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Beyond CA125: the coming of age of ovarian cancer biomarkers. Are we there yet?

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Summary

Ovarian cancer (OC) is the fourth leading cause of cancer deaths among women in the United States, despite its relatively low incidence of 50 per 100,000. Even though advances in therapy have been made, the OC fatality-to-case ratio remains exceedingly high, due to the lack of accurate tools to diagnose early-stage disease when cure is still possible. The most studied marker for OC, CA125, is only expressed by 50–60% of patients with early stage disease. Large efforts have been deployed to identify novel serum markers, yet no single marker has emerged as a serious competitor for CA125. Various groups are investing in combination approaches to increase the diagnostic value of existing markers, but many markers may still lie in under-explored areas of ovarian cancer biology, such as tumor vasculature environment and post-translational modifications (glycomics).

Keywords

Ovarian cancer; biomarkers; early detection; panels; tumor vasculature markers; glycomics

Introduction

Ovarian cancer has a higher fatality-to-case ratio than any other gynecologic malignancy, translating into approximately 22,000 new cases diagnosed annually in the United States [1]. It is thus the most deadly gynecologic malignancy in developed countries. This high mortality rate is largely due to the fact that the majority of women are not diagnosed until the disease is in an advanced, metastatic stage.

It is generally accepted that cancers which arise within the ovary originate in the epithelium of epithelial inclusion cysts, which are derived from the surface epithelium. While most epithelial tumors, including a subset of low grade adenocarcinomas of the ovary (endometrioid and mucinous) follow the adenoma-carcinoma sequence, high grade serous ovarian carcinoma (HGSOCs) often exhibits a unique pattern of progression in which no tumor can be found on the surface of the ovary during the early stages while as the tumor grows the intact ovarian outer surface is disrupted and the cancer extends to the pelvic region [2]. Despite extensive clinical and fundamental research, controversy still exists on the origin of serous female adnexal tumors. Serous ovarian tumors share similar morphological and clinical characteristics with fallopian tube and peritoneal adenocarcinomas. Crum and colleagues suggested that a significant proportion of advanced serous cancers could actually originate from the fimbria of the fallopian tube after the analysis of a consecutive series of prophylactic adnexectomies (bilateral salpingo-oophorectomies [BSOs]) from BRCA-positive women, which could provide a logical explanation for the absence of primary tumor on the ovary surface at early

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stages of cancer [3–7]. In a comprehensive review examining all the published information on the possible cell of origin for serous ovarian carcinoma, Piek et al. could not formally conclude whether HGSOCs arise from the ovarian or tubal surface epithelium [8]. In addition, expression profiling studies have shown that HGSOC cluster separately from low grade carcinomas and borderline tumors, with HGSOC being associated with p53 mutations while low grade tumors are associated with mutations at KRAS, BRAF, PTEN and CTNNB1/*β-catenin* [9], and Lee at al. detected discrete segments of secretory cells with strong nuclear p53 immunostaining in benign-appearing tubal mucosa, (termed p53 signatures) providing more evidence of tubal origin of HGSOC[10]. Environmental agents have not been proven to play a significant role, while epidemiological studies point to possible racial, geographic, social, and hormonal causative factors. Although a strong family history of ovarian cancer and hereditary ovarian cancer syndromes with autosomal dominance have been described, not all of mutation carriers develop ovarian cancer, which suggests a role for interactions with other, as yet unidentified, genetic and epigenetic influences. Finally, although there is convincing evidence that inflammation is a contributing factor in ovarian cancer development [11,12], the role of complement-induced inflammation in tumor initiation or progression remains poorly investigated.

OC cell intraperitonal spreading occurs by direct contact and invasion into the adjacent tissues, such as the uterus, fallopian tubes, bladder, sigmoid colon, or rectum. The exfoliated tumor cells floating in the peritoneal fluid eventually adhere to the mesothelial cells that line the surface of the peritoneal cavity and the outer surface of different pelvic and abdominal organs [13]. Cell binding mediated by CA125, a mucin aberrantly expressed by OC cells, and mesothelin, a GPI-anchored glycoprotein expressed by both OC and mesothelial cells, may contribute to both homotypic (tumor cell-tumor cell) and heterotypic (tumor cell-mesothelial cell) interactions of tumor cells, increasing multicellular spheroid formation in the peritoneal fluid and also increasing tumor load [13], as well as promoting the adherence of the tumor cells to the peritoneum, resulting in micrometastasis [14–16]. This suggests the blocking of CA125/mesothelin-dependent cell adhesion as a therapeutic avenue [17].

The peculiar pattern of progression of ovarian cancer disease combined with nonspecific symptoms such as those related to abdominal bloating and gastrointestinal disturbances [18], provides substantial motivation to identify novel biomarkers that accurately identify ovarian cancer in its early stages, when it is most treatable. Although circulating CA125 antigen still is the only recommended biomarker for clinical use in the US for ovarian cancer, CA125 is expressed in only 50–60% of patients with early-stage disease [19]. Considering that an effective ovarian cancer screening test would require a minimum Positive Predictive Value (PPV) of 10% [20] and a specificity of greater than 99% [21], considerable efforts have been deployed towards this goal, and several other ovarian cancer candidate biomarkers have been identified by various approaches.

Conventional screening tools

In a large scale study in the UK [22] with over 200,000 post-menopausal women enrolled, the sensitivity, specificity and PPV for the CA125/TVU group values for all primary ovarian and tubal cancers were 89.4%, 99.8% and 43.3%, while for the TVU alone group were 84.9%, 98.2% and 5.3%, respectively. For primary invasive epithelial ovarian and tubal cancers, the sensitivity, specificity and PPV were 89.5%, 99.8%, and 35.1% for CA125/TVU, and 75.0%, 98.2%, and 2.8% for TVU alone, respectively. Although the specificity was higher for CA125/TVU than for the TVU alone, both screening strategies resulted in encouraging results and cost-effectiveness analyses are currently underway. The effect of these screens on mortality still remains to be determined. However, other independent reports underline the low sensitivity and specificity of the current means of screening women for early detection of OC [23–31]. In

Netherland, a recent study epitomizing the current state of the art of OC early detection [32] enrolled 241 women with high risk for OC (pathogenic BRCA1 or BRCA2 mutation) in a screening program including pelvic examinations, transvaginal ultrasounds (TVU) and serum CA125 measurements. In the case of abnormal findings in one of these tests, repeated testing within 1 to 3 months was advised. If the abnormality persisted, laparoscopy or laparotomy was performed. For women who underwent prophylactic BSO the screening was stopped. Overall the effectiveness of the screen was disappointing. The PPV for pelvic examination, TVU and CA125 were low (20%, 33% and 3%, respectively), while the Negative Predictive Values (NPV) were high (99.4%, 99.5% and 99.4%) and all detected ovarian cancers were in an advanced stage. Finally, restricting the analyses to incident contacts that contained all 3 screening modalities did not substantially change the outcomes, leading the authors to the grim conclusion that annual gynecological screening of women with a BRCA1/2 mutation to prevent advanced stage ovarian cancer is not effective [32].

Serum biomarkers

Strategies for identification of biomarkers for non-invasive, cost-effective tests, such as ELISA assays, that indicate OC have been in use for decades. Mouse immunizations with whole OC tumor or tumor cell lines have yielded solid successes, including the discovery of CA125 and mesothelin. The thorough understanding of these strategies permits to build on previous achievements for designing novel, successful discovery platforms.

A. Identification of tumor-specific biomarkers through mouse immunization with whole tumor or tumor cell lines

In 1981, Bast and colleagues published the identification of OC125, an antibody obtained through BALB/c mouse immunization with an epithelial ovarian carcinoma cell line (OVCA433) established from a patient with serous papillary cystadenocarcimona [33]. Hybridoma cell lines resulting from the fusion of immunized spleen cells and P3/NS-1 plasmacytoma line were screened against a panel of twenty cell lines, including ovarian, cervical, pancreatic, renal cell, breast, and colon carcinoma, as well as neuroblastoma, melanoma and three types of leukemia cell lines, and against autologous B cell line and enzymatically dissociated cells from allogeneic normal ovary. OC125 was chosen for its specific binding to ovarian carcinoma. Interestingly, OC125 could be used alone in double determinant ELISA assay (sandwich ELISA) [34,35]. It took almost twenty years to understand why a single antibody could perform in a sandwich test assay. OC125 detects CA125, a large glycoprotein, the best studied and still only FDA-approved biomarker for OC detection, that harbors an extracellular domain consisting of SEA domains repeated 7, 12, or 60 times, depending on the variants [36,37]. CA125 ELISA assay was later improved with the identification of another antibody [38]. Yet even the improved assay had serious limitations for the early detection of OC. CA125 was also found to be increased in 1% of healthy blood donors, in 29% of other cancers (lung, breast, pancreas, and colorectum) and in 6% of women with non-malignant conditions (cirrhosis with ascites, acute pancreatitis, ovarian cysts, endometriosis, and pelvic inflammatory disease) (reviewed in [39]).

The identification of Ov569 antibody, an antibody that recognizes a GPI-anchored glycoprotein, mesothelin [40,41], another marker for ovarian carcinoma, was also the result of the same type of strategy, featuring repeated immunizations of BALB/c mice with cells from the malignant ascites of a patient with ovarian carcinoma [42]. Mesothelin was first thought to be exclusively membrane-bound and thus an ideal candidate for targeted therapy against cancer [43–45]. However, the development of several independent double determinant ELISA assays following ours, prepared with the antibodies 569mAb and 4H3mAb [42] (now commercialized by Fujirebio, Inc. as Mesomark®), demonstrated the presence of soluble forms of mesothelin in ovarian and pancreas cancer patients and in mesothelioma patients [46–51].

This and the evidence of a membrane-bound form of mesothelin on the normal lining of peritoneal, pleural and pericardial membranes, strongly suggests the possibility of serious side effects linked to a targeted therapy using an affinity reagent that detects soluble, normal and cancer membrane-bound mesothelin forms equally well.

B. Identification of tumor-specific biomarkers through subtractive screening of cDNA arrays

To discover genes with potential for the diagnosis of ovarian cancer, comparative hybridization of cDNA arrays has been used for the measurement of differences in gene expression between two or more tissues and high-density cDNA array hybridization (HDAH) has proven to be a powerful technology to identify novel biomarkers via the identification of transcripts that show high expression levels in ovarian cancer tissues as compared to ovarian surface epithelium (OSE). In 1999, Schummer et al. discovered HE4 by the screening of an array of 21,500 unknown ovarian cDNAs hybridized with labeled first-strand cDNA from 10 ovarian tumors and six normal tissues [52]. Later, the development of a double determinant ELISA assay [53] permitted validation of HE4 as a biomarker for ovarian cancer [54,55]. As a single tumor marker, HE4 had the highest sensitivity for detecting ovarian cancer, especially Stage I disease. Furthermore, combining CA125 and HE4 is a more accurate predictor of malignancy than either alone [47,51,56]. As for mesothelin, the HE4 ELISA assay has been exclusively licensed out to Fujirebio, Inc. and on Dec 16, 2008 Fujirebio Diagnostics and Roche Diagnostics signed an agreement to commercialize the HE4 test through Roche Diagnostics' Automated Immunoassay Analyzers. Of note, in light of the work of Crum and colleagues [5] demonstrating that at least part of OC may derive from fimbria, it seems reasonable to expect that measurement of differences in gene expression between normal tissues, including fimbria instead of OC only, will lead to novel interesting biomarkers.

C. Identification of tumor-specific biomarkers through proteomic approaches

1) Direct mining of the plasma proteome for identification of tumor biomarkers has been challenging because concentrations of known plasma proteins span nine orders of magnitude, with tumor-derived proteins probably being at the lower end of the range. Tumor immunoproteomics, which defines the subset of proteins involved in the immune response against tumors, has triggered considerable hope as a novel source of markers for early-stage immune response to cancer, as well as of antigens suitable for immunotherapy.

Current proteomic approaches have enabled researchers to interrogate complex proteomes such as body fluids by matching mass spectra to sequence databases to identify proteins [57]. However, despite several systematic approaches exploring tissue samples for serum biomarker discovery, only a few candidate biomarkers emerged from such analyses have been validated as useful biomarkers in the serum [58], probably because the complexities of fluid proteomes cannot be resolved in a single analysis. Current protocols require fractionation of samples and separate analysis of different fractions [59]. Numerous creative ways have been implemented to fractionate samples. They include: a) isotopic labelling of case and control samples followed by directed analysis with limited fractionation or comprehensive analysis with extensive fractionation by ion exchange chromatography and reverse phase chromatography [59]; b) immunoprecipitation of intact tumor-associated proteins by antibodies from cancer patient sera and from cancer-free controls to further identify cancer-specific autoantibody signatures by mass-spectrometry [60]; c) immunoprecipitation of intact tumor-associated proteins by recombinant antibodies (biobodies) [61] selected against cancer patient serum proteins to further identify cancer-specific serum proteins by mass-spectrometry [62]; d) immunoprecipitation of tryptic peptides from a digest of whole human plasma by polyclonal antibodies or the Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) method, to identify cancer-specific serum proteins by mass-spectrometry [63].

2) Immunoproteomics to identify OC-specific autoantibodies. An alternative approach for OC biomarker discovery is based on the detection of serum antibodies directed against tumor antigens (autoantibodies) [64]. Autoantibodies are thought to be more stable and more abundant (especially at low tumor burdens) than tumor antigens. As a matter of fact, many aberrantly expressed proteins during the course of the tumor progression elicit an immune response. For example, antibodies against mutant forms of p53 tumor suppressor protein are present in 24% of cancer patients [64].

Many groups have focused on identifying autoantibodies for cancer diagnostic purposes. Chatterjee at al [65] screened an ovarian cancer phage display library using serum immunoglobulins from an OC patient. By using protein arrays they found 65 clones that interacted with 32 OC sera, but not with sera from healthy women. Similarly, Hudson et al [66] used high density protein arrays of 5005 human proteins to hybridize sera from 30 cancer patients and 30 control individuals. Out of the 94 antigens that showed increased reactivity in cancer patients, three (Lamin A/C, SSRP1, and RALBP1) were validated by immunoblot assays.

In addition to phage display libraries [65,67] and high density protein microarrays [66], autoantibodies specific for cancer have also been sought by serum probing of recombinant proteins [68–70] and denatured proteins resolved by 2-DE [64,71,72]; a number of autoantibodies against tumor antigens have been identified so far. However, collective data shows that only a subset of patients seems to produce antibodies with high affinity to these proteins. Furthermore, autoantibody reaction in these patients exhibited a poor correlation with disease, even when cancer stages were taken into consideration. These results suggest that the function of the autoantibodies found in cancer patients may significantly differ from normal antibody function, including antigen binding and antibody-dependent cell-mediated cytotoxicity ‘ADCC’.

3) Tumor vasculature as a source of biomarkers. Opportunities for identification of biomarkers for ovarian cancer diagnosis can derive from compartments of the tumor other than the tumor islets. Solid tumors need to develop blood vessels at an early stage in order to grow beyond ~1mm [73]. To date there have been a number of studies demonstrating that tumor and normal vascular cells differ at the molecular level in ovarian [74,75] as well as other types of cancers [76–78]. To our knowledge, there are two studies to date that have attempted to identify such molecules for ovarian cancer [74,75]. Both of the studies mined the vascular compartment of ovarian cancer using immunopurification [75] or immunohistochemistry guided laser capture microdissection [74] for the endothelial cell isolation, followed by gene expression profiling using microarrays. Out of the molecules found to be upregulated in both studies, the most promising candidates for diagnostic markers are the transmembrane and secreted ones. Table 1 summarizes these candidate genes from both studies. There are 45 genes which are localized on the cell membrane or are secreted. Nine of the genes in the list are identified in both studies and therefore are more likely to represent consistently highly expressed ovarian tumor endothelial markers. In the absence of studies validating the recently identified TVM as diagnostic markers, we give here a short overview of the genes encoding proteins with differential expression in OC or other tumor types compared to normal tissues, or whose levels correlated with clinical outcome of cancer patients.

Death Receptor 6 (DR6; TNFRSF21) is a member of the TNF-receptor superfamily. It has been shown to activate NF-kappaB and MAPK8/JNK, and induce cell apoptosis. Through its death domain, this receptor interacts with TRADD protein, which is known to serve as an adaptor that mediates signal transduction of TNF-receptors. We were able to detect DR6 levels in the serum of patients by Western blot and found a ~3 fold overexpression in patients with ovarian cancer compared to healthy women [74]. Based on these positive results we are

optimistic that the development of an assay (such as ELISA, luminex etc.) appropriate for accurate measurement of DR6 levels will provide us with a new useful diagnostic test for OC.

CSPG2 (Versican) is a member of the large aggregating chondroitin sulfate proteoglycan (CSPG) family. At its carboxyl terminus, versican has EGF-like, lectin-like and complement regulatory protein-like domains, whereas the amino-terminal domain binds hyaluronan with high affinity. Versican (like DR6) was identified as a tumor vascular marker (TVM) in both ovarian TVM studies [74,75]. High versican expression was associated with poor prognosis in a number of different cancer types such as ovarian [79], endometrial [80] and non-small cell lung cancer [81], as determined with immunohistochemistry.

TEM7 (Tumor Endothelial Marker 7) was first identified by St. Croix et al as differentially expressed by the tumor endothelium in colorectal cancer [78]. TEM7 has been reported to be a regulator of metastasis in osteogenic sarcoma; elevated TEM7 levels, quantified by immunohistochemistry, were associated with poor survival of osteogenic sarcoma patients ($p<0.04$) and it has been suggested to be promising as a therapeutic target [82]

FZD10 (Frizzled homologue 10) is a member of the frizzled family of 7-transmembrane domain receptors. Fukukawa et al. [83] recently reported the therapeutic efficacy of the delivery of an anti-FZD10 monoclonal antibody in a mouse model of synovial sarcoma. A single injection of an isotope labeled Mab increased the median time of tumor progression to 58 days, compared to 9 days for control mice.

SPP1 (Ostepontin, secreted phosphoprotein 1) was initially described as a potential biomarker for ovarian cancer in a MICROMAX cDNA microarray study using RNA isolated from several ovarian cancer cell lines and healthy human ovarian surface epithelial (HOSE) cell cultures [84]. In this study SPP1 was found to be 184 times upregulated. Later the same group reported SPP1's value as a biomarker for ovarian cancer. In particular, osteopontin levels in plasma were significantly higher ($P<.001$) in 51 patients with epithelial ovarian cancer (EOC) (486.5 ng/mL) compared with those of 107 healthy controls (147.1 ng/mL), 46 patients with benign ovarian disease (254.4 ng/mL), and 47 patients with other gynecologic cancers (260.9 ng/mL) [85]. Despite the fact that SPP1 is expressed by tumor cells, it is also found to be overexpressed by ovarian tumor vasculature, as reported by Lu et al. [75]. More recently, other studies have evaluated osteopontin's significance as a biomarker. It has been found to complement expression of CA125 in cases with low or absent expression of CA125 [47] and it may be a clinically useful adjunct to CA125 in detecting recurrent ovarian cancer [86]. Furthermore, SPP1 was found to be differentially expressed in the urine of EOC patients [87]. Osteopontin was also found to be a useful biomarker in multimarker panels for ovarian cancer [88,89]. Higher osteopontin plasma levels were also reported in other cancers such as breast [90], prostate [91] and bladder [92].

In addition to the factors deriving from the vasculature, a number of molecules that promote angiogenesis have been shown to have diagnostic significance [93].

Vascular Endothelial Growth Factor (VEGF; VPF) is one of the most potent mediators of angiogenesis in tumors. Numerous studies have focused on evaluating VEGF as a biomarker. Serum VEGF levels have been reported elevated in EOC patients compared to those of healthy women in a number of studies [94–96]. In addition, VEGF levels correlate with disease progression, but VEGF levels are unrelated to the response to chemotherapy or survival, and thus cannot be used for monitoring treatment efficacy [96]. Serum VEGF levels also seem to be a valuable marker in the subset of patients with low or absent CA125 [47].

Interleukin-8 (IL-8) is an activator of angiogenesis [97] which is secreted by a number of cells, including monocytes, neutrophiles and tumor cells. Elevated IL-8 serum concentrations in

ovarian cancer patients have been reported [98,99]. Lokshin et al. used SEREX technology, which is based on the screening of tumor cDNA libraries for reactivity with cancer patients' serum [100]. With this approach they identified anti-IL-8 antibodies in the serum of ovarian cancer patients, a result that was also confirmed by luminex assay. Combining IL-8 and anti-IL-8 IgG with CA125 resulted in increased classification power as compared to individual markers analyzed separately.

Interleukin-6 (IL-6) is also a cytokine that has been shown to promote angiogenesis [101]. IL-6 is produced by a number of ovarian cancer cell lines and primary cultures. Based on this, Berek et al measured IL-6 in OC patient sera and found that their data support the concept that IL-6 may be a useful tumor marker in some patients with epithelial ovarian cancer, as it correlates with the tumor burden, clinical disease status, and survival [102]. In a study with multimarker panels using multianalyte LabMAP profiling technology, IL-6, IL-8, VEGF, EGF, and CA-125 were used in a classification tree analysis that resulted in 84% sensitivity at 95% specificity [103].

D. CTC single cell detection as a source of biomarkers

In 1990 Cain et al explored for the first time the idea of the spread of OC cells in the bloodstream [104]. Since then, numerous studies have evaluated circulating tumor cell (CTC) levels in the blood stream of OC patients, and technology has evolved to permit the accurate detection of single cells in complex fluids (Microparticle Enzyme Immunoassay (MEIA) detection with LCx® Analyzer (Abbott Laboratories) [105]; CTC-Chip [106]). In a recent study, Fan et al used [107] a cell adhesion matrix to enrich for invasive circulating tumor cells from peripheral blood of 71 patients. Their results show that high numbers of invasive CTCs may predict for shorter disease free survival and later stage disease.

E. Glycosylations

Post-translational modifications are often altered in various human malignancies [92], including phosphorylation [108], sumosylation via Ubc9, [109] and glycosylation, Glycosylation is one of the most common PTM of secreted proteins [110]. Glycoproteins are modified by the attachment and processing of a diversity of glycans at each available glycosylation site. Each glycoprotein is therefore a collection of "glycoforms" that is cell- and protein-specific, highly reproducible in a given physiological state and, importantly, altered in disease [111]. Changes in glycosylation have been described in human carcinomas, including the presence of sialyl Lewis x (SLe^x) epitope, a GlcNAc residue with an alpha 1,3-linked fucose and a beta 1,4-linked galactose which has an alpha 2,3-linked sialic acid [112,113]. In ovarian cancer patients, branching and sialylation increase as haptoglobin, alpha1-acid glycoprotein and alpha1-antichymotrypsin from patients contain elevated levels of subsets of glycoforms, including SLe^x. In addition, patient total serum glycome presents increased levels of core fucosylated, agalactosyl biantennary glycans (FA2) [114]. Specifically, the glycans on OC patient IgG show a trend toward decreasing levels of galactosylation and sialylation, which can be the result of decreased Gal-T activity in plasma cells [115] or increased production of specific subsets of plasma cells with low expression levels of galactosyltransferases [116]. Of note, IgG acquires anti-inflammatory properties upon Fc sialylation, which is reduced upon the induction of an antigen-specific immune response. This differential sialylation may provide a switch from innate anti-inflammatory activity in the steady state to generating adaptive pro-inflammatory effects upon antigenic challenge [117]. General changes in glycosylation in OC patient glycome have the potential to be translated into novel biomarkers. However, direct cancer-derived glycol-biomarkers make up a very small percentage of the total serum glycome, difficult to access by the current technologies. Lectin-and antibody-based assays for glycan epitopes are still problematic due to imprecise specificity of particular glycan structure and low avidities of binding [111]. Probes capable of binding specific glycan epitopes, perhaps

evolved from chemical design (aptamers or peptides [111]) or from recombinant antibodies [118], are urgently needed.

F. Multiplexed assays

Panels of biomarkers have been extensively investigated to improve sensitivity and specificity and have included some of the most promising reported markers such as CA72-4, M-CSF, OVX1, LPA, Prostacin, Osteopontin, Inhibin and Kallikrein [85,119–126]. However, no marker panel reported to date has achieved adequate performance characteristics to be used as a mean for OC screening [127].

One of the first panels was published by Petricoin and colleagues in 2002. The authors used an iterative searching algorithm to analyze the proteomic spectra of a preliminary "training" set and identified a proteomic pattern that seemed to completely discriminate cancer from no-cancer. However, the validation dataset was not representative for the frequency of OC in the general population and the PPV could not be reproduced in further studies.

Several members of the kallikrein-related peptidase (KLK) family have been reported as candidate cancer biomarkers for a wide range of malignancies [128]. KLKs often exhibit coordinated deregulation patterns in cancers, suggesting common regulatory pathways. The role of KLKs in cancer is poorly understood, but as extracellular matrix (ECM) proteases they seem to be involved in ECM remodeling, angiogenesis, invasion and metastasis [129]. Yet, although KLKs have biomarker utility in many cancer types, individually they lack sufficient specificity or sensitivity to be used in clinical practice. Groups of KLKs and other candidate biomarkers have been proposed to improve diagnostic performances. In OC, KLKs 4, 5, 6,10 and 15 are associated with unfavorable prognosis and KLKs 8, 11 and 14 may have diagnostic potential [130,131]. KLKs have been explored in multiparametric analyses and the combination of KLK 7, 10, 11, 13, B7-H4 and CA125 has been shown to be superior to CA125 alone in distinguishing between nonpathologic tissues and metastatic tumors [132]; follow-up experiments are now required to verify the validity of the proposed biomarker paned in serum [128].

Another family of proteins that has been shown to have diagnostic value for OC are the inhibins [133]. The inhibins are TGFβ family members and they consist of two members (inhibin A and B) which are both heterodimers. They are produced by a precursor molecule which is processed to the mature form. Inhibins are attractive molecules for ovarian cancer diagnosis in post-menopausal women where the physiological level should be undetectable since inhibins are normally produced by the ovarian follicles. Assays are available to measure independently inhibin A, B, pro-αC [134] or total inhibin levels [135]. Total inhibin has been shown to improve CA125 performance for granulosa cell tumors and mucinous epithelial carcinomas. The two markers combined detected 95% of tumors with 95% specificity.

More recently, Visintin et al. reported a 6-marker panel (leptin, prolactin, OPN, IGF-II, MIF, CA-125) measured by a multiplex bead-based immunoassay system [136]. The authors reported a sensitivity of 95.3%, a specificity of 99.4% and a PPV of 99.3%. Yet such a high PPV could be reached only if the prevalence of OC were in the vicinity of 50%, while it is actually estimated at 0.036% for women aged 50 and over [137]. As Visintin et al calculated later, for the general population the PPV would be 6.5% or, in other words, with this bead-based assay 14 out of 15 women with a positive test result would experience false-positive test results [138], rendering this test not cost-effective for mass screening.

In conclusion, although multiparametric analyses have the potential to improve diagnostic tests, the careful evaluation of their performances in appropriate sample cohorts is mandatory. Furthermore, validation of biomarkers as true tumor-derived antigens is critical, as metabolic

changes due to the presence of the tumor [139] or potential differences that affect blood chemistry such as psychological stress, time of blood draw, time since last meal, or uncontrolled differences in specimen handling [140,141] can introduce lethal flaws in methods for biomarker discovery.

Conclusion

Over the last few years, the identification of serum biomarkers for OC has mobilized tremendous efforts. Researchers have explored different compartments, from the cancer cell itself to the immune response directed against the tumor, via the extracellular matrix, the vasculature, and the patient's fluids (blood, urine, ascites) using numerous approaches that spanned from transcripts to post-translational modifications. Overall, these studies have yielded numerous markers that unfortunately seem to perform at best similarly to CA125. So far, no novel OC biomarkers seem to stand out.

While the windows of opportunity for unique biomarker identification appear to get narrower, other fields of discovery remain relatively unexplored. The non-cancerous compartment of the tumor (surrounding stroma and blood vessels) is genetically more stable and may represent a less heterogeneous environment among patients that can be mined for biomarkers. Furthermore, the field of post-translational modifications, particularly glycomics, has given rise to promising data, but limitations have arisen, essentially due to the lack of appropriate affinity reagents.

Multimarker panels are currently felt to be the best strategy for sensitive and specific screening for OC. However, some panels have received severe criticism, in part because the panels were not expected to perform well in large populations with heterogeneous genetic backgrounds, and also because the clinical interpretation of results may be confusing. Several studies were also criticized because of a design that did not allow optimal performances in large populations with heterogeneous genetic backgrounds, therefore potentially leading to unmanageably high level of false positive findings.

In conclusion, although current findings emerging from the identification of novel biomarkers may seem discouraging [20], we believe that with the recent development of appropriate technologies, the mining of underexplored yet promising fields like tumor vasculature markers or post-translational modifications (glycomics) will permit the identification of OC biomarkers able to accurately detect OC at early stages.

Executive Summary

- Conventional OC screening tools are ineffective for the general population.
- Mouse immunizations with OC cell lines or patient cells led to hybridoma validation by ELISA, and flow cytometry analysis permitted the discovery of CA125 and mesothelin. Comparative hybridization of cDNA arrays led to the identification of HE4. All three tests are now commercialized.
- Biomarker identification through proteomic approaches is limited by the extremely low biomarker concentration in complex proteomes, generating technical difficulties partially overcome by fractionations.
- Biomarker identification through immunoproteome mining led to the identification of autoantibodies with no or very low affinities for tumor antigens, which is difficult to explain with the current knowledge of cancer biology.

- Biomarker identification through tumor vasculature mining is promising due to the genetic stability of the tumor vasculature to compare with tumor cells. Yet this approach as an alternative source for biomarker discovery is still underexplored.
- Biomarker identification through glycomic approaches is another promising field for biomarker discovery but it has been severely restricted by the lack of adequate high affinity reagents able to bind to glycan epitopes.
- Multimarker panels have the potential for high PPVs, but careful validation with appropriate sample cohorts is mandatory and complex algorithms may be difficult to implement for routine clinical use.

Future Perspective

The high fatality-to-case ratio of OC that contrasts with the disease's favourable outcome when detected early, combined with the lack of specific symptoms, has lead numerous research groups to focus on biomarker discovery for early detection of OC. Although the strong efforts deployed by the field for the identification of novel OC biomarkers have not yet yielded on their early promise, many lessons have been learned that will help to identify genuine biomarkers in the near future.

The most important realization is probably that OC is a highly heterogeneous disease. Molecular analysis revealed a large degree of variability among patients, and understanding this variability may be key for the development of tests able to detect the various phenotypes of OC. These differences are multifactorial and therefore investigating tumor/host interactions such as immune responses and angiogenesis may translate into the next generation of biomarkers for OC early detection.

Yet pinpointing small alterations linked to early stage cancer remains exceedingly challenging. Discovery and detection of such alterations usually stumbles upon the lack of specificity and sensitivity offered by the current technologies. With the launching of the proteomic and glycomics eras, we anticipate many breakthroughs arising from high-throughput approaches. Progress has also been made in the field of affinity reagents, and the limitations inherent to conventional antibody approaches can now be circumvented. For example, and in sharp contrast with more conventional approaches, novel discovery platforms such as phage- or yeast-libraries that display large diversity of recombinant antibodies, require only modest quantities of non-immunogenic molecules, thus making it possible to identify non- or weekly-immunogenic candidates, which is a crucial feature for a disease such as OC that triggers the emergence of tolerogenic molecules.

Lastly, tools for appropriate validation of biomarkers at large, including heterogeneous cohorts that accurately reflect the general population, will become the indispensable companions of tomorrow's marker discovery. Indeed, large biobanks of sera and other fluids or tissues, associated to thorough documentation of patient history, are built up in various places. These costly but necessary efforts will finally give researchers the possibility to quickly and accurately assess novel candidate biomarkers.

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Table 1

Gene	Description	Identified as an ovarian TVM by	Transmembrane	Extracellular
ADAM12	ADAM metallopeptidase domain 12 (meltrin a)	[1] & [2]	✓	✓
CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	[1] & [2]		✓
EGFL6	EGF-like domain, multiple 6	[1] & [2]		✓
FJX1	Four jointed box 1 (Drosophila)	[1] & [2]		✓
FZD10	Frizzled homologue 10 (Drosophila)	[1] & [2]	✓	
MXRA5	Matrix-remodeling associated 5	[1] & [2]		✓
PLXDC1 (TEM7)	Plexin domain containing 1	[1] & [2]	✓	✓
TNFAIP6	Tumor necrosis factor, a-induced protein6	[1] & [2]		✓
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	[1] & [2]		✓
BDKRB2	bradykinin receptor B2	[1]	✓	
C6orf69	potassium channel tetramerisation domain containing 20	[1]	✓	
CD24	CD24 molecule	[1]	✓	
CLDN1	claudin 1	[1]	✓	
COL11A1	collagen, type XI, alpha 1	[1]		✓
COL15A1	collagen, type XV, alpha 1	[1]		✓
COL22A1	collagen, type XXII, alpha 1	[1]		✓
DEFB1	defensin, beta 1	[1]		✓
DSG2	desmoglein 2	[1]	✓	
EPB41L3	erythrocyte membrane protein band 4.1-like 3	[1]	✓	
ESM1	endothelial cell-specific molecule 1	[1]		✓
F2RL1	coagulation factor II (thrombin) receptor- like 1	[1]	✓	
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	[1]	✓	
GPM6B	glycoprotein M6B	[1]	✓	
GPR105	G protein-coupled receptor 105	[1]	✓	
IGSF4	immunoglobulin superfamily, member 4	[1]	✓	
KCNK5	potassium channel, subfamily K, member 5	[1]	✓	
NID2	nidogen 2 (osteonidogen)	[1]	✓	✓
OlfML2B	olfactomedin-like 2B	[1]	✓	✓
PCDH17	protocadherin 17	[1]	✓	
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	[1]	✓	
SLAMF8 (BLAME)	SLAM family member 8	[1]	✓	
SLIT2	slit homolog 2 (Drosophila)	[1]	✓	✓
SPON1	spondin 1, extracellular matrix protein	[1]		✓
STC2	stanniocalcin 2	[1]		✓
TACSTD1	tumor-associated calcium signal transducer 1	[1]	✓	
THY1	Thy-1 cell surface antigen	[1]	✓	
CKLFSF8	Chemokine-like factor superfamily 8	[2]	✓	✓

Gene	Description	Identified as an ovarian TVM by	Transmembrane	Extracellular
COL5A3	Collagen, type V, a3	[2]		✓
DKFZP586- H2123	Regeneration-associated muscle protease, transcript variant 2	[2]		✓
G1P2	IFNa-inducible protein (clone IFI-15K)	[2]		✓
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92- kDa type IV collagenase)	[2]		✓
MUC1	Mucin 1, transmembrane, transcript variant 4	[2]	✓	
POSTN	Periostin, osteoblast- specific factor	[2]		✓
SPP1	Secreted phosphoprotein 1(osteopontin, bone sialoprotein I, early T- lymphocyte activation 1)	[2]		✓
STC1	Stanniocalcin 1	[2]		✓

[1] Buckanovich, R.J., D. Sasaroli, A. O'Brien-Jenkins, *et al.*: Tumor vascular proteins as biomarkers in ovarian cancer. *J Clin Oncol* 25, 852–61 (2007).

[2] Lu, C., T. Bonome, Y. Li, *et al.*: Gene alterations identified by expression profiling in tumor-associated endothelial cells from invasive ovarian carcinoma. *Cancer Res* 67, 1757–68 (2007).