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Proteomics of ovarian cancer: functional insights and clinical applications

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Abstract

In the past decade, there has been an increasing interest in applying proteomics to assist in understanding the pathogenesis of ovarian cancer, elucidating the mechanism of drug resistance, and in the development of biomarkers for early detection of ovarian cancer. Although ovarian cancer is a spectrum of different diseases, the strategies for diagnosis and treatment with surgery and adjuvant therapy are similar across ovarian cancer types, increasing the general applicability of discoveries made through proteomics research. While proteomic experiments face many difficulties which slow the pace of clinical applications, recent advances in proteomic technology contribute significantly to the identification of aberrant proteins and networks which can serve as targets for biomarker development and individualized therapies. This review provides a summary of the literature on proteomics' contributions to ovarian cancer research and highlights the current issues, future directions, and challenges. We propose that protein-level characterization of primary lesion in ovarian cancer can decipher the mystery of this disease, improve diagnostic tools, and lead to more effective screening programs.

Keywords

Ovarian cancer; Proteomics; Biomarker; Drug resistance; Subtypes

1 Introduction

1.1 Epidemiology

Progress concerning prevention, early detection, or overall survival of ovarian cancer has been generally modest over the last four decades. Ovarian cancer (OC) remains the deadliest gynecologic malignancy at advanced age in developed countries, second only to preventable cervical carcinoma in developing countries. According to SEER registry, there have been an estimated 21,980 new cases and 14,270 deaths of ovarian cancer in the United States in 2014. The lack of a practical screening method and the asymptomatic course of the disease

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contribute to the late presentation of the disease. Approximately two thirds of new cases are diagnosed at FIGO stage 3/4 when metastases are already beyond the pelvis, which explains the low 5-year survival rate of 27 % [1]. The early detection of OC is the most essential determinant of survival; it is, nevertheless, still an unachieved goal.

1.2 Disease heterogeneity

Ovarian cancer encompasses a variety of tumors of ovarian origin, each of which has distinctive biological and clinical characteristics. It is a typical example of a heterogeneous disease. In recent years, classification of ovarian tumors has greatly improved due to morphologic, immunohistochemical, and molecular genetic studies [2]. It is now classified into three broad categories based on the main population of cells affected: epithelial cells, germ cells, and stromal cells; of these, epithelial ovarian carcinoma (EOC) represents the majority with 85 to 90 % of all ovarian cancers. EOC is further classified into five subtypes: serous (the most frequent subtype with 70 % of cases), endometrioid, clear cell, mucinous, Brenner tumors, and undifferentiated tumors. There are substantial differences between EOC subtypes regarding genetic risk factors, molecular oncogenesis, mRNA expression, prognosis, and drug response [3]. Furthermore, there is a dualistic model of EOC classification based on clinical and genetic profiles [4]. Type I tumors includes low-grade serous, endometrioid, clear cell, mucinous carcinomas, and Brenner tumors. They are characterized respectively by BRAF, KRAS, PIK3CA, and PTEN somatic mutations, and they are in general indolent, confined to the ovary, and show low sensitivity to chemotherapy. Type II tumors which contain high-grade serous, high-grade endometrioid, carcinosarcomas, and undif-ferentiated carcinomas are, on the contrary, clinically aggressive with late-stage presentation [5, 6]. Genomic instability as well as mutations in TP53 (96 %) and BRCA1/2 (22 %) are common features in high-grade serous ovarian carcinoma (HGSC), which is the most common type in type II, the most common cause of death in OC and the most intensely studied so far [7]. Remarkably, it has been observed that epithelial tumors, namely serous, endometroid, and clear cell carcinomas, share one very interesting feature in that they are morphologically indistinguishable from tissue embryologically derived from Müllerian ducts and not mesothelium which is the epithelium covering of the ovary [8]. Multiple precursor sites have been proposed for every subtype of EOC [9], as if the ovary is acting like a magnet for migrating tumor cells. In the last decade, all studies in which fallopian tubes were examined carefully have confirmed that small *in situ* and early invasive serous tubal intraepithelial carcinomas occurred in women with genetic predisposition for development of HGSC [10]. It is now increasingly accepted among researchers, clinicians, and pathologists that serous epithelial carcinomas arise from the distal fimbriated part of fallopian tube [11, 12]. Startlingly, primary peritoneal carcinoma, which has the same morphological resemblance, genetic drivers, and late clinical presentation as HGSC, is also now hypothesized to originate from tubal epithelium. Therefore, the standard of care for ovarian, fallopian tube, and peritoneal ovarian cancer are similar [13].

1.3 Gene expression patterns

The Cancer Genome Atlas (TCGA) consortium performed integrative genomic and transcriptomic analyses of HGSC specimens and deduced the presence of four subtypes

based on mRNA expression, designated as immunoreactive, proliferative, differentiated, and mesenchymal. Each subtype showed a consistent association with thematic expression markers at the mRNA level. However, these subtypes failed to show any significant association with clinical outcome [6]. Although gene expression studies are a powerful tool for discriminating subtypes, the drivers for these subtypes are still unclear. Published studies have shown a fairly modest correlation between mRNA and protein levels under a variety of conditions [14, 15]. Since proteins are the functional mediators in phenotype characterization, the study of protein expression profiles in genetically annotated tumors was the inevitable next step. This encouraged the National Cancer Institute (NCI) to launch multiple initiatives for employing proteomics in cancer research. The Clinical Proteomic Tumor Analysis Consortium is currently using proteomic techniques to characterize the TCGA series of tumor samples [16]. The first integrative analysis of colorectal carcinoma was recently published [17].

1.4 Decade of proteomic research

The poor prognosis and the lack of a successful treatment in ovarian cancer have driven a decade-long interest in molecular profiling using proteomic technology. Proteomics is the large-scale study of the proteins in order to characterize biologically meaningful subgroups. There are multiple aspects which fall under the scope of proteomics, such as protein identification and quantification, protein–protein interaction, posttranslational modification, and functional analyses. In hindsight, proteomic technology has evolved greatly from gel-based techniques (one- and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE)) [18] to gel-free techniques (reversed-phase protein array (RPPA) [19] and mass spectrometry-based techniques [20, 21]). A shift from the traditional two-dimensional gel electrophoresis to protein microarray and mass spectrometry (MS) techniques has been clear in the last decade. This evolution reflected the trend in modern biology towards sophisticated and comprehensive analyses of biological systems. RPPA is a high-throughput antibody-based technique that provides enhanced sensitivity, quantification, and multiplexing capabilities compared with traditional immunoassays and Western blots. Although TCGA used the RPPA technique in several tumor types, the number of proteins analyzed in a given RPPA experiment is limited by the limited availability of specific antibodies that can detect specific phosophosites or distinguish closely related proteins and protein isoforms with different biological functions [16]. As a result, MS is increasingly becoming the technology of choice for protein identification. Currently, electrospray ionization-MS and matrix-assisted laser desorption/ ionization (MALDI)-MS are the major techniques used in global protein profiling, detection of posttranslational modifications (PTM), as well as global and targeted quantification. In recent years, mass spectrometry-based proteomics made multiple leaps in terms of sensitivity, reproducibility, and reliability. Furthermore, the development of quantitative approaches has opened new avenues for studying the protein differential expression and posttranscriptional and posttranslational modifications in different conditions in an attempt to understand the functional consequences of altered gene expression. Quantitative proteomics has witnessed a breakthrough in absolute and relative quantification techniques: spectral counting, stable isotope labelling by amino acid in cell culture, isotope-coded affinity tags, and isobaric tags for relative and absolute quantification (iTRAQ) (reviewed extensively in [22]). Proteomic

approaches are now uniquely employed in multiple areas of ovarian cancer research: characterization of the mechanism of disease, screening for indicators of the presence of disease in tumor tissue and body fluids, and searching for causes of chemotherapy resistance.

2 Proteomic biomarkers

2.1 Tissue proteomics

Tumor resection surgeries and biopsies serve as an extensive source of tumor tissue for research studies. Due to its practicality for storage in tissue archives, most tissue specimens are formalin-fixed paraffin-embedded (FFPE) blocks. Unfortunately, formalin in these specimens was found to form crosslinks that mask epitopes and reduce the yield of protein from tissue. However, in recent years, analysis of FFPE specimens has improved enormously due to advances in protein extraction protocols (reviewed here [23]). Laser capture microdissection is a novel technique that has been employed to reduce within tumor heterogeneity. Tumor heterogeneity can present a significant challenge within proteomic studies [24, 25]. Furthermore, recent technological developments have enabled global protein analysis of minimum-size specimens. Some proteomic studies exploited these advantages in a retrospective manner to profile proteins in tissue archives of conditions for which clinical outcome and treatment are already known [26].

2.2 Studies of subtype-specific differential expression

The gene expression profiles of EOC subtypes have been extensively studied using mRNA microarrays [27–29]. In contrast, fewer proteomic studies have adopted this approach to measure the protein profile of each subtype [30–33]. Recent studies show less significant changes across grade or stage compared with histologic type [3, 34]. While there have been calls for further classifications based on molecular signatures, protein expression profiling has the power to elucidate the biochemical and cell biologic impact of genomic alterations in different EOC subtypes [35]. An *et al*. used a comparative proteomic approach using 2D PAGE/MALDI-time of flight (TOF)-MS and concluded that the serous subtype showed the most different protein expression profile compared with the normal tissue profile while the mucinous subtype showed the least different [34]. Notably, this study also concluded that morphological changes do not affect the proteomic profile. Using RPPA, Wiegand *et al*. recently identified 50 proteins that are differentially expressed in clear cell carcinoma and endometroid carcinoma compared with HGSC, and that AKT phosphorylation is associated with BAF250a loss in these tumors [33]. Testing markers on different subtypes might pose a problem, since the huge difference in EOC subtypes frequency may lead some markers to be overlooked which may be significant in certain subtypes especially less common ones. Therefore, the identification of tissue-specific markers is essential for diagnosis and prognosis of different subtypes. Some studies have identified subtype-specific markers using proteomic techniques (Table 1). Few markers showed consistent expression in all EOC subtypes [3, 38], indicating that panels of multiple protein markers for different subtypes may be needed to enhance the specificity and sensitivity of EOC diagnosis, prognosis, and therapeutic value.

2.3 Proteomics of Post Translational Modifications - PTM

PTM are the main reason for proteome complexity and are important determinants of cellular functions. Hence, the analysis of PTM is powerful tool to infer the regulatory mechanisms in cells under varied conditions. Covalent modifications of proteins can happen in different ways: glycosylation, phosophorylation, ubiquitination, acetylation, methylation, lipidation, nitrosylation, and proteolysis. MS-based detection of PTM is a growing field in proteomics. The proteomic analysis was extensively discussed by Mann *et al*. [39].

Glycosylation is one of the most common posttranslational modifications and is important in multiple biological processes, including protein folding, localization and trafficking, protein solubility, antigenicity, biological activity and half-life, and cell communication [40]. Protein glycosylation is divided into four main categories: N-linked glycosylation, O-linked glycosylation, C-mannosylation, and glyco-phosphatidlyinositol, anchor attachments. Abbott *et al*. studied tumor-specific glycan changes between tumor and normal ovarian tissue and identified glycoproteins marker that show tumor-specific glycosylation changes [41]. Shetty *et al*. identified 10 N-linked sialylated glycopeptides significantly up-regulated in ovarian cancer patients' serum samples [42]. Kuzmanov *et al*. discovered 13 sialoglycopeptides in ovarian cyst fluid and ascites fluid of ovarian cancer patients [43].

The importance of mapping detected phosphoproteins into networks and pathways in the interactome inspires the growing number of phosophoproteomics profiling studies. Changes in phosphorylated proteins are the most key player in driving signaling pathways. Members of the protein kinase super-family, the second largest family in the human genome (the kinome), catalyze the phosphorylation events that are essential for the regulation of cellular processes like metabolism, proliferation, differentiation, and apoptosis [40]. Due to the low abundance of phosophoproteins and their dynamic nature, their analysis is greatly enhanced by enrichment techniques. Most enriching strategies are based on chemical modifications, affinity chromatography to capture peptides and proteins containing negatively charged phosphate groups onto a positively charged matrix, or immunoprecipitation by phospho-specific antibodies [44]. Genetic studies have identified multiple signaling pathways involved in EOC pathogenesis, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF–κB) pathway, the activator of transcription 3 (Jak-STAT 3) pathway, the mitogen-activated protein kinase (MAPK) pathway, the proto-oncogene tyrosine protein kinase Src pathway, the ErbB activation pathway, the lysophosphatidic acid pathway, the phosphatidylinositol 3-kinases (PI3K) pathway, the Mullerian inhibitory substance receptor pathway, the EGF and VGEF pathways, and the ER beta pathway (reviewed extensively in [45]). These pathways have an important role in cancer cell growth, metabolism, movement, and metastasis. Further proteomic studies are needed to validate genomic inferred pathway information. A pathways-based approach can lead to substantial improvements in the diagnosis and treatment of many cancers.

2.4 Biofluid proteomics

The 5-year relative survival rate of EOC at stage 1 is 92 % while, at stage 3/4, it is less than 30 %. EOC is a curable disease when discovered in its early stages, but unfortunately just 15 % of cases are diagnosed at the first stage [1]. These statistics highlight the importance of

having effective screening and diagnostic tools. Finding a biomarker that has the required sensitivity and specificity to detect ovarian cancer at an early stage is still a challenging issue. Rather, a myriad of biomarker combinations are currently under investigation. Biomarkers can be used for several purposes: diagnostic biomarkers for early diagnosis of disease which may used mainly for screening programs; prognostic biomarkers used to predict disease progression and hence guide the management of disease; recurrence biomarkers are used to monitor the response to a certain treatment.

Cancer Antigen 125 (CA125) is by far the most studied and useful single biomarker in serous and endometroid epithelial ovarian carcinomas till now. It is a glycoprotein which is naturally secreted by Müllerian, coelomic epithelium, and in epithelia of many organs [46, 47]. It is therefore expressed in benign gynecological and abdominal conditions as well as in other malignancies [48]. CA125 is elevated in approximately 70–90 % of women with advanced-stage disease, but just 50– 60% in the early stages [46]. Due to the low prevalence of OC in the population, the positive predictive value of CA125 is only 4 %. In the light of these parameters, it is not practical to use CA125 alone in initial screening for EOC. Meanwhile, the CA125 blood test was granted FDA clearance for use as a monitoring response test in detecting residual or recurrent epithelial tumors in patients after their first-line therapy. As changes in the level of CA125 correlate with the progression of the disease, it has been argued in a recent study that serial CA125 surveillance might identify patients for secondary cytoreductive surgeries [49]. To date, there have been more than 30 markers assessed alone or in combination with CA125 [50], such as HE4, mesothelin, osteoponin, prostasin, macrophage colony stimulating factor, soluble EGF receptor, lysophosphatidic acid, etc. Human epididymis protein 4 (HE4) is a glycoprotein found naturally in the epithelia of reproductive and respiratory systems [51]. It is found to be overexpressed in endometroid (100 %), serous (93 %), and clear cell (50 %), but not in mucinous tumors [52]. When compared with CA125, HE4 demonstrates greater specificity in premenopausal cases and in benign conditions and has higher sensitivity in early-stage tumors [53]. Moreover, it is overexpressed in 32 % of cases with non-elevated CA125 [51]. Currently, HE4 is mainly used in monitoring recurrence or progression of epithelial ovarian cancer [54]. By reviewing the literature, there are discrepancies in the results of combining CA125 and HE4 [55–59]. However, the Risk of Ovarian Malignancy Algorithm, which is a combination of the serum level of CA125, HE4, and menopausal status, was granted clearance from FDA to distinguish benign masses from malignant tumors [58, 60].

One of the first biological applications of mass spectrometry-based proteomics was the identification of new protein-based biomarkers in easily accessible body fluids. Substantial effort has been done to discover early detection biomarkers using the proteomic approach (reviewed in [46, 61, 62]). Proteomic techniques have been utilized in detection of potential biomarkers in a multitude of body fluids: blood, ascites fluid, urine, ovarian cysts, and pleural effusion. Unfortunately, early biomarker studies relied heavily on surface enhanced laser desorption ionization MS (SELDI-MS), a technique which does not initially identify the differentially abundant peptide species and thus has inadequate reproducibility for clinical use. Currently, most proteomic biomarker experiments depend on advanced mass spectrometry platforms which provide peptide identity and quantitative information; additionally, initial observations are routinely validated using targeted MS experiments.

Plasma is one of the most accessible body fluids; hence, it is widely used in disease diagnosis. However, plasma contains a long and complex range of proteins that are different between and within subjects. This hinders proteomic detection of many potential low-abundance protein markers in plasma due to dynamic range issues. Moreover, the changes in plasma proteins that accompany the progression of ovarian cancer are poorly understood. Using SELDI-TOF-MS platform, Zhang *et al*. identified differentially expressed serum proteins for early detection of OC [63]. These findings were later translated to the FDA-approved OVA1 test which is a multivariate index assay made up of five biomarkers: apolipoprotein A1, transthyretin, transferrin, β-2 microglobulin, and CA125 [64]. FDA cleared OVA1 test in 2009 to assist physicians to triage women with suspected pelvic masses. This permits better preoperative management through referral to gynecologic oncologists in a specialized hospital, which is reported to give better outcome [65].

Specific proteins derived from tumors are less abundant in blood because the mechanism of their release is by active secretion or cleavage from cell surfaces by a proteolytic process, or less likely as an end product of apoptosis [61]. Analysis of concurrently expressed proteins in tumor tissue and blood can identify tumor specific protein rather than proteins reflecting the general response to the disease. Wegdam *et al*. recently described a novel approach using proteomic analysis of tissue and serum from benign and malignant serous tumors, and identified two previously known tumor-produced serum biomarkers that are expressed in lower quantities in both plasma and tissue samples [66]. Few studies found lower expressed proteins in blood due to technical difficulties [67, 68]. The majority of extracellular proteins in blood are glycosylated. In addition, glycoproteins were reported to be altered in cancer [69]. In an attempt to overcome the complexity of plasma proteins, multiple studies have aimed at detection of extracellular glycoproteins. Faça *et al*. highlighted the significance of secreted and surface protein shedding in ovarian cancer [70]. Gunawardana *et al*. detected 420 secreted or membrane proteins in ovarian cancer cell lines [71]. Tian *et al*. compared the secretomes across histological EOC subtypes and normal controls using label-free quantification and discovered that versican and periostin are overexpressed in most ovarian tumor subtypes [38]. Zhang *et al*. used LC-MS-MS to analyze primary organ culture from tumor and normal tissue and created a database of 1,261 cancer-related secreted proteins of which three proteins were validated in plasma of diseased and healthy women [72].

Eighty percent of patients are estimated to develop ascites during the course of their disease which makes ascites fluid a good source of tumor cells. Evaluation of ascites is associated with advanced ovarian cancer. The first comprehensive proteomic analysis of ascites of stage 3/4 identified 80 proteins, 18 of which were previously reported in serum and urine [73]. Kuk *et al*. identified 52 potential protein markers in ascites fluid using tandem mass spectrometry [74]. Due to its proximity to the site of the disease, ovarian cyst fluid protein content can reflect changes in tumor tissue before such changes become detectable in the blood. Kristjansdottir *et al*. examined ovarian cyst fluid of benign and malignant serous patients and identified 32 differentially expressed proteins. Among these, serum amyloid A-4 (SAA4) and astacin-like metalloendopeptidase (ASTL) were verified using iTRAQ MS. These finding support the feasibility of using ovarian cyst fluid for biomarker discovery in ovarian cancer [75]. On studying pleural effusion, Davidson *et al*. [76] reported higher

expression of AKT, activated extracellular signal-regulated kinase, activated and total cAMP-responsive element binding protein, and JNK associated with survival, proliferation, and metastasis, and injury pathways. These results should encourage the use of pleural effusion protein profiling as predictive method of patient outcome. Protein profiling of urine was also reported by Petri el al. [77] who performed SELDI-MS on preoperatively and postoperatively collected urine and characterized three significant peaks which produced an area under the receiver operator characteristic curve (ROC AUC) value of 0.88 and compared that with CA125 at 0.96.

2.5 Screening

Weighing the potential benefit and harms of screening strategies, the U.S. Preventive Services Task Force (USPSTF) and the American College of Obstetrics and Gynecologists does not yet recommend screening for ovarian cancer in asymptomatic women in the general population [78, 79]. Different multimodality screening programs have agreed on the impracticality of screening programs applications; (Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial) using transvaginal ultrasound and CA125 absolute cut off [80, 81]. The United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) took advantage of the higher preclinical detection sensitivity associated with Risk of Ovarian Cancer Algorithm to incorporate CA125 levels in cases to detect age-specific incidence of ovarian cancer. With an unprecedented number of participants—over 200,000 post-menopausal women—the results from final mortality analysis will be reported in 2015, which will in turn determine the efficacy of population screening as a strategy in detecting ovarian cancer [82].

Overall, the biggest question remains: Is there any ideal biomarker? A panel of biomarkers identified by different techniques may hold the answer.

3 Drug resistance

In spite of all the evidence of distinctive behavior of EOC subtypes, they are all currently subjected to the same treatment of cytoreductive surgery and paclitaxel and carboplatin therapy. Thus, it is not surprising that EOC subtypes show very different responses to primary chemotherapy. For example, HGSC are initially hypersensitive to primary chemotherapy, which is evident in 70 % of patients. However, 25 % of patients will develop platinum resistance within 6 months, and later, most patients die due to recurrent disease [83]. In contrast, clear cell and mucinous subtypes show very low responses to first-line treatment (18% and 13%, respectively) [84]. In 2006, a NCI meeting into the state of science commissioned separate clinical trials for mucinous and clear cell subtypes [85]. Elucidation of the mechanism as well as risk factors of chemotherapy resistance and monitoring the response to chemotherapy has been investigated by many studies on comparative genome hybridization, expression profiling, and tissue microarray [86–90]. However, prognostic signatures identified in these studies share only a small number of genes in common [27]. In general, drug resistance can be ascribed to three main reasons: pharmacokinetic, tumor microenvironment, and tumor-specific mechanisms [90]. Multiple common mechanisms are thought to contribute partially to chemotherapeutic resistance in ovarian cancer treatment [91].

Few studies have attempted to use proteomic techniques in testing drug resistance of ovarian cancer (Table 2). As most patients do not undergo further surgeries or biopsies, the lack of pre- and post-treatment samples has limited most research studies to cell lines. Yan *et al*. performed the first experiment using 2DE/MALDI-TOF-MS to compare cell lines of multiple resistant cells and their parental cells and identified five differentially abundant proteins [92]. Mitochondrial proteins were investigated for their role in drug resistance as it was hypothesized that they may be able to help cancer cells escape apoptosis [94, 96, 97]. Dai *et al*. used 2-D DIGE/MALDI-TOF-MS to detect five mitochondrial proteins differentially expressed between resistant and sensitive cell lines [94]. Members of the ABC transporter proteins showed in vitro correlation with paclitaxel intracellular concentration, which is thought to be the reason for multidrug resistance, but clinical evidence remains unclear [90]. Li *et al*. examined multiple drug resistance in ovarian cancer cell lines using iTRAQ LC-MS/MS and identified 28 protein markers that might contribute to cisplatin resistance, which were then classified into eight categories: calcium-binding proteins, chaperones, extracellular matrix, proteins involved in drug detoxification or repair of DNA damage, metabolic enzymes, transcription factor, proteins related to cellular structure, and proteins relative to signal transduction [91]. Other mechanisms of drug resistance related to stress response, metabolism, and cell cycle and apoptosis have been revealed by other proteomic studies [95, 98, 99].

Most tumors will eventually develop resistance to different chemotherapies. At the same time, most chemotherapeutic agents have low therapeutic index and are associated with toxicity and severe adverse effects [90], which underlines the importance of monitoring the response to chemotherapeutic agents. In a recent study, Yang *et al*. developed an algorithm (PROVAR) of nine proteins to determine tumor recurrence and progression based on RPPA protein profiles of TCGA samples that still need further validation [100]. Lee *et al*. compared a cohort of pretreatment and post-treatment samples and identified FOXO3a and pS209-eIF4E using RPPA, and found that these strongly predicted the survival among patients treated with olaparib and carboplatin [101]. Carey *et al*. predicted the response to chemotherapy in HGSC by normalizing for CA125 and found that the TGF-beta pathway played a signaling role in platinum resistance through the expression of Smad3, JNK, and EGFR, using reverse-phase protein array [102]. Using a glycoproteomic approach in cell lines, Michele *et al*. too found that, among ten differentially expressed glycoproteins, four showed significant upregulation in chemoresistant state [93]. Overall, a better understanding of the mechanisms underlying drug resistance will offer optimized treatment strategies and improved survival.

4 New approaches in proteomics

4.1 Targeted proteomics

Typically, the design of proteomic experiments is planned as a comprehensive analysis of a sample with no prior knowledge, which is called shotgun proteomics. This is a good exploratory tool for hypothesis generation that can be targeted in the following step. The targeted approach in proteomics is a promising alternative to affinity-based assessments such as ELISA for independent validation of candidate biomarkers. The primary targeted

approach is termed multiple reaction monitoring (MRM) or selected reaction monitoring (SRM), and involves the use of synthetic peptides representing the 'targets' of interest in order to narrow the focus of the MS scans and provide quantitative information. SRM/MRM MS provides an advantage over antibody assays which is its ability for detecting and quantifying multiple peptides in one sample in a throughput manner as well as its flexible nature since it is an antibody-free technique. In the last few years, its sensitivity has improved greatly mainly because of improvement in sample processing techniques and MS sensitivity. SRM experiments typically use triple–quadruple instruments. It is currently widely used in assessment and prioritization of set proteins predetermined from MS-based discovery experiments. SRM emerged as useful tool in biomarker assays assessments. Currently, SRM assays are generated in publicly accessible repositories [103]. SRM/MRM MS also can be applied to simultaneous monitoring of multiple components in singling pathways [104].

4.2 Peptidomics

The serious need for the discovery of biomarkers shed light on the field of peptidomics as a sub-discipline in proteomics. Peptidomics refers to the comprehensive study of peptides to elucidate the exact form of each peptide. As with proteomics, it is used to identify new peptides that exist within tissue and uses quantification techniques to measure the relative level of peptides in different conditions (reviewed extensively in [105]). Villanueva *et al*. showed that serum peptides are significantly different between cancer patients and healthy controls, proving that there is differential protease activity in cancers [106]. In the context of ovarian cancer, there have been a few attempts to identify peptide biomarkers. Lopez *et al*. identified panels of serum peptide biomarkers that differentiate stage 1 ovarian cancer patients from controls [107]. Fredolini *et al*. reported 59 serum peptidome markers that are differentially expressed in ovarian cancer compared with benign gynecological conditions [108]. Our laboratory performed a comprehensive quantitative analysis of ovarian and breast cancer tissue peptidome that could distinguish the molecular subtypes [109]. Bary *et al*. reported peptide markers differentially expressed in ascites fluid between OC patients and controls. Proteolytic processes are deeply associated with cancer pathogenesis. Thus, peptidomic research in cancer is expected to grow in the coming years as methods in technology and bioinformatics improve, providing the necessary improvements in reproducibility and reliability.

4.3 Exosomes

Since its discovery in the 1983 by two groups separately [110, 111], exosomes have raised many questions about the dynamics of vesicular transport and its role in cell–cell communications as well as possible pathophysiological roles in multiple diseases [112]. Exosomes are 40- to 100-nm vesicles, which contain molecular constituents of their cell of origin such as proteins and nucleic acids. They are hypothesized to impact distant cells or promote tumor microenvironments, thus contributing to cancer metastasis [113]. Additionally, emerging studies suggest that exosome play a role in regulation of tumor pathways [114, 115] as well as in development of chemotherapy resistance [116, 117]. Currently, there are more than 4,500 proteins in ExoCarta linked to exosomes in biofluids. A recent review of the role of exosomes in ovarian cancer tumorigenesis can be viewed [118].

In reviewing PUBMED, there are almost 100 publications in exosomal proteomics in cancer; 80 of them in last 5 years, which reflects a growing interest, particularly in refining isolation protocols. MS-based proteomics can offer a large-scale protein analysis for exosome research. As an example, in a recent study, Sinha *et al*. characterized isolated exosome proteomes of ovarian cancer cell lines using an MS-based technique [119]. Pisitkun *et al*. performed proteomic analysis of urinary exosomes to leverage the bioinformatics tools that can later be used in large-scale validation studies [120]. Proteomic profiling of exosomes have been reported in blood [121], urine [122], cerebrospinal fluid [123], saliva [124], and breast milk [125]. Proteomic profiling of cell type-specific proteins in circulating exosomes can provide new diagnostic tools and monitor therapeutic response [126]. Future technological and analytical advancements that ameliorate the difficulties in isolation and purification in vivo will boost research in this field.

5 Challenges

Proteomic strategies are far from being perfect due to multiple reasons related to disease nature, experimental design, and technology limitations. Proteins are of a very dynamic nature, and since there is no means to amplify them yet, their measurements reflect their state in natural sources. One of the biggest challenges that face mass spectrometry detection of biomarkers in body fluids especially plasma is the dynamic range and complexity of plasma proteins. Some plasma proteins vary by up to 12 orders of magnitude. Just 12 proteins constitute 95 % of the total amount of proteins in blood, such as albumin, transferrin, and apoplipoprotein [127]. Development of protein depletion and multidimensional sample prefractionation protocols has enhanced proteome coverage. However, MS sensitivity is still insufficient to detect the minute changes of low abundant proteins (in low picograms per milliliter) in plasma that are of more significance for biomarkers discovery. In ovarian cancer, it is not surprising that biomarkers that are highly expressed in plasma perform poorly in disease detection as ovarian cancer is characterized by loss-of-function mutations and downregulation of tumor suppressor activities. However, detecting decreases in the concentration of low abundance proteins by mass spectrometry is extremely challenging from a technical standpoint. The trend towards identifying tumor-specific biomarkers in tissue and body fluid will offer insights into less abundant biomarkers. Moreover, most samples used in the development of early detection biomarkers are from late-stage post-surgical tumor samples which will distort the result and hamper efforts directed at early detection biomarkers. Biomarker research ought to focus on different biomarkers expressed in different stages and different subtypes of the disease. It is essential to analyze a sufficient number of well-characterized patients' samples with clear inclusion and exclusion criteria that have statistical power to provide reliable results. In addition, follow-up studies are limited and focus on the biomarkers that have an available immunoassay for easier use and availability in clinical laboratories, despite their questioned specificity (hook effect [128]). Development of a new immunoassay constitutes a big pitfall for further investigation of these potential biomarkers because the process is long and costly. To further investigate the robustness of the candidate biomarkers reported in discovery experiments as an accurate surrogate of disease, there is a dire need for assays based on the multiplexing and quantitative abilities of the MRM/SRM MS in independent datasets and by

independent investigators. Statistical analysis challenges address the need for appropriate analysis of acquired data. Failures to validate biomarkers are further discussed in Ioannidis *et al*. [129].

The discordance in protein abundance measurements among some proteomic studies can be ascribed to many reasons. For example, there are many different approaches within proteomics, including different sample preparation protocols, different instruments, different quantification methods, and different analyses. All these differences account for many of the discrepancies in results. Moreover, most proteomic studies in ovarian cancer are based on a small sample size due to the low incidence of the disease. The majority of proteomic studies use the most widely available tumor tissue, which is usually late-stage HGSC. This consequently slows the progress in studying less common subtypes, early-stage disease, and chemotherapy resistance. Additionally, a confounding factor of having an unmatched sample size for each subtype is that it may compromise many results in poorly stratified experiments. The variability and difficulty in experimentation associated with human tissue samples have encouraged the use of less complex samples. Many studies [70, 71, 130] have used cell lines due to the unavailability of tumor tissue and their cell homogeneity. Despite their ease of use, they only represent a subset of proteins that is actually expressed. Recent studies have cast doubt on SKOV-3 and A2780, the most frequently used cell lines in HGSC, as they do not reflect the molecular subtype characteristics [131]. Cell lines need to be selected with caution to minimize the effect of cell line type on the results. Animal models can be also used as a substitute for tumor tissue and have been described by many experiments [132, 133]. Pitteri *et al*. used a mouse model and compared protein expression with cell lines to determine the tumor-specific proteins in blood [134]. Tang *et al*. identified previously known proteins as well as new proteins shed in the plasma of xenograft mouse model [135]. Perhaps the use of genetically modified animal models can overcome the problem of subjects' heterogeneity, yet there is no way to distinguish between host response proteins and tumor-specific proteins [134]. Thus, there is an increasing need for large population-based trials, which will be a great source of molecular knowledge about the early events in tumor development as well as very rare subtypes.

5.1 From proteomics to clinical applications

Resolving the question of primary precursors will have a substantial change in the diagnosis and treatment of OC. For example, if HGSC originates exclusively from fimbria, then elective salpingectomy will be sufficient treatment, instead of the almost 300,000 prophylactic oophorectomy surgeries performed every year and which are associated with premature menopausal complications and increased risk of all-cause mortality [136]. Pushing proteomics towards a study of primary lesion tumors is needed in order to explore early molecular events and the regulating pathways.

All the emerging evidence shows that ovarian cancer subtypes are different diseases. However, the same guidelines for diagnosis and treatment apply for them all. Subtype classification needs more exploration based on molecular characterization using proteomic techniques. Tissue-based markers can be an effective tool to be incorporated with IHC to help in the design of clinical trials and the stratification of subjects for better testing of new

biomarkers or therapeutic agents. It may also yield important information about drug resistance, especially in clear cell and mucinous carcinoma. Phosophoproteomics can offer an insight into underlying pathways of drug resistance and molecular perturbations by specific therapy, which will open the door for personalized tailored approaches and pathway-targeted agents such as kinase inhibitors [137]. Due to the high resistance rate associated with platinum-based chemotherapy, several targeted therapies are being investigated, including targeting the VEGF pathway, DNA repair pathways, platelet-derived growth factor receptor, epidermal growth factor receptor, folate receptor, PI3K–Akt pathway, and Ras–Raf–MEK–MAPK-pathway [138, 139]. Targeted therapies have the ability to change the management of cancer, exploiting the molecular characteristic of each tumor subtype [139]. Use of poly-ADP ribose polymerase inhibitors is a targeted therapy currently being tested for BRCA1/2 deleterious mutations associated tumor, HGSC among them, and is showing promising results in clinical trials 1/2 [140, 141]. Similarly, in breast cancer, prognostic gene markers such as Oncotype-DX and Mammaprint have aided in treatment decisions and have yielded a model of effective tailored therapies [142].

HGSC could be a perfect example where proteomics can add an insight in cancer biology. Aside from frequent TP53 mutations, all nine mutations published by the TCGA article are of low recurrence frequency [6]. This tumor is dominated by loss of function in tumor suppressor genes rather than gain of function in oncogene protein kinases which are the bases of current chemotherapy [16]. The TCGA also reported homologous recombination deficiency in half of the samples and involvement of the NOTCH and FOXM1 pathways [6]. The question arises here: Can these aberrant genetic findings be tested and verified on the proteomic level? Going beyond mere identification and quantification of proteins, proteomic data can be incorporated with multiple other types of biological data to address the complex and multifactorial nature of disease and provide a comprehensive view of its molecular portrait. Applying the systems biology approach with the development of data infrastructure will connect networks and pathways and push towards the search for new biologically interesting patterns in data that could be tested experimentally for targeted therapies [143]. The increasing availability of such data from multiple platforms, which are held in the public domain, will undoubtedly fuel the development of data mining tools.

6 Conclusion

Development of optimal biomarkers for screening and early detection, characterization of the mechanism of disease progression, and predicting chemo-resistance comprise the bulk of ongoing proteomic research into ovarian cancer. Discovery of new biomarkers has been challenging in ovarian cancer. However, using rapidly refined proteomic techniques in studying the hypothesized precursor lesion should add to our knowledge about the early consequences of the disease, which may ultimately lead to early detection biomarkers and potential therapeutic targets. The heterogeneity and the diverse origins of ovarian cancer should be more widely embraced in the design of future basic and clinical research studies. Integrative studies are increasingly emerging, creating comprehensive molecular characterization which can be translated into clinical opportunities.

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Table 1

Ovarian carcinoma-specific overexpressed markers

Subtype	Markers	Method	Reference
Serous	Cellular retinoic acid-binding protein 2 (CRABP2)	2-DE	[36]
	Wilm's tumor 1 (WT-1)	MALDI-TOF	[37]
	Ras-related protein (Rab-3D)	MALDI-TOF	[37]
	Mesothelin	LC-MS/MS	[38]
Endometroid	Estrogen receptor α (ERα)	RPPA	[35]
Clear cell	Annexin-A4 (ANXA4)	2-DE, MALDI-TOF	[36, 37]
	Phosphoserine aminotransferase (PSAT1)	2-DE	[36]
Mucinous	Serpin B5 (SPB5)	2-DE	[36]
	CEA5	LC-MS/MS	[38]
	CEA6	LC-MS/MS	[38]

Table 2

Protein markers in drug resistance discovered by proteomic technology

References	Identified markers	Regulation in resistance
Yan *et al*. (2006) [92]	Annexin A3	Upregulated
	Destrin	Upregulated
	Conflin 1	Upregulated
	Glutathione-*S*-transferase Omeg1(GSTO-1)	Upregulated
	Cytosolic NADP+dependent isocitrate dehydrogenase (IDHc)	Downregulated
Michele *et al*. (2009) [93]	Tumor rejection antigen (gp96) 1	Upregulated
	Triose phosphate isomerase	Upregulated
	Palmitoyl-protein thioesterase 1 precursor	Upregulated
	ER-associated DNAJ	Upregulated
Dai *et al*. (2010) [94]	ATP synthase subunit alpha (ATP-α)	Downregulated
	Peroxiredoxin 3(PRDX3)	Downregulated
	Prohibitin (PHB)	Downregulated
	Electron transfer flavoprotein subunit alpha(ETF)	Downregulated
	Aldehyde dehydrogenase X (ALDH)	Downregulated
Li *et al*. (2010) [91][a]	Isoform M2 of pyruvate kinase isozymes M1/M2 (PKM2)	Downregulated
	Voltage-dependent anion-selective channel protein 1 (VDAC1)	Upregulated
	Talin-1 (TLN1)	Downregulated
	DNA topoisomerase 1 (TOP1)	Upregulated
	Elongation factor 2 (EEF2)	Upregulated
	Peroxiredoxin-1 (PDX1)	Upregulated
Lee *et al*. (2010) [95]	Aldehyde dehydrogenase 1 family, member A1 (ALDH 1A1)	Upregulated
	Annexin A1	Upregulated
	Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2)	Downregulated
	Rho GDP dissociation inhibitor (GDI 2)	Downregulated
Chappell *et al*. (2012) [96]	Activated leukocyte cell adhesion molecule (ALCAM)	Upregulated
	A-kinase anchor protein 12 (AKAP12)	Upregulated
	Nestin	Downregulated

[a]The identified proteins shown here are the only ones to be validated by real-time RT-PCR analysis and/or Western blot analysis. For a complete list, please see the “Reference” section