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Proteogenomic studies in epithelial ovarian cancer: established knowledge and future needs

There has been a concerted effort over the last decade to improve our understanding of the complex biology of ovarian cancer. A linear growth in published proteogenomic studies has addressed a variety of questions regarding its molecular pathogenesis. A number of genes have been identified by transcriptomic approaches, some of which are being investigated as putative tumor markers (*HE4*, *OPN*, *Ep-CAM* and *Mesothelin*), whilst others are potential targets for molecular therapeutic approaches (*VEGF*, *IO4*, *EGFR*, *MUC1*, *CLDN4* and *SLPI*). Proteogenomics has the potential to further change our current characterization and treatment of ovarian cancer. Additional advances will depend on integrated study designs, interdisciplinary collaborations, use of robust high-throughput platforms, as well as uniform guidelines for bioinformatic analyses.

KEYWORDS: biomarkers ■ diagnostics ■ glycomics ■ microarray ■ ovarian cancer ■ ovary ■ post-translational modifications ■ profiling ■ proteomics ■ transcriptomics

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Epithelial ovarian cancer is the fourth most common cause of cancer mortality in women in the western world and the leading cause of death from gynecological malignancies [1]. The majority of women have advanced-stage disease at initial diagnosis and a 5-year survival of 10–30% [2]. The median survival after recurrence is only 2 years despite advances in chemotherapy and secondary debulking surgery in selected patients [1,3,4]. We clearly need better insights into the biology of the disease in order to detect the disease at an earlier, potentially curable stage and to develop more targeted and effective treatment strategies.

Epithelial ovarian cancers are made up of five main histological subtypes that vary in their biological behavior, response to treatment and overall prognosis [5]. However, even within specific histological subtypes there is considerable heterogeneity, particularly within the more common serous cancers [6,7]. Although International Federation of Gynecology and Obstetrics (FIGO) stage and grade are still used to classify all ovarian cancers, it has been recently recognized that there are two separate and quite distinct subgroups of serous cancer: Type 1, which are low grade and indolent and have a distinct molecular pathogenesis, and the more common Type 2 serous cancers, which are high grade and disseminate rapidly [8,9]. Type 1 tumors appear to arise from the ovarian surface epithelium or inclusion cysts [10] and follow a stepwise progression through microinvasive tumors and tumors of low malignant potential.

They commonly have mutations in *KRAS* and *BRAF* [9]. By contrast, Type 2 serous tumors appear to arise from the fimbrial end of the fallopian tube [11–14] and *p53* mutations occur early. *BRCA1/2*-associated ovarian cancers in particular appear to arise from the fimbrial end of the fallopian tube and have a better response to platinum-based chemotherapy [15–17].

Despite the genetic distinction between the various ovarian cancer histological subtypes [18], they are commonly treated in the same manner: namely, with maximal cytoreductive surgery [19–22] followed by platinum- and taxane-based chemotherapy [4,23,24]. It would be valuable if we could use molecular expression profiles to classify ovarian tumors into distinct subtypes based on the biological behavior and to better predict individual patient outcomes based on a such a personal molecular ‘biosignature’ [25,26]. Good examples of this approach have already been provided for breast cancers and lymphomas [27,28].

There has been a concerted effort over the last decade to identify specific molecular profiles of ovarian cancers using cDNA microarrays and oligonucleotide arrays [29,30]. These high-throughput technologies for transcriptomics or genomic expression profiling permit the analysis of the expression of thousands of genes in one experiment examining a specific biological system [31]. The resulting patterns allow researchers to investigate important biological questions that have not been easily addressed with traditional gene expression technologies such as Northern

blots, *in situ* hybridization or RNase protection assays. Following the development of large-scale profiling technologies, the first high-throughput studies examining protein expression levels were performed using serological analysis of autologous tumor antigens by recombinant cDNA expression cloning (SEREX) [32]. Within the growing spectrum of proteomic technologies, most techniques were based on 2D polyacrylamide gel electrophoresis (2D-PAGE) mainly in combination with mass spectrometry techniques such as matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF), electrospray ionization (ESI) or with liquid chromatography (LC).

There has been a linear growth in the number of publications on 'biomic' studies of ovarian cancer. Since the initial studies on transcriptomics of ovarian cancer in 1999 [33–35], 237 studies were published until the end of 2007 using various -omic technologies. A vast amount of data has been amassed over the last decade, providing greater insight into the heterogeneous genetic background of ovarian cancer. This has led to the identification of novel markers as well as new diagnostic, prognostic and therapeutic targets.

The aim of this report is to provide a comprehensive summary of the totality of published studies pertaining to functional genomics of ovarian cancer. We attempt to put the state-of-the-art of proteogenomic studies in ovarian cancer into context, showing the co-evolution of ovarian cancer knowledge and technologies for new assays, which provide the basis for our view of the direction of future research. We also summarize the data and review the clinical questions addressed in the published studies and highlight the most promising novel tumor markers. We briefly describe new technical possibilities [36], which have the potential both to improve the early detection and better predict prognosis of patients with epithelial ovarian cancer, and take a glimpse at the future of ovarian cancer biomarker research.

Identification of genomic profiling studies

We performed an electronic search of the online databases PubMed [101], Cochrane [102] and Medline [103] to identify publications on ovarian cancer proteogenomic profiling during the 9-year period, 1999–2007. Keywords used to search the databases were, in changing combinations: ovarian cancer, ovary, human ovarian;

in combination with: mass spectrometry (MS), serial analysis of gene expression (SAGE), SEREX, cDNA microarray, differential methylation hybridization (DMH), comparative genomic hybridization (CGH), array CGH (aCGH), expression profiles, oligonucleotide array, proteomics, transcriptomics, genomics; or: expression profiling, array, microarray and single-nucleotide polymorphism (SNP) array. Only published studies that used human-derived cell lines or human clinical samples were included. Articles were excluded when the term 'microarray' was related to tissue microarrays without further mentioning expression profiling within the paper; the data were from another, already published proteogenomic study; and publications in languages other than English.

The identified publications were divided into three categories: transcriptomics, proteomics, and other profiling technologies. Transcriptional profiling includes cDNA and oligonucleotide microarray studies and SAGE; proteomic profiling includes studies using SEREX, 2D PAGE, MALDI-TOF, SELDI-TOF, ESI and LC; and other profiling technologies cover DMH, SNP arrays and aCGH studies. The publication data are summarized according to year of publication, high-throughput technology used, number of profiled samples, sample type, sample preparation, aim of study and main study findings (we provide this large summary as supplementary data). 'Numbers of studies' as listed in the supplementary data do not reflect the numbers of published studies but the research questions addressed within studies, with multiple questions possibly arising from individual proteogenomic datasets.

Data summaries were further descriptively analyzed for the overall time of 9 years and for three individual time periods of 3 years each. We evaluated the whole time period in order to study the trend of profiling studies performed per year and method used (FIGURE 1). Individual 3-year periods were used to demonstrate trends within sample types (FIGURE 2), high-throughput technologies (FIGURE 3), and research questions addressed (FIGURE 4). Individual research questions were also further subclassified into 13 main categories (FIGURE 4):

- Tumor marker discovery (cancer vs normal);
- Treatment effects (treatment; wild-type vs knock out or treated vs untreated), resistance (drug resistance); Patient outcome (outcome);

Figure 1. Development of biomimics in ovarian cancer from 1999–2007. Absolute numbers of profiling experiments published over the last 9 years. All incorporated techniques are grouped within the 'Biomimics' graph and individually for 'transcriptomics' (cDNA microarray, oligonucleotide array and SAGE), 'proteomics' (SEREX, 2D PAGE, protein microarray, SELDI-, ESI- and MALDI-TOF MS and their modified techniques) and 'other technologies' (SAGE, SNP array, CGH and DMH). 2D PAGE: 2D polyacrylamide gel electrophoresis; cDNA: Complementary DNA; CGH: Comparative genomic hybridization; DMH: Differential methylation hybridization; ESI: Electrospray ionization; MALDI-TOF: Matrix-assisted laser desorption/ionization–time of flight; MS: Mass spectrometry; SAGE: Serial analysis of gene expression; SELDI: Surface enhanced laser desorption/ionization; SEREX: Serological analysis of recombinant tumor cDNA expression libraries; SNP: Single-nucleotide polymorphism.

- Distinction of different histological subtypes (subtypes), histological grades (grades), the International Federation of Gynecology and Obstetrics (FIGO) stage (stages), primary versus metastatic site (metastasis); ovarian versus nonovarian cancers (epithelial ovarian cancer vs non-epithelial ovarian cancer);
- Aberrant chromosomal patterns (chromosomes), epigenetics (epigenetics), hereditary diseases (hereditary);
- Specificity of technique (technical optimization).

Ovarian cancers subtypes as defined by histology, stage, grade, organ of origin and outcome were further studied in order to assess whether a genetic distinction between groups would be justified. Taken together, FIGURES 1–4 trace the history of clinical biomarker identification using genomic and proteomic technologies. These figures also serve to emphasize the point that although the amount of data

is increasing, we still face the daunting challenge to translate the information contained therein to clinically relevant knowledge that will benefit patients.

Figure 2. Variation of profiled biological samples using high-throughput technologies. Samples used for profiling experiments analyzed within this study: tissue samples ('tissue', n = 114), ovarian cancer cell lines ('cell line', n = 80), and a mix of both ('mix', n = 16). 'Body fluids' (n = 27) stands for biological samples from blood, pleural effusions or ascites.

Figure 3. Distribution in ovarian cancer profiling studies by high-throughput technology applied. Numbers of publications using specific high-throughput technologies are shown. 'Combined techniques' represents the publications using more than one high-throughput technique.

We incorporated published lists of new candidate tumor markers derived from transcriptomic approaches for distinguishing between healthy individuals and patients with ovarian cancer into our previously described automated over-lapping ovarian (OLOV) database [37]. All published candidate gene lists were screened for gene overlaps across individual studies, with overlaps identified by matching gene symbols, accession numbers and UniGene identifiers. We used Gene Cards [104] and the Human Protein Reference database [105] further enhance the list of most frequently identified potential tumor marker genes, with the addition of information regarding cellular localization, translational modifications, molecular class, glycosylation as post-translational modification, available treatment options and clinical testing status (Phase I–III trials). The numbers of studies demonstrating a significant difference between research question groups are presented graphically using OLOV as described previously [37]. OLOV-incorporated genes were ranked by previously described gene identifiers and sorted in decreasing order by number of reoccurrence across studies.

Development of high-throughput profiling studies

Between the years 1999 and 2007, 237 primary studies addressing functional 'biomics' in ovarian cancer have been published. During the first 5 years, there is a roughly linear growth in the number of publications using profiling

techniques, after which the number of publications reaches a plateau (FIGURE 1). Two thirds of studies between 1999 and 2004 used transcriptomic (rather than proteomic or other) methods. After a clear increase in 2005, a steady decline in number of transcriptomic studies can be observed, which is linked to a nearly linear increase in proteomic and other methods since 2004. These other methods were used as frequently as proteomics methods. Although Minimal Information About a Microarray Experiment (MIAME) guidelines state the need to openly distribute microarray raw data for further validation or meta-analysis [38,39], most frequently only lists of significant gene expressions are published, particularly for the earlier time periods. Unfortunately, such lists are of limited utility for integrating information across studies.

Profiling technologies used in ovarian cancer

We have grouped profiling technologies into three major classes:

- Transcriptomics (63.7% of studies): for example, cDNA and oligonucleotide microarrays and SAGE technology, used to study gene expression;
- Proteomics (20.3% of studies): 2D PAGE, typically in conjunction with mass spectrometry technologies, SEREX and protein microarray;
- Other technologies (16.0% of studies), including: (array) CGH, SNP array and DMH.

Multiple techniques were used in 15.6% of studies, most of which combine either transcriptomics with CGH (29.7%), or proteomic technologies with transcriptomics (5%) (FIGURE 3).

The majority (63.7%) of ovarian cancer-specific questions were addressed using technologies to identify gene expression patterns, including cDNA microarrays (51.3%) and oligonucleotide (46.3%) arrays, most commonly produced by Affymetrix (Santa Clara, California, USA; HuGeFL, U95- and U133-series).

Robust transcriptomic technologies and analysis methodologies have led to the detection of several new ovarian cancer biomarkers. By contrast, even with advances in mass spectrometry, proteomic approaches have led to only a few protein biomarkers that are used clinically. Despite technical complexity, various recent reviews have discussed the great potential of proteomics in clinical biomarker discovery [40–44]. Proteomic

approaches applied to detecting clinical biomarkers typically involve a broader spectrum of samples and larger chemical information content on the protein level than other types of biomic studies [45], but technical challenges have hindered development. Owing to the broad and complex diversity of proteins, the field of proteomics has to deal with a wide range of differences in molecular size, relative abundance, concentration, polarization and ionization as well as the high dynamic concentration range of proteins in complex samples like tissues and body fluids. Since there is not at present a single, optimal instrument that satisfies all proteomics research needs, there is a wide variety of mass spectrometry instrumentations, including an extensive assortment of modular arrangements of different types of mass analysers [46]. The current standards in bioinformatical analyses [47] and normalization procedures [48] are rather limited.

Figure 4. Categorization of investigated aims in ovarian cancer proteogenomic studies from 1999 to 2007. Research questions addressed within proteogenomics studies on ovarian cancer are grouped within 3-yearly time periods as (A) tumor marker discovery (Ca vs normal), (B) treatment effects (treatment; wild-type vs knock-out or treated vs untreated), resistance (drug resistance), patient outcome (outcome), (C) distinction of different histological subtypes (subtypes), grades (grades), stages (stages), primary versus metastatic site (metastasis), ovarian vs nonovarian cancers (EOC vs non-EOC), (D) aberrant chromosome patterns (chromosomes), epigenetics (epigenetics), hereditary diseases (hereditary), and (E) specificity of technique (technical optimization). Numbers within graphs represent numbers of studies within time period and question addressed. Ca: Cancer; EOC: Epithelial ovarian cancer.

Recent advances in mass spectrometry-based quantitative proteomics permit the identification of differentially expressed proteins in cells, tissues and body fluids in a high-throughput manner [49,50]. Quantification of proteins by mass spectrometry is currently most commonly performed by differential isotopic labeling of samples, separation of the peptides by liquid chromatography and identification as well as quantification of the peptides by shotgun mass spectrometry and database searches [51–54]. Major improvements during recent years have been achieved by:

- Label-free quantifications that integrate the total ion current of peptide signals as quantitative measure of original peptide concentration [55–58];
- Selected reaction monitoring for quantitative protein identification [59–61];
- Protein microarrays as protein-detecting microarrays, sandwich immunoassays with spotted antibodies, antigen capture immunoassays and direct immunoassays [62].

Variation of biological sample types

The most commonly profiled samples in transcriptomic studies consisted of human tissue (48.3%), cell lines (41.7%) or both tissues and cell lines (9.3%), with a small minority (0.7%) using body fluids. During recent years a change in sample selection has taken place; individual samples were required to be more uniform so that tumors with mixed histological appearances would tend to be excluded. This constraint facilitates laser capture microdissection (LMD) [63] for paraffin-embedded or fresh–frozen tissue specimens in cancer as well as for normal controls [64], particularly ovarian surface epithelium. However, the rate of studies incorporating LMD in order to allow homogenous messenger RNA generation was only 8.9%. Proteomic experiments were performed on tissue specimens (14.6%), cell lines (31.2%), and body fluids (e.g., blood serum, ascites or pleural effusions; 54.2%). The first studies examining ascites and ovarian cyst fluid have only recently been published [65].

The selection of normal control tissue is a major concern in most of the studies published. Whilst in earlier studies whole ovaries were used as a normal control or human ovarian surface epithelial cell lines were used in comparison to tissue samples, improvements have been under way using laser capture microdissection for human ovarian surface epithelium. Whilst

quantities of ovarian surface epithelial cells are a major problem, new proposed precursor lesions such as inclusion cysts and, more recently, the tubal epithelium [10–12,14,66] have so far only rarely been incorporated into biomic studies.

Study aims within genomic profiling

Study aims for all 237 publications were characterized and grouped, and are shown as absolute numbers in a bar graph (FIGURE 4). As study aims have changed during the years, they are presented in 3-yearly intervals (1999–2001, 2002–2004 and 2005–2007), thus demonstrating the general interest shift over time. During these three time periods, most studies investigated the differentiation between cancer versus normal in order to find new biomarkers (82 studies), experimental treatment settings (48 studies), histological subtypes (44 studies), tumor grades (20 studies) and chromosomes (20 studies) aberrant in ovarian cancer (FIGURE 4). The search for a new tumor marker in ovarian cancer (cancer vs normal) was the most studied aspect during 2005–2007 in 38 studies, nearly the same as during the previous 3-year period (2002–2004, 30 studies). The other most commonly addressed topics during the past 3 years included treatment effects (33 studies), drug resistance (31 studies), histological subtype distinction (28 studies), technical optimization (23 studies), grades (15 studies) and chromosomes (13 studies). There is a growing diversity during this most recent period, with the emergence of new fields such as technical optimization of experimental procedures.

Distinct ovarian cancer biology identified by high-throughput profiling

Clinico–pathological characteristics of ovarian cancer were examined on the molecular level to define distinct proteogenomic patterns. Studies aimed to identify a pattern for histological subtypes, stage, grade, organ of origin and disease progression. Distinctions identified for particular subgroups of ovarian tumors are shown in FIGURE 5. These results show that there is a clear molecular difference between histological subtypes of mucinous, clear cell and endometrioid ovarian cancers. Furthermore, endometrioid cancers of either ovarian or endometrial origin seem to have a distinct molecular pattern, even when expressing the same histological phenotype. Serous ovarian cancers of different grades, especially serous borderline tumors, are biologically different from serous ovarian

Figure 5. Genomic profiling studies in ovarian cancer demonstrating distinct entities.

Number of genomic profiling studies showing a genetic difference within groups of diseases studied (arrow with number of studies expressing a difference in brackets). Analyses are performed for (A) histological subtypes of ovarian tumors; (B) organ of disease origin; (C) grade and stage of ovarian cancer; (D) benign and malignant tumors within possible progression models of ovarian cancer.

CICOC: Clear cell ovarian cancer; EnOC: Endometrioid ovarian cancer; EOC: Epithelial ovarian cancer; MBL: Mucinous borderline tumor; MOC: Mucinous ovarian cancer; SBL: Serous borderline tumor; SOC: Serous ovarian cancer.

cancers, which have been further molecularly distinguished as early (FIGO stage I/II) or late (FIGO stage III/IV) stage disease.

Aberrantly expressed genes identified across multiple transcriptomic studies

Candidate genes differentially expressed in ovarian cancer compared with normal controls, detected by all transcriptomic studies independent of specific research question, are listed in order of their number of overlaps across studies (TABLE 1). Most transcriptomic profiling studies identified *OI4*, *VEGF* and *TFCP2*, from which especially *OI4* and *VEGF* have interesting implications. Both gene products are extracellularly localized, are glycosylated and are used as targets for therapies which are either already clinically used; bevacizumab for VEGF; gefitinib for EGFR) or in clinical

Phase II/III trials, targeting VEGF, EGFR, CLDN4 and HER3. Other less frequently identified biomolecules show involvement of either cellular communication, adhesion or immune response. Post-translational modifications, especially glycosylation modifications, can be found in 55% of these biomolecules.

We have also identified overexpressed genes overlapping across studies addressing the same specific research question. Our approach was only possible for analyses of carcinoma versus normal, subtypes, grades and treatment. In this subquestion examination, a maximum of five overlaps across studies could be identified for individual genes (TABLE 2). A reason for this low number is the diversity of clinical questions, which makes it difficult to give question-specific markers. Interestingly, there are some genes which persist across a number of different specific aims, namely HE4 for

Table 1. Overlapping genes most commonly identified in functional transcriptomic studies of ovarian cancer.

OLOV	Gene	Localization	PTMs	Glycan modifications	Treatment	Mechanism of (potential) treatment	Phase
11	<i>O4*</i>	Extracellular	Hydroxylation, phosphorylation, glycosylation	O-glycosylated			I
10	<i>VEGF</i>	Extracellular	Glycosylation	Glycosylated	Bevacizumab (Avastin®)	Monoclonal antibody to VEGF	II and III
10	<i>TFCP2</i>	Nucleus	Unknown	Unknown			
9	<i>BMP7</i>	Extracellular	Disulfide bridge	Unknown	Osigraff®	Regeneration of bone tissue	
9	<i>MUC1*</i>	Plasma membrane	Glycosylation, phosphorylation, proteolytic cleavage	Heavily glycosylated		Immunogenic activity	I
9	<i>HE4</i>	Extracellular	Glycosylation	N-glycosylated			
8	<i>SLPI*</i>	Extracellular	Proteolytic cleavage	Nonglycosylated	SLPI promoter		I
8	<i>POSTN</i>	Extracellular	Phosphorylation	Unknown		PI3K pathway	
8	<i>TOP2A</i>	Nucleus	Phosphorylation	Unknown	Etoposide phosphate (Eposin®, Vepesid®)	Topoisomerase II inhibitor	
8	<i>OPN</i>	Extracellular	Phosphorylation, glycosylation	N- and O-glycosylated		Peroxisome proliferation-activated receptors	
8	<i>EGFR*</i>	Plasma membrane	Disulfide bridge, phosphorylation, glycosylation	N-glycosylated	Gefitinib (Iressa®)	Selective inhibitor of EGFR tyrosine kinase domain	II and III
7	<i>CLDN4*</i>	Plasma membrane	Unknown	Unknown			II
7	<i>CDH6</i>	Plasma membrane	Glycosylation	Glycosylated			
7	<i>ALDH1</i>	Unknown	Acetylation	Unknown			
7	<i>IL6</i>	Extracellular	Disulfide bridge, glycosylation, phosphorylation	N- and O-glycosylated	Tocilizumab (Actemra®)	Monoclonal antibody to inhibit IL6 receptor in rheumatic diseases	
7	<i>Ep-CAM*</i>	plasma membrane	Disulfide bridge, glycosylation	N-glycosylated	Adecatumumab (MT201)	Monoclonal antibody target tumor cells	Prostate and breast cancer clinical trials
7	<i>HER3*</i>	Extracellular	Phosphorylation	Unknown	Pertuzumab, gefitinib	Selective inhibitor of EGFR tyrosine kinase domain	II and III
7	<i>KLK6</i>	Cytoplasm	Disulfide bridge, glycosylation	N-glycosylated			
7	<i>PRAME</i>	N/A	Unknown	Unknown			
7	<i>UBE2C</i>	Nucleus	Unknown	Unknown			
7	<i>p25</i>	Extracellular	Glycosylation	N-glycosylated			
7	<i>CD24</i>	Plasma membrane	Glycosylation	N- and O-glycosylated			

Most commonly identified overexpressed ovarian cancer genes using transcriptomics. Studies in which genes are overlapping, gene symbol (gene), location of analogous protein (localization), post-translational modifications (PTMs), glycan modifications, treatment and treatment options (mechanism of potential treatment) and trial status are listed.

*Genes that are already in clinical trials.

PTM: Post-translational modification; SLPI: Secretory leukoprotease inhibitor.

Table 2. Overlapping overexpressed genes regarding study question.

Research aim	Top candidates and number of overlaps in studies
Carcinoma vs normal (maximum overlaps 5)	<i>POSTN</i> (5); <i>HE4</i> (5); <i>OPN</i> (5); <i>Ep-CAM</i> (5); <i>ERBB3</i> (5); <i>CD24</i> (5); <i>MUC1</i> (5); <i>UBE2C</i> (5); <i>CPLA2</i> (5); <i>KLK7</i> (5); <i>PRAME</i> (5)
Subtypes (maximum overlaps 4)	<i>OPN</i> (4); <i>POSTN</i> (4); <i>C7</i> (4); <i>CR</i> (4)
Grades (maximum overlaps 3)	<i>HE4</i> (3); <i>IBP5</i> (3); <i>Col6A1</i> (3); <i>PAX8</i> (3), <i>ALP</i> (3)
Treatment (maximum overlaps 4)	<i>CCN1</i> (4); <i>CYR61</i> (4); <i>GIG1</i> (4)

carcinoma versus normal and grades, and *OPN* for ‘carcinoma versus normal’ and ‘subtypes’. Genes identified in the ‘treatment’ research question analysis were unique to this question only (TABLE 2).

Established knowledge due to proteogenomics

The aim to improve upon early screening techniques and to develop more effective, targeted treatments for ovarian cancer has driven the field of high-throughput technologies since their inception 10 years ago. The success of the intensive international efforts to improve outcomes, with new targeted therapies being tested in Phase III trials (e.g., VEGF inhibitors as the most advanced therapeutics), has now become evident. Moreover, the first diagnostic marker, *HE4*, is commercially promoted by Fujirebio Inc. and is increasingly being used in conjunction with *CA125*. Particularly notable today is the appreciation of the heterogeneity of ovarian cancer, necessitating its re-categorization into multiple distinct entities. It is thus recognized that we must treat especially mucinous and clear cell ovarian cancers differently from

serous and endometrioid histological subtypes. The careful reflection on the employment of proteogenomic technologies and the critical assessment of the benefits gained from them compared with their costs has also become an unquestionable factor. It is equally important to learn from mistakes and to carefully design future studies in order to maximize the benefit from these costly technologies (TABLE 3).

Future perspective

New, more specific high-throughput technologies under development target the biological products of the gene–protein network, namely glycosylation, phosphorylation and metabolization. These reflect post-translational modifications of ovarian cancer and therefore carry important biological information. Most of our current clinical tumor markers are glycosylated proteins (*CA125*, *CEA* and *CA19–9*), 55% of all newly identified biomarkers show glycosylation (TABLE 3). Technical simplification and standardization of these new methods will improve the uniformity and comparability of results across cohorts and is expected to massively improve our knowledge of ovarian cancer.

Table 3. Optimization for future profiling studies.

Optimization	Study design	Sample selection	Biomics platform selection	Bioinformatical analysis
1	Experimental design in collaboration with statistician	Histological review by a specialized gynecological pathologist	Established /robust technique	Full datasets available for analysis
2	Interdisciplinary team approach involves: biologist, gynecological oncologist, statistician, bioinformatician, pathologist	Avoidance of mixed pathologies (histological subtypes or grades)	Technique able to address the study question	Analysis by a specialized bioinformatician
3	Prospective collection of study cohort	Microdissection of tissue (preferably by a gynecological pathologist)	Reproducible results	Translational communication throughout complete process
4	Result validation within independent cohort (different continent)	Selection of adequate controls in tissue and serum	No inter-personal variation	Distribution of full datasets after analysis
5	Inclusion of adequate statistically significant numbers	No group combination of proven distinct entities		Interstudy validations and meta-analyses
6	Definition of study question reflects the selection of profiling technique			

During this last decade, biomics in the field of ovarian cancer has undergone a paradigm shift away from the single-disease theory towards the acceptance of disease heterogeneity. This implies the presence of different biological mechanisms and precursor lesions for the development of ovarian cancer and the requirement for differential classifications and individualized disease subtype-specific treatments. One significant consequence is that shortcomings of previous studies, such as heterogeneity of samples and methods, application of inadequate methodologies or missing bioinformatics expertise in analysing the datasets, have been largely overcome. Another consequence is that numerous tumor markers have been proposed and are now under evaluation in combination with or without CA125, the still unbeaten tumor marker for ovarian cancer.

Advantages of previous proteogenomic studies include the wealth of data available within the scientific community, the lessons learned from mistakes, and subsequent improvements in technologies and study design and analysis techniques. A far-reaching aspect of increased data acquisition is an accompanying increase in data sharing. There are now several prominent publicly accessible databases of genomic information. These include general microarray data repositories such as the Gene Expression Omnibus (GEO) [67,68], ArrayExpress [69], and the Stanford Microarray Database (SMD) [70] and cancer-specific data repositories such as the Expression Project for Oncology (expO) [106] and ONCOMINE [71,72].

The experimental design and statistical analysis of microarray studies has evolved greatly from the early days of simplistic, single-sample comparisons [73]. In addition to the ordinary considerations of any scientific study, important aspects more specific to design of microarray studies include pairing of samples for dual-channel arrays and sample processing protocols to avoid confounding and increase precision of inferences [74]. The analysis of microarray data generally includes a technology-specific component (so-called 'low-level' analysis) and a study aim-specific ('high-level') analysis. Low-level analysis consists of data preprocessing (image analysis and normalization), required to reliably quantify fluorescence intensities for each transcript. For a review of methods for cDNA arrays, see [75]; for Affymetrix and other single-channel arrays, the most widely used methods are MAS 5 [76] and RMA [77,78]. The most commonly carried out type of higher level analysis is identification of differential expression. Many methods exist for this, but the most reliable ones involve

computing a type of *t*-statistic (or *F*-statistic) based on (log) fold change and a moderated standard deviation. A widely used method is the linear modeling approach [79].

As data sharing becomes the new standard, we gain increased opportunities for knowledge synthesis. Among these is the ability to carry out statistically sound meta-analysis for clinically relevant questions. Integrated analysis of high-throughput transcriptomic studies is complex. The ONCOMINE web tool [107] has some meta-analytic capabilities; however, it provides limited flexibility. We have created a more adaptable approach for combining information across gene expression studies [80,81] but, like ONCOMINE, it still relies on having access to primary data (not just a summary p-value or ranked gene list). An extension of our methodology should allow for combination of heterogeneous data types (such as gene expression and glycomic data) rather than across just gene expression, thereby making fuller use of existing data resources.

Despite widespread acceptance and requirement of MIAME guidelines [38], data sharing is still incomplete, limiting the possibility to quantitatively combine and critically reflect on all research results.

Based on this timeline examination of recent ovarian cancer research and current technological trends, it is of major concern that only a limited number of genes are detectable in an overlap approach. This minimal number of overlaps can be explained by the limited number of available datasets, variation in study design and platforms used and diversity of research questions studied. For this field to be of more efficient assistance in identifying clinically usable biomarkers individual studies need to be comparable within each other. Based on our examination we suggest that future biomic studies meet the following requirements:

- Adequate number of samples, depending on the research question preferably from different continents (in order to avoid or address population-specific effects);
- Prospective inclusion of patients where appropriate for the study question;
- Inclusion of all presumed precursor lesions, namely ovarian surface epithelium, inclusion cysts, fimbrial end of fallopian tube epithelium;
- Inclusion of other cancers to distinguish ovarian specificity;
- Absence of mixed pathological histotypes;

- Clear-cut study question (or hypothesis) and study groups;
- Microdissection in all tissue samples.

Of particular importance is that:

- Qualified analysts (e.g., bioinformaticians and statisticians) perform the data analyses according to internationally accepted guidelines.

Such guidelines need to be developed to establish clear requirements for data analyses. At present, MIAME compliance is widespread but not universal. The research community will need to adapt quickly to new scientific insights in ovarian cancer to avoid costly, unnecessarily repetitive studies. To this end, we encourage the

establishment and support of internationally driven programs and open access platforms that guarantee the sharing of data, especially for the new technologies under current development.

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Executive summary

Epithelial ovarian cancer is the leading cause of death from gynecological malignancies

- Survival rate of 20% in advanced stage disease has not changed over the last 10 years, even with improvements in chemotherapy regimen.
- Increasing acceptance of the heterogeneity of ovarian cancer, which requires a disease subtype-specific treatment regimen.
- Proteogenomics will be the basis for improvements in ovarian cancer diagnosis and management in the future.

High-throughput profiling technologies & their development in ovarian cancer

- A total of 237 proteogenomic primary studies were published on ovarian cancer during 1999–2007.
- Most studies focused on the detection of new clinical biomarkers for ovarian cancer.
- Transcriptomic (gene expression) approaches were most commonly used.
- Proteomic methods are gaining stability and reproducibility during the last few years and will have major potential in the future.

Heterogeneity as the main ovarian cancer biology finding using proteogenomic high-throughput profiling

- A distinct genomic background was found for mucinous, clear cell and endometrioid ovarian cancers as compared with serous ovarian cancers.
- Serous borderline tumors demonstrate a distinct genetic origin from high-grade, high-stage serous ovarian cancers.
- The potential ovarian cancer precursor lesion has been defined as either ovarian surface epithelium, inclusion cysts, fallopian tubal epithelium, or tubal epithelium at the fimbrial end of the fallopian tube.

Overexpressed genes identified across multiple transcriptomic studies

- Most transcriptomic profiling studies identified *O14*, *VEGF* and *TFCP2* as overexpressed genes in ovarian cancer.
- 55% of all identified transcriptomic biomarkers express glycosylation.
- Multiple identified biomarkers are already used as new targeted treatments and for diagnostic purposes.

Conclusion

- New high-profiling technologies targeting the biological products of the gene–protein network are being developed.
- Glycomics will be an area of particular interest in the development of new biomarkers in the future as most so far identified tumor markers show glycosylation changes.
- Establishment and support of internationally driven, multidisciplinary programs joining datasets and cohorts is important.
- Study design requirements are: question-driven prospectively collected clinical samples incorporating all proposed precursor lesions, clear histological subtypes generated using microdissection.
- Future considerations include the definition of bioinformatical analysis standards and integration of different data types, the development of internationally accepted guidelines for bioinformatical analyses using qualified analysts, and open access platforms requiring full data distribution.

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