Angiogenesis biomarkers in ovarian cancer

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Abstract
Epithelial ovarian cancer (EOC) is relatively common. Most patients present with advanced disease and although initial response to chemotherapy is often good, the majority of patients will experience relapse. Relapsed EOC becomes progressively more drug resistant, meaning that more effective therapies are needed to improve quality of life and prolong survival. One such approach is the use of molecularly-targeted agents, many of which are being evaluated in ovarian cancer. The mechanism of action of a number of targeted agents is to interfere with the process of angiogenesis, but despite much research, none have yet been validated for patient selection and monitoring response in any tumor type. The search for candidate plasma- and tissue-based biomarkers for anti-angiogenesis therapy in ovarian cancer has largely focussed on vascular endothelial growth factor (VEGF), although micro-vessel density and others have been proposed and evaluated. Gene expression profiling is another approach to identifying biomarkers, and recent results suggest that this may be useful for predicting the response to bevacizumab. Other genetic and molecular biomarkers are being investigated, with some showing the potential to identify patients who will benefit from anti-VEGF therapy. Identification of biomarkers is now included as part of large, randomized, placebo-controlled, phase III trials, although the number of variables to be taken into account, the requirement to have reliable and specific biomarkers for each therapeutic agent (or class of agents), and the fact that biomarkers probably vary by treatment settings (e.g. neo-adjuvant, adjuvant) makes biomarker identification challenging.

Key words: angiogenesis, biomarkers, epithelial ovarian cancer, gene expression profiling, genetic analysis

Introduction

Epithelial ovarian cancer (EOC) is the 7th most common cause of female cancer death worldwide and the 5th most common cause of female cancer death in the developed world [1]. Despite improvements in the treatment of ovarian cancer, only modest increases in overall survival (OS) have been achieved [2, 3]. Most women present with advanced disease and are treated with maximal surgical debulking and platinum-containing combination chemotherapy. The response rate to chemotherapy is high and complete remission is common, but the majority of women will eventually relapse. Patients with recurrent ovarian cancer often undergo multiple lines of chemotherapy, each with a diminishing duration of response due to progressively more drug-resistant disease. This highlights the need for more effective therapies to improve survival and quality of life.

A greater understanding of the molecular pathways involved in carcinogenesis and tumor growth has led to the development of a large number of novel molecular targeted agents. Many of these agents are under evaluation in ovarian cancer, and several target angiogenesis. The emerging results of these trials are encouraging but the greatest challenge yet may lie ahead as the optimal dose, combination (with chemotherapy or other targeted agents) and scheduling of these drugs is undefined. The cost of these drugs in an unselected population of patients is significant, so there is an urgent need to select the appropriate treatment for patients by identifying and validating predictive and prognostic biomarkers. Preliminary reports have identified potential serological, tissue and imaging biomarkers. However, these will need to be prospectively tested and validated in clinical trials before they can be established in standard practice.

Angiogenesis and ovarian cancer

Many biomarkers currently under evaluation are involved in the regulation of angiogenesis, a complex pro-

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cess that involves multiple pathways, genes, and epigenetic mechanisms. Angiogenesis is regulated by both pro- and antiangiogenic factors and is promoted during tumor development when an imbalance in these factors favors a proangiogenic milieu [4]. While proangiogenic factors such as vascular endothelial growth factor (VEGF) are often overexpressed by cancer cells, including epithelial ovarian cancers (EOCs) [5], receptors for these proangiogenic factors are mostly expressed by tumor endothelial cells and not tumor epithelial cells [6].

In animal models of ovarian cancer, VEGF blockade inhibits ascites production [7] as well as altering tumor vasculature and slowing tumor growth [8]. Clinical studies have demonstrated that high levels of VEGF in pre-operative serum and ovarian cancer tissue are correlated with advanced stage and poor survival [9, 10]. Furthermore, significantly higher VEGF levels have been observed in ovarian cancer patients in comparison to patients with benign ovarian pathology or borderline tumors [9, 10]. In patients with ovarian cancer, markedly elevated serum VEGF levels have been associated with advanced stage, poorly differentiated tumors, increased metastases, presence of large volume ascites and decreased survival [11]. These data form a strong rationale for the development of antiangiogenic therapeutic strategies for ovarian cancer treatment. The VEGF pathway in particular has been subject to intense research with numerous drugs developed to inhibit VEGF itself, or VEGFR at either the ligand binding or tyrosine kinase domain.

Recent randomized clinical trials have demonstrated that combination treatment with chemotherapy and bevacizumab followed by maintenance bevacizumab therapy significantly improved progression-free survival (PFS) but without an improvement in overall survival (OS) in the intention to treat population compared with chemotherapy alone in women with EOC [12-15].

A variety of antiangiogenic agents targeting molecular biomarkers in solid tumors, including EOC, are currently being evaluated. For example, phase III clinical trials have evaluated small molecule tyrosine kinase inhibitors (TKIs; nintedanib, cediranib, and pazopanib) and a fusion peptibody (trebananib) in patients with ovarian cancer (OC).

These agents have the potential to significantly impact the practice of oncology but important questions remain:

1. Is their mechanism of action in patients the same as originally envisioned for antiangiogenic agents and is the mechanism the same as demonstrated in animal models?
2. How can they be used to significantly impact on overall survival?
3. How can these agents be optimally utilisedin the adjuvant setting?
4. Why do some patients develop severe toxicities from antiangiogenic therapy?
5. Why is the benefit from antiangiogenic therapies seen only in some patients and if it is possible to preselect these patients, or the most appropriate therapy?
6. Why do tumors stop responding to antiangiogenic therapy?
7. Is it possible to tailor these new therapies to individual patients?
8. Is it possible to combine antiangiogenic agents in order to increase the extent or duration of their effect?

The answers to these fundamental questions are not fully known for the approved antiangiogenic agents, and will be critical in choosing the appropriate agent(s), and to determine their optimum dose and schedule.

Challenges in biomarker identification

An array of antiangiogenic biomarkers have been studied, including systemic measurements (for example, changes in systemic blood pressure), genotypic analyses (for example, VEGF or interleukin [IL]-8 polymorphisms), circulating protein markers (for example, plasma levels of VEGF), tissue markers (tumor microvessel density) and imaging parameters (for example, Ktrans, the volume transfer constants of gadolinium between plasma and the extravascular space measured by MRI. Although promising candidates have been identified, important challenges limit their translation into practice.

Another challenge is to optimize and standardize various biomarkers assays. For example, different approaches are being used to measure vascular imaging parameters or circulating proteins and cells. Each approach utilises different technology, which makes it difficult to compare trial results.

Angiogenesis biomarkers in ovarian cancer

In ovarian cancer, the glycoprotein CA125 is a well-recognised and validated biomarker for diagnosis and treatment monitoring, with defined criteria of disease response and progression based on changes in its serum levels [16] although the appropriateness of instigating treatment on the basis of CA125-defined progression alone has recently been challenged [17]. For targeted therapies, the identification of specific biomarkers is generally required, as conventional parameters developed for monitoring cytotoxic therapies may not apply.

Identifying predictive biomarkers that can prospectively select patients likely to respond to anti-angiogenic ther-

apy is an absolute priority. Similarly, a biomarker predictive for developing drug-induced hypertension might allow tailored management, with more intensive monitoring or prophylactic anti-hypertensives for high-risk patients.

Currently, there are no validated biomarkers for patient selection or monitoring response to anti-angiogenic therapy in any tumor type, despite multiple studies with these agents. This is due to a number of challenges, not least the complexity of angiogenesis and our limited understanding of the mechanism of action of anti-angiogenic therapies [18]. Furthermore, validation of a predictive biomarker can only be achieved in the context of a randomised controlled trial [19], and in ovarian cancer, data from these studies are only now starting to emerge.

In addition, since anti-angiogenic therapies are employed as maintenance treatment, markers of emerging resistance prior to overt clinical progression are also required to reduce exposure to ineffective treatment. Given the multiplicity of pathways involved in angiogenesis it might also be possible to overcome developing resistance by switching between agents. For bevacizumab, it has been suggested that continuation beyond disease progression may be beneficial. However, this is controversial and biomarkers indicating resistance to bevacizumab would aid clinical decision making.

Although candidate markers of emerging resistance have been proposed, further elucidation of the molecular processes and pathways involved is required.

Candidate biomarkers of angiogenesis in ovarian cancer

Exploratory biomarker studies have been conducted in a selection of the published clinical trials of anti-angiogenic therapies in ovarian cancer [20-27]. All used anti-VEGF agents (either as a single agent or in combination with chemotherapy or other targeted therapies) apart from one study that used thalidomide. The biomarkers studied include general markers of angiogenesis (microvessel density, tumor perfusion and permeability, hypertension, interleukin levels) and components of the VEGF pathway (tissue and circulating VEGF and VEGFR levels, and VEGF-related genetic polymorphisms).

Plasma- and tissue-based biomarkers

Vascular endothelial growth factor

VEGF is one of the most potent pro-angiogenic factors identified to date. VEGF is commonly overexpressed in a number of cancer cell types, including EOC.

Of the 7 members of the VEGF family of ligands, VEGF-A is the best characterized and plays a dominant role in angiogenesis. A recent meta-analysis of 1816 patients with colorectal cancer (CRC), non-small cell lung cancer (NSCLC), and renal cell carcinoma (RCC) participating in phase III trials of bevacizumab found that pretreatment plasma VEGF-A levels were prognostic for patient outcome but were not predictive of response to bevacizumab [28]. Studies included in the meta-analysis used an older version of the VEGF assay that was not selective for any specific VEGF isoform and was thought to predominantly bind VEGF165 due to its higher concentration. A novel enzyme-linked immunosorbent assay (ELISA), with a preference for short VEGF-A isoforms (VEGF110 and VEGF121), found that baseline plasma VEGF was predictive of response to bevacizumab in patients with pancreatic, gastric, and breast cancers, but not in patients with CRC, NSCLC, or RCC [29].

VEGFR-2 is the most important receptor for VEGF-A-mediated angiogenesis [30]. Increased genetic or tissue expression of VEGFR-2 has demonstrated potential prognostic value in pancreatic and breast cancers [31-32]. Plasma VEGFR-2 was not predictive of clinical outcome for women with EOC treated in the randomized GOG-0218 trial that evaluated chemotherapy alone or with concomitant bevacizumab, or with both concomitant and maintenance bevacizumab [33].

The other members of the VEGF family include VEGF-B, -C, -D, and -E, as well as placental growth factors 1 and 2 (PLGF-1 and -2). VEGF isoforms bind preferentially to certain cell surface tyrosine kinase receptors, with VEGF-A binding preferentially to VEGFR-1 and VEGFR-2, VEGF-B to VEGFR-1, VEGF-C and -D to VEGFR-3, and VEGF-E, produced only in viruses, to VEGFR-2 [34].

VEGFR-3 mediates lymphangiogenesis induced by VEGF-C and VEGF-D, and is involved in lymphatic metastases. Importantly, processed VEGF-C and VEGF-D can also bind to and activate VEGFR-2, which is involved in ascites formation [35]. Low levels of tissue-based VEGF-C, delta-like ligand-4, and neuropilin protein expression in women with metastatic breast cancer were associated with a trend toward improved PFS with bevacizumab in a retrospective subset analysis from the AVF2119g trial [34]. However, after correction for multiple hypotheses testing, the associations were not statistically significant. Further evaluation of these biomarkers may still be worthwhile. VEGF-D was found to be an independent prognostic factor in EOC, based on a multivariate analysis of 59 EOCs, 11 borderline tumors, and 20 benign cystadenomas evaluated by immunohistochemistry.

VEGFR-3, α1-acid glycoprotein, and mesothelin VEGFR-3 (also known as fms-related tyrosine kinase-4 [FLT4]), α1-acid glycoprotein (AGP), and mesothelin were identified as candidate biomarkers using mass spectrometry in serum from participants in the ICON7 study which compared standard carboplatin and paclitaxel chemotherapy to carboplatin, paclitaxel and bevacizumab in the first line setting [36]. Mesothelin has been reported to be overexpressed in EOC and to be associated with a more aggressive phenotype manifested by chemoresistance and worse overall prognosis. The individual biomarkers were not predictive of benefit from bevacizumab. However, optimal exploratory cut points for the individual biomarkers, combined with CA-125, were used to develop a signature that was predictive of bevacizumab response. The signature-negative group had an improved median PFS in the standard chemotherapy arm compared with the bevacizumab arm (36.3 *vs* 20 months; p=0.006), while the signature positive group demonstrated improved median PFS in the bevacizumab arm compared with the control arm (17.9 *vs* 12.4 months; p=0.040) [37]. Further validation of the signature is needed to determine the predictive capacity of the biomarkers.

Since VEGF signalling is not the only pathway promoting angiogenesis, evaluating an array of VEGF family and non-VEGF angiogenic factors may provide more predictive power. Many angiogenic factors can be targeted with agents that are currently available or in development; thus, information regarding mechanisms of resistance to anti-VEGF agents could direct novel combination therapies. For example, the sets of factors that predict baseline resistance to bevacizumab, mediate acquired resistance to bevacizumab after initial response, or predict risk of bevacizumab-related toxicity have not been determined. Lack of this information represents a major gap in understanding the clinical mechanisms underlying the efficacy and toxicity of this agent, as well as resistance to it, in patients with EOC.

The inability of pretreatment VEGF family ligands and receptors to predict response to bevacizumab in patients with EOC may reflect the complexity and redundancy of tumor angiogenic pathways.

Micro-vessel density

Micro-vessel density (MVD) has been used to quantify blood vessel formation within tumors and was first identified as a prognostic marker in breast cancer 20 years ago [38]. Increased MVD indicates increased angiogenesis and a decrease in MVD in serial tumor samples during treatment could indicate vascular normalisation in response to therapy. Increased microvascular density has been associated with poor prognosis in ovarian cancer [39, 40] though there are some conflicting reports 52. In a pre-clinical study of VEGF inhibitors in ovarian cancer models, decreased tumor growth was associated with reduction in MVD on serial biopsies. Results from the translational component of GOG 170-D have recently been published [41]. Tumor biopsies were obtained at baseline and after four cycles of bevacizumab. A baseline biopsy was available in 43 (of 62) women and paired samples from 20. In this study, high baseline CD-31 MVD was significantly associated with decreased response to bevacizumab, decreased median survival and an increased risk of death (HR 2.2, 95% CI 1.067–4.467). CD-31 MVD is to be further evaluated in GOG 218.

Others

A number of other proteins have been proposed as putative biomarkers including: neuropilin-1, PIGF, bFGF and ICAM-1. In the study by Han et al. thrombospondin-1 (TSP-1) and p53 expression were examined. No significant association between treatment with bevacizumab and baseline or serial changes in these factors was identified, although there was a suggestion that the presence of p53 and high levels of TSP-1 expression could be associated with decreased risk of death and disease progression, respectively [42]. *In situ* biomarkers can give valuable information but have practical limitations. The assessment of markers on archival primary tissue may not reflect those present in relapsed disease and even at diagnosis there may be heterogeneity between the primary tumor and metastases or within the primary tumor itself. To exploit their full potential, multiple biopsies from primary tumors and metastatic sites are required prior to each course of treatment and serially during therapy to assess dynamic changes. Although this approach is encouraged, particularly in early phase studies, it may not be acceptable to all patients, nor feasible or safe in all cases. Thus, markers that can be obtained by less invasive means may be of greater utility in routine clinical practice.

Circulating cells

Circulating endothelial cells (CECs) are elevated in some patients with cancer and are thought to reflect active angiogenesis with levels returning to normal in patients who achieve a complete response [43]. They can be separated into mature, terminally differentiated CECs of endothelial origin and circulating endothelial progenitor cells (CEPs), which are derived from the bone marrow and mobilise in response to pro-angiogenic factors.

In a small study, CEPs were elevated in patients with epithelial ovarian cancer compared to healthy controls and higher levels of CEPs at diagnosis were related to more advanced stage (III/IV *vs* I/II) and worse prognosis. CEP levels fell following primary debulking surgery but remained higher in patients with >2 cm residual disease [44].

They have therefore been proposed as potential dynamic biomarkers. Currently, the interpretation of CECs/CEPs as biomarkers is limited by a lack of standardisation of the assays and immunological markers used to identify the cells. The potential role of CECs and CEPs as biomarkers in ovarian cancer is being evaluated in ongoing clinical trials [44].

Genetic and molecular biomarkers

Gene expression profiling

Gene expression microarray analysis followed by unsupervised hierarchical clustering has been used to identify meaningful molecular subgroups in a number of cancers including breast [43] and ovarian [45, 46]. In the setting of high grade serous ovarian cancer, The Cancer Genome Atlas (TCGA) study and the study of Tothill et al. identified four molecular subgroups with evidence of prognostic importance in the latter study [45, 46]. Supervised analyses have also been performed in an attempt to identify signatures that better reflect prognosis or resectability [47-49]. Two recently presented studies suggest that these transcriptionally defined molecular subgroups may be of value in predicting the response to bevacizumab.

Gourley et al performed unsupervised hierarchical clustering on gene expression microarray data and identified three molecular subgroups characterised by high expression of angiogenesis genes, immune response genes and both angiogenesis and immune response genes respectively [50]. The patients in the Immune subgroup (high expression of immune response genes but low expression of pro-angiogenic genes) had a superior progression free and overall survival compared to patients in the other molecular subgroups (which were characterised by high expression of angiogenesis genes). More importantly, when this analysis was repeated using translational samples from the ICON7 study (carboplatin and paclitaxel plus or minus concomitant and maintenance bevacizumab) patients in the Immune subgroup who received bevacizumab appeared to have a significantly inferior progression free (HR 1.73; 95% CI 1.12–2.68) and overall survival (HR 2.00; 95% CI 1.11–3.61) compared to those on the control arm. In the other molecular subgroups (high expression of pro-angiogenic genes) there was a trend towards an improvement in progression free survival for bevacizumab treated patients that lasted for the duration of therapy plus about 6 months. The molecular signature for the immune group was able to discriminate patients according to their extent of benefit from bevacizumab (test for interaction, p=0.016).

In a separate analysis, also using translational research samples from ICON7, Winterhoff at al used RNAseq to partition patients according to the original TCGA molecular subgroups. In doing so they demonstrated some difference in the bevacizumab sensitivity between the 4 molecular subgroups. Greater PFS benefit was observed with the addition of bevacizumab to standard chemotherapy in patients with proliferative (median change of 10.1 months; p=0.015) or mesenchymal (median change of 8.2 months; p=0.405) ovarian cancer subtypes compared with immunoreactive (median change of 3.8 months; p=0.080) or differentiated (median change of 3.7 months; p=0.610) subtypes. Similar differences were observed between subtypes for OS.

Taken together, the data from these 2 studies suggest that molecular subtypes may be used to direct bevacizumab therapy in women with OC. Urgent validation in further bevacizumab (and other antiangiogenic) treated populations is urgently required because the ability to stratify patients according to likely benefit from these agents is a pressing need, particularly given the potential that some patients may actually suffer detriment as a result of this therapy.

A study which combined data from TCGA with additional available datasets and newly developed computational algorithms revealed that the PDGF network can stratify prognosis of OC [51]. A more recent study looking at the TCGA dataset showed that low expression of microRNA-378, a microRNA that regulates genes involved in angiogenesis as well as other biological processes, was associated with longer PFS among patients with recurrent OC treated with bevacizumab [52].

Epigenetic biomarkers

Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) is the catalytic core of the Polycomb-Repressor Complex 2 that functions to silence gene expression via trimethylation of histone3 lysine27 [53]. A study on EZH2 demonstrated that high EZH2 expression in EOC or tumor-associated endothelial cells was associated with poor survival, and that EZH2 promotes tumor angiogenesis by silencing vasohibin1 (VASH1), an inhibitor of angiogenesis. Silencing of EZH2 in tumor associated endothelial cells led to a decreased

microvessel density concomitant with reduced orthotopic ovarian tumor growth. Since EZH2 expression is directly regulated by VEGF [54], these results suggest that EZH2 levels or VASH1 promoter methylation may be candidate biomarkers that may predict benefit to anti-VEGF therapy.

Genetic polymorphisms

There is substantial genetic variability within VEGF and VEGFR-2, including multiple single nucleotide polymorphisms (SNPs). Various SNPs were evaluated in the ECOG2100 trial in women with metastatic breast cancer comparing paclitaxel with or without bevacizumab [55]. The VEGF-2578 AA genotype was associated with a superior median OS in the combination arm when compared with other genotypes (HR 0.58; 95% CI 0.36–0.93; p=0.02). The VEGF-1154 A allele also demonstrated improved median OS, with an additive effect of each active allele, but only in the combination arm (HR 0.62; 95% CI 0.46–0.83; p=0.001). Interestingly, the VEGF-634 CC and VEGF-1498 TT genotypes were associated with significantly less grade 3 or 4 hypertension in the bevacizumab arm when compared with the alternate genotypes (p=0.005 and p=0.02, respectively).

Further evaluation is needed to confirm these findings and establish the extent to which they predict benefit from bevacizumab.

Winterhoff and colleagues also examined a subgroup of the ICON7 trial, the German cohort (AGO-OVAR11), to identifymolecular subtypes or features of patients who may benefit from bevacizumab treatment [56].

Biomarker evaluation in clinical trials using new antiangiogenic agents in EOC

Large randomized, placebo-controlled, phase III trials that include acquisition of samples pretreatment, posttreatment, and at the time of tumor progression are ideal studies to address biomarkers. The randomized trial design and use of a placebo control, or at least an arm without antiangiogenic therapy, allow for identification of factors that predict patients who will or will not benefit from antiangiogenic agents. Biomarker assessments are fraught with challenges including sample acquisition, cost, uncertainty regarding biomarker selection, and time.

In AGO-OVAR 16 trial of pazopanib 940 women with FIGO stage II–IV EOC, fallopian tube cancer or primary peritoneal cancer who had not progressed after first-line treatment were randomized to treatment with pazopanib (800 mg/day orally) or placebo for a maximum of 104 weeks [57]. Median PFS was significantly longer in the pazopanib group (17.9 *vs* 12.3 months; HR 0.766; 95% CI 0.64–0.91; p=0.0021), although the first interim analysis for OS showed no difference between groups [57]. Heitz and colleagues presented genomic analyses from a subpopulation of patients from this trial at the 2014 ASCO Annual Meeting, using next generation sequencing to identify potential biomarkers for response to pazopanib [58].

Patients receiving placebo had a greater number of genetic alterations of potential damaging variants from the surgical period to postprogression compared with those receiving pazopanib; 7 BRCA2 and 3 BRCA1 gene changes associated with loss of function were observed in the placebo group versus a BRCA2 gene gain of function in 1 patient in the pazopanib group.

Other interesting trials are AGO-OVAR 12 with nintedanib, ICON6 trial testing cediranib plus standard chemotherapy, TRINOVA-1 trial of weekly paclitaxel plus trebananib, and MITO 16 analyzing potential antiangiogenic biomarkers in advanced ovarian cancer patients receiving carboplatin, paclitaxel plus bevacizuamb.

Translational research data from these pivotal trials have not yet been reported and could potentially yield important information about predictive and prognostic biomarkers.

However, specific biomarkers may be needed for each agent or class of agents, and those predictive in a first-line setting may not apply to treatment of relapsed disease. Appropriate serial sample collection should be an integral component of all trials of antiangiogenic therapy. The increasingly accepted use of neo-adjuvant chemotherapy followed by delayed primary surgery [59] provides an opportunity to undertake ‘window-of-opportunity’ studies [60], evaluating biomarkers of early response, although anti-angiogenic agents have not yet been used in this setting.

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