EXPERT REVIEWS

Ovarian cancer biomarkers for molecular biosensors and translational medicine

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Multiple omics researches in the past two decades have identified over 200 potential biomarkers for ovarian cancer. Discoveries during the 1990s were more focused on clinicopathology-based biomarkers that were targeted to support diagnosis, but the emphasis has shifted to the identification of prognostic biomarkers in the past 10 years. The post-genomic era has opened the door for personalized cancer treatments and the trend of discovery is moving forward to identify more stratified biomarkers to accurately predict the progression of disease, as well as efficacy biomarkers to precisely determine drug response. To better meet future challenges, biomedical research needs the reformed and standardized infrastructure of tissue banks/biorepositories, with national and international initiatives. Of the hundreds of biomarker candidates for ovarian cancer, only a small number are actively being validated with clinical samples, owing to the lack of biomaterials that are linked with accurate clinical data. The purpose of this article is to present selected biomarkers from the past 20 years of ovarian cancer research, placing special emphasis on biomarkers that are strongly associated with positive or negative clinical outcomes. The article also presents a global view of all known potential biomarkers and mutations for ovarian cancer from NCI's Cancer Gene Index developed by Sophic , and Sanger's Catalogue of Somatic Mutations in Cancer database.

Keywords: biomarker • biosensor • ovarian cancer • personalized medicine • tissue bank

Ovarian cancer kills over 125,000 women worldwide each year and kills more women than all other gynecologic cancers combined. In the USA alone, over 15,000 women die every year as a result of ovarian cancer, ranking the disease as the second deadliest cancer for women and the fifth leading cause of cancer death in women [201]. Early stage (I/II) detection has a survival rate of over 90%, but only approximately 20% of all reported cases are caught in the early stages; the 5-year survival rate is approximately 11% when detected in the advanced stages (III/IV) [202]. Symptoms of ovarian cancer are complex and often misdiagnosed as other diseases, but recent developments prescribe more well-defined clinical symptoms for a better diagnosis. Current treatment options, including surgical resection methods and various chemotherapies, have improved for late-stage ovarian tumors, but recent statistics demonstrate that less than a 10% improvement has been made for the 5-year survival rate during the past 35 years [1].

These statistics suggest that more research efforts related to the discovery of ovarian cancer biomarkers are required. Identification of robust and accurate biomarkers for early detection and diagnosis will prevent major misdiagnosis and provide better cancer patient care. A robust detection method based on molecular profiles for ovarian cancer has not yet been established because the disease exhibits a wide range of morphological, clinical and genetic variations during the course of tumor progression. Owing to these cellular and molecular characteristics, identifying appropriate treatments for the disease is a major clinical challenge. Several recently published review articles provide a good overview of ovarian cancer from a molecular and clinical perspective [2–4], and discussions of these perspectives will not be repeated in this article.

Another major challenge for identifying biomarkers is the lack of availability of high-quality normal and cancerous tissue and biofluid biospecimens that are associated with accurately documented clinical data [5]. This is the result of the workflow of the typical clinical setting. Frequently, most of the tumor specimens from surgical resection are used by the pathology

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department to complete the diagnosis of the disease, and the remaining portions are normally archived for up to 20 years, in case they are needed to aid in future patient diagnosis. For larger tumor masses, the leftover tissues are discarded without patient consent, and without the establishment of pre-approved institutional study protocols to salvage and make use of the remaining tissue. Thus, the typical clinical workflow in most regional/community hospitals generates inadvertent situations where only a less than adequate amount of tissue becomes available for biomarker and molecular profiling studies. Efficient tissue banking initiatives can certainly correct these problems.

Multinational efforts in tissue banking (such as those of the Office of Biorepositories and Biospecimen Research and the national biobank initiatives of the NIH/NCI of the USA), close collaboration between academic and community/regional medical centers, and standardization of protocols (spanning from the surgical procedure to the procurement method used by the pathology department) would definitely provide an infrastructure more conducive to biospecimen availability and quality. This kind of reformed infrastructure will enhance biomarker discovery and lay the ground for the construction of the most appropriate assay platforms for ovarian cancer. This article will focus on the molecular diagnostic and prognostic perspective of ovarian cancer by use of potential biomarkers that are already in the literature and in bioinformatics databases, and will discuss how these biomarker candidates can be translated into clinical use.

Clinical methods & molecular profiling

A quantitative and systematic review showed that ultrasonography with color Doppler is a useful preoperative test for predicting the diagnosis of pelvic masses, with a pooled sensitivity of 0.87 and a specificity of 0.92 [6]. Transvaginal sonography is another commonly used detection method designed to provide the size of ovaries via medical imaging technologies. However, the method lacks specificity, is insensitive for early detection and often misses late-stage ovarian tumors [7]. The sensitivity and quantitative design of these technologies tend to depend only on the physical size of the ovary and not on the tumorigenicity of the enlarged mass [8]. The diagnosis of ovarian cancer is complicated due to differences in tumorigenesis origin, including stromal cells (5–10%), germ cells (10–15%) and epithelium-surface cells (>80%) [2]. In addition, many genetic alterations and chromosomal aberrations contribute to the existence of over 100 subtypes for histologic classification of ovarian tumors [9–11]. For this reason, an effective diagnosis would be arrived at more effectively via a combination of robust biomarkers specific for ovarian cancer and current clinical methods.

Over a decade of omics studies have identified many potential biomarkers for ovarian cancer but only a few are currently used in the clinic. Most of this omics research has been carried out at the levels of cancer cell lines and retrospective formalin-fixed, paraffin-embedded biospecimens. Moreover, the newly discovered biomarkers have not yet convinced pathologists, who are the decision makers in the clinic. Many of the molecular signatures/biomarkers from previous reports are related to the differentiation of tumors from normal tissues or the identification of subtypes/clinical phenotypes of cancer. However, most clinicians believe that such clinical phenotypes can be systematically determined by experienced pathologists using established methods, and that only a few of the previously reported biomarkers make suitable adjuncts to help extrapolate on the already established practices of the pathology department. Furthermore, clinicians argue that these limited molecular profiles provide less clinical impact as classifications and nomenclatures continue to evolve on complex clinical phenotypes for ovarian tumors. To counter such arguments, researchers should focus more on identifying stratified and predictive biomarkers for the future. Indeed, there is a need for molecular tools that are based on robust biomarkers specific to clinical outcomes of ovarian cancer subtypes, and for assay platforms that can be easily adapted for routine clinical use. This type of molecular diagnostics can suggest appropriate treatment options based on patients' genomic or proteomic profiling, either from cancer cells or biofluids, including blood and urine. However, developing such diagnostics is a difficult task for researchers that do not have patient biospecimens associated with clinical outcome data. It is thus crucial to raise awareness of the fact that national and international efforts are needed to promote the following: tissue banking; patient and general population education about tissue/blood donations; active participation from patients for donations; and the use of electronic medical records (EMR) to associate scientific data with accurate clinical outcomes.

The task of incorporating molecular profiles into the routine clinical workflow and the diagnostic process may come naturally in the future. To translate molecular signatures into the clinical setting, researchers must identify a set of robust biomarkers that are indisputably accepted by the pathologists in medical centers. These biomarkers can be a combination of genes, proteins, miRNAs, SNPs and mutations that are derived from tissues and biofluids. Biomarkers from biofluids (i.e., blood) would provide more benefits to patients because they are routinely collected in the clinic, are minimally invasive and can be procured and stabilized immediately for future molecular studies. Furthermore, biofluid biomarkers are particularly important for early screening at community events because ovarian cancer is normally asymptomatic until the tumors reach an advanced stage (III/IV). The clinic expects many more potential biomarkers for ovarian cancer to be discovered as we move forward from the postgenomic era to meet the age of the proteome and metabolome. However, simply discovering hundreds of more potential biomarkers will not persuade the clinic; it is not about the quantity but the quality of the clinically relevant biomarkers. The clinic may not accept a profile of hundreds of biomarkers but a handful of the most robust and specific biomarkers that will contribute to accurate diagnosis. This may be the only route to make sure molecular profiling or biomarker assays become an integrative part of the clinical workflow.

Ovarian cancer biomarkers that were discovered in the 1990s are listed in Table 1 and only a couple of these are mainly used in the clinic. In fact, the cancer antigen 125 (CA125/ MUC16) assay is the most used clinical biomarker for ovarian cancer. It has become a semi-mandatory test in the clinic because a rise in

Table 1. Ovarian tumor markers discovered during the 1990s†.

Marker	Full name	Diagnostic/ prognostic	Expression	Localization	Sensitivity	Specificity	Description	Ref.
B2M	β2-microglobulin	Diagnostic	High	Serum	87%	48%	Suitable tool to monitor course of disease when used in combination with CA125	[47]
CA54/61	Mucin-type glycoprotein antigen	Diagnostic	High	Serum	~50–76%	91%	In case of mucinous cystadenocarcinoma Sensitivity 65% (compared with 36% of CA125)	[51]
CA72-4	Cancer antigen 72-4/ TAG-72	Monitoring/ diagnostic	High	Serum/tissue	High	Fair	Discriminates negative serous adenomas from positive serous carcinomas	[108,109]
CA125 II	Cancer antigen 125 II	Diagnostic	High	Serum	Fair	Low	More precise than CA125	[110]
CA602	Cancer antigen 602	Diagnostic	High	Serum	92%	Low	100% sensitivity in serous adenocarcinoma 67% of sensitivity in mucinous adenocarcinoma	[111]
caGT	Cancer-associated galactotransferase antigen	Diagnostic	High	Serum	75%	90%	8/9 in clear cell carcinoma	[112]
Cathepsin B	Cathepsin member B	Preoperative differential diagnosis	High	Serum	100%	Low	Serum level is fairly proportional to FIGO stage; serous > endometroid tumors ($p < 0.001$)	[113]
CD34	CD34 molecule	Prognostic	High	Tissue	Low	Low	Blood vessel count related to lower overall survival ($p = 0.022$)	[73]
COX-1	Cyclooxygenase-1	Diagnostic	High	Serum	68%	Fair	Serum level is proportional to tumor progression	[50]
GAT	Glyphosate *N*-acetyltransferase	Diagnostic	High	Serum	~47.9–52.9%	94%	Differential diagnosis from endometriosis	[114–116]
IAP	Immunosuppressive acidic protein	Diagnostic	High	Serum	89.5%	91.9%	Early detection of recurrence	[117]
M-CSF	Macrophage colony-stimulating factor	Diagnostic	High	Serum	61%	92.7%	Serum level is useful in detecting ovarian cancer	[37]
nm23-H1	Non-metastatic cells 1, protein (NM23A)	Prognostic	Low	Tissue	Fair	Fair	Inverse association with metastatic potential	[74]

†Most of these potential biomarkers can be used for diagnostic purposes but are not currently used in the clinic.
FIGO: International Federation of Gynecology and Obstetrics.

Table 1. Ovarian tumor markers discovered during the 1990s[†] (cont.).

Marker	Full name	Diagnostic/prognostic	Expression	Localization	Sensitivity	Specificity	Description	Ref.
TP53	Tumor protein p53	Prognostic	Low/negative	Tissue	High	Low	p53 expression related to unfavorable prognosis	[104]
Progesterone	Progesterone	Diagnostic	High	Serum	Fair	Low	Mainly related to nonendocrine ovarian tumor volume	[118]
Sialyl SSEA-1 antigen	Sialyl SSEA-1	Diagnostic/prognostic	High	Serum	Low	Fair	Differential diagnosis with other markers	[53]
TNF receptor	p75/p55	Diagnostic	High	Peritoneal fluid	84/54%	60/95%	Proportional to peritoneal fluid quantity and stage of disease	[119]

[†]Most of these potential biomarkers can be used for diagnostic purposes but are not currently used in the clinic.
FIGO: International Federation of Gynecology and Obstetrics.

CA125 during or after treatment correlates with progression of ovarian cancer [12]. The expression of CA125 is elevated above normal (control) serum in early-stage ovarian cancer and is significantly upregulated in late-stage ovarian tumors; however, it lacks specificity (it has been detected in multiple human cancer types) and sensitivity for ovarian cancer (only 23% in stage I ovarian cancer, in contrast to more than 80% in advanced ovarian cancer) [13–15]. For example, CA125 expression correlates with serous ovarian tumors, but the overexpression is also frequent in benign conditions (i.e., endometriosis). Thus, it lacks accurate diagnostic value for early-stage ovarian cancer. Although the US Preventative Services Task Force (USPSTF) recommended against routine screening for ovarian cancer using CA125, it is still most commonly being used to evaluate patients with early signs and symptoms of ovarian cancer [USPSTF recommendation statement]. Recent review articles provide excellent updates on its clinical use and case studies [16,17]. Owing to its limited specificity, a general consensus suggests that CA125 should be used in combination with several other markers and methods, such as mucin and fluorodeoxyglucose (FDG)-PET [18,19]. Undoubtedly, ovarian cancer care will meet a bright future as the combination of advanced imaging technologies and the identification of more ovarian cancer-specific biomarkers continues.

Potential biomarkers for ovarian cancer

Ovarian cancer biomarkers can be found in various tissue origins, including epithelial cells, the sex cord-stromal component, or germ cells. The majority of advanced-stage tumors are of epithelial cell origin, and less than 10% arise from nonepithelial cells; however, over 40% of early-stage tumors are comprised of nonepithelium [20]. Multiple omics research have identified many potential biomarkers for ovarian cancer and these candidates can be extracted through a bioinformatics approach. Supplementary Table 1 [203] shows an extensive list of potential ovarian cancer biomarkers extracted from the NCI Cancer Gene Index [204,205]. The index is the end product of NCI, Sophic (Sophic Systems Alliance Inc., MD, USA), Biomax (Biomax Informatics AG, Munich, Germany), NCI data mining and manual curation project. Bioinformaticians used the Biomax BioLT Linguistic Tool to mine 18M Medline abstracts, 94 million sentences and validate and manually annotate the role codes and evidence codes for 6955 cancer genes. For this project, NCI specified that a 'cancer gene' is any gene that co-occurs in a sentence with a cancer term from the NCI thesaurus. Each suspect cancer gene identified in the automated mining process was, in turn, manually validated and annotated by the NCI's role codes and Karp's evidence codes [21]; Karp's codes were used to specify the methods used by the authors to generate data and conclusions in publications. The use of the NCI Cancer Gene Index is an excellent approach for generating a shortlist of candidate cancer biomarker genes and to extrapolate further in studying cancer gene–disease subtypes and cancer gene–compound/treatment relationships.

Supplementary Table 1 was generated by the collaborative efforts of the Cancer Research Program of the Cancer Center of Hackensack University Medical Center (HUMC, NJ, USA) and the Sophic

Systems Alliance, Inc. Biomax BioXM Knowledge Management module was used to mine and further curate the shortlist of candidate biomarker genes in the Cancer Gene Index. Ovarian cancer genes were generated through automated methods combining search terms such as 'ovarian, cancer, tumor, biomarker, overexpression, upregulation, downregulation and differential expression'. This combination of automated search, step process curation methods using high-quality data sources allows researchers to identify and test candidate biomarkers without 'reinventing the wheel' (i.e., without repeating omics experiments). This approach can reduce the time, effort and cost of exploring clinically relevant cancer biomarkers. However, the biomarkers derived from such informatics methods require further analysis and rigorous validation steps with clinical biospecimens. The biomarker role codes that are assigned during the manual annotation process do not guarantee the status of a 'true' biomarker. It only means that the annotation scientists found enough evidence in the paper to assign the 'biomarker' role code to a suspect 'cancer biomarker gene'. The actual biomarker designation can only be achieved through sufficient bench work and clinical validation.

In addition to the differential expression signatures of genes, various genetic alterations (i.e., mutations) in signaling proteins can be excellent biomarkers to link with clinical outcomes. Similar to NCI's Cancer Gene Index, the Catalogue of Somatic Mutations in Cancer (Sanger Institute, Hinxton, UK) database is extremely useful to help initiate mutation/genetic aberration-based biomarker projects. The database is a compilation of 130,000 genetic abnormalities found in human diseases and in hundreds of cancer types. To generate a systematic and organized readout from the database, BioXM was used to extract the sites of genetic alterations specific to ovarian cancer, and the output was then manually organized (Supplementary Table 2 [203]). The main BioXM query used to search for was 'ovary' as the primary site and only reports with specified histology or subhistology were included in the list (i.e., reports with not specified [NS] histology or subhistology were omitted). Furthermore, only known protein mutations were included while mutations listed as (p.?), (p.(=)), (p0) or (p.0?) were omitted. The analysis of a chart of disease-specific histology/subhistology mutations in the table can help researchers easily identify gene mutations and their relationship to specific ovarian tumor subtypes. One cautionary note here is that more than half of the 137 mutations reported for ovarian cancer are derived from one cell line or tumor sample. To establish clinical values, a large number of biospecimens with clinical outcome data and rigorous validation processes are required.

It would be interesting from the systems biology perspective to identify clusters that may represent common signaling pathways, transcription activations, metabolic end points and common functional outputs from the list of putative biomarkers (Supplementary Tables 1 & 2) for ovarian cancer. At a larger scale, cross-examination of the list of the putative biomarkers with other known high-quality sources, such as the Sophic Druggable Genome Database, USC Toxnet toxicology database, compound, clinical trials and state/national clinical outcome or EMR, may generate target molecules that are more relevant to clinical use. The discovery of clinical biomarkers will be more efficient and productive as more public and private scientific databases become available for integration into bioinformatics platforms.

Several mutation studies have been reported for ovarian cancer and are associated with clinical phenotypes or outcomes. From the compilation of the mutation data in Supplementary Table 1, and from the findings of a recent report [11], some gene mutations are associated with specific subtypes of ovarian cancer: *TP53* mutations are very common in serous carcinoma; *KRAS* mutations are prevalent in adenocarcinomas; *CTNNB1* mutations are common in endometrioid carcinomas, but rare in serous, mucinous and clear cell carcinoma (CCC); and *PICK3CA* mutations are most frequent in CCC. From the biomarker perspective, the compiled data suggest that the different subtypes of ovarian cancer could be distinguished by gene mutations, with the exception of endometrioid adenocarcinomas, which seem to share gene-expression changes with serous carcinomas. The subtypes with deregulated Wnt and/or P13K/Akt signaling (i.e., low-grade endometrioid adenocarcinomas) are distinguished from serous carcinomas [11]. As documented previously in many cancer types, p53 is also an independent marker for poor prognosis in ovarian cancer where 88% of p53 mutations are single amino acid substitutions in exons 5–8 (functional domain) and the rest are frameshift or nonsense mutations [11]. Additionally, mutations on *KRAS*, *BRAF*, *PTEN*, β-catenin (*CTNNB1*) and *TGFBR2* have been reported for mucinous, endometrioid, and low-grade serous tumors [22]; and mutations on *TP53*, *BRCA1*, *BRCA2*, *MLH1* and *MSH2* have been reported for high-grade ovarian cancer subtypes (*MLH1* is not listed in Supplementary Table 1 because its amino acid mutation was listed as (p.?)) [23]. It should be noted that the mutations in Supplementary Table 1 have been linked mostly to tumor staging and malignancies, but not with response to known clinical treatments for ovarian cancer.

Although many potential biomarkers are under development and undergoing preclinical and clinical trials, an established panel composed of multiple ovarian cancer biomarkers for the purpose of diagnostic, prognostic, and efficacy platform does not yet exist. In the near future, a combination of various biomarkers from genomics (genes, miRNA, mutations, SNPs), proteomics (peptides, proteins, modifications) and metabolomics (small-molecule intermediates, hormones, systemic compounds) will provide the clinic with assay platforms to select cancer patients that suit specific treatment options. Biomarker candidates in Table 1 & Supplementary Table 1 can be used to develop multiplex assay platforms that can be used for targeted therapies that are directly associated with the molecular circuitry of individual ovarian cancers as 'personalized' treatments.

The US FDA approved the OVA1 test (Vermillion, Inc., CA, USA) in September 2009 as an adjunct diagnostic assay, and the assay kit has a panel of five biomarkers consisting of transthyretin, apolipoprotein A-1, β2-microglobulin, transferrin, and CA125 [206]. This multiplexed assay platform is designed to guide the referral of women 18 years or older who have an operable pelvic mass to either a gynecological oncologist or a generalist obstetrician-gynecologist for surgery [207]. This kind of multiplex assay for the point of early-screening diagnosis can

reduce the mortality rate of ovarian cancer patients because those who have their initial operation performed by a gynecological oncologist have better survival rates [24]. In preclinical approval trials, OVA1 correctly identified all patients by stage, and the assay has high sensitivity (92.5%) with moderate specificity (42.8%) and positive (42.3%)/negative (92.7%) predictive values [25,208]. Addition of more robust biomarkers that are specific for ovarian cancer in multiplex assays would provide better patient care.

Biomarkers for early detection, diagnosis & disease progression monitoring

Most biomarkers discovered for ovarian cancer are based on clinicopathology (i.e., tumor staging and progression) and not truly intended for early detection. However, biomarkers that could lead to an accurate early detection and diagnosis would significantly increase the disease-free survival. Researchers often select potential biomarkers based on established molecular pathways of ovarian cancer without considering the fact that ovarian tumor-specific biomarkers are not required to be linked to mechanisms of the disease itself, *per se*. Some robust biomarkers may be second or tertiary byproducts in signaling cascades of ovarian tumor progression but not directly involved in molecular circuitry of tumorigenesis of primary ovarian tumors. Nonetheless, clinical contributions from these types of biomarkers can be highly valuable for the sake of early detection and diagnosis. A foremost important development that will benefit patients is the construction of molecular diagnostic/assay platforms that can be used at common community events (i.e., Mother's Day Walkathon) and can routinely screen female populations by simple and minimally invasive methods, such as a finger-prick test or a urine analysis. From the perspective of point-of-screening biomarkers, serum markers that can be used for global screening purposes would enhance preventative cancer care at an early stage of ovarian cancer. For example, creatine kinase B (CKB) is highly expressed in early-stage (since stage I) of ovarian tumor tissues and is significantly elevated in the sera of ovarian cancer patients [26]. Some of the potential biomarkers for the purpose of early detection and community screening are listed in **Boxes 1 & 2**.

Recently, vascular smooth muscle growth-promoting factor (VSGP/F-spondin) was identified to be specifically expressed in advanced ovarian tumors, including metastatic ovarian carcinomas [27]. VSGP/F-spondin is well characterized for its role in exonal guidance, differentiation of nerve cells and neuronal developmental pathways [28], but has not yet been established in cancer. Thymidine phosphorylase (TP; aka platelet-derived endothelial cell growth factor, [PD-ECGF]) activity has also been shown to have significant correlation with malignant and advanced-stage ovarian tumors and to have high activity in the sera of high-grade ovarian patients. This indicates its value as a tissue and serum diagnostic marker for advanced ovarian tumors [29]. This protein is also upregulated in other solid tumors and is involved in tumor growth, metastasis (by inhibiting apoptotic pathways) and inducing angiogenesis; thus, it contributes to aggressive tumor progression and metastasis and poor prognosis [30].

A combination of point-of-care diagnostic biomarkers (i.e., biomarkers that provide timely results for clinical decision-making and patient care), such as serum and urine biomarkers, and pathology reports would generate significantly more accurate diagnosis and prognosis, and it may provide important clues for drug dosing or alternative treatment options for oncologists. Prostasin/PRSS8 is an example of a biomarker that is overexpressed in the sera of patients with epithelial ovarian cancer, and the combination of prostasin and CA-125 provides a detection sensitivity of 92% and specificity of 94% for ovarian tumors [31]. This tryptase/serine protease is also found in other major human cancers, including prostate, bladder, colorectal and pancreatic tumors [32], and recent data suggest that the protein is involved in proteolytic cleavage of the extracellular domain of EGF receptor (EGFR), causing a constitutively phosphorylated receptor that could potentially participate in fueling tumor growth [33]. Furthermore, prostasin is overexpressed in ovarian cancer cell lines and it has been detected in the serum of ovarian cancer patients at levels significantly higher than that of normal controls [33]. Other serum markers that have been reported to monitor ovarian cancer regardless of subtype are MMPs [34], MIF [35], EGFR [36], macrophage colony-stimulating factor (M-CSF) [37] and follicle-stimulating hormone (FSH) [38]. The roles of these proteins in cancer are well documented as signaling proteins that are globally (often not specific for ovarian cancer) involved in tumor progression; thus, extended discussions will be omitted from this article.

The use of urine biomarkers is another noninvasive method to diagnose ovarian cancer. TGF-α was excreted by 79% of patients with ovarian cancer, whereas it was only excreted by 17% of patients with benign tumors and 23% of healthy controls [39]. TGF-α is reported to cross-talk with the EGFR pathway and is involved in the transformation of ovarian tumors [40]. Additionally, its physiological role as a hormonal peptide may be to regulate inflammation and cell proliferation [41]. Furthermore, the level of urinary angiostatin (uAS; a proteolytic fragment of plasminogen) was high in patients with epithelial ovarian cancer (EOC) and may serve as a diagnostic or prognostic biomarker [42]. Its role in cancer may be associated with the anti-inflammatory and nuclear factor (NF)-κB pathways [43,44] and inhibiting migration of immune cells [45]. The level of urinary mesothelin (SMRP) also correlates with early and late disease and shows greater sensitivity to early-stage ovarian cancer [35]. A recent report suggests its use in combination with HE4 or CA125 for detection of ovarian carcinoma [46].

Several reports have suggested advantages of combining CA125 with other candidate biomarkers. β2-microglobulin alone has the same sensitivity as CA125 but with a lowered specificity of 48%; however, the sensitivity and the specificity were significantly greater when both biomarkers were used in combination [47]. A multiplex panel consisting of apolipoprotein A1, a truncated form of transthyretin, a cleavage fragment of inter-α-trypsin inhibitor heavy chain H4 and CA125 is used to detect early-stage ovarian cancer; the panel can achieve a sensitivity of 74% (65% CA125 alone) and a specificity of 94% (52% of CA125 alone) [48]. Another multiplex panel uses a combination of CA125, C-reactive protein (CRP), serum amyloid A (SAA), IL-6 and IL-8 to achieve a sensitivity of

92.3% and specificity of 91.3% for early-stage ovarian cancer [49]. Similarly, the sensitivity of COX-1 is 68% when used alone but increases to 87% when used as a duplex assay consisting of COX-1 in combination with CA125 [50]. The clinical value of CA54/61 is also greater for ovarian cancers when used with CA125, since this duplex method can distinguish between cancer and benign tumors [51]. IL-7 used in combination with CA125 , also, has an advantage over CA125 alone in that it can correctly identify ovarian cancer versus a benign pelvic mass [52]. Furthermore, expression levels of serum sialyl SSEA-1 antigen have proven to be correlated with the stage and effect of therapy, especially when used with TPA, IAP, ferritin, CA19-9 and CA125 [53].

Human epididymis protein 4 (HE4) is a promising biomarker. Reported data from the NCI's Early Detection Research Network (EDRN) and the Prostate, Lung, Colorectal and Ovarian (PLCO) at the American Association for Cancer Research (AACR) in 2009 concluded that HE4 nearly matched CA125 for early detection of ovarian cancer [209]. However, some reports present opposing views regarding HE4 [54]. In general, HE4 either matches or exceeds CA125 in sensitivity and specificity, and appears to surpass it in terms of distinguishing cancer from benign tumors [25,55,56]. For example, HE4 has better sensitivity (96.9% compared with 85.7%) and specificity (96.3% compared with 79%) than CA125 at the time of diagnosis, and the overexpression of HE4 preceding that of CA125 is associated with a relapse of the disease [57]. A combination of HE4 with CA125, carcinoembryonic antigen (CEA), and VCAM-1 in an assay panel has been proposed for detecting early stage ovarian cancer versus benign tumors owing to its sensitivity of 86% [58]. Another study measured the combined levels of CA125, HE4, and mesothelin in patient sera to predict impending cancer as early as 1 year prior to clinical diagnosis [59]. In all cases, HE4 expression was present in only specific subtypes of ovarian cancer; 93% in serous, 100% in endometrioid EOC, 50% in carcinomas, and 0% in mucinous tumors [60].

Some biomarker developments focus on identifying diagnostic biomarkers for specific ovarian cancer subtypes. For example, Wilms tumor protein (WT-1) has been documented to be a highly sensitive and specific marker of serous carcinomas of ovarian surface epithelial origin with nuclear staining (negative for other subtypes) and mostly negative for other major tumor types (including breast, colon and lung cancer tissues), indicating that such markers would fit well with molecular pathological work in the clinical setting [61]. Moreover, positive expression of this marker is directly associated with an unfavorable prognosis in all the patients with ovarian carcinomas (risk ratio [RR]: 1.7; 95% CI: 1.2–2.3%), but is a favorable prognostic marker within the high-grade serous subtype (RR: 0.5; 95% CI: 0.3–0.8%). This indicates that this marker can also be used as a specific prognostic marker for serous carcinomas subtype [58]. As for other ovarian subtype biomarkers, hepatocyte nuclear factor (HNF)-1β and immunosuppressive acidic protein (IAP) have been reported to be diagnostic markers for CCC of the ovary, where HNF-1β overexpression is specific for CCC and detected in peritoneal fluid, and IAP is implemented to be used as a follow-up marker, as well as in early detection for recurrence when nuclear staining is positive [62,63].

Box 1. Diagnostic biomarkers.

Stage-nonspecific biomarkers: diagnosis & follow-up

Serum
- CA125
- HE4
- Prostasin
- IAP

Tissue
- hK6,7
- HNF-1β
- WT-1

Urine
- TGF-α
- uAS
- Mesothelin

Tumor stage-specific biomarkers: early stage

Serum & tissue
- CKB

Tumor stage-specific biomarkers: later stages

Serum & tissue
- TP

Tissue
- CLIC4
- VSGP/F-Spondin

Screening of ovarian cancer relapse
- FDG-PET with CA125

Serum
- IAP

CA125: Cancer antigen 125; CKB: Creatine kinase, brain; CLIC4: Chloride intracellular channel 4; FDG-PET: Fluorodeoxyglucose-PET; IAP: Immunosuppressive acidic protein; TP: Thymidine phosphorylase; uAS: Urinary angiostatin; VSGP/F-spondin: Vascular smooth muscle growth-promoting factor; WT-1: Wilms tumor 1.

Prognostic biomarkers

An unfortunate statistic of ovarian cancer is that one out of 57 women who visit the clinic have ovarian tumors and approximately 60% of them are already in advanced stage (National Cancer Institute). To provide more effective cancer care, validated prognostic and stratified biomarkers are needed to predict the course of the disease and the responses to specific drugs used for treatments, especially for front-line therapies on chemo-naive patients.

Recent reports suggest several prognostic end point biomarkers that can be implicated in multiple ovarian subtypes and can thus be used as global prognostic tools to predict survival as the final outcome. For example, overexpression of topoisomerase II, correlates more with rapidly progressing late-stage (mean 2-year survival) ovarian cancer than with long-term survival (mean 11 years; $p = 0.0001$) with 88% specificity and 94% sensitivity, suggesting that it has prognostic value for advanced ovarian cancer [64,65]. Increased protein level of tissue plasminogen activator (tPA) in plasma is another global prognostic biomarker

Box 2. Prognostic biomarkers and biomarkers related to drug efficacy.

Prognostic: favorable

Serum
- hK8

Tissue
- hK13
- hK11
- PR

Prognostic: unfavorable

Serum
- hK10
- IGFBP-2
- YKL-40
- tPA

Tissue
- $\alpha_v\beta_6$ integrin
- α-V integrin
- β III tubulin
- CD24
- c-Ets1
- EMMPRIN
- GEP
- Indoleamine 2,3-dioxygenase
- M-CAM
- p-glycoprotein
- Topoisomerase II
- WT-1

Drug efficacy

Serum
- CASA
- Tetranectin
- YKL-40

Tissue
- Indoleamine 2,3-dioxygenase
- ATP7B

CASA: Cancer-associated serum antigen; EMMPRIN: Extracellular matrix metalloproteinase inducer; GEP: Granulin-epithelin precursor; PR: Progesterone receptor; tPA: Tissue plasminogen activator.

associated with shorter survival ($p = 0.0003$) for multiple subtypes of ovarian cancer, but the expression pattern does not discriminate between benign and malignant tumors [66]. Similarly, high expression of YKL (chitinase 3-like 1)-40 is a prognostic tumor marker in recurrent ovarian cancer ($p < 0.001$), where relapsing patients with high plasma level of YKL-40 had significantly shorter survival than patients with low level of YKL-40 in plasma [67]. Patients with negative COX expression also have significantly shorter survival compared with patients with COX2-positive tumors [68].

Positive VEGFA and inducible nitric oxide synthase-negative tumors are global prognostic ovarian cancer biomarkers that have been associated with improved progression-free survival in patients with macroscopic complete tumor recession [69]. The cytokines IL-6, IL-7, IL-8, IL-10, MCP-1 and IFN-γ-inducible-protein (IP)-10 and CA125 are also associated with disease-free and overall survival in univariate analysis; and, in the case of multivariate analysis, IL-7 and IP-10 were independent predictors of overall survival [52]. The frequency of α_V integrin expression, on the other hand, is higher in short-term survival ovarian carcinoma cells and correlates with poor survival based on multivariate survival analysis, suggesting that this subtype of integrin can predict outcomes in advanced-stage ovarian cancer [70]. The expression of $\alpha_V\beta_6$ integrin increases gradually as ovarian tumor progresses from benign to late and metastatic stages, suggesting that the levels of expression can be used for prognosis at different stages of disease progression [71]. A meta-analysis demonstrated that overexpression of CD24 is related to advanced clinical stages (odds ratio: 1.59; 95% CI: 1.244–2.032; $p < 0.001$) and shortened overall survival (hazard ratio: 2.13; 95% CI: 1.656–2.730; $p < 0.001$) [72]. Additionally, CD34 correlates with ovarian neovascularization [73] and the nm23-H1 gene product correlates inversely with metastatic potential [74].

In addition to those already mentioned, several other global ovarian cancer serum prognostic biomarkers have been reported. Although not specific for ovarian cancer, HER-2 is overexpressed in over 50% of ovarian carcinomas and the overexpression correlates with reduced survival with patient age and tumor stage ($p = 0.021$ for early stage and $p = 0.0054$ for advanced stages) [75]. The level of tPA does not associate between tumor stages of ovarian cancer, but it has prognostic value because its level in serum is significantly higher (>9 ng/ml) in shorter survival, particularly in patients with poorly differentiated tumors [76]. Similarly, the level of endostatin in serum has moderate correlation with advanced ovarian tumors when compared with normal controls, but it would not be a robust prognostic marker [77]. Haptoglobin and CRP levels in preoperative serum in ovarian cancer patients have significant correlation with poor outcome for overall survival in multivariate analyses ($p = 0.036$), and the association with prognostic value is stronger in late-stage ovarian cancer [78]. Serum tetranectin and CA125 have reciprocal prognostic values for advanced stage ovarian tumors versus early and localized ovarian cancer, respectively [79]. For combined biofluid analyses, high serum FSH and low ascites estradiol levels correlate with recurrent ovarian cancer, and FSH in ascites has prognostic value for a favorable outcome [80]. Low levels of IL-1 RA in serum ($p = 0.004$) and ascites ($p = 0.05$) correlates with favorable clinical outcome and increased post-operative progression-free survival in ovarian cancer patients, particularly in serous-papillary subtypes [81].

Several reports on prognostic biomarkers are associated with specific subtypes of ovarian cancer. Increased expression of indoleamine 2,3-dioxygenase is associated with advanced-stage serous-type ovarian cancer that is resistant to paclitaxel, and with impaired overall survival in patients ($p = 0.0001$) with serous-type ovarian cancer [82,83]. Progesterone receptor (PR) overexpression is associated with a favorable prognosis and better survival ($p < 0.001$) in multiple ovarian cancer subtypes but has stronger

prognostic value with endometrioid types [84,85]. Extracellular matrix metalloproteinase inducer (EMMPRIN) is an adhesion protein that is overexpressed in advanced-stage ovarian cancer and has shown strong correlation with progression of metastatic serous ovarian tumors when compared against early-stage tumors [86].

In some cases, the overall cellular expression of biomarkers does not significantly change, but mislocalization in subcellular compartments will either cause loss or gain of functions. Conventional omics methods are not designed to identify such types of biomarkers, but the progress in high-throughput imaging technologies would enhance the discovery of biomarkers that are represented by proteins that have altered subcellular localizations. For example, an increased cytoplasmic expression of CD24 correlates ($p = 0.0002$ from 69 ovarian tumors) with short patient survival with a mean of 98 versus 37 months, suggesting significant prognostic value in altered localization [87]. Although lacking association with robust clinical outcomes, intracellular chloride channel-4 (CLIC4) has been reported to have reciprocal expression along with altered subcellular localization (nuclear in normal to cytoplasmic in cancer) in a progressive manner in ovarian tumors [88].

Expression of proteins in tumor-activated stroma/fibroblasts in advanced-stage ovarian cancers can serve as excellent biomarkers for prognosis. Interestingly, CLIC4 expression is dramatically downregulated in primary tumor tissues while significantly upregulated in neighboring tumor stroma as tumors progress to advanced stages, suggesting that this reciprocal expression pattern of CLIC4 may have a role for both diagnosis and prognosis [88]. Similarly, granulin–epithelin precursor (GEP) expression is preferentially low in metastatic ovarian carcinomas, where the expression is downregulated in malignant effusions, and its reciprocal overexpression in the stromal component is strongly associated with poor overall survival in invasive ovarian tumors [89]. Expression of the proto-oncogene *c-Ets1* is strongly associated with papillary ovarian cancer tissue that has invaded the stromal portion within the tumor microenvironment, providing prognostic value in evaluating progression of metastasis for ovarian cancer [90].

Kallikrein family members are serine proteases that are regulated by steroid hormones and have both diagnostic and prognostic values. Among the favorable markers are human kallikrein 8, 13 and 11 (hK8, hK13, and hK11). The expression of hK8 is high/positive (tumor cytosol protein >26 ng/mg total protein) in ovarian cancer patients with longer progression-free survival and overall survival ($p < 0.05$) and reduced risk of relapse ($p < 0.001$) [91]. Patients with a high expression of hK13 in tumor cytosol in the early stage had an advantage for longer progression-free survival and overall survival ($p < 0.05$), and reduced risk of relapse ($p < 0.007$) and death ($p < 0.002$) [92]. Although not specific to ovarian cancer, hK11 is found in the serum of early-stage patients and its overexpression is significantly associated with decreased risk of relapse ($p = 0.007$) and death ($p = 0.005$). Furthermore, hK11-positive patients have longer progression-free survival ($p = 0.005$) and overall survival ($p = 0.003$) [93,94]. Among the unfavorable kallikrein markers are hK10, hK6 and hK7. The level of hK10 is significantly high in the serum of advanced-stage ovarian cancer patients with high specificity (90% when hK10 >700 ng/l), and it is associated with increased risk for relapse and death ($p < 0.003$) in both Cox and Kaplan–Meier survival analyses [95]. Overexpression of hK6 and hK7 in advanced-stage ovarian cancer patients suffer a progressive form of the disease and frequently have shorter disease-free survival and overall survival ($p = 0.01$), indicating both markers have stronger diagnostic values than prognostic values [96,97]. The expression of hK6 is not a strong prognostic indicator for ovarian cancer, but it can be used in conjunction with other kallikreins, as well as non-kallikrein biomarkers, to create a multiparametric prognostic test for ovarian cancer [98].

Most published ovarian tumor markers are diagnostic or prognostic, but very few are stratified biomarkers. The discovery of stratified biomarkers is based more on drug response and clinical outcomes, and these markers can be used to predict, for example, those that will respond to first-line therapy and experience disease-free survival for over 5 years versus those that will undergo continuous and multiple relapses. Several examples of stratified prognostic biomarkers for ovarian cancer are leading the trend. Similar to indoleamine 2,3-dioxygenase's relation to paclitaxel [82,83], βIII tubulin overexpression is associated with a short period of progression-free survival and with resistance in several paclitaxel treatment models, suggesting its usefulness in identifying patient candidates with worse prognosis for more aggressive therapy options [99]. Another stratified prognostic biomarker, M-CAM, showed a progressive increase in expression as ovarian cancer advances to late stage or in subgroups of patients that relapse post-frontline therapy, indicating a fairly accurate gauge of time-of-progression (positive vs negative; $p = 0.001$) and overall survival ($p = 0.0003$) for advanced ovarian cancer, as well as prognostic and stratification value in the clinical setting [100]. Still another stratified prognostic biomarker is overexpressed p-glycoprotein, which correlates with unfavorable prognosis for progression-free survival ($p = 0.006$) and better response to chemotherapy ($p = 0.001$) when tumor tissues stain negatively in a uniformly treated and followed-up cohort of advanced ovarian cancer patients [101]. CYFRA 21-1 assay (cytokeratin 19) is a stratified prognostic biomarker predictive of the response to chemotherapy in patients with advanced ovarian cancer but is not suited for the prognosis of survival [102]. Furthermore, annexin A3 is overexpressed in patient resistant ovarian cancer [103] and serum CRP levels in ovarian cancer patients have high correlation with treatment resistance, while ovarian tumors with p53-negative status correlated with an overall negative prognosis [104].

It should be noted that a combination of FDG-PET technology and validated serum biomarkers can enhance detection of recurrent ovarian cancer for patients. For example, elevated levels of CA-125 antigen in sera was used in conjunction with FDG-PET to accurately diagnose recurrent ovarian cancers and benign lesions at which the diagnostic sensitivity, specificity, accuracy, and positive- and negative-predictive values reach 100, 85, 94, 92 and 100%, respectively [105].

Efficacy biomarkers

Identifying biomarkers that predict patient responses to specific drugs (i.e., efficacy biomarkers) is challenging since tumors are frequently resected from patients prior to therapies, and

follow-up surgeries are not normally required until the disease recurs. Consequently, the only types of biospecimens that can be collected in a sequential manner to monitor drug responses during treatments are biofluid-related. Although most protocols of early-stage clinical trials involve the collection of all blood- or tissue-derived biospecimens by the sponsoring pharmaceutical companies for detailed molecular analyses, obtaining these biospecimens as shared reagents is often difficult for scientists in academia. More collaborative efforts between industry and academia would enhance discoveries of efficacy biomarkers. Despite this challenge, several important efficacy biomarker findings have been reported. ATPase-copper transporting β polypeptide (ATP7B), for example, has been documented to have a correlation with chemoresistance to cisplatin-based drugs by having its expression significantly increased in patients with moderately to poorly differentiated ovarian carcinomas. Here, cisplatin was found to bind to the NH-terminal copper-binding domain of ATP7B, which might be a contributing factor to cisplatin resistance [106]. In more general terms, high levels of cancer-associated serum antigen or YKL-40, or low levels of tetranectin in patient serum have predictive value for second-line chemoresistance at the time of ovarian cancer relapse [107].

Conclusion

The fit-for-purpose practice of the 1990s was mainly targeted for diagnosis. In the past decade, the focus has shifted to prognosis, with the use of more stringent biomarker development and validation methods. The ovarian cancer community can benefit significantly by looking back to the past 20 years of omics research to find biomarkers that are robust, specific and directly related to clinical outcomes. Through the use of cell lines and patient biomaterials, numerous omics research have produced hundreds of potential biomarkers for ovarian cancer. The front-line practice of using well-established 'knowledge management'-based informatics software will reduce 'reinventing-the-wheel' types of discovery projects and allow for a wealth of new potential biomarkers for ovarian cancer to be managed effectively. Some of the biomarkers mentioned in this article welcome further validation with more samples by independent laboratories. They have a high potential to be translated to the stages of assay and clinical developments for diagnosis and prognosis purposes.

The 'junk-in, junk-out' awareness (i.e., the awareness that the use of low-quality experimental samples will lead to low-quality results) in the biomarker discovery process has helped stir the research community to set up tissue bank/biorepositories, and to focus on biospecimen stability, on the integrity of collection protocols, on the discovery phase quality assurance, and on the establishment of correct reference standards for assays. The national and international initiatives to standardize the procurement, storage and distribution of biospecimens will significantly enhance the identification of clinically relevant biomarkers for ovarian cancer. In addition, the setup of an appropriate informatics infrastructure is required to discover more clinically associated genetic biomarkers (i.e., SNP and mutations), since we are approximately 10 years away from facing an explosion of the personal genome data that will come from every patient that will be treated in the clinic.

Expert commentary & five-year view

Future molecular assay platforms will have different types of biomarkers derived from multiple omics data, including genomics, proteomics, metabolomics, glycomics, lipidomics and toxicomics. Significantly improved and faster validation methods may be available for ovarian cancer research owing to increased regulatory and investigational efforts on behalf of biorepositories and biospecimen research groups at community/regional medical centers, as well as at the national level. A majority of the population (i.e., >80% of the US population) receives medical treatments at community and regional hospitals, and not at university-based academic medical centers; however, patient participation in tissue/blood donations in community clinics is extremely low (typically <2%), and this is a major drawback to all cancer biomarker discovery efforts. The research community only hopes that the national government will provide more prudent measures to increase the availability of biospecimens for biomedical research in the near future.

On a positive note, it is only a matter of time until engineers, physicists, bioinformaticians, biologists and clinicians work together to create biosensors/detection devices that will use a multitude of established biomarkers to measure various clinical end points. Multiple assay platforms will accurately predict the course of disease progression and drug responses; will quantitatively measure the efficacy and toxicity of treatments; and will be able to propose evidence-based alternative treatment options, such as the dosing of chemotherapy regimens or types of drug combinations. The future expects high-throughput automated assay platforms for microfluidic (i.e., biofluid related) and solid-phase biomarker analyses. This format is likely to be multiplexed (e.g., multiplex immunoassays) and to employ multivariate methods that use a set of biomarkers to predict specific clinical end points of interest.

Biosensors are expected to be miniaturized for point-of-screening purpose, and highly advanced robotics would be used for point-of-care assay platforms with less complexity in instrumentation. This, in turn, is to be highly integrated with the ongoing efforts with EMR infrastructure in the clinic. Sensitive biomarkers that are intrinsically inexpensive to detect by assays will have significant advantages owing to their better chances of obtaining insurance coverage and FDA endorsement; thus, they will likely reach the clinical market quickly. Each set of future biomarkers will be specifically designed to measure clinical end points or predict clinical outcomes and simultaneously contribute to clinical decision-making. With all of the advancements mentioned, we expect to provide better care for patients with ovarian cancer in the future.

As electronic health records become semi-mandatory for all medical centers, clinical outcome databases will be readily accessible by researchers and the identification of clinical biomarkers will become more effective. With national and institutional initiatives to develop tissue banks, more patient biospecimens that are associated with clinical data/outcome may become available for biomarker discovery research. Owing to more health regulations and reimbursement policies related to medical treatments, the biomarkers that will be used in the clinic need to be rigorously validated and certified at the national, state and institutional levels. The changes in reimbursement policies

would also require simple, accurate and fast assays rather than comprehensive assay types that are laborious and expensive to perform. Owing to these reasons, future assay platforms will become more multiplex and may include a combination of genes, miRNA, proteins, metabolites and genetic alterations/mutations. The next-generation of deep sequencing in terms of personalized genome sequencing of known mutation spots and known oncogenes) and other newly developed technologies will help to accelerate assay time and the accuracy. One important aspect of all of these biomarker discoveries is that the discoveries start with high quantity and quality of biospecimens. To prepare for better patient care in the future, the population at the national level must be educated about donating their tissues and/or blood for biomedical research, otherwise all leftover tissues at the time of post-diagnoses will be discarded in the pathology department without patient consent.

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Key issues

- A bioinformatics approach can be used to extract putative biomarkers for ovarian cancer and to validate them with biospecimens derived from patients to discover clinical biomarkers.
- The discovery phase of biomarkers should be focused more on stratification of clinical prognosis and efficacy of drugs rather than on the clinical phenotyping (i.e., the ovarian cancer subtype identification) that is currently part of the clinical workflow in pathology departments.
- Only robust, consistent and well-validated biomarkers will be accepted by clinicians; thus, potential biomarkers must go through rigorous validation steps with high-quality clinical samples that may be available from tissue banks.
- The research data of many candidate biomarkers for ovarian cancer are available in the literature (many mentioned in this article), and they may be tested together to formulate a multiplex panel that would provide accurate diagnoses for the clinic.
- The biomarkers that can be identified from patient's biofluids, particularly from blood, should receive more attention from researchers because they are routinely collected in the clinic by minimally invasive procedures and it is relatively easy to receive consent from patients.
- A panel of highly robust biomarkers that represent genes, proteins, microRNA, mutations and metabolites in multiplex format will be valued in the clinic.
- Easy to use biosensors/devices are needed to assay a set of robust clinical biomarkers and the assay procedure must be user-friendly and with fast turnaround-time, allowing nurses and clinical laboratory personnel to deliver better cancer care for our patients.

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