Ovarian cancer prognosis based on gene expression profiling

Ph.D. thesis

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1. INTRODUCTION

With ~43,000 cases in Europe and ~22,000 cases in the USA each year, ovarian carcinoma is the eighth most frequent malignant tumor in the female population. While some improvements were achieved in the 5-year survival due to improved efficiency of surgery and treatment with empirically optimized combinations of cytotoxic drugs, the overall cure rate today still remains as low as 30%. The most likely explanation for this is the high heterogeneity of ovarian carcinomas.

Subtypes of ovarian cancer are recognized based on grade and on histological subtypes. While high-grade malignancies grow rapidly, are relatively chemo-sensitive, and evolve without a definitive precursor lesion, low-grade tumors grow more slowly, are more resistant to chemotherapy, and share molecular characteristics with other low-malignant potential neoplasm. Expression profiling studies have shown that high-grade tumors cluster separately from low-grade carcinomas and borderline tumors. About90% of epithelial ovarian cancers are clonal. This is also reflected in their classification into four different main histotypes of high-grade serous (resembling normal cells of the fallopian tube), endometrioid (cells of the endometrium), mucinous (endocervix), and clear cell (vagina) cancers. The correlation between the different subtypes and their precursor cells were already confirmed by altered gene expression patterns. These subtypes show further differences regarding their epidemiology, genetic changes, gene expression, tumor markers and chemotherapy response. Meanwhile, similarities were also described between high-grade serous and endometrioid cancers and between endometrioid and clear-cell cancers. High-grade serous and endometrioid cancers respond better to platinum- and taxane-based chemotherapeutic regimens than the other subtypes. Mucinous and endometrioid carcinomas are less aggressive and have a better overall survival than high-grade serous tumors. Mucinous, and endometrioid ovarian carcinomas have low malignant potential. Mucinous but not clear cell histology is associated with significantly worse prognosis in advanced ovarian cancer treated with combination platinum/paclitaxel. Clinicians also recognize that the behavior of endometrioid adenocarcinoma is quite different from that of clear cell or high-grade serous carcinoma. The aggressive high-grade serous tumors account for approximately 60% to

80% of ovarian cancer cases. It is of outmost interest to identify markers of histology subtypes, disease progression and aggressiveness.

2. AIM OF THE STUDY

It was previously hypothesized that analysis of global gene expression in ovarian carcinoma can identify dysregulated genes capable of serving as molecular markers, provide insight into the molecular characteristics of the disease and provide the basis for development of new diagnostic tools as well as new targeted therapy protocols. Gene expression analysis has identified ovarian carcinogenesis-, histology subtype-, therapy response-, prognosis- and progression-related gene signatures. In a recent study, a databank of single genes published as components of gene expression profiles specific for ovarian carcinoma was constructed with utilizable data sets that used different array technology platforms. In these studies, 463 genes were associated with histological subtypes, but none of them was identified in more than a single study. The discrepancy and low reproducibility of these studies also led to the limited predictive values of these signatures which have not yet been sufficient to affect patient management.

One of the main weakness of previous studies was the low sample number used for analysis. As currently several datasets are available in the Gene Expression Omnibus, we decided to perform a true meta-analysis of these data. Our further aim was to overcome the differences in previous studies and to establish a new predictor which is capable to discriminate between the four most frequent histology subtypes as well as predict prognosis in ovarian cancer. To achieve these goals, we accumulated a sizeable collective of public microarray datasets, analyzed the data and then used samples from our ovarian cancer biobank for subsequent RT-PCR based validation.

In brief, my aims were the following:

1. Are the previously published gene sets **correlate to histological subtypes** of ovarian cancer capable to classify histology on independent datasets?
2. Are the previously published gene sets **correlate to prognosis** of ovarian cancer capable to predict prognosis on independent datasets?
3. Can we **identify genes correlated to tumor progression** with gene expression profiling based on microarray study?
4. Can we **identify genes correlated to histological subtypes** with gene expression profiling based on microarray study?

3. METHODS

3.1. Included raw microarray studies

I systematically searched Pubmed (http://www.pubmed.com) and GEO (http://www.ncbi.nlm.nih.gov/geo/) using the keywords "ovarian", "normal", "cancer" and "GPL96" and "GPL570" (platform accession names for Affymetrix HGU133A and HGU133A+2 microarrays). Only studies publishing raw microarray expression data were considered and included in our present analyses.

3.2. Statistical analyses

First, the platform GPL570 was mapped to the platform GPL96 using the best match tables available at the Netaffx analysis center (http://www.affymetrix.com).The downloaded data was MAS 5.0 normalized in the R statistical environment (http://www.R-project.org) using the Bioconductor package Affy (http://www.bioconductor.org). MAS 5.0 applies normalization on an individual chip; it has excellent specificity and good sensitivity. As MAS 5.0 it is the factory-default normalization method, in the future even single microarrays can be added to our table. To eliminate the effects of different factory-default settings for average expression on the GPL96 and GPL570 platforms, a second scaling normalization was performed on the matched gene set to set the average expression for each array to 1000. Then, gene expression data was imported into BRB-ArrayTools 3.7.0(developed by Dr. Richard Simon and Amy Peng Lam, http://linus.nci.nih.gov/BRB-ArrayTools.html). Thresholding the

intensity at the minimum value was performed if the spot intensity was below the minimum value of 10. If less than 20 % of expression data had at least a 1.5 -fold change in either direction from gene's median value or the percent of data missing or filtered out exceeded 50 %, then the gene was discarded. All together 21,377 genes passed these filtering criteria. Then, gene set expression comparison using LS/KS test were performed to compare different histology subtypes as well as normal and cancerous tissue. In these, the significance threshold was set to 0.01. In the survival gene set analysis only samples with available survival data were used (n=199).

3.3. Clinical sample collection

Ovarian cancer samples were collected at the 1st Department of Gynecology of the Semmelweis University Budapest and the National Institute of Cancer between 2005 and 2008. Ethical approval for the clinical sample collection was granted by an Institutional Ethical Commission. Samples were snap frozen and stored at –80ºC until RNA isolation.

3.4. RNA isolation and quality control

RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). Frozen biopsy samples were lysed and homogenized in the mixture of 300µl GITC-containing lysis buffer and 3µl β-mercaptoethanol by Polytron homogenizator for 30-40 sec. The lysed samples were digested in Proteinase K solution at 55ºC for 10 min. After silica membrane cleaning and DNase I. treatment (in order to absolutely remove genomic DNA), the total RNA was eluted in 50µl RNase-free water.

Quantity and quality of the isolated RNA was tested by using a Nanodrop1000 system (BCM, Houston, TX, USA) and by gel electrophoresis using an Agilent Bioanalyzer system (Agilent Technologies Inc., Santa Clara, CA, USA). RNA (A260) protein (A280) concentrations and sample purity (260/280 ratio) were also measured. Only high quality, intact total RNA was accepted for samples that showed regular 18S and 28S ribosomal RNA bend pattern on the Bioanalyzer analysis. RNA was kept in a deep freezer at -80ºC until RT-PCR measurement.

3.5. TaqMan RT-PCR measurements

TaqMan real-time PCR was used to measure the expression of 40 selected genes using a Micro Fluidic Card System (Applied Biosystems, Foster City, CA, USA). Of the top genes correlated to survival and related to histology subtypes those with available taqman probes were selected. Additionally, the genes had to have an average MAS 5.0 expression over 1000 in at least one class to be included. A set of genes correlated to chemotherapy resistance (tubulins and ABC transporters) and breast cancer (mammaglobin-A and synuclein gamma), and two housekeeping genes were also added for additional analyses. The measurements were performed using an ABI PRISM® 7900HT Sequence Detection System as described in the product user guide.

3.6. Data analysis of the RT-PCR measurements

For data analysis the SDS 2.2 software was used. The extracted delta Ct values (which represent the expression normalized to the average expression of the ribosomal 18S and the RPLP0 expression) were grouped according to the clinical characteristics (survival and histology subtypes) into groups. Then, comparison of two classes and survival analysis was performed using Significance Analysis of Microarrays (SAM). In these, two groups (e.g. high-grade serous carcinomas vs. all other samples; or borderline and low grade serous carcinomas vs. all other serous carcinomas) were compared in one setting. The statistical significance was set to achieve a false discovery rate (FDR) below 10%. Kaplan-Meier survival plots were generated for genes correlated to survival using WinSTAT 2007 for Microsoft Excel (Robert K. Fitch Software, Germany). Finally, multivariate analysis was performed using WinSTAT to assess whether the genes alone are more powerful than known clinical parameters (stage, grade, histology).

4. RESULTS

4.1. Meta-analysis of microarray data

829 microarrays of ovarian samples were downloaded, 806 ovarian cancer samples (from datasets GSE9891, GSE14001, GSE2109, GSE6008, GSE14764, GSE3149 and GSE15578) and 23 normal samples (from datasets GSE15578, GSE14001, GSE3526, GSE1133, GSE2361, GSE7307 and GSE6008). The complete normalized database containing the MAS5 expression values and clinical characteristics for all microarrays is available at http://www.kmplot.com/ovar/@ovary_normalized.txt.

Gene lists of 38 previously published ovarian-cancer associated publications were used in the gene set analysis**.** After mapping of the published gene sets to Affymetrix microarrays only those having at least 50% of their genes present on Affymetrix platform were retained (n=16). Gene sets were analyzed as being capable to predict the difference between normal and tumorous and between different histology subtypes in independent analyses. At $p<0.005$ eight gene sets were capable of discriminating between tumor and normal tissue and different histology subtypes.

Survival information was published only for 2 studies (GSE3149 and GSE14764) comprising 199 samples altogether. None of the previously published gene sets was capable to significantly predict survival in these patients.

The downloaded combined microarray dataset was used as a new training set to identify new genes correlated to histology subtypes and survival.

According the meta-analysis, the serous carcinoma could be identified the following gene set: **TSPAN8** (tetraspanin 8), **WT1** (Wilms tumor 1), **NPR1** (natriuretic peptide receptor A), **MSLN** (mesothelin), **GAS1** (growth arrest-specific 1), **MUC16** (mucin 16), **SPON1** (spondin 1), **LYPD1** (LY6/PLAUR domain containing 1).

The endometroid carcinioma: **WT1** (Wilms tumor 1), **GAS1** (growth arrest-specific 1), **IGF2BP2** (insulin-like growth factor 2), **ARHGAP29** (Rho GTPase activating protein 29), **GAS6** (growth arrest-specific 6), **MSLN** (mesothelin), **SCGB2A1** (secretoglobin, family 2A), MLPH (melanophilin).

The clear cell carcinoma: **PBX1** (pre-B-cell leukemia homeobox 1), **MEIS1** (Meis homeobox 1), **CLIC5** (chloride intracellular channel 5), **CXADR** (coxsackie virus receptor), **WT1** (Wilms tumor 1), **TCF7L2** (transcription factor 7-like 2), **FXYD2** (FXYD domain containing ion transport regulator 2), **SLC3A1** (solute carrier family 3 member 1).

At least the mucinous carcinoma: **EMX2** (empty spiracles homeobox 2), **CHI3L1** (chitinase 3-like 1), **PAX8** (paired box 8), **TFF1** (trefoil factor 1), **SPINK1** (serine peptidase inhibitor, Kazal type 1), **CEACAM6** (carcinoembryonic antigen-related cell adhesion molecule 6), **TFF3** (trefoil factor 3).

Using appropriate statistical analysis, the prognosis of ovarian cancer can be predicted by gene expression profiling. The top 15 genes are the following: **PRPS2** (phosphoribosyl pyrophosphate synthetase 2), **ZYX** (zyxin), **DOPEY2** (dopey family member 2), **PHF1** (PHD finger protein 1), **HSDL2** (hydroxysteroid dehydrogenase like 2), **FANCL** (Fanconi anemia, complementation group L), **LYPLA2** (lysophospholipase II), **MYO9B** (myosin IXB), **HDGFRP3** (hepatoma-derived growth factor, related protein 3), **MYRIP** (myosin VIIA and Rab interacting protein), **220388_at** (unknown), **GIPC1** (GIPC PDZ domain containing family, member 1), **REST** (RE1-silencing transcription factor), **ARMCX1** (armadillo repeat containing, X-linked 1), **CTNNAL1** (catenin, alpha-like 1).

4.2. Clinical sample collection

Altogether 64 ovarian cancer samples were collected from patients aged 60±11 years. The median relapse-free survival was 24.5 months with 31 relapses and the median overall survival was 29 months with 23 deaths. Forty-four of the patients had high-grade serous, three low-grade serous tumors and six patients had serous borderline tumors. Four of the patients had a secondary breast cancer.

4.3. TaqMan RT-PCR measurements

As our goal was to use microarray data to establish consensus discriminative genes, we included the top meta-analysis-identified genes in the TaqMan analysis. Besides significant genes, we also selected a set of

literature-based genes associated with hormone therapy and chemotherapy response.

The expression of the selected genes was measured in three settings: genes associated with survival, with histology subtypes and with breast cancer pathogenesis were assessed in independent analyses. Due to the low number of samples in other than the high-grade serous histology subtype, only the high-grade serous samples were compared to a pool of all other samples. Of the clinical variables, only stage was associated with survival (p=0,02).

The high malignant potential serous ovarian cancer can be identified on clinical samples the following gene set: **GAS1** (growth arrest-specific 1 protein), **WT1** (Wilms tumor 1), **MSLN** (mesothelin), **NPR1** (natriuretic peptide receptor A/guanylatecyclase A), **TSPAN8** (tetraspanin 8), **ARHGAP29** (Rho GTPase activating protein 29), **MUC16** (mucin 16, cell surface associated), **ZYX** (ESP-2, HED-2), **MYO9B** (myosin IXB), **SNCG** (synuclein, gamma/breast cancer-specific protein 1), **TUBB1** (tubulin, beta 1), **MAP4** (microtubule-associated protein 4), **TUBA1B** (tubulin, alpha 1b).

Due to gene expression profiling, the **TOP2A** gene (topoisomerase (DNS) II alpha) can differentiate the high malignant serous tumor from the low malignant potential borderline serous tumor.

Genes associated with survival were used to construct Kaplan-Meier survival plots. In these, samples were divided based on comparison to the average expression of the genes across the entire dataset; samples having lower than average expression (0) and samples having higher expression (1) were defined as two separate groups. The analyses were performed for both relapse-free survival and overall survival. Finally, the genes ESR2 and PGR were also investigated in the microarray datasets and both were significantly associated with overall survival (p=0,007 for ESR2 and p=0,03 for PGR). The **TSPAN8** (tetraspanin 8) is also a significant gene according to the overall survival.

The relapse-free survival can be predicted by **MAPT** (microtubule-associated protein tau) and **SNCG** (synuclein, gamma) genes.

The relapse-free survival after taxol and carboplatin treatment can be predicted by **MYRIP** (myosin VIIA and Rab interacting protein) and **SNCG** (synuclein, gamma) genes.

5. DISCUSSION

Current molecular profiling data of ovarian cancer are already providing new insights into the genesis of ovarian cancer. To overcome limitations of previous studies, we gathered several datasets from Gene Expression Omnibus to perform a true meta-analysis of ovarian-cancer signatures. I assessed previously published datasets related to ovarian carcinogenesis, histology subtypes and survival. I also established new predictors for the discrimination of histology subtypes and for prediction of prognosis. The results were validated using RT-PCR in 64 ovarian cancer patients.

While my study was designed to identify ovarian cancer-associated gene sets that are clinically relevant, the analysis of available transcriptomic studies dealing with ovarian cancer demonstrated merely a low efficiency. In fact, only eight of the 16 published gene sets analyzed in our study were capable to deliver significant discriminative power, and none of the gene sets was capable to predict survival. The most likely explanation for this lack of reproducibility is the use of different technology platforms for generating the gene expression profiles. However, several other factors can contribute: the clinical ovarian carcinoma samples included in the various studies did not exhibit identical clinico-pathological parameters, different methodologies were used for evaluating the primary data and many studies were based on experimental results obtained *in vitro* studies. The fundamental differences in these factors can explain the ineffective confirmation by different studies. Interestingly, studies capable to discriminate normal and cancerous ovaries were also capable to discriminate histology subtypes.

Genomic studies have demonstrated that mucinous adenocarcinomas are similar to borderline tumors and to benign cystadenomas. Additionally, mutations in *K-RAS* are specific for borderline tumors, low-grade tumors, and mucinous adenocarcinomas. These results lead to the speculations of malignant transformation following a sequence of adenoma to borderline tumor to invasive adenocarcinoma more frequently than to high-grade serous carcinomas. I have investigated a set of top genes using RT-PCR in our patients and were capable to validate almost all genes hypothesized as being related to histology subtypes by either

microarray-analysis or literature search. These results support the hypothesis of distinct molecular characteristics of the different histology subtypes described in earlier studies.

In my patients only three genes (ESR2, PGR and TSPAN8) were correlated to overall survival and two genes (MAPT and SNCG) to relapse-free survival. A future study with significantly more patients (preferably over 1000 samples) could deliver a much more robust estimation of predictive power.

Previously, expression of the estrogen receptor (ER) was found more frequently in low-malignant potential and low-grade ovarian cancers, suggesting that hormonal treatment might be effective for controlling these ovarian cancers. While my meta-analysis of microarray datasets did not identify ER as a top candidate gene, I have found differential expression of ER in high-grade serous carcinomas as well as a correlation to survival in my ovarian cancer patients.

At the moment, prognostic and predictive parameters as described are far from precise, nor are the current chemotherapy regimens highly effective, which emphasizes the need to identify new biomarkers. My results deliver validation as a true meta-analysis for several previously published gene sets and individual genes. Additionally, I was able to confirm the power to discriminate histology subtypes in a clinical cohort for a set of RT-PCR measured genes. New analyses in the future, like RNA-seq, will enable to directly link gene expression, genotype, and phenotype, thereby making a more complex meta-analysis possible at different stages of biological processes.

6. PUBLICATIONS

Overall impact factor: **19,587**.
Impact factors of related articles: **6,858**.

Related articles

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