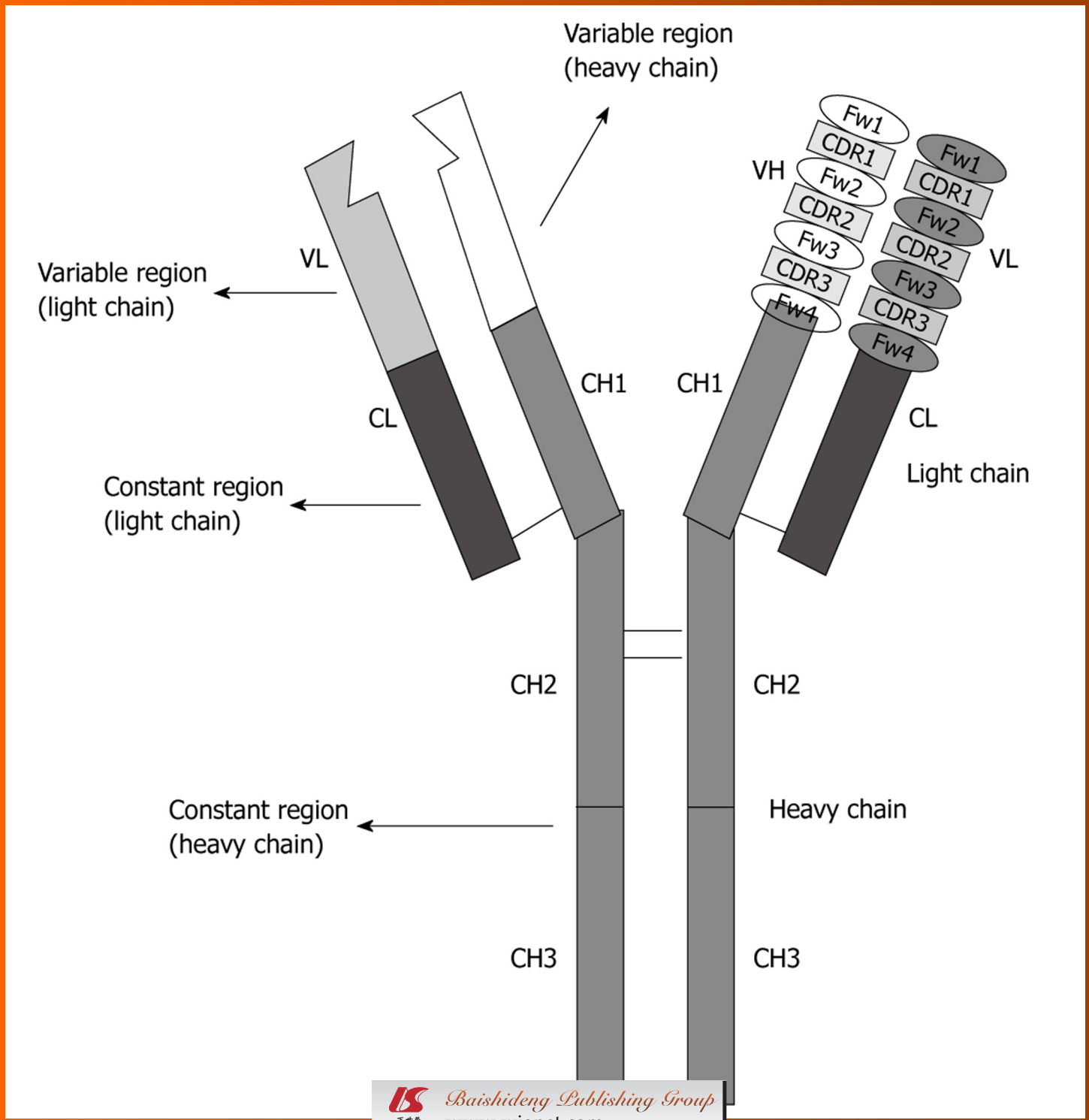
ISSN 2218-4333 (online)

World Journal of *Clinical Oncology*

World J Clin Oncol 2011 June 10; 2(6): 237-280



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WJCO World Journal of Clinical Oncology

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

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2010-2014

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WJCO World Journal of Clinical Oncology

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Monthly Volume 2 Number 6 June 10, 2011

World Journal of Clinical Oncology
Volume 2 Number 6 June 10, 2011

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NAME OF JOURNAL
World Journal of Clinical Oncology

LAUNCH DATE
November 10, 2010

SPONSOR
Beijing Baishideng BioMed Scientific Co., Ltd.,
Room 903, Building D, Ocean International Center,
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http://www.wjgnet.com

PUBLISHING
Baishideng Publishing Group Co., Limited,
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
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http://www.wjgnet.com

PUBLICATION DATE
June 10, 2011

ISSN
ISSN 2218-4333 (online)

EDITORIAL OFFICE
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World Journal of Clinical Oncology
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http://www.wjgnet.com

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/2218-4333/g_info_20100722172206.htm

ONLINE SUBMISSION
http://www.wjgnet.com/2218-4333office

World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v2.i6.237

World J Clin Oncol 2011 June 10; 2(6): 237-244
ISSN 2218-4333 (online)

EDITORIAL

Idiotype vaccines for lymphoma: Potential factors predicting the induction of immune responses

Susana Inoges, Ascension Lopez-Diaz de Cerio, Helena Villanueva, Fernando Pastor, Elena Soria, Maurizio Bendandi

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Received: January 15, 2011 Revised: March 8, 2011
Accepted: March 15, 2011
Published online: June 10, 2011

Abstract

Over the last two decades, lymphoma idiotype vaccines have been the first human cancer vaccines to show striking evidence of biological and clinical efficacy on the one hand, as well as clinical benefit on the other. More recently, however, three large-scale, independent, randomized clinical trials on idiotypic vaccination have failed to achieve their main clinical endpoints for reasons likely to depend more on flaws in each clinical trial's study design than on each vaccination strategy *per se*. Independently of these considerations, a major hurdle for the development of this substantially innocuous and yet potentially very effective type of treatment has been the fact that, even to date, no factors ascertainable before vaccination have been prospectively singled out as predictors of subsequently vaccine-induced, idiotype-specific immune as well as clinical responses. The aim of this review article is precisely to analyze what has been and what could be done in this respect in order to give a greater chance of success to future trials aimed at regulatory approval of idiotype vaccines.

Key words: Clinical outcome; Clinical trial; Idiotype; Immune response; Lymphoma; Vaccine

Peer reviewer: Charles H Lawrie, DPhil, University Research Lecturer, PI, Lymphoid Malignancy Research Group, Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Rm 4834, Level 4, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom

Inoges S, Lopez-Diaz de Cerio A, Villanueva H, Pastor F, Soria E, Bendandi M. Idiotype vaccines for lymphoma: Potential factors predicting the induction of immune responses. *World J Clin Oncol* 2011; 2(6): 237-244 Available from: URL: http://www.wjgnet.com/2218-4333/full/v2/i6/237.htm DOI: http://dx.doi.org/10.5306/wjco.v2.i6.237

THE IDIOTYPE

The term idiotype refers to the entire collection of antigenic determinants called idiotopes, which are displayed on an individual immunoglobulin molecule. Idiotopes can be found solely in the hypervariable regions of the immunoglobulin variable domain, are somatically generated and are recognized as foreign because the limited amount of them normally present in an individual is intrinsically insufficient to elicit the activation of any self-tolerance mechanism[1]. Although it seems plausible that most immunologically relevant idiotopes should structurally encompass, completely or in part, the complementarity-determining regions of the immunoglobulin's variable regions[2], it is important to stress that the two terms are not synonymous, insofar as the former are involved in

the definition of the antigenic properties of the immunoglobulin, while the latter take part in the definition of its specificity as an antibody (Figure 1). In this respect, idiotopes are more thoroughly classified in two categories, that is, public and private idiotopes. The former are largely derived from the immunoglobulin's framework region sequences, whereas the latter mostly arise from the unique immunoglobulin's complementarity-determining region sequences. The implications of this different localization are extremely important, particularly when we consider the whole immunoglobulin no longer in functional terms, that is as an antibody, but rather as an immunological target itself, that is as a collection of antigens in the context of a cancer vaccine-induced, anti-idiotypic immune activation. In particular, only humoral responses against the private idiotopes will have value for tumor suppression, since other antibodies, if at all raised, will be absorbed by the serum immunoglobulins. Similarly, only private idiotopes will ultimately function as a collection of clonal markers for each tumor[3]. Since each immunoglobulin features its own idiotype and identical idiotypes define identical immunoglobulins, the clonal idiotype of a B-cell malignancy can serve as a complete, tumor-specific antigen for vaccine therapy, as long as the tumor cells express it intact as their functional B-cell receptor on the cell membrane and in the form of idiotopes associated with the HLA molecules for epitope presentation[4].

IDIOTYPE PRODUCTION METHODS

Although a number of different procedures are currently employed to reproduce the clonal, patient- and tumor-specific idiotype in the lab, most of them are based on one of the following general methods: large scale culture of hybridomas, recombinant technology and DNA vaccines. While this last option aims at generating a vaccine based on the idiotype-encoding DNA sequence, and has so far found little application in human clinical trials[5], the first two methods aim at reproducing the soluble protein idiotype, which is subsequently integrated in the vaccine formulation, as has been the case in several phase- Ⅰ, -Ⅱ and -Ⅲ clinical studies[6]. It goes without saying that DNA vaccines imply the generation of the idiotype by the patient himself, as some of his somatic cells are transfected through the administration of the DNA sequences encoding only for the idiotype. In contrast, the ultimate product of hybridoma methodology or recombinant technology applications is a whole, idiotype-containing immunoglobulin. Given the specific topic of this review, only studies conducted with soluble protein, whole-immunoglobulin idiotype vaccines will be analyzed, as no clinical trial on idiotype DNA vaccines or on soluble idiotype fragments would allow any conclusions to be drawn on the role of potential factors predicting the induction of specific immune responses because of the very limited number of patients enrolled[7,8].

Most, if not all idiotype vaccine-related achievements in terms of proofs of principle have been achieved using

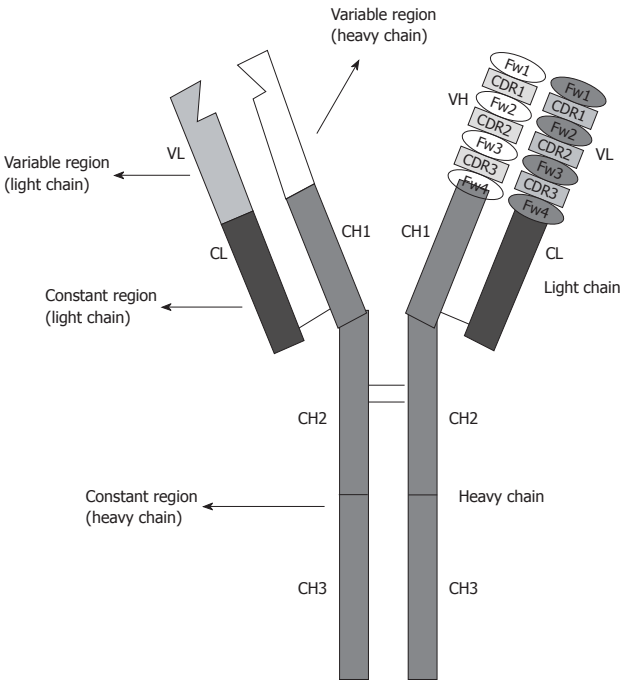

Figure 1 Schematic representation of a monomeric immunoglobulin. Idiotopes are scattered throughout the heavy and light chains' variable regions. Fw: Framework region; CDR: Complementarity-determining region.

idiotype-containing, clonal immunoglobulins obtained through hybridoma-based methodology[9,10]. Among the growing hybridomas, one is ultimately selected according to a number of morphological, genetic, immunological and quantitative features. In particular, this hybridoma should grow relatively rapidly, possess an idiotype molecular fingerprint fully overlapping with that of the corresponding tumor cell[11], and also secrete a sufficient amount of the tumor-specific immunoglobulin to guarantee enough material for all vaccine doses, as well as for the post-vaccine immunological tests[12]. It is intuitive that this methodology, as valuable as it has been from a scientific point of view, can often be time-consuming, logistically demanding, and overall very expensive[6].

A viable methodological alternative to hybridomas is represented by recombinant technology aiming at a molecular rescue of the idiotype, which involves polymerase chain reaction-based amplification and cloning of the genes encoding for the tumor-specific immunoglobulin variable regions, followed by their ligation into plasmid or viral vectors for protein expression in mammalian (e.g. murine lymphoma), insect (e.g. sf9), bacterial (e.g. *Escherichia coli*) or plant (e.g. tobacco) cells[13]. Depending on the specific method utilized, the ultimate recombinant, custom-made idiotype can still be embedded into a full-length tetrameric immunoglobulin in which a common heavy chain backbone may consist of either a human IgG3 or IgG1 scaffold. All in all, it is important to note that when hybridoma methodology is employed, the whole immunoglobulin obtained is virtually identical, at least in terms of amino acid sequence, to that featured by the original tumor, while when recombinant technology

is utilized this can be said only for the idiotype itself, as the heavy chains differ quite substantially.

THE MAIN IDIOTYPE VACCINE FORMULATION

Independently of the method used to reproduce the tumor-specific, soluble protein idiotype in the lab, the most widely used vaccine formulations employ chemical conjugation with the powerful immunogenic carrier keyhole Limpet Hemocyanin (KLH)[14]. The function of this molecule is indeed that of enhancing idiotype immunogenicity, which despite the specificity of the private idiotope collection remains substantially low. Furthermore, clinical grade granulocyte-monocyte colony-stimulating factor (GM-CSF) is used as an immunologic adjuvant to be added to the ultimate vaccine formulation[15]. The typical dose of both idiotype and KLH has been 0.5 mg of each per vaccination since the inception of the use of this experimental approach in humans[16], while the dose of GM-CSF has ranged from 100 to 500 mcg per vaccination in different clinical trials, but with no evidence of substantially different outcome among them[17]. So far, the most impressive clinical results with idiotypic vaccination have been obtained in patients with follicular lymphoma[18]. However, given the peculiar focus of this review, some inferences will also be made based on the sole idiotype vaccine clinical trial ever conducted in patients with mantle cell lymphoma[19].

POTENTIAL FACTORS PREDICTING RESPONSE TO IDIOTYPIC VACCINATION

In principle, potential factors predicting whether idiotypic vaccination may induce clinically-relevant immune responses could independently depend on several biological aspects involving each patient *per se*, the type of lymphoma, the tumor-specific immunoglobulin as a whole, each idiotype, the pre-vaccine treatment administered to patients, the number of vaccine doses administered and the duration of the vaccination program in which patients are enrolled. Moreover, it has to be underlined that any of these factors which may be retrospectively singled out from closed clinical trials should also undergo further subsequent verification in a prospective manner, as it is not unusual that the latter ends up disproving the validity of the former's conclusions. Finally, it has to be borne in mind that each patient's tumor-specific idiotype is a weak antigen that normally does not elicit any immune response in the autologous setting.

Factors directly related to the patient himself or herself may play a role before, during and even after the vaccine administration program takes place. For instance, age could be a crucial factor insofar as elderly patients may feature a lymphoma-harboring immune system that is *per se* weaker than that of younger patients, or might become weaker due to the pre-vaccine therapy. Yet, no study has ever focused on ascertaining whether an age threshold might or should be established, above which vaccination may be meaningless and even detrimental to the success of clinical trials, particularly in follicular lymphoma[20], a disease affecting and recruiting in clinical trials many elderly patients. Another factor depending on each patient and virtually impossible to both assess and chart is his or her immune function status at the time of each vaccination, as most if not all clinical trials allow postponing vaccine dose administration by 1 wk or so in case of concomitant disease (e.g. bacterial or viral infections), but it is not known whether either the acute illness or the vaccination schedule modification might affect idiotype vaccine effectiveness.

The type of lymphoma may also affect idiotype vaccine efficacy, although too few attempts other than against follicular lymphoma have been made to draw firm conclusions. For instance, it is possible that small lymphocytic lymphoma, with its lower expression of tumor surface immunoglobulin than that of other B-cell malignancies, may be less amenable to this vaccination strategy. It suffices to say that, in principle, any B-cell lymphoma[21] whose cells express a full immunoglobulin on their surface may potentially benefit from the otherwise innocuous[22] idiotypic vaccination. However, further studies would be required to convert this speculation into a factual statement, particularly in relapsed/refractory large B-cell as well as in mantle cell lymphoma.

A recent report[23], not yet fully published, has focused on the idiotype-containing, whole tumor immunoglobulin as the source of a potentially very important factor predicting the ultimate clinical outcome of patients undergoing idiotypic vaccination, that is the original heavy chain isotype of the immunoglobulin. In particular, as discussed in depth below, retrospective data from an incomplete randomized clinical trial seem to indicate that follicular lymphoma patients, whose tumors featured an idiotype-containing IgM, experienced a better post-vaccine clinical outcome than their peers whose tumor featured an idiotype-containing IgG. However, it has to be remembered that similar results originated from retrospective studies, namely those focusing on the prognostic role played by different Fc γ receptor genotypes featured by follicular lymphoma immunoglobulins (Table 1) on the outcome of patients receiving idiotypic vaccination[24,25] have previously generated the same understandable excitement, but were not confirmed in subsequent prospective trials[26].

Being a weak antigen, each tumor-specific idiotype may also be more or less prone to function as a valuable vaccine core product. Moreover, depending on the type of lymphoma, each idiotype may or may not contain acquired potential glycosylation sites[27], which are indeed present in most, if not all, cases of follicular lymphoma[28,29] but may be lacking in many cases of the other B-cell malignancies still expressing a tumor-specific immunoglobulin on the surface of the tumor clone[30]. It goes without saying that, when present, these acquired potential glycosylation sites may or may not actually be

Table 1 Relevance of potential factors predicting idiotype vaccine-induced clinical outcome in major clinical trials

No. of patients	Comparison	Type of study	Results	Ref.
136	FcγRIIIa 158	Retrospective analysis	Better outcome for V/V	[24,25]
289	FcγRIIIa 158	Prospective analysis	No outcome difference	[38]
117	Idiotype/Isotype	Retrospective analysis	Better outcome for IgM	[23]

FcγRIIIa: Fc γ receptor IIIa; V/V: Valine/valine.

Table 2 Overview of randomized clinical trials addressing the clinical benefit of idiotypic vaccination in patients with follicular lymphoma

Pre-treatment	Pre-vax status	Random	Endpoint	Results	Ref.
6-8 × PACE q 3w	CR	2:01	DFS	Better outcome if vaccinated	[34,35]
8 × CVP q 3w	CR, PR	2:01	PFS	No difference	[38]
4 × rituximab q 1w	CR, PR, SD	1:01	TTP	No difference	[39]

PACE: Cyclophosphamide, doxorubicin, etoposide and prednisone; q 3w: 1 cycle every 3 wk; CR: Complete response; DFS: Disease-free survival; CVP: Cyclophosphamide, vincristine, prednisone; PR: Partial response; PFS: Progression-free survival; q 1w: 1 dose weekly; SD: Stable disease; TTP: Time to progression.

glycosylated on the original tumor cell, and when they are, it is virtually impossible that the idiotype contained in the vaccine formulation, however obtained in the lab, will feature the same glycosylation pattern. In fact, no matter the idiotype production method utilized, the glycosylation machinery involved is not derived from human cells, but rather from other mammalian, insect, or plant cells[18]. Whether all these features depending on each single idiotype and on its production technique, if thoroughly studied, may ultimately allow researchers to predict which idiotype vaccine could and could not be employed in patient after patient for clinically-successful immunization remains to be determined.

As briefly mentioned above, the overall idiotype vaccine formulation utilized in most large clinical trials has remained unchanged over the years. The only component that has been subjected to substantial dose changes is the adjuvant, that is, GM-CSF. However, even when employed within the same trial[17] at different doses, such differences have not emerged as a potential factor predicting better or worse clinical and immunological outcomes.

Pre-vaccine treatment and its enhancing or detrimental role towards successful immunization by means of idiotype vaccines has been the subject of much speculation, but no trial has actually been conducted to confirm and quantify, for instance, the alleged negative role of pre-vaccine rituximab, which causes a complete and relatively long-lasting normal B-cell depletion, and is thought to prevent the immune system from fully or partly responding to idiotypic vaccination[20]. Similarly, it is thought to be desirable that patients receiving pre-vaccine chemotherapy should be allowed to recover, at least from a quantitative standpoint, their normal immune function status[26]. Yet, most past trials have been designed to include a predetermined duration of the off-therapy period between the end of chemotherapy and the administration of the first idiotype vaccine dose[6]. Therefore, it is not known whether patients who responded to vaccination from an immunological and clinical response may have simply been those with a fully recovered immune function before vaccination start, or whether this is a detail of no importance.

Finally, another potential factor predicting the clinical outcome of patients undergoing idiotypic vaccination might be the length of the vaccination program. However, no clinical trial has ever been designed to assess whether prolonged idiotypic vaccination is intrinsically more or less efficacious than a program relying on the administration of only a few vaccine doses. In the former case, this could be theoretically true either because some patients start responding to the vaccine later than others[22] or because long-term boost may prevent the loss of the immune response elicited against the tumor-specific idiotype. In the latter scenario, this might be hypothesized as the result of a potential induction of immunologic tolerance against the same weak antigen through an extended vaccination schedule[22].

MAJOR CLINICAL TRIALS AND POTENTIAL RESPONSE PREDICTING FACTORS

Over the last decade, four independent clinical trials have attempted to formally prove the clinical benefit of idiotypic vaccination: a phase-Ⅱ, non-randomized proof-of-principle study not aimed at regulatory approval, which achieved its main clinical endpoint, and three large-scale, phase-Ⅲ randomized studies (Table 2) designed to achieve such a goal, although, as predicted well before their conclusion[31], they ultimately failed to achieve their main clinical endpoint[6]. All these studies have been designed based on the assumption that a vaccine-induced, idiotype-specific immune response is crucial to improve

disease-free survival in lymphoma patients. However, it is still theoretically possible that the simple capacity of responding to an idiotype vaccine is sufficient to achieve that clinical goal, irrespective of whether the elicited immune response is idiotype-specific or not[32]. Moreover, the only way to formally prove that the idiotype specificity of a vaccine-induced immune response is the key to an improved clinical outcome would consist of randomizing lymphoma patients to receive either their own, tumor-specific idiotype vaccine or a control vaccine formulation containing an irrelevant idiotype produced in the same way[6].

The first study reported the outcome of 25 patients with follicular lymphoma after induction of a second complete response with standard chemotherapy without rituximab and subsequent extensive idiotypic vaccination[26]. In this case, the tumor-specific idiotype was reproduced in the lab through hybridoma methodology. A vaccine-induced, humoral and/or cellular, idiotype- and/or tumor-specific immune response was elicited in 20/25 patients. The median duration of the second complete response among these patients was statistically significantly longer than the median duration of their first complete response. Moreover, in all cases it was also conspicuously longer than 13 mo, which is the unchanged median duration of a second complete response induced by standard chemotherapy without rituximab over the last three decades. On the contrary, the five patients who did not respond to vaccination from an immunological standpoint had a second complete response shorter than both 13 mo and their first complete response. All these findings, both combined and in isolation, were unprecedented in follicular lymphoma treatment[33], and even in a non-randomized context, clearly proved for the first time the clinical benefit associated with the use of a human therapeutic cancer vaccine[32]. Besides allowing vaccine administration only to patients in second complete clinical response after uniform salvage chemotherapy not including rituximab, this study's design prevented initiation of vaccination until a documented quantitative recovery of each patient's immune status was documented in terms of normal numbers of circulating CD19-, CD3-, CD4- and CD8-positive cells, independently of the time required to achieve such recovery in each case. However, given the single-arm nature of the study, it is not possible to conclude whether this detail may in the future predict or explain a greater chance of favorable immune and clinical outcome.

The only phase-Ⅲ, randomized clinical trial based on hybridoma-rescued idiotype vaccines was launched at the National Cancer Institute 10 years ago[34]. Based on a previous phase-Ⅱ study[17] that had succeeded in proving clinical efficacy[6] of idiotypic vaccination in patients with follicular lymphoma, this trial was also designed to provide actual immunizations with the idiotype vaccine or the control only to patients achieving a clinical complete response following pre-vaccine chemotherapy. However, the cyclophosphamide, doxorubicin, etoposide and prednisone (PACE) regimen was not widely used for follicular lymphoma treatment even before the advent of rituximab, and as soon as it became evident that the addition of rituximab to any chemotherapy regimen dramatically improved response rates in follicular lymphoma patients[20], the absence of this monoclonal antibody in the pre-vaccine treatment schema made patient enrollment in this trial virtually unethical. In any case, while the study was open, it randomized newly-diagnosed follicular lymphoma patients achieving chemotherapy-induced first complete response to receive the vaccine formulation either with or without the customized, tumor-specific idiotype component. While waiting for the full report of this largely incomplete trial to be published, it is worth underlining that this study has shown for the second time evidence of clinical benefit associated with the administration of the bona fide customized vaccine. In particular, only one hundred and seventeen patients have received either vaccine formulation instead of the three hundred and seventy-five patients that were supposed to be effectively randomized[35]. Moreover, the statistically significant ($P = 0.045$) advantage in disease-free survival achieved by the patients receiving the bona fide vaccine (44.2 mo *vs* 30.6 mo for the control arm) falls decisively short of the threshold ($P < 0.01$) originally stipulated by the company with the Food and Drug Administration as the main clinical endpoint for regulatory approval[6].

In this study, patients were vaccinated after post-chemotherapy off therapy of preset duration. Therefore, it is not clear whether the immune status of each patient at the time of vaccination was somehow assessed. However, as briefly mentioned above, an unexpected, retrospective finding has been preliminarily reported from this study[23]. Of the seventy-six patients actually receiving their bona fide idiotype vaccine, thirty-six featured an IgM, while forty featured an IgG tumor immunoglobulin isotype. Of the forty-one patients in the control arm, twenty-five featured an IgM, while fifteen featured an IgG tumor immunoglobulin isotype and one had a mixed IgM/IgG isotype. No difference in disease-free survival was observed when comparing vaccinated and control patients whose tumor idiotype displayed an IgG. The IgM subgroup of patients receiving the bona fide vaccine fared significantly better than those in the control arm (median time to relapse: 50.6 mo *vs* 27.1 mo, $P = 0.002$). All these subgroups of patients presented with numbers too small, and the study design ensured that this statistical difference could be confirmed in a prospective, randomized study adequately powered to address this issue. Of course, it will be important to assess the existence or lack of possible correlation between tumor-associated immunoglobulin isotype on the one hand, and both outcome results and specific immune responses elicited by vaccination on the other. Similarly, it could be of interest to retrospectively try to confirm or disproof these findings in all concluded, large-scale trials featuring a common pre-vaccine treatment for all enrolled patients. In any case, should this outcome difference between patients

with idiotype-bearing IgM or IgG isotype be confirmed in more sizeable studies, hybridoma-derived idiotype vaccines may once again regain scientific supremacy over recombinant idiotype vaccines, even if patients with an IgG-borne tumor-specific idiotype are excluded from vaccination protocols. In fact, nowadays the production of recombinant idiotype vaccines reproduces an idiotype systematically mounted on a shared IgG scaffold, and this might be seen as detrimental with respect to the ultimate idiotype immunogenicity.

As mentioned above, previous studies have tentatively singled out factors that seemed to predict, with the confidence derived from highly statistically significant differences in clinical outcome, which patients are more likely to respond to idiotypic vaccination[24,25]. However, no confirmatory evidence has subsequently emerged from prospective trials meant to put such preliminary findings to the test. In particular, the extensive experience in idiotypic vaccination at Stanford University[36,37] had led to the retrospective conclusion that immunoglobulin G Fc receptor (FcγR) polymorphisms might accurately predict the clinical response of lymphoma patients to idiotypic vaccination. In particular, in a group of 136 patients, it was found that those with FcγRIIIa 158 valine/valine (V/V) genotype had a longer progression-free survival [16] than those with valine/phenylalanine (V/F) or phenylalanine/phenylalanine (F/F) genotypes (V/V, 8.21 years *vs* V/F, 3.38 years, $P = 0.004$; V/V 8.21 years *vs* F/F, 4.47 years, $P = 0.035$). When the researchers analyzed whether such a statistically significant correlation could be related to the pre-vaccine response to chemotherapy [36], they also found that in patients with pre-vaccine complete response, the 5-year progression-free survival was 69% for those with a subsequent idiotype-specific humoral response and/or V/V genotype, but only 40% for patients with neither. The median time to progression was 10.47 years *vs* 3.46 years ($P = 0.012$). In patients with pre-vaccine, chemotherapy-induced partial response, the 5-year progression-free survival was 57% for patients with the specific humoral response and/or V/V genotype, but only 17% for patients with neither. The median time to progression had not been reached in the former *vs* 1.31 years ($P = 0.001$) in the latter group.

However, these strong and consistent retrospective results were not prospectively confirmed by the same scientists in their large, randomized, phase-Ⅲ clinical trials employing a recombinant idiotype vaccine[37]. Even more disappointingly, this study showed no statistically significant differences in progression-free survival between vaccinated patients and those in the control arm, possibly because vaccination was administered not only to patients who had achieved a pre-vaccine, chemotherapy-induced complete response, but also to those with pre-vaccine, post-chemotherapy partial response.

Finally, another independent study based on a novel recombinant idiotype vaccine also failed to show statistically significant differences in time to progression between vaccinated patients and those in the control arm[38]. As rituximab had meanwhile become part of the standard of care for patients with follicular lymphoma, the goal of this trial was to assess whether idiotypic vaccination could further improve survival of follicular lymphoma patients solely pre-treated with four weekly doses of rituximab. As such, most patients were ultimately vaccinated with both active disease and severe B-cell depletion, and this potentially double-negative status at the time of vaccination is likely to have influenced the disappointing outcome of the trial far more than the quality of the recombinant vaccine *per se*[6]. In particular, patients in the control arm seemed to have experienced a statistically significant better outcome than those in the experimental arm, a difference that disappeared when standard follicular lymphoma prognostic factors were retrospectively applied to both groups and factored in the analysis[38].

As inferred above, despite lacking comparative data to make a stronger case, the use of rituximab without allowing conspicuous B-cell recovery prior to initiation of idiotypic vaccination is likely to diminish and perhaps even abolish the likelihood of a vaccine-induced, idiotype-specific immune response. We do not know for sure whether idiotype specificity is crucial for the vaccine to exert a clinical effect, but it seems quite established that patients with no vaccine-induced immune response at all are less likely to experience any clinical benefit[26]. In a study of idiotypic vaccination for patients with mantle cell lymphoma, it was concluded that pre-vaccine chemoimmunotherapy containing rituximab delays humoral responses, but does not affect cellular responses[19]. However, it should be underlined that the delayed humoral responses observed in that study were all directed against the highly-immunogenic carrier [14] contained in the vaccine formulation, not against the idiotype, which is a far weaker and yet the sole vaccine formulation antigen that matters[39].

CONCLUSION

There are many questions still unanswered regarding idiotypic vaccination. We do not know whether there may be substantial outcome differences when using the whole immunoglobulin or the sole Fab[7], when reproducing the idiotype through hybridoma or recombinant methodology, when vaccinating newly-diagnosed or relapsed patients, when treating patients with follicular or other types of lymphoma. Similarly, many potentially crucial details concerning this immunotherapeutic approach remain to be determined, such as the number of doses, the pre-vaccine treatment, and the type of indispensable immune response that it should be induced *via* vaccination. Finally, it is now paramount to verify whether patients with IgM- and IgG-borne idiotypes undergoing vaccination have indeed a critically different outcome, particularly taking into account that, outside of idiotype vaccine trials, most standard diagnostic protocols for surface immunoglobulin-positive B-cell lymphomas do not include a routine determination of the immunoglobulin isotype.

All in all, the quest for one or more reliable factors both assessable prior to starting the production of an idiotype vaccine and capable of predicting its clinical usefulness remains a crucial element in the continuing development of this active immunotherapy strategy. It is desirable that new trials, including those currently ongoing and based on recombinant idiotype vaccines produced in tobacco plants[40,41], are able to close the current knowledge gap in this field.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v2.i6.245

World J Clin Oncol 2011 June 10; 2(6): 245-261
ISSN 2218-4333 (online)

REVIEW

Potential of soluble CD26 as a serum marker for colorectal cancer detection

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Supported by (in part) A grant from Xunta de Galicia (10 PXIB 310 215 PR) and FEDER founding
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Received: January 21, 2011 Revised: March 28, 2011
Accepted: April 5, 2011
Published online: June 10, 2011

Abstract

Colorectal cancer is characterized by a low survival rate even though the basis for colon cancer development, which involves the evolution of adenomas to carcinoma, is known. Moreover, the mortality rates continue to rise in economically transitioning countries although there is the opportunity to intervene in the natural history of the adenoma–cancer sequence through risk factors, screening, and treatment. Screening in particular accounted for most of the decline in colorectal cancer mortality achieved in the USA during the period 1975-2000. Patients show a better prognosis when the neoplasm is diagnosed early. Among the variety of screening strategies, the methods range from invasive and costly procedures such as colonoscopy to more low-cost and non-invasive tests such as the fecal occult blood test (guaiac and immunochemical). As a non-invasive biological serum marker would be of great benefit because of the performance of the test, several biomarkers, including cytologic assays, DNA and mRNA, and soluble proteins, have been studied. We found that the soluble CD26 (sCD26) concentration is diminished in serum of colorectal cancer patients compared to healthy donors, suggesting the potential utility of a sCD26 immunochemical detection test for early diagnosis. sCD26 originates from plasma membrane CD26 lacking its transmembrane and cytoplasmic domains. Some 90%–95% of sCD26 has been associated with serum dipeptidyl peptidase IV (DPP-IV) activity. DPP-IV, assigned to the CD26 cluster, is a pleiotropic enzyme expressed mainly on epithelial cells and lymphocytes. Our studies intended to validate this test for population screening to detect colorectal cancer and advanced adenomas are reviewed here.

Key words: Antibody arrays; Bioinformatics; Biomarkers; cancer; Clustering; Cytometric beads; Diagnosis; Dipeptidyl peptidase Ⅳ; Enzyme linked immunosorbent assay; Prognosis; Soluble CD26; Screening; Serum

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Cordero OJ, Imbernon M, De Chiara L, Martinez-Zorzano VS, Ayude D, Paez de la Cadena M, Rodriguez-Berrocal FJ. Potential of soluble CD26 as a serum marker for colorectal cancer detection. *World J Clin Oncol* 2011; 2(6): 245-261 Available

from: URL: http://www.wjgnet.com/2218-4333/full/v2/i6/245.htm DOI: http://dx.doi.org/10.5306/wjco.v2.i6.245

EARLY DIAGNOSIS AND POPULATION SCREENING FOR COLORECTAL CANCER

Colorectal cancer (CRC) clearly meets all the required conditions for the adoption of a screening policy.

First, because it is an important issue for public health since it is one of the most common cancers (ranking third both in men and women) worldwide and because it is characterized by a low survival rate due to diagnosis in advanced stages, which leads to high mortality rates. For example, in the United States the American Cancer Society estimated that in 2010 there were 142 570 new cases and 51 370 related deaths from colon cancer[1] and more than 1 million new cases and about 530 000 deaths worldwide[2]. Moreover, globally, while in all developed countries CRC rates have stabilized or are declining[3], CRC incidence in economically transitioning countries continues to rise both in its incidence and in mortality because of increased exposure to risk factors[2, 3].

The second condition is that the basis of colon cancer development is well known and involves the evolution of adenomas to carcinoma[4,5], therefore, individuals with a history of adenomas have a higher risk of cancer[6] and removal of polyps results in a reduction in colon cancer incidence[7]. However, we have the opportunity to intervene in the natural history of the adenoma–cancer sequence[8].

Third, there are precise and feasible diagnostic methods that allow detection of the disease in early stages (non-metastatic tumors), which could be surgically cured by removal (reduction in the mortality rate of CRC), as well as the identification and removal of polyps (reduction in the incidence rate of CRC)[9-11]. Moreover, treatment is more effective and patients show a better prognosis when the neoplasm is diagnosed early[12].

Interestingly, it has recently been reported that screening accounted for 53% of the decline in CRC mortality observed between 1975-2000 in the USA (26% less mortality); the other two facts being changes in risk factors (35%) and treatment regimes (12%)[8]. Moreover, the decline in CRC mortality in the USA can be enhanced if current trends, including screening, against cancer are accelerated; for example, only approximately 50% of its population older than 50 years have been screened[8]. Needless to say that in most countries, including many developed countries, no screening strategy has been proposed.

There are a great variety of screening strategies available for the average risk population, that is, individuals of, or over, 50 years with no other known risk factors for the development of CRC. These methods range from invasive and costly procedures such as flexible sigmoidoscopy, double contrast barium enema, and colonoscopy to more low-cost and non-invasive tests such as the fecal occult blood test (FOBT). All these methods have advantages and disadvantages regarding their sensitivity, specificity, risk, availability and cost but they have been shown to decrease CRC incidence and mortality[8, 13-15].

Colonoscopy is the gold standard[15] and multiple studies have provided indirect evidence regarding the higher benefits of colonoscopy compared with other methods[16]. However, the costs and risk of complications, besides discomfort, have made this and other invasive tests such as flexible sigmoidoscopy[17], poorly accepted for screening in an asymptomatic population[18-23].

The benefit of CRC screening using a non-invasive test for blood in stool (Hemoccult) was established in 1993[13]. Subsequently, this result was corroborated in two other randomized controlled trials, leading to recommendations in many countries for CRC screening[24, 25]. The FOBT is the simplest and least expensive non-invasive approach to CRC screening available, however, it has several disadvantages. The most common method is the non-rehydrated guaiac FOBT[26], based on the detection of peroxidase activity in the stool sample. Consequently, reagents also bind to nonhuman hemoglobin-like substances in feces, such as animal myoglobin and plant peroxidases. As the presence of these substances in the colon and rectum are related to diet, important dietary restrictions are required to minimize false negative results[13, 27]. Notwithstanding, its sensitivity and specificity are 30%-40% and 96%-98%, respectively[28], with lower percentages for the detection of adenomas[29, 30].

In the United States, the current recommendations include a number of screening tests in addition to Hemoccult. Immunochemical tests (iFOBT), which have not been evaluated in a randomized controlled trial, have performed similarly or even better in some studies, with generally higher compliance rates compared to Hemoccult or other guaiac-based tests[27, 31-34], involving no dietary restriction, and resulting in fewer false positives[35]. The use of an immunochemical test in patients scheduled for colonoscopy[36] showed the advantages of a quantitative test to determine the cutoff for positivity to adjust the screening program according to the resources available. Moreover, this test can be automated and two instead of three samples can be used for quantification[37].

In Japan, more than 6 million people have been screened with immunochemical tests, with a positivity rate of 7.1%[35, 38-40]. With 60% of positive tests complying with the diagnostic protocol, the CRC detection rate was 1.6 per 1000. More than 70% of the cancers were classified as Duke's A or Duke's B, suggesting that the program worked well in detecting early stage cancer; CRC mortality and incidence were reduced by 72% and 59%, respectively[41]. Somewhat puzzling is the fact that guaiac is more sensitive than immunochemical for advanced adenomas (41.3% *vs* 29.5%)[42], this may be because peroxidase sensitivity of the guaiac test detects lower levels of bleeding as some authors speculate. However, other explanations must also be considered.

This last study does illustrate the utility of comparing

different tests rather than conducting long-term and expensive randomized controlled trials to evaluate each new FOBT. There are considerable data on Hemoccult, therefore comparing performance, outcome, compliance, and cost with new blood or fecal occult tests, as was done in this study[42], should be enough for the acceptance of new tests.

As blood could be present in the stool for other reasons, such as hemorrhoidal bleeding, iFOBT was also tested in combination with protein stool markers like hemoglobin-haptoglobin, calprotectin, carcinoembryogenic antigen, and the novel fecal markers S100A12 and tissue inhibitor of metalloproteinase-1 (TIMP-1), the latter allowing the detection of CRC at significantly higher rates than can be obtained with iFOBT alone[43]. Genetic markers are also promising tools, such as the DNA-based stool test *PreGen-Plus* from EXACT Sciences, which shows a sensitivity of 51%-91% for CRC, with an average of 65%, and specificity between 93%-98%[30,44].

However, non-invasive biological serum markers would be of great benefit for screening, because blood-based diagnostics can additionally classify tumors into distinct molecular subtypes and monitor disease relapse and response to treatment. Increasingly, biomarker strategies are becoming critical to identify a specific patient subpopulation that is likely to respond to a new therapeutic agent. The improved understanding of the underlying molecular features of common cancers and the availability of a multitude of recently developed technologies to interrogate the genome, transcriptome, proteome and metabolome of tumors and biological fluids have made it possible to develop clinically applicable and cost-effective tests for many common cancers[45,46].

SERUM BIOMARKERS IN CRC SCREENING

Other advantages over stool testing are: sampling may be more convenient and acceptable for the patient, there is no microflora which could degrade the biomarker or hamper analysis, and sample processing may be easier. In addition, as it will be commented later, information on the very early pathways of carcinogenesis, such as immune system cross-talk, can only be found in serum.

A meta-analysis evaluating blood markers for early detection of CRC reported in 2007 summarizing the performance characteristics of various approaches[47] found that seventy different markers fulfilled the inclusion criteria with an overall sensitivity that ranged from 18% to 65%. The markers included cytologic assays, DNA and mRNA markers, and soluble proteins.

Three studies investigated cytologic assays, an inhibition of *in vitro* leukocyte adherence by incubation with tumor antigens, and the detection of circulating tumor cells by a membrane array[48-50]. Sensitivity was above 70% for early stages and specificity ranged from 94% to 98%, however, the number of cases by tumor stage was very small. Notwithstanding, cellular mechanical properties have recently received increasing attention as a potential biophysical marker for cancer cells[51].

Four studies[52-55] with DNA markers for the early detection of CRC were reported in that review[47]. Free DNA, as well as mRNA, was isolated from circulating cells. Blood samples were analyzed for both genetic and epigenetic alterations of genes involved in the adenoma-carcinoma sequence, such as K-ras, tumor suppressor protein p53, APC (adenomatous polyposis of the colon), hMLH1 (human MutL homologue 1) or HLTF (helicase-like transcription factor). Sensitivity reported for this group of markers was about 60% and lower, whereas specificity ranged from 73% to 100%. The potential of detecting adenomas was investigated only for mutations in the *K-ras* gene in one study which showed a sensitivity of 35% for adenomas[54]. A recent review evaluated four commercialized biomarker tests based on that information (K-ras and B-raf mutation analyses, mismatch repair protein testing, and the Oncotype DX Colon Cancer Assay) for inclusion in the NCCN Guidelines Panel for Colon Cancer. In two cases, the available evidence was inconsistent to be included in the specific NCCN Guidelines[56].

Novel data on genetic and epigenetic mechanisms of CRC and how these alterations relate to emerging biomarkers for early detection, risk stratification, prognosis and prediction of treatment responses, are reviewed in[57-60]. Potential markers waiting to undergo clinical validation for response to therapies are hypermethylation of *septin-9* and *DPYD* (dihydropyrimidine dehydrogenase) genes.

Many relevant studies[47] applied reverse transcription-PCR to detect mRNA expressed in circulating tumor cells. Blood samples were analyzed for mRNA molecules coding for CEA, cytokeratins (CK) 8, 9, and 20, human telomerase reverse transcriptase (hTERT), guanylyl cyclase C (GCC), carcinoembryonic gene member 2 (CGM2), melanoma-associated antigen family A (uMAGE-A), tumor-associated antigen L6, mucins (MUC) 1 and 2, protease M (ProtM), and thymidylate synthase. The most promising performance characteristics in this group of markers were reported for GCC mRNA[61], showing above 80% sensitivity for early stages.

Recent research has shed light on the biological importance of microRNAs(miRNAs). Their association with formation, angiogenesis, metastasis, and chemo-therapy resistance of tumors has become one of the core issues in epigenetics of cancer, including CRC. miRNAs serve as micromanagers, negatively regulating gene expression. The potential utility of miRNAs in the preclinical stage has been explored, since manipulation of miRNAs may offer an alternative therapy for chemo- and radio-resistant CRCs[62-64].

The discovery that aberrantly expressed miRNAs vary among different tumor types and some of them are secreted in highly stable, cell-free form into blood[65] led to the hypothesis that circulating (and fecal) miRNAs might potentially serve as non-invasive markers for early diagnosis

of CRC[63]. For example, 69 miRNAs were detected in CRC but not in control group sera; of these, 12 were not found in the serum of lung cancer patients[64]. Circulating miRNAs are packed in complexes, either called exosomes or microvesicles, and emerging evidence has indicated that such external miRNAs are involved in cell-to-cell signal transduction and genetic information exchange[64,66]. Two miRNAs significantly elevated in plasma and CRC tissues, but reduced in postoperative samples when compared with preoperative samples are miR-17-3p and miR-92a, both belonging to the miR-17-92 cluster. At a cut-off value of 3.6 for miR-17-3p (relative expression in comparison with RNU6B), the sensitivity was 64% and the specificity was 70%; at a cut-off value of 240 for miR-92a, the sensitivity was 89% and the specificity was 70%. In addition, miR-92a can distinguish CRC from other gastrointestinal cancers and inflammatory bowel diseases as well as advanced adenoma from normal controls, with a sensitivity of 64.9% and a specificity of 81.4%, whereas its expression levels were not correlated with tumor-node-metastasis (TNM) stages[66,67].

Fifty two protein markers in the meta-analysis of 2007[47] were analyzed by common standard procedures, still more easy-to-use and quicker than the nucleic acid methods, like ELISA, RIA, or activity assays, or by chromatographic and mass spectrometric (MS) assays based on surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS, and matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. This group of markers can be further subdivided into carbohydrate antigens, carcinoembryonic antigens, other antigens, antibodies, cytokines, and other proteins. Sometimes, different markers were analyzed in parallel. For example, combinations including carbohydrate antigens and carcinoembryonic antigens were very common.

Carcinoembryonic antigen (CEA) was the first blood marker proposed in connection with CRC[47, 68]. Although overall sensitivity ranged between 43% and 69%, there was a clear increase in sensitivity by tumor stage, ranging from 8% for Duke's A up to 89% for Duke's D. Specificity was above 90% in nineteen studies.

Carbohydrate antigens (defined by monoclonal antibodies against colon carcinoma cell lines) include CA 19-9, CA 195, CA M26, CA M29, CA 50, CA 72-4, CA M43 and CA 242. Many studies evaluated CA 19-9, with an overall sensitivity from 18% to 65%, and specificity above 90% in most studies. Sensitivities greater than 50% were only observed for nonlocalized disease. For other carbohydrate antigens, the observed sensitivity, its stage dependency, and specificity were comparable.

Early approaches for other antigens investigated sialylated Lewis X antigen (sLeX) and CO 29.11, another sialylated Lewis antigen. sLeX was originally found on tumor tissues by immunohistochemistry and CO 29.11 is expressed and shed by carcinoma cells of colon and other cancer types[69-77]. Later studies investigated the potential of PSA, PA 8-15 (another tumor-associated antigen that was originally observed in a pancreatic cancer cell line), small intestinal mucin antigen (SIMA), and urokinase-type plasminogen activator (u-PA). For the latter, a sensitivity of 76% (82% for non-mestatasized disease) and a specificity of 96% have been reported[69, 70].

Among various circulating autoantibodies against antigens such as DEADbox protein 48 (DDX-48), p53, sFasL (the death receptor ligand of CD95), or NCC-ST 439 (a tumor-related carbohydrate)[78-83], sensitivities for the detection of CRC hardly reached 30%, although specificity was 100% in all studies.

In studies[84-89] evaluating cytokine markers such as vascular endothelial growth factor (VEGF), insulin-like growth factor II (IGF-Ⅱ), IGF-binding proteins (IGFBP-2), stem-cell factor (SCF), and interleukin-3 (IL-3) which can reflect several immune system-related pathways of carcinogenesis[90], if specificity was high (between 90% and 100%), sensitivity was low (37% for VEGF in TNM Ⅰ stage patients), or *vice versa*.

Among the other proteins, subgroup examples are the α-defensins[91], the nicotinamide N-methyltransferase[92], the α-L-fucosidase[93] and the tumor M2-pyruvate kinase (M2-PK), an isoform of the glycolytic enzyme pyruvate kinase[94]. Recent works also studied other potential markers in relation to polyp characteristics: for serum sulfatase activity, differences regarding the number of adenomas (single or multiple) were significant[95]; serum leptin, adiponectin and resistin also differed between controls and patients with adenomas or CRC, although there was no relationship with dysplasia, histopathology or polyp localization[96].

One of the signatures of a cancer cell is the change in the nuclear structure and architecture, and alterations in the composition of nuclear structural proteins are associated with various types of cancer such as breast, prostate, bladder, lung and ovarian, as well as squamous cell carcinoma of the neck[97, 98]. Nuclear proteins, colon cancer–specific antigen (CCSA)-2, CCSA-3, and CCSA-4 were recently identified as serum biomarkers that are specific for colon cancer[99,100].

As a summary of these and more recent studies, the more promising results, for both sensitivity and specificity, were observed with u-PA (76% and 96% respectively[61], M2-PK[101] (69% and 90%), TPA-M (70% and 96%), CP (cancer procoagulant; 86% and 82%)[102], sCD26 (soluble cluster of differentiation 26) (90% and 90%[103], fibrin degradation (DR-70) (80% and 93%)[104], prolactin (77% and 98%)[105], laminin (89% and 88%)[106], BSP (bone sialoprotein; 88% and 100%, although similar results were found in breast and prostate cancers)[107] and CCSA-2 (78% and 97%), but sCD26 has been the most studied, as commented in later subheadings[108-111].

SOLUBLE CD26

Dipeptidyl peptidase Ⅳ (DPP-Ⅳ), assigned to the CD26 cluster, is a multifunctional or pleiotropic protein expressed particularly on epithelial cells and lymphocytes. CD26/DPP-IV has been consistently associated with

cancer since it was known as ADCP, or the ADA-2 / Large isoform[112,113]. Many reviews have discussed the non-enzymatic role of CD26/DPP-IV as an extracellular anchorage for ADA in cancer and the potential usefulness of this protein in therapeutics and diagnostics[114-119]. The ADA-CD26 complexes may participate in cell-to-cell contacts[120-122] or, more probably in this context, through the catalysis of adenosine to inosine[121,123,124]. Proliferating cells accumulate high extracellular concentrations of adenosine, a purine nucleoside found within the interstitial fluid of solid tumors, which may be toxic or influence the proliferative potential of a cell, depending on the relative expression and type of adenosine receptor (AR). Therefore, the different levels of the cell-surface CD26-ADA complex and relative expression of ARs on a tumor cell may lead to the generation of tumor subclones, as well as its participation in the well-known adenosine inhibition of cell-mediated immune responses to tumor cells[115,116,119,124-127]. Other pro-oncogenic activities may be related to the recently described CD26-ADA-plasminogen ternary complex. Binding of plasminogen to cell-surface receptors promotes its conversion to plasmin, which is required for proteolysis of the ECM in several physiological and pathological processes, including cell migration, tumor cell invasion and metastasis[127].

CD26, also present at the invadopodia, together with other ectoproteases and metalloproteases (MMPs)[128-130], can participate in malignant transformation and cancer progression through its ability to bind collagen and fibronectin[81,116,117,128,129,131,132]. MMPs and FAPα(a CD26 homologous protein expressed in tumor cells) digestion of ECM components will allow passage of the malignant cells through basement membranes and stromal barriers. This pro-oncogenic behaviour is thus consistent with the non-enzymatic interactions with cell-surface ADA and plasminogen mentioned above, and the formation of FAP-CD26 heterodimers[129, 133].

However, there is a fundamental difference between CD26 and the other proteases involved in cancer development and progression as executors of ECM degradation: CD26 is constitutively expressed in the tissues mentioned at the beginning of this heading, and its enzymatic activity regulates the biological activity of regulatory peptides, such as incretins secreted by the enteroendocrine system (DPP-IV has therefore become a novel therapeutic target for inhibitors that extend the endogenously-produced insulin half-life in diabetics [114,115,134-141], and similarly the half-life of growth factors and chemokines[142]).

In addition, glypican-3 has recently been reported as the first natural inhibitor of CD26/DPP-IV enzymatic activity, in *in vitro* experiments[143]. Glypicans are basically absent in adult tissues, but up-regulated in many tumor tissues[144]. If glypican-3-dependent local DPP-IV inhibition can be confirmed in a physiological context, this indicates a natural protective role for the enzyme that should be blocked in the tumorigenic process.

This anti-oncogenic role, first contrasted in 1999 by Houghton's group[145-148], together with many data -differences in the cellular staining pattern with respect to the normal tissue, significant intratumor heterogeneity and changes in CD26 expression linked to the transition of tumor stages- already reviewed[115], indicate a quite complex situation in the physiological microenvironment of cancer niches. The possibility that the tumorigenic process may manipulate the functions of CD26/DPP-IV, for example evading the immune system by modifying local chemokine gradients (and therefore, immune cell homing), and by modulating cytokines and angiogenic or immunosuppressive factors[90,149-153] deserve to be studied in more detail[142].

In this context, the role of serum DPP-IV activity, first discovered in 1968 by Nagatsu's group in Japan[154], is not known. Within normal plasma/serum, some 90%–95% of DPP-IV activity has been associated with a relatively high concentration of serum (or soluble, in contrast to transmembrane) CD26 (sCD26) in human serum (570 μg L-1)[115,118,155-157]. Since sCD26 is heavily glycosylated, its molecular weight is similar to that of transmembrane CD26[156,158] although it lacks transmembrane and cytoplasmic domains (the sequence starting at the 39th position)[156].

There is no direct correlation between serum CD26 protein concentrations and serum enzymatic activity assays, for three reasons: (1) There are some circulating proteins other than CD26 with DPP-IV activity (DPP-Ⅱ, FAPα,...); (2) sialylation (a type of glycosylation) of sCD26 [114,118,135-137,158] is strongly enhanced in elderly individuals[159], and certain type of hypersialylation can inhibit DPP-IV activity[160], consistent with the fact that serum/plasma DPP-IV enzymatic activity tends to decrease with age[118]; (3) it has recently been suggested that the serum protein attractin, which enhances the enzymatic activity of tolloid proteases[161,162], may regulate the DPP-IV activity of CD26/sCD26 in the same way [115]. Serum attractin is actually frequently co-purified with sCD26[163-166].

Iwaki-Egawa *et al*[156,167,168] suggested that sCD26 must be shed from any plasma membrane on CD26 expressing cells that are in contact with blood, by proteolytic cleavage. The fact that only one CD26 mRNA form is usually reported[169-171], and that it is transported from its site of synthesis in the rough endoplasmic reticulum to the microvillar membrane of enterocytes, and in some cell lines in a membrane-bound state[135,136,172-174], also suggest that it is not secreted. It must be pointed out that the shedding of most integral membrane proteins is often regulated by a PKC-dependent mechanism[175-177].

However, CD26 has been found to be soluble in the lumen of secretory granules, undergoing exocytosis to the interstitial space of endocrine pancreatic A cells, where sCD26 may act on secretory products of neighbouring islet cells[178,179]. Autolysis of the protein by the acidic pH conditions inside the granules has been observed *in vitro*[179,180]. In addition, another possibility related to the intracellular sorting is the secretion of soluble proteins through MMP-dependent shedding from exosomes. Exosomes are small membrane vesicles derived from

intracellular multivesicular bodies that can undergo constitutive and regulated secretion from cells upon fusion with the PM[181-183]. Exosomes with CD26/DPP-IV have been found in human saliva, released at the basolateral surface of enterocytes, and in ram epididymal fluid[184-186].

In addition, the origin of sCD26 is also unknown. The hepatobiliary system was the first to be suggested[187]. Liver epithelium is often cited as the most likely potential source[113, 116, 137, 188-192] and at least in some conditions, sCD26 originates from the brush border of hepatocytes[190]. However, CD26 is predominantly located in the bile canaliculi[190, 193, 194], and a recent study found that in chronic hepatitis C and other liver viral infections, DPP-IV activity levels were not correlated with several markers of bile duct injury or hepatocyte injury[195]. These authors suggested that the increased activity in these diseases may originate directly from its shedding from the peripheral blood T cells involved in the control of viral infections or, indirectly, by stimulating other cells such as hepatic stellate cells. The involvement of T cells had already been suggested in studies of liver regeneration[137,196]. In fact, Kasahara *et al*[197] suggested a possible origin of sCD26 from the immune system, although they also identified serum isoforms from liver, spleen or kidney. Kidney, an obvious potential source because it contains large amounts of CD26, was rejected early on[156] because anephric individuals have normal amounts of sCD26, and because sCD26 contains approximately twice as much sialic acid as kidney CD26. However, several data suggest that serum CD26 is at least partly shed from T cells[142, 158, 161, 194, 198-204], although these data do not preclude the possibility of sCD26 also being shed from the endothelium of venules or the capillary bed of several organs such as lung, myocardium and striated muscles, spleen and pancreas[134, 194, 199, 205-210]. Moreover, this fraction of serum CD26 which originated from immune system cells can be regulated[158, 211-213] and causes an imbalance among specific sCD26 isoforms in the serum of patients.

As it is not known to which CD26 functions regulation of this proteolytic or secretory process is related, the physiological role of soluble CD26 in biological fluids with respect to the transmembrane CD26 can only be hypothesized. Current data support three potential biological functions, which may be partly responsible for the different roles of CD26 in various clinical settings. (1) Involvement in the activation–deactivation of some chemokines, and therefore in inflammatory processes. Extracellular proteases, many shed (or ripped, from a process called "ripping"[175]), which alter the chemokine gradients, participate in this crucial early step of the immune response. For CD26, the modulation of SDF-1 and the CXCR4 axis of cell homing has been particularly well studied[214, 215]; (2) Circulating sCD26 may also participate in the clipping or inactivation of the biologically still active blood substrates such as vascular regulatory peptides (substance P or bradykinin)[216-224], growth factors or hormones (e.g. only 20% of incretins GLP-1 and GIP, which originated in the gastrointestinal duct, are still active in the blood pool)[139,140] and (3) In the case of oncogenic processes, in addition to possible involvement in both immunosuppressor[122,136] and angiogenic mechanisms[122,136], the process of shedding may initiate or dampen CD26 involvement in cell-adhesion processes through fibronectin, ADA or collagen binding[121,123-126,144,154,225-227].

sCD26 PATHOPHYSIOLOGY AND CRC DIAGNOSIS

Many studies have demonstrated altered serum levels of enzymatic DPP-IV activity (see review[142]) and soluble CD26 protein in several diseases. Some studies show contradictory results, probably related to the stage of the disease considered (or in which a particular patient has been recruited)[103,110,111,158, 228-230]. However, other discrepancies between enzymatic activity and protein concentration measurements can be explained by putative changes in the glycosylation pattern (leading to a lack of immunorecognition of sCD26), the putative presence of the DPP-IV activator attractin, inhibitor glypican-3 or the secretion of other dipeptidyl peptidases such as DPP-II or soluble FAPα (DASH). For example, in myocardial infarction patients treated with streptokinase, the enzyme concentration is reduced to more than 50% after 90 d of therapy, while measurements of DPP-IV enzymatic activity did not change during that period[211]. On the contrary, the same authors found that there was no change in sCD26 concentrations between healthy donors and patients with rheumatoid arthritis and lupus erythematosus, although a lower enzymatic activity was detected[158].

Reference values of DPP-IV specific activity show no differences in serum and plasma[118,142,187], but most reports do not use the same assay conditions or the same definition of specific activity -the same applies to the units of catalytic activity-, making it difficult to compare results even from the same authors. However, the amount of sCD26 antigen found in normal serum with the most commonly used commercial ELISA kit (Bender MedSystems), corresponds well with the expected values based on the specific activity of purified serum DPP-IV[118,142]. Together, these findings support the use of immunodetection techniques for the quantification of these molecules because they are more specific.

DPP-IV enzymatic activity is high in patients with hepatic cancer, hepatitis, osteoporosis, cholestasis and other liver diseases. On the other hand, the mean DPP-IV activity remains unchanged in metastatic bone disease, esophagus, gall bladder, chronic myelocytic leukemia or leiomyosarcoma cancers, in allergic asthma, celiac disease,and adult T-cell leukemia, although serum DPP-IV in the latter is strongly correlated with the percentage of CD26+ T cells. However, decreased levels of DPP-IV were observed in patients with acute lymphocytic leukemia, thyroid and oral cancer, advanced gastric carcinoma, HCV infections, inflammatory bowel diseases, type II diabetes, in healthy smokers, in pregnancy, and in alcoholics and patients suffering from major depression.

Table 1 Performance characteristics of the sCD26 test

	Cohort *n*	CRC risk	sCD26 cut-off (ng/mL)	CRC in sCD26+ patients	Advanced adenomas in sCD26+ patients	Polyps in sCD26+ patients	Other findings in sCD26+ patients	Sensitivity (%)	Specificity (%)
1st Case-control study[89]	175	Diagnosed with CRC	410	99/110	-	-	6/110 Crohn´s 1/110 GC	90% (CRC)	90% (CRC)
1st Case-finding study[97]	170	Average-risk	410	-	-[a]	8/21	2/21 diverticula	-	-
2nd Case-finding study[95]	2673	Average- and increased-risk	410	2/140	4/140[2]	46/140[2]	12/140 diverticula	100% (CRC)[1]	89.9% (CRC)[1]
2nd Case-control study[94]	299	Increased-risk	460	27/110	20/110	13/110	18/110 IBD; 18/110 non-IBD; 14/110 anemia, diarrhea, rectal bleeding	81.8% (CRC) 58% (CN)	72.3% (CRC) 75.5 (CN)

n: Number of individuals; CR: Colorectal; CRC: Colorectal cancer; CN: Colorectal neoplasms including CRC and advanced adenomas; GC: Gastric cancer; IBD (inflammatory bowel diseases, includes colitis and Crohn's); non-IBD (includes hemorrhoids and diverticula). [a]No data on polyps´ pathology could be obtained; [1] : Data obtained after one-year follow-up for the detection of interval cancers. [2]: Pathological anatomy information obtained only for 16 of 46 polyps. The four advanced adenomas are also included in the polyps' column.

A reduction in DPP-Ⅳ activity has been related to symptoms of depression and anxiety under certain circumstances. Contradictory results were reported for psychologically-related eating disorders such as anorexia or bulimia, CRC, rheumatoid arthritis, lupus erythematosus and Sjögren syndrome.

Many studies have used sCD26 as a soluble marker of Th1 cellular immune activation, together with sCD30 and sometimes sCD23 as markers of Th2 (humoral response)[231-234]. The concentration of sCD26 increases in HIV-1 patients, leishmaniasis, myocardial infarction and atopic dermatitis. It does not change in asthmatics, osteoarthritis and gastric cancers. In many, but not all studies, it decreases in rheumatoid arthritis and particularly in lupus erythematosus and Sjögren syndrome, while results from hepatitis C virus (HCV) are not consistent. In summary, low levels of DPP-Ⅳ/sCD26 occur concurrently with impaired immune status -some hematological and solid malignancies can be included-, whereas increased levels occur in inflammatory and infectious diseases (enhanced immune status), other hematological tumors, and liver diseases[142].

We were the first to report reduced levels of sCD26, using immunodetection, in the serum of CRC patients, compared with healthy donors[103]. Reduced levels of enzymatic activity were reported for a small group of patients in 1987[235], although other authors found increased DPP-Ⅳ activity in a cohort of CRC patients comparable to ours[229]. We have already made some putative explanations to clarify this lack of correlation. From a biological point of view, further research studies are needed, but this issue does not affect the focus of this article. Our most important finding was that lower concentrations were found particularly in the early stages of the disease. Sensitivities higher than 80% (Table 1) were found for Dukes' stages A, B and C, whereas it was impaired in Dukes' stage D, in which CEA levels diagnosed better. Interestingly, it was in stage D where the DPP-Ⅳ activity actually increased in the study mentioned[229]. In this first study, we also found that sCD26 as a variable is not related with Dukes' stage classification, age, gender, tumor location or degree of differentiation, which also suggested the potential usefulness of this molecule for early diagnosis of CRC. We also showed preliminary data on the potential prognostic value with a follow-up of 2 years until recurrence; additional data has not yet been published. Moreover, we did not find changes in related diseases such as gastric-tract carcinomas, and in two of four blood cell cancers the concentration was raised (n = 4); impaired levels of sCD26 were observed only in some cases with gastric tract benign pathology and with Crohn's disease[103, 228].

These last results on specificity agreed with published works, and Crohn's disease data may be irrelevant for screening since these patients should have been detected years before the CRC screening procedure. To establish the feasibility of the sCD26 test for the diagnosis of CRC, we decided to perform a first pilot case-finding study that tested 170 persons of both genders at average risk for CRC (older than 50 years and asymptomatic for bowel disease), excluding individuals with a family history of CRC, or colorectal polyps, or personal history of CRC. From 29 individuals positive for the marker (with serum levels below or at the cut-off of 410 ng/mL), as previously studied[113], 21 underwent the colonoscopic procedure with colorectal findings in ten individuals (47.6%) against 3 out of ten individuals negative for the marker (30%)[113], showing an additional value of sCD26 for the detection of premalignant lesions (Table 1).

The aim of a later case-finding study in a large cohort (2754 presumably healthy individuals) was to evaluate its association with epidemiologic parameters as well as certain common digestive-related symptoms or pathologies[109]. Personal questionnaires were completed for data such as personal and familial history of colorectal polyps or cancer, bowel diseases (non-inflammatory benign pathologies: anal fissure, hemorrhoids, diverticula, irritable bowel syndrome and spastic colon; and inflammatory bowel diseases: colitis

or Crohn's disease), symptoms (rectal bleeding or fecal blood and changes in bowel habits), and smoking status. Individuals with a personal history of CRC, personal history of a cancer other than CRC, personal history of colorectal polyps, and familial history of cancer and/or colorectal polyps were excluded.

The mean sCD26 concentration in this cohort corresponded to 555.9 ± 181.7 ng/mL, similar to that previously reported for 52 healthy donors (559.7 ± 125.5 ng/mL). However, the range in this large cohort was considerably broad compared to that of the healthy donors (118–3062 ng/mL and 273–863 ng/mL, respectively)[109]. Information concerning the smoking status was also obtained. 63.8% of the individuals were non-smokers, 27.8% were current smokers, while 8.4% were former smokers. Former smokers showed statistically significant higher values of sCD26, and current smokers lower than non-smokers, the latter fact correlating with data on enzymatic activity[236-240], however, this small difference (20 and 10 ng/mL, respectively) was not statistically significant when grouped by the number of cigarettes per day.

According to the cut-off point 410 ng/mL, 273 individuals (10.2%) were sCD26+[109]. To extend the validation of sCD26 as an early biomarker for CRC, a colonoscopic procedure was recommended to these individuals. Among the 140 individuals that underwent colonoscopy, one case of CRC was diagnosed, resulting in a very high prevalence (0.7%) for this cohort. In addition, there were 46 cases of colorectal polyps (32.9%), 12 cases of colorectal diverticula (8.6%) and 81 individuals without apparent colorectal pathology (Table 1). The PPV for the sCD26 test considering all the findings was 42.1%[111]. The sCD26+ individual diagnosed with CRC after colonoscopy received surgery after three months, finding a tumor in Dukes' stage B. Interestingly this patient had a negative FOBT two weeks before the measurement of sCD26. Another case was a sCD26+ individual who had a second positive test three months afterwards, and later was diagnosed by colonoscopy with a 1-cm villous polyp in the transverse colon, which was not extirpated. After seven months, the patient was diagnosed and operated on for a moderately differentiated adenocarcinoma at Dukes' stage A (data not published). The 46 individuals diagnosed with colorectal polyps represent a percentage similar to those diagnosed in the first case-finding study, also elevated considering the average risk[30]. Information regarding the pathological anatomy of polyps was obtained only for 16 cases. Of these, 75% presented neoplasic histology (adenomas). Trying to explain the high number of colorectal polyps diagnosed, no differences were found between the mean age of the individuals with and without polyps (data not published).

Although the most accurate means of measuring sensitivity and specificity is to perform colonoscopies in all the screened patients regardless of the test result, when this is not possible, several authors use a follow-up period to detect interval cancers[24,214,241]. Therefore, it is assumed that a false negative becomes clinically apparent through subsequent screening or the appearance of symptoms. According to this approach, with a one year follow-up of our individuals, a sensitivity of 100% and a specificity of 89.9% for CRC were obtained (Table 1)[111].

These results, with special interest on the absence of correlation among all the parameters analyzed, particularly the personal and familial history of CRC and polyps together with rectal bleeding and changes in bowel habits, proved that the sCD26 test can be easily offered and evaluated in a large population cohort. Additional data also support the usefulness of serum sCD26 levels for patient monitoring because four of the patients diagnosed with polyps requested a second sCD26 test after polypectomy, which showed normalized values (> 410 ng/mL) in the new measurement in all patients[111].

However, accurate clinical values suggesting that a serum CD26 test is an improvement on the current non-invasive screening tests recommended was lacking. Therefore a case-control study with 299 symptomatic and asymptomatic patients, who were to undergo colonoscopy, was performed[108]. Colonoscopy indication was mostly due to symptoms such as rectal bleeding, abdominal pain, diarrhea, constipation, anemia, colorectal polyp or cancer surveillance, and CRC screening. Patients were classified into groups as follows: no colorectal pathology (symptomatic with rectal bleeding, abdominal pain, diarrhea, anemia, constipation, or asymptomatic with personal history of polyps or CRC, and family history of polyps); non-IBD (hemorrhoids and diverticula); IBD (colitis or Crohn's disease); colorectal polyps (hyperplastic polyps, non-advanced adenomas and advanced adenomas); and CRC.

The average sCD26 level for the group of patients with no colorectal pathology or benign colorectal pathology was 641.2 ± 241.2 ng/mL, higher than the cut-off point obtained with healthy donors as the control cohort. Therefore, we chose to calculate a new cut-off, 460 ng/mL. According to this, the sCD26 test has a sensitivity and specificity of 81.8% and 72.3%, respectively, for CRC (Table 1), (a specificity of 79.3% when the group of symptomatic patients with no colorectal pathology was considered). The mean sCD26 concentration decreased, although non-significantly, as the pathology diagnosed was more severe, that is, from no colorectal pathology to CRC, with a noticeable decrease in the group with IBD and anemia. Interestingly, individuals with anemia showed a substantially elevated sCD26 positivity rate (71.4%), as well as the IBD group (69.2%), both responsible for the specificity value. IBD is associated with at least a 5-fold increased risk for CRC, representing one of the highest risk groups based on the inflammation-dysplasia-carcinoma sequence[242]. However, these individuals are usually diagnosed at the age of the potential CRC screening procedure, as commented, and its impact on the specificity data can be avoided. When considering only asymptomatic individuals, specificity increases to 90%, which agrees with our previously published results[103,104,113].

On the other hand, as no carcinomas *in situ* were detected in the patients included in the study, the decrease in sensitivity in this context is probably related to altered frequencies in CRC stages (sCD26 is a poorer marker in Duke's A than in B or C stages)[103].

In this study, we also analyzed the relationship of this biomarker with advanced adenomas. Defining advanced adenomas as those larger than 10 mm, with tubulovillous or villous histology, or with high-grade dysplasia, and classifying patients with more than one polyp according to the most advanced lesion, sensitivity for the detection of CRC and advanced adenomas was 58.0%, with a specificity of 75.5% (Table 1). We found no statistical differences, according to the sCD26 positivity rate, with regard to the number of polyps, their size, location, morphology or histology, but differences closely significant were observed with the grade of dysplasia, a morphological marker of neoplastic lesions. The positivity rate increased gradually with the degree of dysplasia: 22.2% for non-dysplastic polyps, 32.5% for low-grade dysplastic adenomas and almost double (60.0%) for high-grade dysplastic adenomas. Concerning advanced adenomas, a term commonly used to group adenomas that have an increased likelihood of malignant transformation, the sCD26 positivity rate was statistically significant.

As commented, iFOBT is now preferentially offered for average-risk screening. A highly sensitive FOBT (guaiac-based) test (Hemoccult SENSA®) reached 71-79% sensitivity with single testing, and 85% with multiple testing, with corresponding specificities of 86% and 95%[241-243], although these parameters are probably overestimated as these studies lacked colonoscopic examination of the negative cases. For both pathologies together (CRC and advanced adenomas) in an asymptomatic high-risk cohort, however, higher sensitivity, 65.3% (and 87.5% for specificity), resulted with Hemoccult SENSA®[244] compared to iFOGT (33.1% sensitivity and specificity of 97.5%, parameters obtained with flexible sigmoidoscopy). For other experimental serum biomarkers, the CCSA-2 has shown 97.3% sensitivity and 78.4% specificity, although hyperplastic polyps and non-advanced adenomas were considered as findings, while IBD patients were absent in their cohort[100]. Therefore, sCD26 seems to perform adequately as a blood biomarker for CRC and advanced adenomas, and is independent of the frequent but intermittent bleeding, unlike guaiac FOBT or iFOBT.

In hepatocarcinoma, a loss of membrane CD26 is correlated with higher DPP-IV levels. This fact is not seen in CRC, as almost all CRC patients show reduced serum levels of sCD26[103,111], but loss of membrane CD26 expression only occurs in 11% of colorectal tumor[181]. In conclusion, for CRC, sCD26 is not correlated with cell proliferation, or with the alteration of CD26 expression in CRC tumor cells. Nor is there any direct correlation between sCD26 levels and tumor location, degree of histological differentiation, type of metastasis or Dukes' stages of CRC[245], which may affect the hepatic production of sCD26. Therefore, sCD26 is also independent, if not of the tumorigenic locus, at least of the tumorigenic tissue. In addition, as it seems immune-related[142], the sCD26 decrease in the plasma of patients should appear sooner in the adenoma-carcinoma development compared to the presence of fecal blood.

CONCLUSION

As commented previously, it has recently been reported that screening accounted for 53% of the decline in CRC mortality observed during 1975-2000 in the USA (26% less mortality). Moreover, decline in CRC mortality in the USA could be enhanced if current trends against cancer were accelerated[2]. Therefore, any kind of screening strategy should be proposed in advanced and developing countries. For FOBT Hemoccult, which is a non-invasive and relatively cheap test, there are considerable data from many prospective studies in different countries of the world; however, not many countries include this screening method in their public health systems.

In the context of this review, dealing with an experimental CRC screening test that is easier to monitor in the health system, or with a better clinical value, the idea of comparing different tests rather than conducting long-term and expensive randomized controlled trials to evaluate each new test is very important, as suggested by Mandel[37] in the commentary on the Allison study that compared performance, outcome, compliance, and cost of guaiac FOBT and iFOBT[42] fecal occult blood tests. In this way, it will be much easier to study and promote new fecal or blood tests. With this aim, we are currently initiating a multicentric, prospective, double-blinded study in an average-risk population, where the performance of the quantitive iFOBT and the sCD26 assay will be assessed and compared with the gold standard colonoscopy.

Another important idea, as we have previously proposed[110], is the combination of biomarkers for the management of cancer, since it is difficult to achieve a simple test to detect early-stage tumors that is useful for screening purposes. For example, we have tested sCD26 levels, α-L-fucosidase activity and CEA in the same patients[109], and while, at a specificity of 100%, α-L-fucosidase activity did not enhance the sensitivity value obtained with sCD26 alone in TNM stage II patients, the sensitivity obtained from the combination of both markers was 65% *versus* 33% for sCD26 alone in TNM stage I patients. In the same way, a very recent work assessed the combination of CEA with three other biomarkers, sCD26, DR-70 and MMP-9, previously selected from 26 candidates, for the detection of CRC[246]. This study confirmed that sCD26 and DR-70 (fibrin and fibrinogen degradation products[104]) are the more promising of the available serum markers, although DR-70 showed a significant correlation with age. Values of the area under the ROC curve, and sensitivity and specificity for sCD26 were similar to our latest study mentioned above[108] using a similar cohort of case-control patients who attended

colonoscopy. The same study[246] also showed that a combination of sCD26, DR-70 and CEA detected CRC, particularly at the early stage of disease, significantly better than CEA alone or other biomarker combinations at certain specificities[246]. Nevertheless, our data on sCD26 for the detection of the earliest stages were much better (and data of CEA worst)[103,109]. This discrepancy perhaps may be due to the differences in the cohort composition in each study, to the statistical method employed for the combination of biomarkers, or to a development of the ELISA for the measurement of CEA levels (the kits used for CEA, but not for sCD26, were different in each study).

Therefore, the approach tries to increase the clinical value of each biomarker or yield a test more able to distinguish between patients and healthy individuals, and ideally also among different kinds of tumors, in the way we have tested for head and neck cancer *versus* non-small cell lung cancer[247] at a low scale, and others for the screening of Alzheimer disease[248], or to identify lymph node metastases in non-small cell lung cancer patients[249]. In this case, a panel of six serum biomarkers classified the patients better than conventional clinical methods.

To measure several biomarkers at a time, ELISAs for key serum markers are being arrayed or multiplexed based on immunoblot technology or flow cytometric beads[110,247,249].

These techniques, in relation with other genomic or proteomic techniques, are more transferable to practical application in clinical decision-making. In this sense, it is interesting to note that the multiplexed diagnostics market has grown rapidly and generated sales of approximately $2.4 billon in 2009, and is expected to tip in favor of continued rapid growth, reaching almost $5.8 billion in 2015. Moreover, the multivariate data obtained from such a test can easily be managed with new statistical methods already developed for the fields of genomics and proteomics in general.

However, as multiple cancer screening tests are been advocated for the general population, clinicians and patients are not always well-informed of screening burdens. For example, in the ongoing Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, a randomized controlled trial to determine the effects of prostate, lung, colorectal, and ovarian cancer screening on disease-specific mortality, an individual has an approximately 50% or greater risk of a false-positive finding by the 14th test[250]. Physicians should educate patients about the likelihood of false positives and resulting diagnostic interventions when counseling on cancer screening.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Tian L

World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v2.i6.262

World J Clin Oncol 2011 June 10; 2(6): 262-271
ISSN 2218-4333 (online)

REVIEW

Review of the treatment of metastatic non small cell lung carcinoma: A practical approach

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Received: March 12, 2011 Revised: June 2, 2011
Accepted: June 7, 2011
Published online: June 10, 2011

Abstract

In recent years, as we have a better knowledge and understanding of the biology of non small cell lung carcinoma (NSCLC), which leads us to targeting biomarkers driving the NSCLC carcinogenesis and metastatic potential, we now have an increased number of options to offer our patients with NSCLC. We also realize the importance of distinguishing squamous and non squamous histology to guide our treatment decisions of NSCLC. The palliative care concomitant with therapies from the very start of the treatment also showed an impact on survival. This review examines the treatment options in all lines of therapy for metastatic NSCLC that have been approved in Canada, the United States, or Europe.

Key words: Metastatic; Non small cell lung carcinoma; 1st Line; 2nd Line; 3rd Line; Treatment

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Hirsh V. Review of the treatment of metastatic non small cell lung carcinoma: A practical approach. *World J Clin Oncol* 2011; 2(6): 262-271 Available from: URL: http://www.wjgnet.com/2218-4333/full/v2/i6/262.htm DOI: http://dx.doi.org/10.5306/wjco.v2.i6.262

INTRODUCTION

Lung cancer remains one of the most common cancers worldwide and a leading cause of mortality, with an estimated 1.6 million new cases and nearly 1.4 million deaths annually. The majority of patients with non small cell lung carcinoma (NSCLC) present with advanced stage disease at diagnosis. A large number of patients who are diagnosed at an early stage will eventually experience disease relapse and will also need treatment for a metastatic disease. The 5-year survival rate of lung cancer patients remains only about 15%. Furthermore, advanced lung cancer causes debilitating symptoms which can seriously affect the quality of life (QOL) and survival.

Historically, the treatment of NSCLC has involved a finite number of cycles of first-line chemotherapy, the most commonly-used regimens being platinum doublets[1] for patients with a good performance status (PS) and no significant comorbidities, after which patients with tumour response or stable disease were observed for evidence of disease progression; at this point, suitable patients would start second-line therapy. We learned that the introduction of a third chemotherapeutic agent only increased toxicity, but not efficacy. We also realized that only about 50%-60% of patients go on to receive second-line therapy and of those, only 50%-60% will receive third-line therapy. It is therefore important to ensure that patients receive the best therapeutic option in each line of therapy[2].

In recent years, two new concepts have been introduced in the treatment of metastatic NSCLC: maintenance therapy and targeted biologic agents. Maintenance therapy after first-line therapy can be with either chemo-

therapeutic or biologic agents, it may include drugs given in the induction regimen, or different agents (i.e. "early" second-line treatment) with the aim of preventing progression and prolonging progression-free survival (PFS). Targeted agents, when compared with chemotherapeutic agents in this setting, show fewer toxicities, especially cumulative toxicities such as myelosuppression; thus the possibility of a longer duration of therapy[3].

Two main groups of targeted agents for NSCLC, which are presently approved in the United States, Canada, and Europe, based on the results of clinical trials, including their efficacy and safety profiles, are the inhibitors of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF). Erlotinib or gefinitib and bevacizumab are the respective representatives of these groups. Another EGFR inhibitor, cetuximab, is not currently approved in Canada and the United States. Gefitinib was granted marketing authorization for the treatment of EGFR mutation-positive metastatic NSCLC.

The options and lines of treatments in metastatic NSCLC are increasing. The understanding of the development of resistance to different therapeutic agents will help us to decide on the sequence of therapies i.e. the choices for first, second, third, and further lines of treatment. Our decisions will not only depend on age, gender, comorbidities, smoking history, racial origin, and PS of patients, but also on the tumour characteristics and the toxicity profile of the therapies.

The goal of the treatments of advanced NSCLC is only palliative for now, thus QOL remains a very important factor. Early control of symptoms such as nausea, diarrhoea, constipation, pain, or prevention of cytopaenias and bone metastases enables patients to maintain good PS and QOL, enabling them to receive now available numerous lines of treatments. We now better understand various prognostic and predictive factors which can guide our decisions regarding the different treatment options and help us to deliver a personalized, individualized treatment for our NSCLC patients, leading to increased treatment efficacy, decreased toxicity and improved QOL.

FIRST-LINE TREATMENT OF METASTATIC NSCLC

Chemotherapy in first-line

The third-generation chemotherapy agents such as paclitaxel, docetaxel, gemcitabine, vinorelbine, irinotecan, and pemetrexed in platinum-based doublets are more effective in terms of response rates and survival and are better tolerated than the older platinum-based combinations[4,5]. The overall benefit obtained by modifying chemotherapy regimens has been small and has yielded no tangible improvement in overall survival (OS)[6]. Median OS reached with chemotherapy plateaus at 8-10 mo, even with pemetrexed, as demonstrated in per protocol population in a phase Ⅲ trial[7] comparing first-line cisplatin-pemetrexed to cisplatin-gemcitabine, showed a median OS of 10.3 mo for each treatment arm.

In a pre-specified analysis, the median OS was significantly longer for cisplatin-pemetrexed than for cisplatin-gemcitabine in patients with adenocarcinoma histology [n = 847, 12.6 mo *vs* 10.9 mo, hazard ratio (HR) = 0.84, P = 0.03] and large-cell carcinoma histology (n = 153, 10.4 mo *vs* 6.7 mo, HR = 0.67, P = 0.03). The median survival of patients with squamous histology assigned to cisplatin-pemetrexed (n = 244) was only 9.4 mo; and was 10.8 mo on cisplatin-gemcitabine (n = 229, HR = 1.23, P = 0.05). For patients with NSCLC without further subtype classification (n = 252), no significant differences were observed between the two arms[7]. Thus, cisplatin-pemetrexed should not be given for squamous tumours. Carboplatin-pemetrexed demonstrated efficacy similar to that of carboplatin-gemcitabine in first-line treatment of metastatic NSCLC[8]. No comparison is yet available of the platinum-taxane regimens with the platinum-pemetrexed regimens. Carboplatin is favoured in certain centres and countries, especially in the more frail patients with different comorbidities, due to less toxicity.

Targeted therapies in first-line

The first targeted agent which when added to a platinum doublet in first-line metastatic NSCLC resulted in an improved efficacy, was the anti-VEGF monoclonal antibody, bevacizumab. VEGF has multiple roles in tumour angiogenesis. It has been shown to promote survival[9] and to increase permeability of existing tumour vasculature[10], while stimulating the growth of new tumour vessels[9]. In addition, VEGF is known to have a direct effect on tumour cells, including survival, migration, and invasion[10]. Two early effects of anti-VEGF therapy include regression of existing tumour microvasculature, and normalization of the remaining microvasculature, helping to better deliver chemotherapy to the tumour[11]. A third effect is the continued inhibition of the formation of new tumour vasculature[12].

Bevacizumab was tried in a phase Ⅱ trial (Figure 1), where it was added to carboplatin/paclitaxel. It significantly improved response rate and PFS in patients with advanced NSCLC[13].

The ECOG 4599 (Eastern Cooperative Oncology Group) phase Ⅲ trial demonstrated significant improvement in median OS (12.3 mo *vs* 10.3 mo, HR = 0.79, P = 0.003), median PFS (6.2 mo *vs* 4.5 mo, HR = 0.66, P < 0.001), and response rates (35% *vs* 15%, P < 0.001) for bevacizumab in combination with carboplatin-paclitaxel as compared with chemotherapy alone[14]. Bevacizumab is the first agent combined with chemotherapy to improve survival beyond 1 year for patients with non-squamous pathology of NSCLC. In the same trial in patients with adenocarcinoma, median OS was 14.2 mo *vs* 10.3 mo for control.

The AVAIL (AVASTIN in lung) trial was the second, randomized phase Ⅲ trial with cisplatin-gemcitabine and bevacizumab 7.5 mg/kg or 15 mg/kg *vs* cisplatin-gemcitabine only, in a three-arm study design. This study was conducted 4-5 years later than the ECOG study,

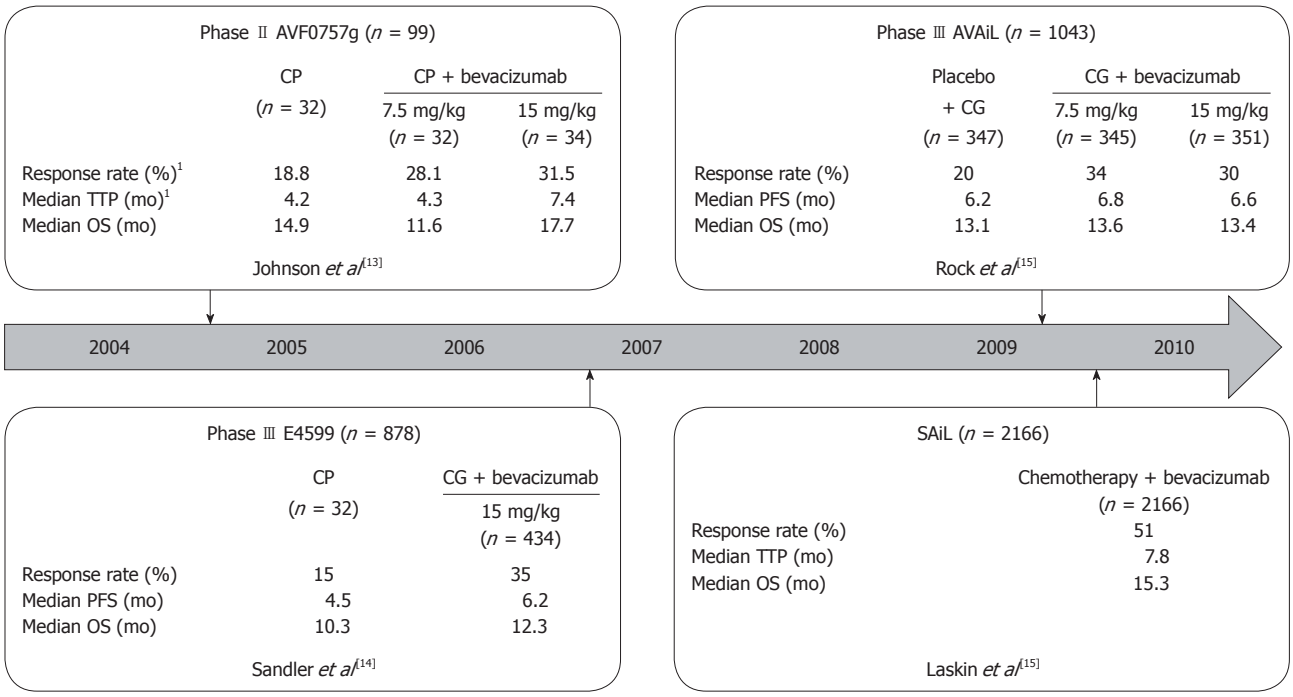

Figure 1 First-line bevacizumab data in non small cell lung carcinoma. CP: Carboplatin, paclitaxel; CG: Cisplatin, gemcitabine. [1]Investigator assessment.

when more lines of treatments were available and they could confound OS, and crossover to bevacizumab was possible, thus median PFS was a primary endpoint. PFS was significantly prolonged with bevacizumab 7.5 mg/kg plus chemotherapy compared with chemotherapy alone (6.7 mo *vs* 6.1 mo; HR = 0.75, $P = 0.003$) and an objective response rate of 34.1% compared to 20.1% for chemotherapy alone ($P < 0.0001$). PFS was also significantly improved in patients receiving bevacizumab 15 mg/kg plus chemotherapy as compared with placebo (6.5 mo *vs* 6.1 mo; HR = 0.82, $P = 0.03$).

The SAIL (Safety of Avastin in Lung) trial examined the safety of bevacizumab in a broad patient population[15,16]. More than 2000 patients demonstrated a clinical benefit with bevacizumab, not only with different cisplatin, but also carboplatin doublets - regimens according to the investigators' choice. In this trial, median PFS was 7.8 mo and median OS was 15.3 mo[15].

A 2000 patient registry trial in the United States ARIES, (Avastin Registry: Investigation of Effectiveness and Safety), showed similar results as the SAIL trial even though 647 patients were elderly > 70 years old. Some had hypertension, central tumour location, central nervous system (CNS) metastases, or receiving anticoagulation therapy. Median PFS was over 6 mo, and median OS was 13.3 mo[17]. A meta-analysis of more than 13000 bevacizumab-treated patients provided reassurance that the risk of CNS bleeding in patients with brain metastases is not increased[18].

In contrast, phase Ⅲ trials with cetuximab plus taxane-carboplatin (BMS - 099) and cetuximab plus cisplatin-vinorelbine in the FLEX (First line Erbitux) trial, failed to demonstrate a PFS benefit in patients with NSCLC (4.4 mo *vs* 4.2 mo and 4.8 mo, respectively)[19,20]. A marginal OS benefit was observed in FLEX (11.3 mo *vs* 10 mo), which raises the question of the benefit of subsequent post-induction therapies.

A large, phase Ⅲ trial ESCAPE, (Evaluation of Sorafenib, Carboplatin And Paclitaxel Efficacy in NSCLC) of sorafenib, a multikinase inhibitor in combination with carboplatin-paclitaxel, showed no benefit in patients with NSCLC. Moreover, the addition of sorafenib had a detrimental effect in patients with squamous cell histology. The trial was stopped prematurely and did not meet its primary OS endpoint[21].

The NCIC, (National Cancer Institute of Canada) BR.24 phase Ⅱ/Ⅲ study of cediranib in first-line NSCLC was also discontinued because of unacceptable toxicity. A follow-up, randomized phase Ⅲ trial (NCIC BR.29) is currently ongoing, testing cediranib at the lower dose of only 20 mg orally daily with carboplatin-paclitaxel compared to carboplatin-paclitaxel alone in patients with metastatic NSCLC. Many other randomized trials of targeted therapies combined with chemotherapy have failed to demonstrate clinical benefit.

Evidence-based medicine: a practical approach in first-line

A number of factors will affect the choice of first-line therapy in metastatic NSCLC, including available clinical data, patient characteristics (age, smoking history, histology, racial origin, tumour mutation status, patient preference, and physician's experience with certain agents. Although pemetrexed has demonstrated an OS benefit in patients with non-squamous NSCLC, that benefit was restricted to the sub-analysis of a subgroup of patients who

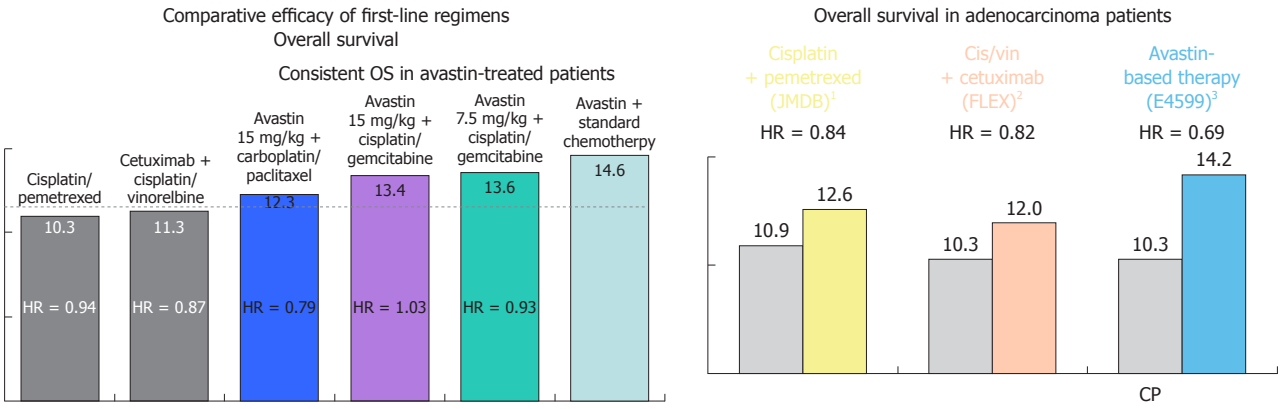

Figure 2 Comparison of overall survival with the most frequently given regimens in first-line treatment of metastatic non small cell lung carcinoma. [1]Scagliotti *et al*[7]; [2]Pirker *et al*[20]; [3]Sandler *et al*[14]. HR: Hazard ratio; OS: Overall survival.

received cisplatin. No comparison of platinum-taxanes with platinum-pemetrexed is available. Thus, patients not eligible for bevacizumab should receive platinum-containing doublet chemotherapy, of which cisplatin-pemetrexed is the most promising for non-squamous histology. Results from phase Ⅲ trials will help to determine the role of pemetrexed-platinum with bevacizumab in the first-line setting. A summary of OS with the most frequently used regimens in first-line treatment of NSCLC is shown in Figure 2.

The evidence suggests that EGFR tyrosine kinase inhibitors (TKIs) are particularly effective agents in patients with EGFR mutation-positive tumours. A phase Ⅲ trial, open-label study (the IRESSA Pan-Asia Study - IPASS)[22] examined the efficacy of gefitinib in first-line as compared with carboplatin-paclitaxel in clinically selected patients with NSCLC. The results revealed significantly longer PFS, increased objective response rates ($P < 0.0001$), and improved QOL among EGFR mutation-positive patients who received gefitinib than among those who received carboplatin-paclitaxel, but median OS was not statistically different. The difference in the rates of objective response with gefitinib was remarkable at 71.2% and 1.1% for EGFR mutation-positive and negative patients, respectively, median PFS was 9.5 mo on gefitinib compared to 6.3 mo on chemotherapy (HR = 0.48, $P < 0.0001$), and median OS was 21.6 mo *vs* 21.9 mo, respectively, in mutation-positive patients (HR = 1.00, $P = 0.99$).

IPASS was the first study to demonstrate the high incidence of EGFR mutation-positive tumours in female Asian patients who were never or light ex-smokers, with adenocarcinomas.

The presence of an EGFR mutation can be both a predictive and prognostic factor of improved efficacy and outcomes. We now have similar results from Korean[23] and Japanese trials[24], which also showed very positive results in patients with EGFR mutation-positive tumours who received gefitinib. The same results were recently presented with erlotinib *vs* carboplatin-gemcitabine in the OPTIMAL trial, (previously known as CTONG 0802)[25,26], where EGFR mutation-positive patients had median PFS on erlotinib of 13.1 mo *vs* 4.6 mo on chemotherapy (HR = 0.16, $P < 0.0001$). The Spanish Lung Cancer group demonstrated similar results in a phase Ⅱ trial[27].

In mutation-positive patients (exon 19 deletion and 21 point mutation), EGFR-TKIs are the treatment of choice in the first-line for metastatic NSCLC. Oral administration is more convenient and less toxic contributing to a better QOL and excellent efficacy in many patients. In the case of unknown mutation status, patients should receive chemotherapy treatment. Education on the necessity of an adequate tumour biopsy is of utmost importance for optimal patient management. Currently, there are no predictive markers for anti-VEGF therapy.

Maintenance therapy

A number of studies have evaluated regimens using either sequential or maintenance chemotherapy as post first-line treatment for NSCLC patients who have not experienced disease progression. A review of those studies suggests that the optimal regimen remains unclear[2,28].

Chemotherapy in maintenance

A phase Ⅲ trial[29] compared the efficacy and safety for docetaxel administered to patients either immediately after first-line gemcitabine-carboplatin or only at the time of disease progression. The study showed a statistically significant improvement in PFS of 3 mo for patients receiving immediate docetaxel therapy and a non-significant trend toward an improved OS. Ninety-five percent of patients in the immediate arm received docetaxel, but only 63% of patients in the delayed-therapy arm received docetaxel. When OS was compared only for patients who received docetaxel, median OS was 12.5 mo in both arms.

The JMEN trial evaluated maintenance pemetrexed plus best supportive care (BSC) against placebo plus BSC. With maintenance pemetrexed, the PFS in the overall patient population was 4.0 mo as compared with 2.0 mo for placebo (HR = 0.60, $P < 0.0001$)[30]; however, patients with squamous histology did not benefit from pemetrexed therapy. The trial excluded patients who had previously

Table 1 Efficacy (progression-free survival) outcomes of trials in the maintenance setting in patients with non small cell lung carcinoma

Trial	Treatment	*n*	Median PFS (mo)	HR
AVAiL[31]	Placebo	41	3.2	NR
	Bevacizumab 7.5 mg/kg	174	4.6	
	Bevacizumab 15 mg/kg	162	4.6	
ATLAS[32]	Bevacizumab + erlotinib	370	4.76	0.722
	Bevacizumab + placebo	373	3.75	$P = 0.0012$
SATURN[33]	Erlotinib	437	NR	0.71
	Placebo	447	NR	$P < 0.0001$
JMEN[30]	Pemetrexed	441	4.0	0.5
	Placebo	222	2.0	$P < 0.0001$

PFS: Progression-free survival; HR: Hazard ratio.

Table 2 Efficacy data in the second-line setting

Outcome	Erlotinib[41] (150 mg daily)	Docetaxel[38-40,46] (75 mg/m^2 every 3 wk)	Pemetrexed[40] (500 mg/m^2 every 3 wk)
RR (%)	8.9	6.7-8.8	9.1
Median duration of response (mo)	7.9	5.3-9.1	4.6
Median PFS (mo)	2.2	2.7-6	2.9
Median OS (mo)	6.7	5.7-7.9	8.3
1-year survival (%)	31	30-37	30
2-year survival (%)	13	0	0
Median OS (mo) in PS 0/1 patients with one prior regimen	9.4	9.1	9.4

PFS: Progression-free survival; OS: Overall survival; PS: Performance status.

received pemetrexed with cisplatin. The lack of a delayed pemetrexed arm means that it is difficult to ascertain the true benefit of immediate compared to second-line pemetrexed. Only 19% of patients in the placebo arm received pemetrexed in the second-line, raising the question of whether the observed survival benefit would have been maintained if more patients had received second-line pemetrexed. Patients on pemetrexed require folic acid and vitamin B12 to reduce treatment-related toxicities. The most frequent adverse events related to pemetrexed are neutropenia and fatigue.

Targeted therapies in maintenance

In all bevacizumab trials, bevacizumab was administered as a maintenance therapy, followed by first-line chemotherapy with bevacizumab, if there was no disease progression or unacceptable toxicity. In the maintenance phase of AVAIL, (Avastin in Lungs), there was a significant increase in PFS in the bevacizumab arm as compared with the placebo arm (4.6 mo *vs* 3.2 mo, Table 1)[31]. The Atlas trial demonstrated that the benefit is further improved with the addition of erlotinib (4.76 mo *vs* 3.75 mo, HR = 0.722)[32], but OS was not improved and the toxicity was more severe on the two-drug arm. In the SATURN trial, a 41% improvement in PFS was observed for erlotinib as compared with placebo[33]. In addition, maintenance with erlotinib demonstrated a survival benefit in all subgroups of patients, including those with squamous tumour pathology. This benefit was independent of EGFR mutation status[34]. For the mutation-positive patients, a HR = 0.1 for median PFS was unprecedented.

Future directions

A phase Ⅱ trial reported by Patel *et al*[35] demonstrated excellent results with first-line pemetrexed plus carboplatin and bevacizumab followed by maintenance with pemetrexed and bevacizumab in non-squamous NSCLC patients. The overall response rate was 55%, median PFS was 7.8 mo and OS was 14.1 mo. Another phase Ⅱ trial demonstrated that bevacizumab plus pemetrexed and oxaliplatin followed by bevacizumab maintenance achieved a median PFS of 7.8 mo and a median OS of 16.7 mo[36]. These trials suggest an improved efficacy when bevacizumab and pemetrexed are combined in different regimens. Phase Ⅲ trials are ongoing.

Clinical trial data in colorectal cancer patients suggest an advantage in maintaining clinical benefit by continuing bevacizumab beyond progression to keep VEGF levels down[37], in bevacizumab eligible patients.

Patients who are not eligible for bevacizumab and/or want a more convenient, oral treatment, causing mainly rash or diarrhoea, can be maintained by erlotinib, which is also effective in squamous histology, unlike pemetrexed. For non-squamous histology, depending on patient preference or ineligibility for bevacizumab, pemetrexed also remains an option.

Palliative therapies, especially early prevention of skeletal-related events, such as fractures, spinal cord compression, radiotherapy, and surgery to bone should be an integral component of active treatments[38,39].

SECOND-LINE THERAPY

Chemotherapy in second-line

Several chemotherapy agents, including docetaxel and pemetrexed, have demonstrated efficacy in the second-line treatment of NSCLC patients[40-43]. Pemetrexed is approved for non-squamous histology only. Both drugs offer similar efficacy in randomized, phase Ⅲ trials[42], with median OS of 8.3 mo for docetaxel and 7.9 mo for pemetrexed, however, pemetrexed has a milder toxicity profile than docetaxel[41].

Targeted therapies in second-line

Erlotinib is an EGFR-TKI that suppresses intracellular signalling pathways, which promote cell growth and proliferation[44,45]. Unlike chemotherapy, it causes no cumulative hematologic toxicities, allowing for a longer treatment duration. The toxicities associated with chemotherapy allow for only a limited number of cycles, median of approximately 4 cycles. Table 2 compares clinical data for erlotinib, docetaxel, and pemetrexed.

In a randomized, placebo-controlled study (NCIC BR.21), erlotinib demonstrated improvement in median

OS (6.7 mo *vs* 4.7 mo) and QOL across all subgroups[43,46]. Fifty percent of patients were treated in second-line, and 50% in third-line; some patients even had PS of 3.

The safety and efficacy of erlotinib were confirmed in the phase Ⅳ trial, TRUST (TaRceva LUng Cancer Survival Treatment), in a broad patient population[47], where median OS was 8.1 mo, and 1-year survival was 38.6%.

Gefitinib, another EGFR-TKI, failed to demonstrate a survival advantage in the overall population of the phase Ⅲ trial, ISEL (Iressa Survival Evaluation in Lung Cancer), where patients had to be refractory to previous chemotherapy. A phase Ⅱ study of a single-agent, sorafenib (targeting mainly angiogenesis), in second-line suggests only modest benefits and some specific toxicity, such as hand-foot syndrome[48]. Vandetanib (ZACTIMA), targeting VEGF receptor and EGFR, has demonstrated only a modest benefit[49-51] in phase Ⅲ second-line trials alone or in combination with pemetrexed or docetaxel; and was withdrawn from the market for NSCLC treatment.

A practical approach in second-line

A good response to first-line chemotherapy may warrant further chemotherapy in second-line. A meta-analysis of single agents *vs* doublet chemotherapy demonstrated improvement in response rate, but it did not translate into a PFS or OS benefit, only being associated with an increased toxicity[52]. If patients tolerated first-line chemotherapy poorly, an EGFR inhibitor may be the preferred choice for second-line.

Non-inferiority in terms of OS for gefitinib compared with docetaxel, was demonstrated in the phase Ⅲ trial INTEREST (Iressa NSCLC Trial Evaluating Response and Survival versus Taxotere)[53]. Non-inferiority was shown regardless of a patient's EGFR protein expression, EGFR gene mutation, or K-RAS gene mutation status. The only advantage for OS was for patients who received docetaxel in third-line treatment. Given the lack of difference in clinical benefit relating to the sequence of chemotherapy *vs* EGFR-TKI in the second and third lines (INTEREST), as well as reduced toxicity and easy, convenient oral administration (sometimes for longer periods of time), EGFR-TKIs are preferred second-line agents for NSCLC. Obtaining EGFR (exon 19 and 21) mutation status of the tumour for second-line treatment of NSCLC is not a necessity. Numerous randomized trials for second-line treatments of NSCLC are ongoing with different targeted agents. Patients who received EGFR-TKIs in first-line as their tumours were positive for EGFR mutations, could receive a platinum doublet in second-line, if their PS and comorbidities permit. More data are needed for this patient population. We now have data from many trials with bevacizumab and EGFR-TKIs, see Table 3.

THIRD-LINE TREATMENT

A number of trials are investigating the role of anticancer therapies in the third or fourth-line setting. The phase Ⅲ Zephyr trial (Zactima Efficacy trial for NSCLC Patients with HistorY of EGFR and chemo-Resistance), investigated the role of Vandetanib in the third and fourth-line setting. Median PFS was significantly prolonged - 1.9 mo on Vandetanib *vs* 1.8 mo on placebo ($P < 0.0001$, HR = 0.63)[54].

BIBW 2992 (Afatinib), a dual irreversible inhibitor of EGFR and Her-2 demonstrated encouraging results in a randomized, phase Ⅲ trial (Lux Lung 1), involving 585 patients who had progressed after 1-2 chemotherapy regimens (one had to be platinum-based) and who had to be at least 3 mo on EGFR-TKI without disease progression. The patients received afatinib 50 mg po daily plus BSC or BSC plus placebo (randomization was 2:1). Median time on EGFR-TKI was 10.2 mo, 81% patients were receiving EGFR-TKIs for more than 24 wk. Complete or partial response on prior EGFR-TKI treatment was 45% suggesting a very high tumour EGFR mutation rate. Afatinib extended median PFS, tripling it over PFS with placebo (3.3 mo *vs* 1.1 mo, $P < 0.001$, HR = 0.38)[55], however, median OS, the primary endpoint, was not significantly different, 10.78 mo with BSC plus afatinib *vs* 11.96 mo with BSC plus placebo (HR = 1.077, $P = 0.7428$). The disease control rate was higher on afatinib (58% *vs* 18%, $P < 0.0001$). Moreover, afatinib significantly improved cough, dyspnea and pain, and delayed the time of deterioration of these symptoms[54]. The main side effects as expected were diarrhoea and rash, which were manageable. OS was confounded by further lines of treatment and their imbalance. Seventy nine percent of patients in the placebo arm received further chemotherapies or targeted agents. One hundred and forty four patients in the afatinib arm and 43 patients in the placebo arm did not receive further lines as no treatment was available in these centres, and here OS favoured the afatinib arm ($P = 0.02$, HR = 0.65). Patients who clinically benefited from prior EGFR-TKI (i.e. response rate, DCR > 6 mo) had PFS 4x longer on afatinib *vs* placebo (4.4 mo *vs* 1.1 mo) and there was a trend for better OS (HR = 0.9).

A phase Ⅲ trial of sorafenib (a multikinase inhibitor) *vs* placebo, the MISSION trial (Monotherapy Administration of Sorafenib in patientS with non-small cell Lung cancer), in third or fourth-line therapy has finished accrual and results are expected soon. Combining an insulin-like growth factor (and receptor) inhibitor with erlotinib to try to prevent development of resistance to erlotinib is also under investigation.

Practical approach in third-line

Erlotinib is a viable third-line treatment option for patients who have not yet received it. In spite of an exquisite sensitivity of EGFR mutation-positive tumours to EGFR-TKIs such as erlotinib or gefitinib, eventually all patients progress, as they develop resistance to EGFR-TKIs. The most frequent mutation is T790M on exon 20, and is found in about 50% of such patients. Afatinib showed preclinical evidence of activity for this mutation

Table 3 Selected trials of erlotinib and bevacizumab

Study	Phase	*n*	Eligibility	Regimen	Line of therapy	Primary endpoint
PASSPORT (AVF3752g)	II	110	Previously treated or untreated non-squamous NSCLC with treated CNS metastases	Chemo or erlotinib followed by bev	First/second	Grade ≥ 2 symptomatic CNS haemorrhage
BRAIN (AVF21823)	II	115	Stage Ⅳ non-squamous NSCLC with asymptomatic brain metastases in first and second line	First line: bev + carbo/pac Second line: bev + erlotinib	First/second	PFS
EAGLES	II	78	Patients aged > 70 yr without important comorbidities	Bev + gem or bev + gem/cis	First	PFS at 6 mo
ML21896	II	~250	Patients aged ≥ 65 yr with advanced metastatic or recurrent non-squamous NSCLC	Bev + pem or bev + pem/carbo	First	Proof of non-inferiority of bev + pem
BRIDGE (AVF2744g)	I/II	40	Previously untreated squamous NSCLC	Bev + carbo/pac	First	Grade ≥ 3 pulmonary haemorrhage
ABIGAIL (BO21015)	II	~300	Locally advanced, metastatic or recurrent non-squamous NSCLC	Bev + carbo/gem or carbo/pac	First	Correlation of biomarkers with response
MIMEB (ML21803)	II	40	Histologically confirmed advanced non-squamous NSCLC stage ⅢB/Ⅳ	Bev + erlotinib	First	Evaluate accuracy of FDG-/FLT-PET and DCE-MRI for early prediction of non-progression
EURTAC	III	146	EGFR mutation-positive NSCLC	Erlotinib	First	PFS in patients with
SATURN (BO18192)	III	1949	Previously untreated advanced NSCLC	Erlotinib	First	PFS in all patients and in patients with EGFR IHC+ tumours
RADIANT		945	Advanced NSCLC	Erlotinib *vs* placebo	Adjuvant	DFS
FASTACT-2	III	450	Asian patients with previously untreated advanced NSCLC	Erlotinib + chemo *vs* placebo + chemo	First line	PFS
ATLAS (AVF3671f)	III	1150	Previously untreated advanced NSCLC	Bev + carbo/pac, gem/cis or carbo/doc) Non-progressing patients randomized (1:1) to bev + erlotinib or bev + placebo	First line maintenance	PFS
TARGET	II	428	EGFR mutation-positive NSCLC	Erlotinib	First	PFS
TORCH	III	900	Previously untreated advanced NSCLC	First-line erlotinib second-line gem/cis *vs* first-line gem/cis second-line erlotinib	First/second	OS

NSCLC: Non-small cell lung cancer; CNS: Central nervous system; bev: Bevacizumab; carbo: Carboplatin; pac: Paclitaxel; PFS: Progression-free survival; gem: Gemcitabine; cis: Cisplatin; pem: Pemetrexed; FDG: [18F]-2-fluoro-deoxy-D-glucose; FLT: F-fluorodeoxythymidine; PET: Positron emission tomography; DCE-MRI: Dynamic contrast-enhanced magnetic resonance imaging; EGFR: Epidermal growth factor receptor; IHC: Immunohistochemistry; DFS: Disease-free survival; chemo: Chemotherapy; doc: Docetaxel; OS: Overall survival.

and Lux Lung 1 showed significant activity of afatinib, especially in patients with a high possibility of EGFR mutations on the basis of clinical criteria. Thus, afatinib is likely to be a possible option for third or fourth line treatment of metastatic NSCLC patients. Lux Lung 2 (60 patients in first line, and 60 patients in second-line, only EGFR mutation-positive NSCLC) showed very exciting results, median PFS of 15 mo, median OS of 24 mo for patients with EGFR exon 19 and 21 mutations.

Two phase Ⅲ trials in EGFR mutation-positive patients with adenocarcinoma treated in first-line, comparing afatinib to cisplatin-pemetrexed, are ongoing.

Only 3%-5% of patients with NSCLC have the ALK fusion gene. Crizotinib is an oral, potent and selective small-molecule ATP-competitive inhibitor of ALK and MET kinases and their oncogenic variants. Overall response rate was 56%, DCR at 8 wk was 88% and median PFS was 9.0 mo in heavily pre-treated NSCLC patients[56].

Trials are now ongoing in first-line treatment, comparing crizotinib to pemetrexed/cisplatin or carboplatin in a phase Ⅲ study of non-squamous NSCLC and in second-line comparing crizotinib to pemetrexed or docetaxel again in a phase Ⅲ study[6].

CONCLUSION

The main goal should be to provide the best possible treatment in terms of both efficacy and safety in each line of therapy. As compared with chemotherapeutic agents, targeted agents may offer reduced toxicity, especially with prolonged use. By increasing the agent's specificity, and possibly combining different agents in order to target different pathways, we will increase the treatment efficacy[57]. New agents, such as PARP inhibitors for squamous cancers, and IGFR, HDAC, HSP 90 and C-MET inhibitors are being tested in clinical trials, especially in combination with the already established targeted agents or with chemotherapy.

Predictors of response may help to guide individual treatment decisions. We need to identify the biomarkers of response and resistance (old and newly developed) at every step, and every line of treatment. A personalized,

targeted approach is the future of treatment in all lines, and a re-biopsy of tumours will be required for analysis of biomarkers, including newly developed markers of resistance to EGFR-TKIs, but also sensitivity to other agents, such as afatinib. Analysis of circulating tumour cells and blood biomarkers to define predictors of tumour response and treatment benefit is needed for the future.

ACKNOWLEDGMENTS

The author wishes to thank Ms. Stavroula Kalantzis for her assistance in the preparation/development of the manuscript.

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S- Editor Tian L **L- Editor** Webster JR **E- Editor** Zheng XM

World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v2.i6.272

World J Clin Oncol 2011 June 10; 2(6): 272-280
ISSN 2218-4333 (online)

REVIEW

Role of endoglin and VEGF family expression in colorectal cancer prognosis and anti-angiogenic therapies

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Received: February 9, 2011 Revised: March 2, 2011
Accepted: April 5, 2011
Published online: June 10, 2011

Abstract

Colorectal cancer (CRC) is one of the cancer models and most of the carcinogenic steps are presently well understood. Therefore, successful preventive measures are currently used in medical practice. However, CRC is still an important public health problem as it is the third most common cancer and the fourth most frequent cause of cancer death worldwide. Nowadays, pathologic stage is a unique and well-recognized prognostic indicator, however, more accurate indicators of the biologic behavior of CRC are expected to improve the specificity of medical treatment. Angiogenesis plays an important role in the growth and progression of cancer but its role as a prognostic factor is still controversial. Probably the most important clinical implication of tumor angiogenesis is the development of anti-angiogenic therapy. The goal of this review is to critically evaluate the role of angiogenic markers, assessed by either endoglin-related microvessel density or expression of vascular endothelial growth factor family members in the CRC setting and discuss the role of these angiogenic markers in anti-angiogenic therapies.

Key words: Angiogenesis; Colorectal cancer; Colorectal cancer treatment; Endoglin; Prognosis; Vascular endothelial growth factor

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Martins SF, Reis RM, Mesquita Rodrigues A, Baltazar F, Longatto Filho A. Role of endoglin and VEGF family expression in colorectal cancer prognosis and anti-angiogenic therapies. *World J Clin Oncol* 2011; 2(6): 272-280 Available from: URL: http://www.wjgnet.com/2218-4333/full/v2/i6/272.htm DOI: http://dx.doi.org/10.5306/wjco.v2.i6.272

COLORECTAL CANCER EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer death worldwide[1-3]. Globally, CRC incidence varies widely, with higher rates in North America, Australia and Western Europe and lower rates in developing countries [4], although, in recent years, high CRC rates have also been reported in these countries[5]. In terms of mortality, geographic disparities have also been observed[6]. In Western countries, CRC is

the second most common cause of death from malignant disease, and despite improvements in treatment mortality remains high with metastatic spread to the liver occurring in about 50% of patients[7].

European countries rank highest in the global statistics, both in terms of CRC incidence and mortality. From 1998 to 2002, the incidence of CRC in Europe for men and women was 38.5 and 24.6 (world age standardization (ASR-W)) per 100 000 inhabitants and mortality over the same period was 18.5 and 10.7 (ASR-W) per 100 000 inhabitants, respectively[8]. However, over the past twenty-five years, mortality rates among Caucasians have steadily declined[9]. Data from the World Health Organization (WHO), between 1997 and 2007 have revealed that mortality from CRC declined by around 2% per year from 19.7 to 17.4/100 000 for men (world standardized rates), and from 12.5 to 10.5/100 000 for women, and these recent decreases in CRC mortality rates in several European countries are likely due to improvement in earlier diagnosis and treatment, with a consequent higher survival[10].

CRC incidence is generally higher in men, and the risk increases with age, as the majority of cases are diagnosed in patients older than 50 years[1, 3, 8], with only 5% of cases recorded in patients younger than 40 years[1]. A large nationwide study identified CRC as one of the 10 most commonly diagnosed cancers among men and women aged 20-49 years[11]. The prevalence of advanced CRC also increases with age and is higher among men than women[12].

COLORECTAL CANCER PROGNOSIS AND DISEASE PROGRESSION

The main prognostic factors in CRC are tumor size (T), lymph node involvement (N), grade of differentiation (G) and distant disease spread (M)[1-3,9,13,14]. Other important factors include invasion of blood and/or lymphatic vessels and penetration or perforation of the bowel wall[14].

Long-term survival correlates with stage of the disease[9, 15-17], and this is the most important predictor of mortality. The five-year survival rate for localized disease is 90.4%, but only 39% of CRC is diagnosed at this early stage[9, 16]. Approximately 15-20% of patients die as a consequence of CRC in early stages compared with 40-80% in advanced stages[15]. The overall 5-year survival rate varies among studies but is approximately 60%[9, 15, 16]. Stage-specific survival rates are 96%, 87%, 55%, and 5% for TNM stage Ⅰ, Ⅱ, Ⅲ, and Ⅳ, respectively[9,17,18].

One third of the patients submitted to curative intent surgery die of local and/or distant tumoral recurrence [15]. Among the sites of metastasis, liver is the organ most frequently involved (38%-60% of cases), followed by abdominal lymph nodes (38%), lung (38%) and peritoneum (28%)[14]. Of those diagnosed with metastatic disease, less than 10% are still alive after 5 years[16]. The 5-year overall survival rates for patients in whom hepatic resection was technically feasible and who had metastasis confined to the liver was only 25%-40%[7,19,20]. Better results were reported by Abdalla *et al* and Choti *et al*, with a 5-year overall survival rate of 58% following resection[21] and a rate of 67% described by de Haas *et al*[22]. These higher survival rates likely reflect improvements in patient selection, perioperative and postoperative care, multidisciplinary treatment, and an appropriately aggressive approach to safe hepatic resection[21]. Therefore, early diagnosis is critical to improve survival rates in CRC[23] and owing to its typically slow growth, there is a large potential for reducing the burden of the disease by early detection and removal of precancerous lesions or early cancer stages[24].

On the other hand, the pathologic clinical stage is currently the single most well-established prognostic indicator, but it does not fully predict individual clinical outcome[7, 25, 26]; also, the response of clinically-identical tumors to the same treatment may be vastly different[1]. This is particularly contentious for those tumors with intermediate stage disease (Stage Ⅱ, T3-T4N0M0)[7], where one third of patients with tumor-free lymph nodes have recurrences, and therefore, adjuvant chemotherapy may be beneficial[27]. In this group, carcinoma cells are not detected in lymph nodes by conventional staging methods in 24% of patients. Surgical technique and specific pathological staining may improve staging accuracy and the appropriate selection of patients for chemotherapy[27]. Furthermore, the identification of cancer penetration or perforation is particularly important in defining CRC aggressiveness[14]. Accordingly, identification of prognostic molecular markers capable of categorizing those patients at high-risk, would be very helpful for improving treatment strategies mainly in lymph node negative patients, determining the characteristics of patients' outcome, predicting cancer dissemination and recognizing which patients might benefit most from adjuvant chemotherapy and those unlikely to benefit thus sparing them the toxicities of treatment[14, 27-29].

Molecular markers may improve clinicopathologic staging and provide a basis to guide novel therapeutic strategies which target specific tumor-associated molecules according to individual tumor biology[1, 2, 7, 14], however, so far, no ideal molecular marker has been found to predict disease progression[29].

HIGHLIGHTS OF THE ANGIOGENESIS PHENOMENON

Angiogenesis plays a key role in tumorigenesis and metastatic processes[1, 28, 30]. It consists of the formation of new blood vessels from the endothelium of pre-existing vasculature[2, 30]. Sprouting from existing blood vessels is the principal process of angiogenesis and involves proliferation of activated endothelial cells, migration of endothelial cells to reach remote targets, assembly of endothelial cells into new capillary tubes, followed by synthesis of a new basement membrane and maturation of vessels with formation of a vascular lumen[30]. However, recruitment and *in situ* differentiation of bone marrow-derived endothelial

progenitor cells are also involved[30].

Tumor angiogenesis is essential to allow neoplastic mass development favoring access to the blood components, and also strengthening the vascular routes in the metastatic process[25, 31-33]. Neovascularization as a whole promotes tumor growth by supplying nutrients, oxygen and releasing growth factors that promote tumor cell proliferation[25, 30, 34-36]. Hypoxia in solid tumors occurs at a distance of ≥ 70 μm from functional blood vessels and it is generally accepted that tumors do not exceed a volume of 1-2 mm^3 without induction of angiogenesis[36]. Intratumoral vasculature density is believed to be associated directly with cancer cell entrance into the systemic blood circulation, with the ability of cancer cells to invade locally normal anatomic structures, and the establishment of blood-borne metastases in distant organs[32, 37]. Regulation of tumor angiogenesis is the result of a complex balance between many stimulatory and inhibitory factors, which are secreted by both tumor cells and host-infiltrating cells as well as by tumoral stroma-cells activity[2, 30, 34]. Malignant neoplastic cells promote angiogenesis by secreting growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF), among others that stimulate endothelial migration and proliferation[2,25,31,33,37,38].

The role of angiogenesis as a prognostic factor, however, is still controversial[13, 39]. Weidner *et al* first reported a direct correlation between the incidence of metastasis and the number and density of blood vessels in invasive breast cancers. Similar studies have endorsed this correlation in gastrointestinal cancers[33] and in a variety of malignancies[2, 7, 13, 25, 35, 37]. An association between increased angiogenesis and an increased incidence of metastases and a subsequent decrease in survival curve rates was observed for the vast majority of solid tumors[2, 7, 3, 25, 35, 37].

Several studies revealed high angiogenic activity in CRC, which was more likely correlated with aggressive histopathological features that included parietal invasion, tumor stage, grade of tumor differentiation, metastatic potential and poor patient survival[1, 13, 32]. Tanigawa[35] *et al* confirmed this premise, although a significant variation in patient populations and techniques was used, which can explain, in part, the inverse relationship between tumor vascularity and patient survival observed by these authors. Gurzu[13] *et al* added that augmented angiogenesis in CRC was higher in early-stages of tumoral proliferation but was not a progressively increasing process, having rather an oscillating character.

However, other studies revealed that angiogenesis does not provide any significant information[13, 28, 30]. These controversial statements may be credited to the lack of standardization of the different methods of counting tumoral blood vessels and to the different cut-offs used to define relevant parameters to consolidate the results and, lastly, to the different antibodies used to highlight the blood vasculature[13, 28, 30].

Despite the debates, assessment of tumor angiogenesis may be particularly useful in prognostic classification of patients with apparent early cancer by conventional tumor staging, some of which may still develop early recurrence or metastasis (despite being staged as having early cancers by conventional parameters such as tumor size)[30].

De Vita[37] *et al* observed that highly angiogenic tumors were associated with the presence of lymph node invasion . Nevertheless, a higher percentage of patients with node-positive colon cancer than those without will experience recurrence and might benefit from anti-angiogenic adjuvant therapy. Thus, angiogenesis can be used to identify a subset of patients at high risk for recurrence regardless of their lymph node involvement[35].

There is evidence that blood vessel density is also important in predicting cancer response to chemotherapy or radiotherapy[20]. Angiogenic tumors have a more aggressive phenotype and the degree of intra-tumoral microvessels is significantly predictive of poor response to platinum-based chemotherapy in terms of complete response, as seen in two studies, one in squamous cell carcinoma patients[40] and the other in patients with epithelial ovarian cancers[41]. In addition, Takagi[42] *et al* observed that blood vessel density was a valid predictor of the effects of intra-arterial targeted carboplatin chemotherapy and concurrent radiotherapy for treating human oral and oropharyngeal squamous cell carcinomas. Zhang[43] *et al*, trying to identify reliable predictive factors for local control of hypopharyngeal cancer (HPC) treated by radiotherapy, observed that microvessel density (MVD) in biopsy specimens was closely correlated with local control of HPC treated by radiotherapy. In one study of 28 patients with advanced gastric cancer treated by paclitaxel and carboplatin, tumors with medium MVD showed a significantly higher response rate compared with those with either a high or low MVD[44]. Long course of radiotherapy significantly decreased angiogenesis in rectal cancer tissue. MVD have been found to be a favorable marker for tumor behavior during radiotherapy and a predictor of overall survival after a long course of radiotherapy. Further investigations are now needed to determine the changes in angiogenesis during a shorter course of radiotherapy[1]. However, the most important clinical implication of tumor angiogenesis is probably the development of anti-angiogenic therapy, targeting tumor vessels instead of cancer cells[30].

ENDOGLIN AND ASSESSMENT OF MICROVESSEL DENSITY AS ANGIOGENIC MARKERS

Microvessel density (MVD) assessment is the most common technique used to quantify intratumoral and peritumoral angiogenesis in cancer[2, 7, 28, 30, 39]. It was first developed by Weidner *et al* in 1991 who used pan-endothelial immunohistochemical staining of blood microvessels, mainly with Factor VIII related antigen (F. VIII Ag or von Willebrand's factor), CD31 or CD34, and rarely CD105[2].

Measurement of angiogenesis is complicated by the

fact that it is a dynamic process. Intra-tumoral microvessels can be identified by immunostaining of endothelial cells by two categories of human endothelial cell-specific antibodies: the pan-endothelial cell markers and specific antibodies that bind selectively to proliferating endothelium[44, 45]. CD31 is utilized as the pan-endothelial marker of choice; it is characterized by equal intensity of staining for small and large vessels. The disadvantages associated with staining for CD31 antigen include co-staining of inflammatory cells. The selective antibodies, such as endoglin, distinguish quantitatively between tumor neovascularization and pre-existing vessels with no or poor staining of lymphatics and normal quiescent blood vessels[46]. Most studies revealed that high MVD predicts occurrence of metastatic disease[2, 7, 13, 25, 32, 35, 37], and although tumor angiogenesis is unlikely to be the only factor responsible, it provides large numbers of leaking blood vessels for vascular invasion[25].

Endoglin (CD105) is a receptor for the TGF-β1 molecule that is up-regulated in tumor angiogenesis [13, 25, 29]. Its secretion is induced by hypoxia[29] and, as it is present mainly in new vessels, it is very useful in the assessment of newly formed vessels in malignant neoplasms[13, 25, 29]. It is also currently accepted as a potential target for anti-angiogenic therapy, especially in cancer patients at risk of developing metastases[29]. The endoglin antibody binds preferentially to the activated endothelial cells that participate in tumor angiogenesis, however, endoglin expression is weak/or negative in vascular endothelium of normal tissues; accordingly, it is a more specific and sensitive marker of tumor angiogenesis than the others commonly used such as pan-endothelial markers[25, 29]. Intra-tumoral MVD determined by immunohistochemical staining for endoglin has been reported to be an indicator of poor prognosis in many types of solid neoplasia such as breast carcinoma, cervical cancer, endometrial carcinoma, gastric carcinoma, melanoma, some testicular tumors, non-small cell lung cancer, prostate cancer, renal cell carcinoma and squamous cell carcinoma[29].

In CRC, many reports indicate that endoglin assessed immunohistochemically correlates not only with MVD, but also with survival curves, and it has also been identified as a valuable parameter for predicting increased risk of developing metastatic disease[25, 29,42]. Yan[47] *et al* reported that MVD was higher in CRC patients with metastases than in those without and observed that the specificity and sensitivity of MVD in predicting metastatization in CRC was 66.22% and 51.72%, respectively. In other studies, the presence of endoglin also had a prognostic meaning, showing a positive correlation with the presence of angio-lymphatic invasion, lymph node metastases, tumor stage and hepatic metastases, reinforcing the premise that endoglin might be considered for further therapeutic trials as anti-angiogenic therapy[25, 29].

Endoglin is not only expressed on the cell surface but its soluble form can also be detected in the blood[29, 48]. Myśliwiec[29] *et al* demonstrated an apparent continuous endoglin rise in plasma from patients with metastatic colorectal cancer, and Li[48] *et al* reported that circulating endoglin levels positively correlated with CRC Dukes' stage and survival; patients with a high MVD, above the median 3.10 × 250, showed the worst prognosis. Takahashi[49] *et al* observed that increased serum endoglin was associated with metastasis in patients with solid tumors including colorectal and breast carcinomas; and, in CRC patients, the difference in endoglin levels between the metastasis-negative patients and the metastasis-positive patients was statistically significant. Conversely, it was recently demonstrated that assessment of endoglin in plasma is not a useful maker of CRC, but might be helpful in selecting patients with metastatic diseas[29].

VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY AND CRC

Quantification of angiogenic factors in solid malignant tumors provides an alternative to MVD evaluation in assessing tumor angiogenic activity[28, 30]. Numerous studies have demonstrated that tumor overexpression of vascular endothelial growth factor (VEGF) correlates with high tumor MVD and is associated with advanced tumor stage or tumor invasiveness in various common human cancers[30, 37, 50, 51] and, its overexpression in colon cancer tissue indicates poor prognosis[51]; although paradoxically, some data showed that MVD might have a significant prognostic value in colon cancer tissue, whilst VEGF has not[52].

VEGF is the most widely studied angiogenic factor; it increases vascular permeability and is the most potent, direct acting, angiogenic protein known[28, 29, 36, 37, 52]. Normally, VEGF is weakly expressed in a wide variety of human and animal tissues; however, high levels of VEGF expression can be detected at sites where physiologic angiogenesis is required, such as fetal tissue or placenta, or in the vast majority of human tumors and other diseases i.e., chronic inflammatory disorders, diabetes mellitus, and ischemic heart disease[37]. Furthermore, both VEGF and its receptors are expressed at high levels in metastatic human colon carcinomas and in tumor-associated endothelial cells, respectively[37]. Consequently, VEGF is recognized as a prominent angiogenic factor in colon carcinoma and the assessment of VEGF expression may be useful for predicting metastasis from CRC[37]. In fact, VEGF expression was found to be higher in patients with metastatic tumors than in those with non-metastatic tumors[37,38], and high levels of VEGF expression were associated with advanced cancer stage and related with unfavorable prognosis[51-53].

De Vita *et al* [37] reported that preoperative serum VEGF levels might be useful for predicting the outcome of colon cancer patients following surgery. After surgery, VEGF levels tend to decrease compared with preoperative concentrations[30, 37]. Conversely, elevated VEGF levels after surgery may indicate significant residual disease, even

if it is not evident macroscopically[37].

Other studies have shown that VEGF is also a useful marker for prognosis by significantly correlating with angio-lymphatic invasion, lymph node status and depth of invasion, notwithstanding it was not an independent prognostic factor[25, 29].

Although numerous publications dealing with the measurement of circulating VEGF for diagnostic and therapeutic monitoring have been published, the relationship between the production of tissue VEGF and its concentration in blood is still unclear[31]. Some of the controversies regarding the clinical value of VEGF serum level measurement are related to the well-known fact that circulating VEGF is largely found in platelets, and as a consequence an open debate is ongoing to clarify if VEGF serum levels truly reflect tumor expression of VEGF or whether there are other potential sources of circulating VEGF, such as blood cells[30]. Cressey[31] *et al* noted that the cell-associated isoform (VEGF189), but not the soluble isoforms (VEGF121 and VEGF165) appear to play an important role in tumor progression. In addition, Serum VEGF protein levels are a prognostic parameter for progression-free and overall survival in CRC. Patients with high soluble VEGF levels might have a more aggressive disease, and the improved outcome observed in their series might be a reflection of the disease biology[54,55].

The effect of VEGF depends not only on tumor cell expression of VEGF, but also on the VEGF receptors in the endothelial cells[30]. The ligands of the VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E; and the receptors are VEGFR-1, R-2 and R-3[56].

VEGF-A is commonly overexpressed by a wide variety of human tumors, and this overexpression has been correlated with progression, invasion and metastasis, MVD, and poorer survival and prognosis[56]. In CRC, VEGF-A is the ligand of the VEGF family most abundantly expressed[29]. VEGF-A promotes angiogenesis through enhancement of permeability, activation, survival, migration, invasion, and proliferation of endothelial cells[57]. VEGF-A and VEGF-B play a role in early tumor development at the stage of adenoma formation[7, 58].

Myśliwiec[29] *et al* found a strong positive association with VEGF-A plasma concentrations assessed postoperatively and the presence of distant metastases. Zlobec[59] *et al* also correlated high VEGF expression with response to preoperative radiotherapy in patients with rectal tumors.

VEGF-C and -D are glycoproteins structurally similar and sharing areas of sequence homology with VEGF-A. In CRC, augmented VEGF-C expression has been found to correlate with lymphatic invasion and lymph node metastasis[60]. Elevated levels of serum VEGF-C have been found in patients with breast cancer, lung cancer and cervical cancer and it appears to be an independent marker for early diagnosis of cancer metastasis. Moreover, increased VEGF-C mRNA expression in tumor tissues correlates positively with lymphatic metastasis and poor prognosis[61]. A correlation between VEGF-D expression levels in the primary tumor and lymph node metastasis is still disputable, with controversial data reported[62].

Another important fact is that through the development of anti-angiogenic therapy, CRC prognosis is improving[30, 63-65]. Median survival of patients with metastatic CRC (mCRC) treated with best supportive care is approximately 6 mo. Palliative chemotherapy considerably improves treatment outcome, with fluorouracil (FU) plus irinotecan and/or oxaliplatin extending median overall survival to approximately 20 mo[66]. Thus, in the past decade, the median overall survival of patients with mCRC has increased from 12 mo to approximately 20 mo, mainly due to the development of new combinations with standard chemotherapy[67]. Currently, anti-angiogenic treatment can prolong the survival time by some months, however, the results are not reproducible for all cases[13]. There have been clinical trials which show as many as 94% of invasive carcinomas and 88% of *in situ* carcinomas having a complete response[68]. Unfortunately, there are no tumor characteristics or molecular markers at present that help to identify patients who are likely to benefit from anti-angiogenic treatment[69].

Bevacizumab (BV) is a monoclonal antibody against VEGF with anti-angiogenic properties, and several clinical trials supported the use of BV in the first-line treatment of mCRC[70]. BV is typically used in combination with other chemotherapeutic agents such as oxaliplatin, irinotecan, leucovorin, and 5-fluorouracil (5-FU) for treatment of patients with mCRC[70, 71]. In addition to its direct anti-angiogenic effects, BV may also improve the delivery of chemotherapy by changing tumor vasculature and decreasing the elevated interstitial pressure in tumors[69]. When combined with standard chemotherapy regimens, it has been associated with significant improvements, compared with chemotherapy alone, in the efficacy end points of overall survival, progression-free survival, and response rates in patients with mCRC and for some facilitates secondary resections[72]. Jubb[73] *et al* demonstrated that in patients with mCRC, the addition of BV to irinotecan, 5-FU/leucovorin (IFL) improves survival regardless of the level of VEGF expression, or MVD. In a review by Tappenden[74]*et al*, the addition of BV to IFL resulted in a statistically significant increase in median overall survival (OS) of 4.7 mo, and in a median progression-free survival (PFS) of 4.4 mo. An overall tumor response rate of 44.8% was reported for BV plus IFL compared with 34.8% for IFL plus placebo within one study. In a pivotal, placebo-controlled, phase III trial in patients with mCRC (Genentech Study 2107), the addition of BV to IFL resulted in a significantly longer survival time (20.3 *vs* 15.6 mo) and progression-free survival time (10.6 *vs* 6.2 mo) than with IFL plus placebo[73, 75-78]. In a placebo-controlled, phase II trial (Genentech Study 2192), adding BV to 5-FU plus LV resulted in a significantly longer progression-free survival time than with 5-FU and LV plus placebo in

Table 1 The main results of CD105 and VEGF studies

Study	*n*	High levels of CD105 were associated with	High levels of VEGF were associated with
Barozzi *et al*[7]	101	M1	M1
Saad *et al*[25]	150	M1, N1 and angiolymphatic invasion	N1, angiolymphatic and depth of invasion
De Vita *et al* [37]	81	NE	NCs (serum levels)
Cascinu *et al*[38]	121	NE	RR
Myśliwiec *et al*[29]	48	M1	Colorectal cancer patients (plasma levels)
Li *et al*[48]	111	Dukes' stages and survival	NE
Takahashi *et al*[49]	34	M1	NE
Liang *et al*[51]	114	NE	N1, TNM staging and poor prognosis
Zheng *et al*[52]	97	NE	Poorly differentiated adenocarcinoma
Cressey *et al* [31]	76	NE	TNM
Cao *et al*[53]	71	NE	N1, M1, TNM, and OS
Miyazaki *et al*[58]	127	NE	RR, DF, OS (plasma levels)

DF: Disease-free; M1: Positive distant metastasis; N1: Positive lymph node metastasis; NCs: Non-curative surgery; NE: Not evaluated; OS: Overall survival; RR: Recurrence rate

patients with mCRC who were unsuitable candidates for first-line therapy with irinotecan (9.2 *vs* 5.5 mo). There was also a trend towards a longer survival time in patients receiving 5-FU, LV, and BV (16.6 *vs* 12.9 mo)[77]. BV was also tested in mCRC combined with an oxaliplatin-based regimen in the second-line setting. In this randomized phase Ⅲ trial (E3200), patients with previously treated CRC were randomized into 3 arms: FOLFOX4 plus BV, FOLFOX4 and BV only. Results showed superior survival and progression-free survival in the FOLFOX4 plus BV arm. In this study, BV was equally effective with the oxaliplatin-based regimen[78].

BV ultimately achieved FDA approval in 2004 as a first-line treatment for mCRC in combination with chemotherapy, based on its statistically and clinically meaningful benefits on progression-free survival and OS and has since garnered additional approval[79]. BV is the most used VEGF inhibitor with clear proof of efficacy in CRC, however, optimal use of this agent at various stages of the disease is still under investigation. Additionally, there are numerous other angiogenic agents targeting VEGF and other pro-angiogenic systems in clinical development[80]. These novel targeted agents inhibit the VEGF pathway by targeting the VEGF ligand, its receptors or by blocking downstream signaling pathway components. Anti-angiogenic agents include antibodies, small molecule tyrosine kinase (TK) inhibitors, antisense oligonucleotides and aptamers[81].

Table 1 summarized the main results of CD105 and VEGF studies.

CONCLUSION

Despite major advances, in terms of knowledge and treatment of CRC in recent years, the single most well-documented prognostic marker of pathologic stage remains the gold standard for disease stage at diagnosis. Angiogenesis plays an important role in the growth and progression of cancer but its role as a prognostic factor is still controversial. Most studies report that endoglin and vascular endothelial growth factor family expression are indicators of poor prognosis in CRC patients. Beyond these controversies, the ultimate clinical implication of tumor angiogenesis is the development of anti-angiogenic therapy, targeting tumor vasculature.

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World J Clin Oncol 2011 June 10; 2(6): I
ISSN 2218-4333 (online)

Acknowledgments to reviewers of *World Journal of Clinical Oncology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Clinical Oncology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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January 15, 2011
Current Trends in Breast Cancer: Updates From the 2010 San Antonio Breast Cancer Symposium, Dallas, TX, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA, United States

January 21-23, 2011
8th Meeting of the EAU Section of Oncological Urology, London, England, United Kingdom

January 27-28, 2011
2nd National Conference: Recent Advances in Renal and Bladder Cancer, London, United Kingdom

January 27-28, 2011
8th Annual Cancer Drugs Research & Development, San Diego, CA, United States

February 10-12, 2011
17th Annual NOCR Meeting, Las Vegas, NV, United States

February 19-22, 2011
Scripps Cancer Center's 31st Annual Conference: Clinical Hematology and Oncology, San Diego, CA, United States

February 24-26, 2011
European Multidisciplinary Conference in Thoracic Oncology (Lung 2011-EMCTO), Lugano, Switzerland

February 25-27, 2011
7th European Congress on Hematologic Malignancies: From Clinical Science to Clinical Practice, Budapest, Hungary

March 02-05, 2011
64th Society of Surgical Oncology Annual Cancer Symposium 2011, San Antonio, TX, United States

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March 07-09, 2011
9th International Symposium on Targeted Anticancer Therapies, Paris, France

March 09-13, 2011
16th National Comprehensive Cancer Network Annual Conference (NCCN 2011), Hollywood, FL, United States

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12th European Congress: Perspectives in Lung Cancer, Torino, Italy

March 14-18, 2011
Oncology Imaging Update in Costa Rica, Guanacaste, Costa Rica

March 17-19, 2011
International Cancer Prevention Update Symposium, New York, United States

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April 29-30, 2011
Cancer Survivorship Conference, Minneapolis, MN, United States

May 23-24, 2011
4th International Conference on Ovarian Cancer Screening, London, United Kingdom

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47th American Society of Clinical Oncology Annual Meeting, Chicago, IL, United States

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7th EADO Congress European Association of Dermato-Oncology, Nantes, France

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ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 23-25, 2011
"MASCC/ISOO 2011 International Symposium, Athens, Greece

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September 1-3, 2011
Tri-Society Head and Neck Oncology, Singapore, Singapore

September 7-10, 2011
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September 23-27, 2011
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October 06-07, 2011
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World J Clin Oncol 2011 June 10; 2(6): I-V
ISSN 2218-4333 (online)

INSTRUCTIONS TO AUTHORS

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

Organization as author

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

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

Volume with supplement

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

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

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

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

Patent (list all authors)

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

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