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Review

Update on Biomarkers in Development of Anti-angiogenic Drugs in Gastric Cancer

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Abstract. *The treatment of advanced gastric cancer remains challenging as the outcomes achieved with surgery alone or adjuvant or neoadjuvant chemotherapy and radiotherapy are poor. New treatment strategies are emerging and being tested in advanced gastric cancer. Vascular endothelial growth factor (VEGF) inhibitors have been confirmed as important therapeutic agents in randomised clinical trials in multiple solid tumour settings. Until now, results of phase II and phase III clinical trials of anti-angiogenic agents on gastric cancer have been relatively modest, with moderate improvement in overall survival. The effects of these drugs are limited due to development of resistance to them and the increased risk of tumour invasion and metastasis. If we are to optimise or develop combination regimens for advanced gastric cancer with VEGF inhibitors that build on their efficacy, it is critical to identify and validate biomarkers in order to enable selection of those patients who are prone to benefit and monitor their response to the drugs. Validated biomarkers can help to further personalise VEGF inhibitors and dosage determination for advanced or metastatic gastric cancer, particularly as these drugs can be toxic and expensive. Although no biomarker is validated for routine use for this purpose, several candidates are currently under investigation. In this review, we aim to give an overview of the recent developments in biomarkers for anti-angiogenic therapy in gastric cancer tumour angiogenesis.*

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Gastric cancer affects more than one million people per year worldwide (1). Despite advancement in early detection, most patients with gastric cancer present with advanced stages and metastasis at the time of diagnosis (2). Currently, surgery removal remains the only therapeutic option for the disease. However, management of advanced gastric cancer is shifting from standard surgical resection, adjuvant and neoadjuvant regimens to combination strategies with targeted therapies, including anti-angiogenic therapies [especially vascular endothelial growth factor (VEGF) inhibitors] (3).

A biomarker can act as a diagnostic indicator that is objectively measured and evaluated for a biological of pathogenic process or of pharmacological response to a therapeutic intervention (2). Targeted therapies theoretically target essential element(s) of signaling pathways known to be involved in tumorigenesis. For targeted therapies, identifiable corresponding biomarkers related to those pathways that help guide treatment are generally required, as general parameters developed for monitoring cytotoxic therapies may not apply. In gastric cancer, well-recognised and validated biomarkers for diagnosis and treatment monitoring include the carcinoembryonic antigen (CEA), and carbohydrate antigens (CA). These markers exhibit defined criteria of disease response and progression based on changes in their serum levels, although the appropriateness of instigating treatment by such biomarker-defined progression alone has recently been challenged (4). In addition, pharmacodynamic biomarkers are used in the development of targeted therapies to indicate target modulation (proof of mechanism) and define the optimal biological dose in early-phase clinical trials.

Based on the simplified assumption of the homogeneity of vasculature, anti-angiogenic therapy targeting tumour vasculature has been designed to apply to most solid tumour types, irrespective of the genetic makeup of each tumour. Since the clinical development of anti-angiogenic agents has

not been biomarker-based, the overall efficacy of current anti-angiogenic drugs has suffered greatly from the practice of indiscriminately applying those medicines to all patients with cancer. The same tumour type responds differently to the same anti-angiogenic therapy. For example, in two clinical trials of bevacizumab (3) and ramucirumab (5), a subset of patients with gastric cancer responded transiently, whereas another group had essentially no response. Bevacizumab (Avastin®) is a monoclonal antibody targeting VEGF, whereas ramucirumab is another human monoclonal antibody selectively binding to the extracellular region of VEGF receptor-2 (VEGFR2). These findings raise the importance of: i) predicting whether a patient will benefit from an anti-angiogenic agent, and monitoring its efficacy or the development of resistance to it; and ii) exclusion of patients who do not response to anti-angiogenic therapy, in order to avoid the toxicities and cost of these drugs; and iii) the development of new anti-angiogenic strategies in which current anti-angiogenic drugs have failed in unselected populations.

At the point in the development of VEGF inhibitors for cancer treatment, the complexity of tumour angiogenesis, with the various intrinsic/extrinsic regulatory and adaptive mechanisms suggest that multiple and different biomarkers are essential for comprehensively examining angiogenesis and its therapeutic response. There is an urgent need to identify related biomarkers (*e.g.* predictive, pharmacological and surrogate response), and in particular, to discriminate them from prognostic biomarkers which inform on the progression of disease irrespective of treatment. The focus of this review is to report on recent advances in these areas, where many hypothesis-driven biomarker candidates have been tested in experimental models and clinical studies, even though to date none has been validated for routine use in patients bearing advanced gastric cancer. We arbitrarily clustered them into two groups: systemic and local (*in situ*) biomarkers for the sake of discussion.

Systemic Biomarkers

Hypertension. The rapid induction of hypertension in a significant minority of patients appears to be a classic adverse effect of VEGF inhibitors. The results obtained from the RAINBOW trial, which began in 2010 and involved 665 patients with advanced gastric cancer, showed hypertension occurring at a higher rate in patients treated with ramucirumab, a human antibody targeting VEGFR2, in combination with paclitaxel regimen (5). The level of hypertension is dose-dependent, although the exact mechanisms behind it might be multifactorial and remain to be elucidated. One plausible explanation is that VEGF signaling regulates nitric oxide synthesis (6). Inhibition of VEGF signaling causes a reduction in the synthesis of nitric oxide, increasing vasoconstriction and therefore hypertension. Nevertheless, it provides the rationale for using a change in blood pressure as a surrogate system biomarker to evaluate successful inhibition of VEGF signalling. For instance, it may be practically appropriate to increase the dose of VEGF inhibitors, if tolerated, until hypertension is observed. The report of a multicentre phase III trial including 355 patients bearing advanced gastric or gastro-oesophageal junction adenocarcinoma seems to corroborate this hypothesis (7). It was the first to report that a single VEGF inhibitor can exert survival benefit in patients with advanced gastric cancer. The rates of hypertension were higher in the ramucirumab-treated group than the placebo-treated group, whereas the other adverse effects occurred in both groups at similar rates (7).

Circulating biomarkers. Circulating biomarkers of VEGF signalling: Circulating molecules associated with angiogenesis are the most accessible potential biomarkers. Blood sampling is convenient and less invasive than tissue sampling, allowing multiple samples to be taken. Techniques of isolation are quantitative, simple and relatively inexpensive, while concentration measurement is quantitative. These markers consist of circulating angiogenic factors, cell surface receptors, adhesion molecules, circulating proteins or peptides, downstream signalling molecules and even cells (8, 9).

Serum VEGF levels are thought to reflect the activity of VEGF-mediated tumour angiogenesis and an increasing circulating VEGF level has been shown to be an adverse prognostic factor in gastric cancer (10). The majority of clinical trials that have evaluated VEGF inhibitors in cancer treatment have involved investigation of circulating VEGFA (plasma or serum), although urine measurement has been studied (11, 12), even though the effects of VEGF inhibitors on gastric cancer are controversial and inconclusive. For example, one study demonstrated that VEGF inhibition using bevacizumab combined with chemotherapy might be a promising option for patients with metastatic or unresectable gastro-oesophageal junction adenocarcinoma (13), whereas a randomised phase III study of bevacizumab plus capecitabine failed to provide evidence of the efficacy of bevacizumab in gastric cancer (3).

Correlation between circulating VEGFA levels and response to VEGF inhibitor treatment has also been examined in patients with gastric cancer. However, the results were complex and inconclusive. Of note, in patients with extensive-stage gastric cancer patients from non-Asian regions who received bevacizumab in addition to chemotherapy agents, baseline levels of VEGFA and neuropilin-1 (NRP1) predicted bevacizumab effect in terms of overall survival. Patients who had high baseline levels of both VEGFA and NRP1 had a higher risk of progression to death compared to those who had a low baseline of these (14). Thus, a hypothesis would be that the change in serum

VEGF levels induced by VEGF inhibition might predict patient benefit. However, patients with Asian origin tended to have a low baseline of serum VEGFA and those with high VEGFA levels did not respond to the VEGF inhibitor. However, additional similar trials with more patients might help us clarify the apparent race-based discrepancy. One possible reason is that the high serum VEGF level in the patients from the two populations come from different sources. One study conducted using analysis of VEGFA expression in Japanese patients with advanced gastric cancer (both *in situ* lesions and peritoneal metastases) showed that a significant fall in serum VEGFA level was only observed in patients with peritoneal metastases treated regionally (peritoneally) with bevacizumab (15). That study indicated that malignant and metastatic peritoneal ascites may be the primary source of VEGFA for gastric cancer, and the antibody neutralizing VEGFA reduced its entry into circulation, resulting in a low blood concentration, thereby enhancing the efficacy of systemically administering VEGF inhibitors by removing ascites before the treatment.

In addition to VEGFA, other members of the VEGF family exhibit differential effects on gastric cancer vasculature. Patients with high expression of both VEGFC have a worse prognosis, with higher microvessel density (MVD), local invasion and metastasis (16), while VEGFB does not seem to be involved in the progression of gastro-oesophageal cancer (17), and VEGFD is mainly responsible for lymphogenesis in gastric cancer (18). Most consistently, placenta growth factor (PlGF) expression levels are significantly higher in gastric cancer (19). Intriguingly, PlGF levels are increased in patients with different types of cancer treated with VEGF inhibitors, irrespective of inhibitory mechanisms (20). This finding led to the hypothesis of a critical role of PlGF in tumour vasculature rescue and the development of resistance to VEGF inhibitors, that need to be further examined. Of interest, changes in PlGF levels may serve as pharmacodynamic systemic biomarkers for VEGF inhibition in gastric cancer treatment.

Various alternative strategies inhibiting VEGF signaling include targeting VEGFRs. Several small-molecule inhibitors have been investigated in combination with chemotherapy agents in patients with advanced gastric cancer. For instance, sunitinib and sorafenib are designed to target the binding of ATP to the tyrosine domain of VEGFRs in a reversible manner. Unfortunately, both drugs exert off-target effects due to lack of specificity. Recently a specific VEGFR2 tyrosine kinase inhibitor, namely apatinib, replaced two aforementioned drugs in clinical trials for gastric cancer. Ramucirumab is an example of alternative method of anti-VEGFR strategy; it is a fully humanized monoclonal antibody targeting the VEGF-binding domain of VEGFR2. Cediranib is a highly potent inhibitor targeting both VEGFR2 and VEGFR1. However, none of these receptor tyrosine kinases have yet been evaluated as molecular biomarkers of response in gastric cancer, at least they have not yet been formally reported.

Apart from the full-length membrane receptors, soluble VEGFRs (sVEGFRs) are presented in serum as the result of alternative splicing or membrane shedding (21). sVEGFR1 has a high affinity for VEGFA, leading to development of sVEGFR1 in patients with gastric cancer as endogenous VEGFA inhibitor (22). The circulating sVEGFR1 level is being explored as a predictive biomarker of response to VEGFA inhibitors in cancer, including gastric cancer (14). One hypothesis is that patients with cancer with pre-existing high levels of sVEGFR1 are prone to development of resistance to bevacizumab. sVEGFR2 is abundant in blood circulation. A clinical study showed that foretininb, an oral VEGFR2 inhibitor, induced a significant decrease in serum sVEGFR2 in patients with metastatic gastric cancer (23). The presence of sVEGFR2 has been associated with improved outcome in other solid types of tumour, but its value as a predictive or pharmacodynamic biomarker for VEGF inhibition in patients with gastric cancer is currently unknown.

To date, a circulating biomarker signature for anti-VEGF treatment of solid types of cancer has been recognized to include an increase in serum VEGFA and PlGF, and a reduction in soluble VEGFRs. We propose that there is a necessity for expansion in the evaluation of this biomarker signature to gastric cancer clinical trials involving VEGFR inhibitors since this biomarker signature represents the largest repertoire of VEGF signalling targeted by VEGFR inhibitors.

Circulating cells: Circulating endothelial cells (CECs) are believed to be derived from the turnover of blood vessel walls, either of the mature vessel ortumour vasculature. A sub-population of CECs originating from the bone marrow that have a progenitor-like phenotype are called circulating endothelial progenitor cells and contribute to vascularization of late-stage cancer. Healthy adults have 1-20 CECs per millilitre of their peripheral blood and the changes in the number of CECs significantly increases in patients with advanced gastric cancer (24). After successful chemotherapy, the number regresses to normal levels in patients with advanced gastric cancer and is a predictive biomarker (25). However, results of studies evaluating changes in CECs and circulating endothelial progenitor cells in patients with cancer receiving VEGF inhibitors are controversial and inconclusive due to a significant diversity of techniques found in their isolation, as well as the lack of a clear consensus on antigen profiling. In general, the CEC number tends to decrease upon treatment with VEGF inhibitors in patients with cancer (26, 27). Interestingly, one study showed that in patients with gastrointestinal stromal tumours treated with sunitinib, a transient increase in CECs was associated with a better outcome (28).

Other notable circulating cells are circulating tumour cells (CTCs) that are derived from metastatic cancer cells or even cancerous stem cells. Efforts have been made to detect, isolate and enumerate CTCs. Mesenchymal epithelial transition factor for cancer (c-MET) is a receptor tyrosine kinase encoded by the MET oncogene and identified as a prognostic marker in gastric cancer (29). Interestingly, *c-MET* has been used to capture and identify circulating gastric cancer cells (30). Higher levels of c-MET positive circulating gastric cancer cells were only detected in patients with gastric cancer. We speculate here that this approach may be useful in follow-up of patients who are under anti-VEGF therapy.

Other circulating biomarkers. Recently, microRNAs (miRNAs), possessing excellent stability in the bloodstream, have been detected in the serum or plasma from patients with a variety of solid tumour types. In gastric cancer, some miRNAs, including miRNA-378, -451, 486, -17-5p, -21, 106a, -106b, 199a-3p and -200c displayed higher serum expression (31-33). Among them, high serum levels of miRNA-199a-3p and -200c were significantly associated with gastric cancer and declined after gastrectomy (34, 35).

Energy metabolism changes are one of the cancerous hallmarks (36). Energy metabolism reprogramming results in increased glycolytic activity and reduced mitochondrial respiration, even in the presence of oxygen, a phenomenon called the Warburg effect (37). As a result, change in circulating mitochondrial DNA has emerged as a potential biomarker for gastric cancer (38).

Circulating cell-free DNA (cfDNA) is released into blood circulation during tumour metastasis, necrosis, and apoptosis (39). A decrease in cfDNA has been associated with reduced tumour size on treatment. A clinical study demonstrated the possibility of usage of cfDNA as a sensitive predictive biomarker to assess tumour burden and residual tumour after treatment (40). The authors found that changes in the level of cfDNA are significantly correlated with gastric cancer development.

Annexin A2 (ANXA2) has been found to be involved in tumour growth, invasion, angiogenesis and metastasis in autocrine and paracrine manners in patients with gastric cancer (41). ANXA2 belongs to annexin family of calcium and phospholipid-binding proteins that regulate a range of molecular and cellular activities during carcinogenesis. Clinical investigation revealed that an elevating serum level of ANXA2 in patients with gastric cancer was significantly associated with the development of chemoresistance (42).

Unfortunately, these circulating biomarkers have not been incorporated into the clinical evaluation of VEGF inhibitors in patients with gastric cancer. However, it is now important that their potential as biomarkers for anti-VEGF therapy for gastric cancer treatment is researched.

Genetic biomarkers. Genetic studies have suggested that inherited variations in genes related to angiogenesis, at least partially, cause the documented mixed response to VEGFA inhibitors. Few studies have reported an association between clinical outcome of gastric cancer and single-nucleotide polymorphisms (SNPs) in genes for VEGFA. When patients with advanced gastric cancer were treated with oxaliplatin, 5-fluorouracil (5-FU), and leucovorin (FOLFOX), SNP analysis demonstrated that *VEGFA* -634G/G gene polymorphism was related to higher serum VEGFA levels (43). Unfortunately, no study has reported that patients with VEGFA -634G/G genotype attained a better response rate and progression-free survival (PFS) regarding VEGF inhibitors. Interestingly, patients bearing advanced colorectal cancer with *VEGF* -A634G/C genotype had a significant response rate to the regimen of 5-FU, irinotecan and bevacizumab (44). Moreover, patients with recurrent ovarian cancer with *VEGFA* +937T polymorphism C/T genotype had a longer PFS when treated chemotherapy with bevacizumab (45), while *VEGFA* 2578AA genotype was significantly associated with the response rate in patients with advanced breast cancer, with a superior median overall survival after receiving paclitaxel combined with bevacizumab (46).

More recently, the effects of multiple genetic variants on the efficacy of bevacizumab were evaluated by a meta-analysis of six randomized phase III trials in colorectal, pancreatic, lung, renal, breast, and gastric cancer (9). The study identified that three genetic variants in *VEGFC*, endothelial PAS domain-containing protein 1 (*EPAS1*) and interleukin 8 receptor α (*IL8RA*) were predictive of bevacizumab treatment outcome. These variants were significantly associated with PFS in bevacizumab-treated patients ($p<0.05$). Interestingly, one variant in VEGFA had predictive value in bevacizumab-treated patients but failed to reach significance in the interaction analysis ($p=0.091$). Furthermore, the VEGFC variant does not affect VEGFC expression and its predictive role in bevacizumab treatment is not yet clear. In contrast, *EPAS1* (hypoxia-induced factor-1 α) is a well-established regulator of angiogenesis as an oxygen-sensitive transcription factor in response to hypoxic stress conditions (47). The *IL8RA* variant has been reported to regulate angiogenesis independently of VEGFA signalling by altering chemokine (C-X-C motif) receptor 1 (CXCR1) expression. In addition, the altered expression of CXCR1 was shown to activate a particular immune response, which could exert a synergistic effect on outcome of bevacizumab treatment (48). In terms of a biomarker for anti-VEGF therapy in patients with gastric cancer, these genetic variants should be explored in further prospective gastric cancer trials, and it will be important to define the structure–functional relationships for particular variants associated with best or worst outcome.

Local (*in situ*) Biomarkers

Tissue-based biomarkers. As emerged from the AVAGAST trial, intratumoral levels of VEGFA in patients have so far not been shown to predict a value for bevacizumab treatment in patients with gastric cancer (14), although its positive impact on overall survival has been reported (49). For most types of solid tumours, the only way to make a definitive diagnosis is to examine a tumour specimen, whenever available, for example, when biopsies can be performed or when tissues are obtained at surgery. However, there are limitations to identifying tissue-based biomarkers related to the effects of anti-VEGF therapy because of invasiveness and the difficulty in standardizing immunohistochemical analysis. The conclusion from AVAGAST, a recent phase III trial of bevacizumab in combination with chemotherapy in advanced gastric cancer, are exemplary of such limitations (14). For instance, different sample methods such as slides or blocks confounded the interpretation, leading to inconsistent results. Loss of immunoreactivity more often happens to paraffin-embedded tumour tissue stored on slides rather than blocks. Moreover, the main pitfalls of applying immunohisto-chemistry as a quantitative analysis without the consensus of standardized tests include pre-analytic tissue processing and subjective scoring. Given the disappointing results so far, and considering the limitations of tissue biomarker evaluation, tissue-based VEGFA does not appear to be a promising biomarker unless the above limitations are improved considerably.

Tumour MVD has often been evaluated as a biomarker relating to blood vessel formation within tumours and was first reported in breast cancer more than two decades ago (50). Increased MVD has been associated with poor prognosis in gastric cancer (51), although there are some conflicting reports (52). In a study of VEGF inhibition in a gastric cancer model, decreased tumour growth was associated with a reduction in MVD on serial tumour biopsies from gastric cancer xenografts in animals (53). The translational component from this pre-clinical study was that a decrease in high basal MVD was significantly associated with the response to bevacizumab in the bevacizumab-sensitive models, whereas no change was detected in the bevacizumab-insensitive models at any time point after treatment. Most interestingly, the decrease of MVD in response to the antitumour activity of bevacizumab was detected before the reduction of tumour volume. Unfortunately, no correlation between the basal MVD and the response to bevacizumab was identified in the landmark phase III trial of bevacizumab in addition to capecitabine/5-FU in advanced gastric cancer (14).

Imaging biomarkers. Anti-VEGF agents are primarily cytostatic and functional imaging techniques can measure changes in tumour vasculature or metabolic process associated with angiogenesis. Endoscopic imaging can monitor the pre-neoplastic, pre-malignant, and cancerous stages in gastrointestinal lesions. However, conventional optical white-light endoscopy (WLE) appears to be of limited use in evaluating gastrointestinal vasculature. A major challenge in the screening of gastric tumour vasculature is the large surface area of the gastric lumen and the need to perform image-enhanced wide-field imaging that can highlight small abnormal mucosal areas. Several new imaging techniques to overcome limitations of WLE have emerged, including probe-based confocal laser endomicroscopy (pCLE) (54), narrow-band imaging and magnification endoscopy (NBI) (55), autofluorescence imaging (AFI) (56) and i-Scan (57). A recent study demonstrated that pCLE seems to hold the greatest promise as a prognostic and predictive biomarker for VEGF inhibitors in patients with gastrointestinal cancer (58-60). In pCLE, a confocal scanning microscope is integrated into a conventional flexible endoscope, providing cellular and subcellular resolution in the horizontal plane of the targeted tissues. By using fluorescein isothiocyanate-labelled antibodies against angiogenesis-related proteins, such as VEGFA, VEGFRs or cluster of differentiation 31(CD31) (an endothelial-specific antigen), pCLE is capable of *in vivo* assessment of the morphological alterations and the abnormal microvasculature of the (mucosa) gastric cancer. By combined the morphological pCLE data with the histological data, an arbitrary angiogenesis scale (Cannizzaro-Spessoto scale) has been proposed to reflect the increased intratumoral tortuous and large vessels, fluorescein leakage, and defect flux (58). At the moment, pCLE use has been limited to a small number of specialist centres because of the requirement for well-trained endoscopists (61). We firmly believe that new design strategies incorporating with these new imaging techniques will significantly increase biomarker-driven clinical trials of VEGF inhibitors in patients with gastrointestinal cancer.

In spite of the use of endoscopy, endomicroscopy is a most useful diagnostic tool for gastrointestinal lesions; repeated endoscopy can cause discomfort and other side-effects. Capsule endoscopy (CE) was originally designed for relatively non-invasive measurement of bowel movement without causing pain to the patient (62). The latest development in CE has led to novel wireless capsule video endoscopy (WCVE), consisting of CE with a camera, battery, and attached LED lamps for illumination (63). After being swallowed, the WCVE device emits a radio signal to an external meter. A video is recorded at a rate of two frames per second as the WCVE device travels along the digestive track. Once downloaded, specialists examine the video to visualize the zone of interest and lesions. To our knowledge, there is no study yet reporting the use of WCVE for studying tumour vasculature in gastrointestinal cancer. In our opinion, however, one of the greatest advantages of WCVE is that it is

much less invasive, without need for hospitalisation and expert support through the process. Thus, WCVE will be very useful for monitoring the effects of VEGF inhibitors administered to patients with gastrointestinal cancer in clinical trials of VEGF inhibitors.

Summary

Many of the biomarker studies described above are exploratory and observational. There is considerable heterogeneity between different studies, assays and outcomes. Therefore, high-quality biomarker studies should be conducted to test candidate biomarkers in optimised randomised clinical trial designs, particularly some of the above-mentioned biomarkers that have not been evaluated in patients with gastric cancer receiving VEGF inhibitors. We believe that a specific group of different biomarkers (*i.e.* a signature) may be needed for each VEGF inhibitor. Since neoadjuvant chemotherapy followed by delayed primary surgery does not benefit overall survival in patients with gastric cancer (64), identification of biomarkers of early response to VEGF inhibitors is difficult. Nevertheless, identifying a proper biomarker signature for currently approved VEGF inhibitors is a priority.

One major challenge for the interpretation of biomarker studies is to make a distinction between prognostic and predictive values from a vast amount of data. Predictive biomarkers in the first-line setting may also not be suitable for patients with relapse. Another difficulty is determining whether a given effect is the result of anti-angiogenic activity or of affecting other oncogenic functions. Another layer of complexity in interpretation is that most clinical studies are in combination with chemotherapy agents, making it more difficult to extract the effects of each VEGF inhibitor.

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