to contrast agents, which presented as prolonged cholestasis following "successful" therapeutic ERCP for an obstructing distal CBD stone and an enhanced-MRCP that excluded residual stones. Clinicians must be aware that ERCP and MRCP with the contrast agents iopromide and gadoterate meglumine may have the possibility of inducing prolonged hyperbilirubinemia.

REFERENCES

- 1 Aiso M, Yagi M, Tanaka A, Miura K, Miura R, Arizumi T, Takamori Y, Nakahara S, Maruo Y, Takikawa H. Gilbert Syndrome with Concomitant Hereditary Spherocytosis Presenting with Moderate Unconjugated Hyperbilirubinemia. *Intern Med* 2017; **56**: 661-664 [PMID: 28321066 DOI: 10.2169/internalmedicine.56.7362]
- 2 Tziatzios G, Gkolfakis P, Papanikolaou IS, Dimitriadis G, Triantafyllou K. An unusual case of prolonged post-endoscopic retrograde cholangiopancreatography jaundice. *Hepatobiliary Pancreat Dis Int* 2016; **15**: 220-222 [PMID: 27020640]
- 3 Luketic VA, Shiffman ML. Benign recurrent intrahepatic cholestasis. *Clin Liver Dis* 2004; **8**: 133-149, vii [PMID: 15062197 DOI: 10.1016/S1089-3261(03)00133-8]
- 4 Dourakis SP, Mayroyannis C, Alexopoulou A, Hadziyannis SJ. Prolonged cholestatic jaundice after endoscopic retrograde cholangiography. *Hepatogastroenterology* 1997; **44**: 677-680 [PMID: 9222670]
- 5 Chavalitdhamrong D, Donepudi S, Pu L, Draganov PV. Uncommon and rarely reported adverse events of endoscopic retrograde cholangiopancreatography. *Dig Endosc* 2014; **26**: 15-22 [PMID: 24118211 DOI: 10.1111/den.12178]
- 6 Niriella MA, Kumarasena RS, Dassanayake AS, Pathirana A, de Silva Hewavisenthi J, de Silva HJ. Worsening cholestasis and possible cefuroxime-induced liver injury following "successful" therapeutic endoscopic retrograde cholangiopancreatography for a distal common bile duct stone: a case report. *J Med Case Rep* 2016; **10**: 371 [PMID: 28003028 DOI: 10.1186/s13256-016-1123-0]
- 7 Ramalho J, Ramalho M, Jay M, Burke LM, Semelka RC. Gadolinium toxicity and treatment. *Magn Reson Imaging* 2016; **34**: 1394-1398 [PMID: 27693607 DOI: 10.1016/j.mri.2016.09.005]
- 8 Lee HM, Bonis PA, Kaplan MM. Persistent cholestatic jaundice after ERCP. *Am J Gastroenterol* 2006; **101**: 204-205 [PMID: 16405561 DOI: 10.1111/j.1572-0241.2006.00393_7.x]
- 9 Saritas U, Aydin B, Ustundag Y. Plasmapheresis and corticosteroid treatment for persistent jaundice after successful drainage of common bile duct stones by endoscopic retrograde cholangiography. *World J Gastroenterol* 2007; **13**: 4152-4153 [PMID: 17696241 DOI: 10.3748/wjg.v13.i30.4152]

P- Reviewer: Gkekas I, Isaji S, Kitamura K S- Editor: Ma YJ
L- Editor: Filipodia E- Editor: Huang YJ





Published by **Baishideng Publishing Group Inc**
7901 Stoneridge Drive, Suite 501, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: <http://www.f6publishing.com/helpdesk>
<http://www.wjgnet.com>



ISSN 1007-9327

