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Review Article

Gene-expression signatures in ovarian cancer: Promise and challenges for patient stratification

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HIGHLIGHTS

- Ovarian cancers have different gene expression signatures with different outcomes.
- New correlative studies will improve clinical utility of the classification.
- New platforms will facilitate accurate and cost-effective classification.

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ABSTRACT

Microarray-based gene expression studies demonstrate that ovarian cancer is both a clinically diverse and molecularly heterogeneous disease compromising subtypes with distinct gene expression patterns that are each associated with statistically significant different clinical outcomes. The information provided by gene expression based assays is promising and deserves incorporation into clinical decision-making. Further studies are needed to determine which subtype signatures are most appropriate to select patients for a given therapy. This process will require the development of standardized molecular diagnostic assays that can be used for retrospective correlative studies and prospective validations of their clinical utility. Recent advances in assay development for FFPE tissues will facilitate accurate and cost-effective classification of ovarian cancer and help move the evolving molecular classification to clinic. The current review will summarize the development of gene expression based assays in ovarian cancer and will describe how the results of studies to date have expanded our appreciation of the heterogeneity of ovarian cancer. We discuss difficulties in the development and validation of molecular classifications in ovarian cancer and we provide future directions how we may be able to soon classify the disease in a manner that might have greater clinical utility.

Contents

1. Introduction

Ovarian cancer is the second most common gynecologic malignancy in the United States [1]. Despite radical surgery and initial high response

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rates to platinum- and taxane-based chemotherapy, most patients experience a relapse, with a median progression-free survival of only 18 months [2]. Therefore novel therapies are urgently needed to improve outcomes. However, the success of new drug development in ovarian cancer will strongly depend on biomarkers able to identify women likely to benefit from a given new therapy. The development of microarray-based gene expression profiling has raised high expectations for rapid advances in ovarian cancer classification, prognostication and prediction. The ability to analyze thousands of transcripts in parallel by microarray-based technologies has been perceived as a way to objectively classify tumors into molecular subgroups [3–5]. These subgroups are thought to have distinct biological features that can translate into different therapeutic implications. Indeed, several prognostic and predictive molecular classifications have emerged in the last 10 years in ovarian cancer. However, their clinical impact is yet unclear and existing studies have not yet yielded definitive answers to many of the questions critical for the successful clinical implementation of gene expression profiling in ovarian cancer.

The current review will summarize the development of gene expression based assays in ovarian cancer and will describe how the results of studies to date have expanded our appreciation of the heterogeneity of ovarian cancer. We discuss difficulties in the development and validation of molecular classifications in ovarian cancer and we provide future directions how we may be able to soon classify the disease in a manner that might have greater clinical utility.

2. Gene expression signatures with prognostic relevance

The future value of molecular characterization in ovarian cancer lies in the potential that one may be able to identify those patients that need therapy (prognostic value) or those that are most likely to benefit from a given therapy (predictive value). Here we conducted a systematic Medline search of the literature to identify published gene expression studies in ovarian cancer. Moreover, the reference lists of major publications were further screened for additional trials. Studies focusing on reanalyses of publically available data sets were included if they provided clinically relevant new information. Many early generation ovarian cancer gene expression profiling studies focused primarily on the prognostic value of gene expression signatures. Results of these early prognostic gene expression studies [6–14] and those following [15–25] are summarized in Table 1 (Table 1). Most of these studies have identified a group of prognostically relevant genes in relatively small training sets but did, to their credit, validate the prognostic relevance of the respective gene signatures in independent cohorts. However, limitations of many of these studies are the use of different gene expression platforms, different methods for analysis, over fitting the data, restriction to mostly high grade serous ovarian cancer histology and, most importantly, all studies to date were retrospective in nature. Most studies which all intended to define an independent prognostic gene signature were not able to define a common gene signature that would be robustly reproducible across different studies. Moreover, there was no or minimal overlap in the genes identified to be of prognostic relevance between studies, no matter whether they were early prognostic gene expression studies or those performed more recently.

Waldron et al. undertook a systematic validation of gene expression-based prognostic models for late-stage, high grade serous ovarian cancer published between 2007 and 2012 [26]. They identified 14 prognostic models for late-stage ovarian cancer and validated these in 10 published datasets comprising 1251 primarily high-grade, late-stage serous ovarian cancer patients. They assessed each model for concordance of risk scores with overall patient survival by determining a concordance index (C-index). The C-index is interpretable as the probability that a patient predicted to be at lower risk than another patient will survive longer than that patient: its expected value is 0.5 for random predictions and 1 for a perfect risk model. How high of a C-index is needed for a useful prognostic tool depends on the clinical context. For example, the C-index of the Gleason score for prostate cancer has been estimated at 0.74–0.76 and that of the American Joint Committee on Cancer colorectal cancer staging system at 0.62. The ovarian cancer analysis showed a wide range of accuracy of published prognostic models and signatures. The top-ranked three models were those of the TCGA consortium [18, 23], a signature by Yoshihara et al. [20] and one by Bonome et al. (optimally debulked patients) [10]. These achieved summary C-indices between 0.57 and 0.60. Of the remaining 11 models only 9 predicted slightly better than chance, with summary C-indices varying between 0.54 and 0.56 [26]. These results suggest that most prognostic models published prior to 2012 require further improvements to be of clinical value.

One of the main questions in this context is whether this disparity can be attributed only too trivial reasons such as different technologies,

Table 1
Gene expression studies in ovarian cancer assessing the prognostic relevance of gene signatures.

N	FIGO stage	Gene expression assay	Signature	Prognostic significance P value		Ref.	Year
				Training set	Validation set		
68	III/IV	Affymetrix array	115 genes associated with OS	0.004	0.01	Spentzos [6]	2004
65	I–IV	Affymetrix array	26 genes associated with OS	NA	0.007	Berchuck [7]	2005
45	I–IV	cDNA array	85 genes associated with OS	<0.001	NA	Jazaeri [8]	2005
79	III/IV	cDNA array	14 genes associated with PFS	<0.001	<0.05	Hartmann [9]	2005
95	III/IV	Affymetrix array	57 genes associated with OS	0.018	0.015	Bonome [10]	2008
157	III/IV	Neth. Cancer Institute oligonucleotide array	86 genes associated with OS	0.015	0.007	Crjins [11]	2009
80	I–IV	Affymetrix array	300 genes associated with OS	0.009	0.007	Denkert [12]	2009
53	III/IV	Affymetrix array	166 genes associated with OS	0.003	NA	Mok [13]	2009
43	I–IV	Affymetrix array A	14 genes associated with OS	<0.001	<0.001	Jochumsen [14]	2009
110	III/IV	Agilent array	88 genes associated with PFS	<0.001	<0.001	Yoshihara [15]	2010
70	I–IV	Affymetrix array	60 genes (BRCA signature) associated with OS	NA	0.006	Konstantinopoulos [16]	2010
285	I–IV	cDNA array	C1–C6 subtype signatures	<0.001	0.354	Tothill [17]	2008
215	III/IV	Affymetrix and Agilent arrays	193 genes (TCGA prognostic signature) associated with OS			TCGA [18]	2011
35	I–IV	Affymetrix array	14 genes associated with OS	0.003	<0.001	Sabatier [19]	2011
260	III/IV	Agilent array	126 genes associated with OS	<0.001	0.003	Yoshihara [20]	2012
246[b]	I–IV	cDNA array	14 genes associated with OS	<0.001	0.031	Kernagis [21]	2012
304[a]	III/IV	Affymetrix and Agilent arrays	14 DNA repair genes associated with OS	<0.001	<0.05	Kang [22]	2012
481[a]	III/IV	Affymetrix and Agilent arrays	100 genes (CLOVAR survival signature) associated with OS	<0.001	0.004	Verhaak [23]	2013
121	I–IV	Agilent array	200 genes (POSTN/TGFBI signature) associated with OS	0.009	0.001	Karlan [24]	2014
174	III/IV	Agilent array	TCGA subtype signatures	0.004	0.04	Konecny [25]	2014

[a] Re-analyses of the TCGA data set [18].
[b] Re-analyses of the Tothill data [17].

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different patient populations and different types of analysis. The biostatisticians Ein-Dor et al. provide a compelling answer to this question [27, 28]. They show that many equally predictive gene lists can be produced from the same analysis simply because very many genes (often >1500) can be correlated with survival and the rank order of the top candidate genes is influenced by the subset of patients used in the training set. The large statistical fluctuations in gene rank indicate that small gene lists from early publications are not robust and cannot be reproduced in independent experiments. However, this also implies that reproduction of the exact same specific gene list is less important and that it appears to be of greater relevance to develop a consensus between investigators which set of genes should be used for future validation studies. To improve reproducibility of prognostic gene signatures in ovarian cancer perhaps one has to identify the much sought after master genes that control the metastatic potential and include these master genes in the gene list. However, such master genes will not necessarily be top ranked with respect to correlation with survival. Nevertheless, the construction of prognostic or predictive tools on the basis of a short gene list may still be possible but will require a consensus which genes should be used in retrospective and prospective validation studies.

In this respect breast cancer microarray-based gene expression profiling assays set an example for a strategy in ovarian cancer. The MammaPrint is a microarray-based gene expression profiling assay based on the Agilent chip and analyzed data from 78 patients with node negative breast cancer [5]. The 70 genes that compromise the MammaPrint assay are proliferation genes and genes associated with invasion and angiogenesis. Since 2002 this signature has been validated on numerous cohorts of node negative breast cancer patients [29, 30] and has been shown to provide independent prognostic information beyond standard clinic-pathological variables. MammaPrint was also the first gene expression based assay to receive clearance by the FDA to be sold in the U.S. as a prognostic test for women with node negative breast cancer.

3. Gene expression signatures for molecular classification of ovarian cancer

In 2003 a Stanford group was the first to publish specific gene expression patterns that distinguish ovarian cancer subgroups using an unsupervised hierarchical clustering approach [31]. This early study was limited by its sample size ($n = 42$) and mixed histologies (serous grade I–III, clear cell). Nevertheless, it was the first study to identify distinct gene expression profiles using an unsupervised classification approach. The authors described an immunoreactive ovarian cancer subgroup ("Lymphocyte Cluster") enriched with genes characteristic of immune cell infiltrates. Moreover, they also identified a cluster with very high expression of cytokeratins ("Epithelial Cluster") and one with high expression of genes characteristic of extracellular matrix formation ("Extra cellular matrix/stromal cluster"). In addition, a clear cell subtype of ovarian cancer displayed a distinct signature of genes that were differentially expressed when compared to the other histological types of ovarian cancer. Despite of the studies size limitation this was the first report to suggest that ovarian cancer tumors may be classified into molecular subgroups with distinct biological features that could translate into different therapeutic implications [31]. In 2004 an Italian Group performed gene expression assays and used an unsupervised class discovery approach in 59 tumor specimens from patients with ovarian cancer [32]. They identified distinct molecular signature related to epithelial-mesenchymal plasticity. Notably 40% of the genes associated with this mesenchymal signature overlap with the genes upregulated in the extracellular matrix/stromal cluster previously published by the Stanford group. The authors emphasize that the observed mesenchymal signature was unlikely to be caused by stromal cell infiltrates since all of the samples analyzed had a tumor cell content of >70%. These early classification studies and those that followed are summarized in Table 2 (Table 2). Many of the earlier classification studies in ovarian cancer focused on defining gene signatures of known traditional histologic ovarian cancer subtypes including the rare histologic types such as clear cell, mucinous or endometrioid ovarian cancer. These analyses show that clear cell and mucinous ovarian cancers can be readily distinguished from serous ovarian cancers based on their gene expression profiles, regardless of tumor stage and grade [33–36]. One of these studies also confirms that low-grade endometrioid cancers are distinctly separated from the other groups of ovarian tumors [37]. In contrast, high grade endometrioid adenocarcinomas show significant overlap with the other molecular high grade serous subtypes [17]. Wu et al. identified two distinctive subgroups of endometrioid ovarian cancer, based on their global gene expression patterns [38]. One of these subgroups was highly similar to serous cancer and tended to be of higher tumor grade [38]. Genetic annotation of the dataset also revealed that p53 mutations were common among those endometrioid cancers with a serous-like gene expression profile. Moreover, deregulated β-catenin signaling and defects in the PI3K–PTEN pathway were shown to be typical among those endometrioid cancers that did not share gene expression homology to serous cancer and which also tended to be low-grade [38]. In a further analysis of the same dataset, WT1 gene expression was demonstrably associated to those endometrioid carcinomas with a serous-like gene expression profile [37]. Earlier studies also demonstrate that the gene expression profile of low grade serous ovarian cancers is very distinct from the expression profiles of high grade serous ovarian cancers [39]. Moreover, low grade serous ovarian cancers have activating mutations in KRAS, BRAF, and HER2, suggesting MAP kinase activation which is reflected by increase expression of genes characteristic for that signaling pathway [40].

The first comprehensive molecular classification study using gene expression analysis of high-grade serous and endometrioid ovarian cancers was conducted by the Australian Ovarian Cancer Study Group [17]. In this study 285 ovarian cancer specimens of serous ($n = 246$), endometrioid ($n = 20$), low malignant potential ($n = 18$), and adenocarcinoma ($n = 1$) histology were examined. Using an unsupervised clustering approach the authors identified distinct molecular subtypes that have been designated with neutral descriptors (C1, C2, C3, C4, C5 and C6). Clustering of subtype C1 was driven primarily by enhanced expression of a stromal gene cluster. The C2 group was characterized by an immune signature. C5 was defined by genes expressed in mesenchymal development and samples assigned to the C4 group were simply distinct from the aforementioned signatures. A small number of low grade endometrioid ovarian cancers formed a cluster C6 and cluster C3 was defined by ovarian tumors of low malignant potential and serous ovarian cancers of low grade [17].

The main four high grade molecular subtypes (C1, C2, C4 and C5) were validated in a cancer genome atlas research TCGA Network study [18]. Analysis of data from 489 high-grade serious ovarian cancers identified 1500 intrinsically variable genes that were used for unsupervised clustering using consensus non-negative matrix factorization (NMF) clustering. In this study high-grade serious ovarian cancers were classified into four distinct molecular subtypes which were termed immunoreactive (=C2), differentiated (=C4), proliferative (=C5) and mesenchymal (=C1) on the basis of gene expression in the clusters. T cell chemokine ligands characterized the immunoreactive subtype. The differentiated subtype was associated with high expression of MUC16 (CA125) and MUC1 as well as other genes suggesting a more mature stage of development. The proliferative subtype was characterized by high expression of transcription factors and proliferation markers but low expression of differentiated ovarian tumor markers (MUC16, MUC1). The mesenchymal subtype was characterized by increased expression of genes suggestive of increased stromal components such as fibroblasts and vascular pericytes [18]. Surprisingly, however, survival time did not differ statistically significantly for the TCGA subtypes in the 489 tumor samples studied [18]. This result was unexpected, because considerable variation in outcome can be observed in high-grade serous ovarian cancer patients matched for stage and the

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Table 2
Gene expression studies classifying ovarian cancer into distinct molecular subgroups potentially to be used to stratify patient groups.

N	FIGO stage	Gene expression assay	Morphology[a]	Signature	Tissue source	Ref.	Year
42	I–IV	cDNA array	HGS, CC, Endo,	Lymphocyte, epithelial, extracellular matrix/stromal and proliferation clusters	Fresh frozen	Schaner [31]	2003
59	I–IV	cDNA array	HGS, CC, Endo,	Mesenchymal-like versus epithelial-like	Fresh frozen	De Cecco [32]	2004
113	I–IV	Affymetrix array	HGS, CC Muc	Clear cell, mucinous and serous gene signatures	Fresh frozen	Schwartz [33]	2002
50	I–IV	Affymetrix array	HGS, CC, Endo, Muc	Clear cell, mucinous, endometrioid and serous gene signatures	Fresh frozen	Marquez [34]	2005
44	II/IV	NCI cDNA array	HGS, CC, Endo,	Clear cell, endometrioid and serous gene signatures	Fresh frozen	Zorn [35]	2005
31	I–IV	cDNA array	CC	Clear cell signature	Fresh frozen	Anglesio [36]	2011
74	I–IV	Affymetrix array	HGS, Endo	Endometrioid signature	Fresh frozen	Madore [37]	2010
285	I–IV	cDNA array	HGS, LGS, LMP, Endo	C1–C6 subtype signatures: C1, stromal (TCGA = mesenchymal) signature; C2, immune signature; C3, LMP, LGS; C4, differentiated signature; C5, mesenchymal (TCGA proliferative) signature; C6, early stage Endo signature	Fresh frozen	Tothill [17]	2011
489	III/IV	Affymetrix and Agilent arrays	HGS	TCGA subtype signatures: Immunoreactive, differentiated, proliferative and mesenchymal signatures	Fresh frozen	TCGA [18]	2012
129	II–IV	DASL on Illumina BeadChip arrays	HGS	100 genes: 4 subtypes, angiogenic and non-angiogenic	FFPE[a]	Bentink [51]	2013
489	III/IV	Affymetrix and Agilent arrays	HGS	CLOVAR subtype signatures: Immunoreactive, differentiated, proliferative and mesenchymal signatures	Fresh frozen	Verhaak [23]	2014
121	I–IV	Agilent array	HGS, LMP, Endo, CC, MMMT, benign	POSTN/TGFBI and estrogen receptor/WT1 signatures	Fresh frozen	Karlan [24]	2014
174	III/IV	Agilent array	HGS	TCGA subtype signatures: Immunoreactive, differentiated, proliferative and mesenchymal signatures	Fresh frozen	Konecny [25]	2013
114	III/IV	Affymetrix and Agilent arrays	HGS	Nuclear factor kappa B (NFkB) transcription and extracellular signal-regulated kinase (ERK) signaling networks	Fresh frozen	Barlin [43]	2013
106	I–IV	DASL on Illumina BeadChip arrays	HGS, LGS, Endo, CC, Muc	C1–C6 subtype signatures: C1 (TCGA = mesenchymal) signature, C2 immune signature, C3 LMP, LGS signatures, C4 differentiated signature, C5 (proliferative) signature, C6 early stage Endo signature	FFPE[a]	Sfakianos [52]	2015
111	I–IV	TaqMan, Fluidigm, Illumina, Nanostring	HGS, Endo	C1 (mesenchymal), C2 (immunoreactive), C4 (differentiated) and C5 (proliferative)	FFPE[a]	Leong [54]	

[a] HGS, high grade serous; LGS, low grade serous; CC, clear cell; Endo, endometrioid, Muc, mucinous; FFPE, formalin fixed paraffin embedded.

amount of residual tumor following primary debulking surgery, suggesting that molecular determinants of survival may nonetheless be very important. Thus the TCGA Network recently modified their molecular classification of high grade serous ovarian cancer by reducing and integrating the original TCGA subtype gene signatures (~1500 genes) with pure prognostic gene signatures, thus creating a combined classifier named "Classification of Ovarian Cancer" (CLOVAR) with a smaller number of genes (~100 genes) that may allow a more robust survival classification and enrichment strategy for new treatment approaches [23].

To confirm the presence of four high-grade serous ovarian cancer expression subtypes we performed an independent study and applied the pre-specified TCGA Network gene signatures to a cohort of 174 well annotated high grade serous ovarian cancers from Mayo Clinic with long-term clinical follow up available for each case. Unsupervised clustering confirmed stable clustering of high grade serous ovarian cancer into four molecular subgroups. Moreover, we were able to show that the distinct gene expression patterns were each associated with statistically significantly different clinical outcomes where patients whose tumors expressed the immunoreactive signature had the best and those patients whose tumors expressed the mesenchymal signature had the worst overall survival [25]. In our study using the Mayo Clinic samples the unsupervised clustering by consensus NMF also demonstrated stable clustering of high grade serous ovarian cancers into three or two subgroups. However, unlike clustering based on four subgroups, neither classification into three or two subgroups had prognostic relevance [25]. A comparison of group assignments by cross tabulation suggested that the expression matrix of the immunoreactive and mesenchymal groups were merged when three clusters were depicted [20]. Moreover, the differentiated and proliferative subtypes appeared to merge into one subgroup when only two NMF clusters were depicted. These findings may indicate some commonality in the biological underpinnings between the immunoreactive and mesenchymal and between the differentiated and proliferative subgroups. A similar observation was recently made by the TCGA which provides updated information on their ovarian cancer research projects through a web portal [41]. In the most recent updated consensus NMF clustering of 569 ovarian cancer samples the TCGA also identified three clusters as a robust classification solution [41]. However, data on the prognostic relevance of these three expression subtypes or comparison of group assignments by cross tabulation has not been presented yet.

Further research will be necessary to better understand which classification model will be most useful for clinical practice. Ultimately the predictive value of each transcriptional subtype will need to be validated in future correlative studies preferably using medium-throughput expression profiling platforms useful for expression profiling on formalin fixed paraffin embedded tissue specimens.

A recent overview analysis combined publicly available gene expression array data from 1538 ovarian cancers from 16 data sets [42]. The cohort included predominantly serous histologies, but also included 27 mucinous, 25 clear cell, 96 endometrioid and 55 other histological subtypes and included high grade as well as low grade tumors. Using an unsupervised clustering approach the authors identified six groups, confirming the presence of a differentiated (Epi-A), immunoreactive (Epi-B), mesenchymal (Mes), and proliferative subtype. The authors subdivided the proliferative group into a stem-like A (Stem-A/Proliferative) and stem-like B (Stem-B) group as both expressed markers typical for stem cells. Using this approach 95 cases remained unclassified (Other). A comparison of this grouping scheme with the results of the previously published studies mentioned above (TCGA, Australian ovarian cancer group) demonstrated that the Stem-B subtype had been described earlier in the multi-histologic subtype ovarian study from Australia and that it resembles the C6 subtype (predominantly low grade early stage endometrioid tumors). The stem-like B (Stem-B) group was not seen by the TCGA which was, however, limited to high-grade serous ovarian cancers.

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In contrast to using an unsupervised computational biology approach described in the preceding studies other groups elected to characterize and cluster gene expression patterns through a supervised approach based on pathways that are thought to be biologically relevant in ovarian cancer development and progression. A recent study by Karlan et al. yielded two distinct molecular subgroups of high-grade serous ovarian cancer: a subgroup that expressed TGF-β-correlated genes and a subgroup that expressed ESR1/WT1-correlated genes, which each demonstrated distinct prognostic relevance [24]. Similarly, Barlin and colleagues recently performed a supervised class comparison of gene expression signatures between ovarian cancer patients that recurred before or after five years [43]. Pathway analysis identified networks indicative of nuclear factor of kappa B (NFkB) and extra cellular signal related kinase (ERK) signaling [43].

In the landmark paper of the TCGA an integrated data analysis was performed on 316 fully analyzed cases (DNA sequencing, DNA methylation, DNA copy number and gene expression analysis) [18]. This analysis identified novel cancer associated pathways commonly deregulated in high-grade serous ovarian cancer. The RB1 (cell cycle) pathway and PI3K/KRAS pathways were deregulated in 67% and 45% of the cases, respectively. Moreover the NOTCH signaling pathway was altered in 22% of high-grade serous ovarian cancer. Approximately 50% of high-grade serious ovarian cancer cases exhibit defects in homologous recombination pathway components, causing chromosomal instability [18]. This subgroup of patients has been shown to be more responsive to platinum-based chemotherapy and PARP inhibitors when compared to patients with high-grade serous ovarian cancer with an intact homologous recombination pathway [18, 44, 45]. In the remaining 50% of cases CCNE1 (cyclin E1) has been suggested to be a possible driver of cell cycle progression. Interestingly CCNE1 amplifications are mutually exclusive with the BRCA1/2 mutations suggesting that their respective impacts on genomic stability are either redundant or synthetically lethal [46]. All of the aforementioned pathways which are each deregulated in specific subgroups of ovarian cancer provide novel enrichment strategies for targeted therapies for ovarian cancer. Nevertheless, future studies and more in depth analysis of available data bases are needed to better define the overlap of these deregulated pathways with the evolving transcription based classification.

4. Clinical impact of gene expression profiling in ovarian cancer

The TCGA paper on the genomic analysis of high grade serous ovarian carcinoma was published in 2011. Unexpectedly this wealth of information on the molecular heterogeneity of high-grade serous ovarian cancer has had very little impact on clinical practice or clinical trial design to date. The lack of an apparent overlap between reported signatures, the shortage of independent validations and the absence of prospective studies may have contributed to the low adoption of the evolving classification in clinical practice. Moreover, the predictive value of these evolving signatures, e.g. showing an improved treatment response to a targeted therapy, has yet only been studied in very select instances [47, 48].

A further point of confusion may be the fact that in contrast to breast cancer where published molecular subtypes (HER2-enriched, luminal A, luminal B, basal-like) show a very high degree of mutual exclusivity, it's been proposed that individual ovarian tumors may express multiple subtype signatures at once. For the TCGA data set gene set activation scores for each individual sample were calculated and 82% of the 489 tumor samples could be assigned to at least two subtypes and 44% to at least three subtypes when dichotomous cut offs were used for the gene set activation scores. In our own independent studies using the same method of single sample gene set enrichment analysis (ssGSEA) 40% of high grade serous ovarian cancers samples could be assigned to two subtypes and 2% to three subtypes [25]. Although most gene expression studies have been performed on the samples with high tumor content, present host cells such as immune cells, fibroblasts, endothelial cells or vascular pericytes may nevertheless contribute to the TCGA signatures such as those seen in the immunoreactive and mesenchymal (stromal) subgroups and thus be a reason that individual samples can harbor gene expression profiles that would allow an assignment to two different subgroups at the same time. Importantly, this level of gene expression that is primarily derived from host cell infiltrates (immunoreactive and stromal) may be distinct from a tumor cell related epithelial-mesenchymal plasticity (differentiated and proliferative/mesenchymal) [49, 50]. Future studies are needed to better understand what contributions of gene expression signatures in ovarian cancer can be attributed to host cells. However, the development of treatments that target the stromal cells of ovarian cancers including immune cells (anti-CTLA mAbs, anti-PD1/PDL1 mAbs) or tumor vasculature (anti-VEGF mAbs) underscores the notion not to interpret contributions of stromal cells to ovarian cancer gene expression signatures as "contamination" but rather appropriate image of the actual tumor biology which may help clinicians to better guide patients to the best and most appropriate treatment option.

5. Overcoming limitations

It is clear that before the evolving molecular classification of ovarian cancer can be converted to clinical use, further validation of the prognostic and predictive importance is required and associations between subtype signatures and treatment responses will need to be assessed, preferably using samples from controlled randomized clinical trials. An obstacle to the rapid clinical implementation of microarray-based prognostic or predictive assays is that fresh frozen tissue samples are often required. This hinders access to large series of archived specimens for validation and also poses challenges for prospective testing. Studies on the associations between subtype signatures and treatment responses will preferably be possible when using assays that can be performed on available formalin-fixed paraffin-embedded (FFPE) tissues. Importantly, several other RNA quantification methods (real-time reverse transcription PCR [qRT-PCR], cDNA-mediated Annealing, Selection, Extension, and Ligation [DASL] and NanoString) can be applied to archival FFPE tissue samples. Therefore, the later analytical platforms are more readily amenable to large-scale validation for future use in clinical practice. Use of these assays will also help develop a standardized molecular diagnostic assay that can be prospectively assessed for clinical utility. A recent study was able to confirm the presence of four molecular subtypes using DASL gene expression profiling in FFPE tissues of 129 patients diagnosed with advanced high grade serous ovarian cancer [51]. Another more recent study confirmed that the TCGA and Australian Ovarian Cancer Group signatures could be reliably detected in archival FFPE tissues using the DASL assay, which was chosen as it was the most widely used approach for whole genome profiling in FFPE tissue at the time of analysis [52]. Concordance of gene expression at the individual gene level was assessed by comparing array data from the same cancers (matched pairs of fresh frozen and FFPE of 30 patients). The authors found that although individual probes of genes were weekly correlated between FFPE and frozen samples the combination of larger sets of probes had robust ability to predict survival and the molecular subtypes among 106 patients with advanced stage high-grade serous ovarian cancer [52]. We were recently able to perform DASL gene expression arrays in ovarian cancer tissue samples from 380 patients that were treated in the ICON7 study which evaluated the addition of the bevacizumab to carboplatin and paclitaxel in front-line therapy ovarian cancer [47]. In this retrospective study we were able to confirm that molecular classification of ovarian cancer based on gene expression signatures was feasible using FFPE tissue. Moreover, these study results suggest that the mesenchymal and proliferative subtypes preferentially benefited from the addition of bevacizumab when compared to the differentiated and immunoreactive subtypes [47]. Newer studies report the successful development of RNA-Seq methods suitable for biomarker discovery in fixed clinical tissue [53] and may

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have led to the discontinuation of the first generation whole transcriptome profiling assays using the DASL platform.

6. Moving into the future

The Australian Ovarian Cancer Study Group recently developed a minimal signature for classification, including 39 differentially expressed genes and nine control genes. Three methods were compared for their ability to correctly classify fresh and FFPE samples: 1) PCR-based assays (low density arrays and Fluidigm), 2) the fluorescent oligonucleotide array NanoString and 3) a targeted RNA sequencing assay (Illumina) [54]. In this study the Illumina targeted RNA sequencing assay and the NanoString assay were superior to the PCR-based assays [54]. This is actually the first non-microarray-based molecular classification study of high-grade serous ovarian cancer. The 48 gene based assay correctly classified 100% and 80% of fresh frozen and FFPE high-grade serous ovarian cancer samples, respectively. Based on the authors assessment the NanoString assay may be able to provide the most accurate and cost-effective classification of ovarian cancer when using FFPE tissues. The utility of the Nanostring platform is currently being validated in a larger study including tissue specimens from more than 3000 ovarian cancer cases that were collected through the Ovarian Tumor Tissue Analysis (OTTA) consortium [55].

Very recently the first whole genome sequence analysis of 92 high-grade serous ovarian cancers has been published which included transcriptome sequencing (RNAseq) to support the whole genome sequencing data (56). First and foremost this study confirmed previous reports that p53 mutations are prevalent in high grade serous papillary ovarian cancers and that inactivating mutations in genes associated with homologous recombination are seen collectively in half of all primary tumors. However, using a next generation sequencing approach provided novel insights in many areas. For example although NF1 and RB were inactivated by truncating point mutations in only 6% of the samples, inclusion of gene breakage raised the frequency of inactivating mutations to 20% for NF1 and 17.5% for RB which was supported by expression levels obtained by transcriptome sequencing data (56). Moreover transcriptional profiling showed an enrichment of the immunoreactive subtype in patients with BRCA1 mutations [56]. Of note, in 23 of the presented 92 cases a second tissue/ascites sample was available for comparison with the primary tumor specimen allowing the authors to study drug resistance mechanisms. In the recurrent tumors reversions of BRCA1/2 mutations were found in five cases and mutations in the gene encoding the multidrug-resistant protein MDR1 (ABCB1) with increased MDR1 expression were found in two cases [56]. This study highlights the importance of obtaining serial samples to gain a better understanding of the changes that occur in the development of tumor progression and drug resistance. This study also provided preliminary but provoking information regarding the question whether intra-patient tumor heterogeneity may limit the data generated form a single tissue biopsy in patients diagnosed with advanced ovarian cancer. Contrary to earlier findings in other tumor types this study showed that ovarian cancer tumor samples had a dominant signature that was stable across multiple tumor deposits that were obtained from different locations with the exception of one ovarian cancer molecular subtype [56]. The consistency of subtypes in most patients suggests that evaluation of one or at most two samples per patient may be sufficient to robustly stratify patients for subtype-specific therapeutic approaches.

In summary, these newer studies highlight the feasibility and potential of new assay platforms such as Nanostring or next generation RNA sequencing for transcriptome analysis. The advances in assay development paired with their use in retrospective and prospective clinical correlative studies will facilitate accurate and cost-effective classification of ovarian cancer and hopefully help move the evolving molecular classification of ovarian cancer into clinic rather sooner than later.

Conflict of interest

The authors declare that there are no conflicts of interest.

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