Identification of Prognostic Groups in High-Grade Serous Ovarian Cancer Treated with Platinum-Taxane Chemotherapy

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ABSTRACT

Disseminated high-grade serous ovarian cancer (HGS-OvCa) is an aggressive disease treated with platinum and taxane combination therapy. While initial response can be favorable, the disease typically relapses and becomes resistant to treatment. As genomic alterations in HGS-OvCa are heterogenous, identification of clinically meaningful molecular markers for outcome prediction is challenging. We developed a novel computational approach (PSFinder) that fuses transcriptomics and clinical data to identify HGS-OvCa prognostic subgroups for targeted treatment. Application of PSFinder to transcriptomics data from 180 HGS-OvCa patients treated with platinum-taxane therapy revealed 61 transcript isoforms that characterize two poor and one good survival associated groups ($p = 0.007$). These groups were validated in eight independent data sets, including a prospectively collected ovarian cancer cohort. Two poor prognostic groups have distinct expression profiles and are characteristic by increased hypermethylation and stroma related genes. Integration of the PSFinder signature and BRCA1/2 mutation status allowed even better stratification of HGS-OvCa patients’ prognosis. The herein introduced novel and generally applicable computational approach can identify outcome-related subgroups and facilitate the development of precision medicine to overcome drug resistance. A limited set of biomarkers divides HGS-OvCa into three prognostic groups and predicts patients in need of targeted therapies.
Introduction

Epithelial ovarian cancer is the eighth most common cause of female cancer deaths worldwide. The 5-year survival rate for high-grade serous ovarian cancer (HGS-OvCa), the most common ovarian cancer sub-type, is only 35-40% (1). The standard treatment for HGS-OvCa is surgery and platinum-taxane combination therapy, and while most HGS-OvCa patients at the advanced stage of the disease initially respond to it, and some patients experience extended and repetitive responses, the treatment is very seldom curative and the majority suffers relapse within 18 months (2). This is mostly due to the fact that despite being a single morphological and clinical entity, genomic alterations in HGS-OvCa are complex (1,3), and there are no known targetable mutations that could serve as uniform therapeutic targets. Accordingly, HGS-OvCa cannot yet be managed optimally, often leading to adverse side-effects and health costs without reasonable benefit.

Little is known on factors that predict outcome of HGS-OvCa platinum-taxane therapy at treatment naïve stage. Currently the best biomarkers to predict chemotherapy response and improved 5-year survival odds are germline mutations in BRCA1 or BRCA2, detected in 15-22% of HGS-OvCa patients (4). Furthermore, several proteins, such as STAT3 (5) and MUC16 (6), and pathways, such as PI3K-AKT and VEGF (3), have also been suggested to contribute to platinum resistance in ovarian cancer. Still, there are very few clinically relevant treatment options for platinum resistant ovarian cancer patients available at the moment. Thus, biomarkers that are able to predict the response for platinum-taxane therapy at treatment naïve stage are urgently needed.

Establishing reliable prognostic or predictive biomarkers requires a coherent subset of patients that share common clinical and molecular characteristics, such as short survival and activation of a certain pathway. Furthermore, molecular data from tumors with identical anatomical location, stage and grade can be very heterogeneous. Thus, reliable biomarker identification requires molecular data from large cohorts of treatment naïve tumors. A major bottleneck in translating large-scale
molecular data into robust biomarkers is the paucity of cost-efficient and robust computational methods (7). Many existing approaches to identify biomarkers use a two-step approach where computational methods, such as unsupervised learning (8,9), Bayesian methods (10) or dimension reduction (11,12), are used to identify clusters of patients that share clinical or molecular characteristics. This is followed by assessment of biological or medical relevance for the identified clusters using Gene Ontology, pathway or clinical data. Such approaches often result in clusters of patients that are formed without association to a clinical endpoint. Furthermore, several studies have identified clusters of gene or protein markers associated with prognosis of ovarian cancer patients (13-15), but these clusters may contain hundreds of loosely correlated genes, which makes the choice of putative biomarkers challenging.

Here, we introduce a novel computational approach called Prognostic Subgroup Finder (PSFinder) to stratify patients into prognostic groups by co-expressed isoform- and gene-level markers identified from the large-scale transcriptomics and clinical data. The major novel aspect in the PSFinder approach is that the identified groups of patients stratified by co-expressed markers are directly associated with a clinical endpoint, which facilitates interpretation of the groups as well as planning subsequent experiments. We applied PSFinder to HGS-OvCa patients treated with the standard platinum-taxane combination therapy from the TCGA HGS-OvCa cohort, and identified three prognostic groups with distinct expression profiles stratified by 61 transcript isoforms. Two of the groups (Poor I and Poor II) were associated with short overall survival and one (Good) with prolonged survival. Linking the BRCA1/2 mutation status to subgroup information further increased the prognostic value.

Materials and Methods

PSFinder approach

Prognostic Subgroup Finder (PSFinder) identifies patient groups that 1) consist of features (e.g.,
transcript isoforms or genes) whose expression correlates strongly across the samples and which exhibit distinct expression patterns between patient groups, 2) have statistically significant association with the clinical data, such as survival time. The overall workflow of PSFinder is shown in Fig. 1.

PSFinder uses an iterative rule-based approach to search for co-expressed features which are able to divide the samples into groups that have significant association to the clinical data. The iterative process starts from all the samples in the cohort proceeding to a subset of samples and is executed until there are no features fulfilling the requirements to further divide the subset of samples into groups. When given a set of samples, PSFinder feature selection starts with seed search (Step1), continues with initial clique collection (Step2) and iterative clique merging (Step3), as shown in Fig. 1. After iterations, the samples at the leaf nodes form subgroups, and all the features used in the sample divisions are returned as markers for prognostic subtype stratification. The detailed steps of PSFinder are in the Supplementary material. PSFinder is available upon request by filling the registration form https://elomake.helsinki.fi/lomakkeet/59036/lomake.html.

**Transcript isoform and gene level expression for TCGA ovarian cancer patients**

Exon array data (level 1; Affymetrix Human Exon 1.0 ST v2) and associated clinical information of two separate HGS-OvCa cohorts were downloaded from The Cancer Genome Atlas (TCGA) repository (12). The discovery cohort included 180 patients (from 13 different hospitals) treated with platinum and taxane combination, and the validation cohort included 327 ovarian cancer samples from patients (from 14 different hospitals) who are reported to have been given various treatments in addition to platinum-taxane based chemotherapy according to the TCGA patient treatment annotations. Of note, it is possible that some patients in the discovery cohort may have also received other treatments due to lack of treatment annotations.
Exon array data were processed at transcript isoform level and gene level by Multiple Exon Array Preprocessing (MEAP) algorithm (16). Transcripts differentially expressed between Poor I and Good patients and between Poor II and Good patients were collected by t-test (adjusted $p < 0.05$) and fold change (absolute log2-scale FC $\geq 1$).

**Global hypermethylation in PSFinder subgroups**

All probes ($n=27,578$) on HM27 methylation array from 180 and 326 HGS-OvCa patients in the discovery and validation sets were used in this analysis. Probes overlapping with SNP, repeats, mapping to multiple locations or with poor detection $p$-values were masked. By using the same parameters as in the study of clear cell renal cell carcinoma (17), we investigated 16,355 CpG loci that are unmethylated in normal ovary tissues (average DNA methylation $\beta < 0.2$) for cancer-specific hypermethylation. Percentage of hypermethylation loci ($\beta > 0.2$) were calculated and visualized by boxplots for the trend of increasing DNA promoter methylation in Poor I patients. Two-sided $t$-test was used to evaluate the significance of the global DNA hypermethylation changes between the groups.

**Genes affected by promoter hypermethylation in Poor I patients**

16,355 cancer-specific CpG hypermethylation loci described above were used in this analysis. A gene is said to be affected by promoter hypermethylation based on the following criteria: 1) significant negative correlation between its promoter CpG hypermethylation and gene expression (Spearman correlation coefficient $< -0.2$ and adjusted $p < 0.05$), and 2) significant CpG hypermethylation changes in Poor I versus Poor II and Poor I versus Good comparisons (two-sided $t$-test $p < 0.05$), and 3) significant expression changes in Poor I versus Poor II and Poor I versus Good comparisons (two-sided $t$-test $p < 0.05$).

**HGS-OvCa cell lines**

Three primary cell lines, M022i, M068i and OC002 were created, characterized using RNA
sequencing, and used in drug treatment experiments. M022i and M068i originated from ascites fluid collected during interval surgery after primary platinum and taxane chemotherapy, whereas OC002 originates from samples taken during primary surgery before chemotherapy. Each patient had rapidly progressing HGS-OvCa (overall survival 4.0, 7.4 and 12.0 months for M022i, M068i and OC002, respectively). All three primary cell lines and the established OVCAR-8 cell line (NCI), which is classified as “possibly high-grade serous” in (18), were grown as spheroids. OVCAR-8 was obtained from DCTD Tumor/Cell Line Repository (NCI) on 2011, grown for 2-4 passages before freezing and assayed within six months after thawing.

**RNA sequencing and expression analysis for HGS-OvCa cell lines**

RNA was extracted using RNeasy kit (Qiagen) with DNase I treatment. The sequencing libraries were constructed in the Beijing Genomics Institute (BGI) using a modified protocol similar to the TrueSeq Stranded Total RNA with RiboZero kit (Illumina Inc) and sequenced with HiSeq2000 (Illumina Inc) with the standard protocol (pair-end sequencing with 100 bp read length).

The RNA-seq data were processed and analyzed using an in-house pipeline (Icay et al. manuscript in preparation). Briefly, 15 bp from the head and 20 bp from the tail of the raw reads were trimmed using Trimmmomatic (19), followed by alignment to the human genome (hg19, Ensembl annotation version 75) using STAR (20) 2-pass alignment. Gene expression was quantified by FPKM values using the Cufflinks-Cuffmerge-Cuffquant-Cuffnorm pipeline (21).

**Evaluation of DNA methylation inhibitor effect on cell viability**

To evaluate the role of methylation, the primary and established cell lines were treated with Decitabine (Selleckchem), which is an inhibitor of DNA methyltransferase. Cells were plated on 96-well plates with 2500 cells/well. Decitabine was plated on the two following days in final concentrations of 0.01, 0.1, 1.0 and 10 μmol/L. Cell viability was measured 72h after the first treatment using the ATP assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega) in at
least three experiments with triplicate wells each. The compound effect was assessed by activity area (area above drug response curve, AA), as suggested by Barretina and colleagues (22), and by percentage of AA out of total curve area A(max) (AA%).

**Multivariate analysis of prognostic factors**

We used a Cox proportional hazard regression model (23) containing known prognostic indicators including age (continuous variable), stage (continuous variable), grade (continuous variable), residual disease (continuous variable), BRCA1/2 mutation (categorical variable wild-type versus mu-type) and PSFinder prognostic classification (categorical variable combined Poor I and Poor II versus Good prognostic groups) on 429 HGS-OvCa patients from the discovery and validation sets with clinical survival data available. Ovarian cancer BRCA1/2 mutation data were downloaded from cBioPortal for Cancer Genomics (24).

**Prospective ovarian cancer patient cohort (MUPET cohort)**

As an independent validation set we used a prospective ovarian cancer cohort consisting of 29 high-grade serous ovarian cancer (HGS-OvCa) patients treated at the Department of Obstetrics and Gynecology, Turku University Hospital (ClinicalTrials.gov Id: NCT01276574). The study and the use of all clinical material have been approved by 1) The Ethics Committee of the Hospital District of Southwest Finland (ETMK): ETK 53/180/2009 § 238 and 2) National Supervisory Authority for Welfare and Health (Valvira): DNRO 6550/05.01.00.06/2010 and STH507A.

Patients were treated with either primary surgery followed by six cycles of platinum and taxane based chemotherapy or three cycles of neoadjuvant chemotherapy (NACT) followed by interval debulking surgery and 3-6 chemotherapy cycles. The treatment modality for each patient was determined based on preoperative imaging studies and diagnostic laparoscopy. Treatment response was determined with clinical examination, CA125 level and contrast-enhanced CT utilizing Recist1.1 criteria. Tumor and ascites samples were collected during operation. One part of each
specimen was snap-frozen and another part was directly prepared for cell culture to generate primary cell lines. Detailed clinical information is shown in Table 1.

Quantitative real time RT-PCR

RNA was extracted with RNeasy kit (Qiagen), and reverse-transcribed to cDNA using Tetro cDNA synthesis kit (Bioline, UK) and oligo dT primers. Expression of selected markers was determined in triplicate samples using TaqMan qRT-PCR with Applied Biosystems 7900HT instrument (Finnish DNA Microarray Centre, Turku Centre for Biotechnology, University of Turku, Finland).

The primers and probes (listed in Supplementary Table S4) were designed using Universal ProbeLibrary Assay Design Center (Roche Applied Science). To select the optimal endogenous controls for the normalization of gene expression data, we studied the expression of ACTB, GAPDH, GUSB, HPRT1, RPL19, PPIA, and TBP. Based on SLqPCR R-package (25) and BestKeeper (26), PPIA and TBP were selected as the best reference genes in both MUPET patient samples and cell lines. Raw qRT-PCR Ct values were thus normalized against the geometric mean of PPIA and TBP.

Results

Expression signature identified by PSFinder predicts prognosis of platinum-taxane combination treated HGS-OvCa patients

We analyzed isoform expression data from 180 treatment naïve HGS-OvCa cancer samples available in the TCGA repository (12) using PSFinder (Fig. 1). The samples originated from patients who had a disseminated disease and were first treated with surgery and then with first-line platinum-taxane combination therapy (clinical data in Table 1).

PSFinder analysis resulted in 61 co-expressed transcripts in 51 genes that divide the discovery
cohort of 180 samples into three groups (Fig. 2A and Supplementary Table S1). Two groups (Poor I and Poor II) are associated with relatively short overall survival and have markedly distinct expression profiles, whereas one group (Good) is associated with longer survival (log-rank test $p = 0.007$, Fig. 2B). The median overall survival times for patients in Poor I, Poor II and Good groups were 45, 50 and >120 months, respectively. The five-year survival rates for Poor I, Poor II and Good groups were 42%, 40% and 68%, respectively. There were 3,724 differentially expressed transcripts (DETs) between samples in Poor I vs Good groups, whereas between Poor II vs. Good there were only 657 DETs.

To further evaluate the reproducibility of the three subgroups identified from the isoform-level expression data, we applied PSFinder to gene-level expression data of the same 180 TCGA HGS-OvCa samples. The gene-level analysis resulted in 32 genes that characterize three groups (Supplementary Table S2, Supplementary Fig. S1), which are very similar to the isoform-level results, although four samples identified into a poor prognosis group in the isoform-level analysis were characterized as good prognosis samples in the gene-level analysis. This small discrepancy is most likely due to the differences between isoform-level and gene-level expression. Of the 32 genes, 18 were also identified from the isoform-level, including six Poor I (AC083884.8, RP11-793H13.3, RP11-867G23.3, CERS6-AS1, MAP1S and KLF16) and 12 Poor II (ADAM12, ADAMTS12, AEBP1, COL3A1, COL5A1, COL5A2, COL6A1, COLEC12, FAP, ITGA5, MMP14 and MMP2) genes. The 32 gene-level characterization markers allow the use of several HGS-OvCa cohorts in the validation of Poor I, Poor II and Good subgroups as the expression data from current available cohorts are mostly measured by conventional microarrays.

We then compared the PSFinder results with two recently published ovarian cancer prognostic signatures. Firstly, Tothill and colleagues used a two-step approach where they obtained gene expression data to identify molecular subtypes in serous and endometroid ovarian cancer and
identified six subtypes (C1-C6) which were linked to clinical outcome (8). When we compared the 61 transcripts identified by PSFinder to the genes characterizing C1-C6, we found that the Poor II subtype is almost identical to the C1 (high stromal response) molecular subtype. Poor II is characterized by 25 co-expressed transcripts, and 88% of them belong to the genes that define the C1 group. As expected, the similarity is even more pronounced at the gene level: all except one out of 24 genes characterizing Poor II are in the gene list defining C1. Patients in C1 have short overall survival (8), which is also in accordance with the expected survival in the Poor II type. Secondly, we compared PSFinder results to the CLOVAR survival signature, which is based on univariate Cox regression analysis followed by correlation analysis resulting in 100 genes, whose gene expression was correlated with survival (14). None of the 100 CLOVAR signature genes belonged to the isoform- or gene-level results emerging from PSFinder. The lack of overlap is not unexpected considering the different design principles in CLOVAR and PSFinder; CLOVAR uses univariate Cox regression to identify genes with lowest p-values whereas PSFinder finds groups of co-expressed genes with survival association. We tested univariate Cox regression on genes identified by the CLOVAR approach using the 180 TCGA HGS-OvCa patients treated with platinum and taxane combination. From the 100 genes identified by CLOVAR, only 22% were significantly associated ($p < 0.05$) with overall survival.

**Eight independent HGS-OvCa validation cohorts confirm the robustness of the PSFinder results**

In order to test the universality of the PSFinder identified poor and good survival types we used two independent cohorts for transcript-level validation and six independent cohorts for gene-level validation.

The first transcript-level validation cohort (TCGA validation cohort) consists of transcript level data from 327 ovarian cancer patients from the TCGA repository who have received some other
chemotherapy in addition to platinum-taxane based treatment. Patients from this cohort have been subjected to varying treatment regimens, which may introduce a bias to the survival times, as some drugs can prolong and other drugs can shorten the overall survival of the patients independent of the platinum-taxane treatment effect predicted by the PSFinder signature. The subgroup prediction using the 61 transcript predictor (linear predictor score (LPS) in Supplementary material) revealed three subgroups sharing similar expression profiles as the TCGA discovery set (log-rank test $p = 0.015$, Fig. 2C and Supplementary Fig. S2). The Poor I group has only 30 patients in the TCGA validation cohort, which may cause unstable estimates in the Kaplan-Meier analysis. Therefore, we combined the Poor I and Poor II groups, leading to 123 poor responding patients, with the available survival data and observed a clear difference between poor and good survival ($p = 0.014$, Fig. 2D).

In the second transcript-level validation we used transcript-isoform specific qRT-PCR in a prospective cohort (MUPET) consisting of primary cancer samples from 29 HGS-OvCa patients who received platinum-taxane based chemotherapy (27) (clinical data in Table 1). There were specific assays available for 24 transcripts in Universal ProbeLibrary Assay Design Center (lifescience.roche.com; Roche Diagnostics), and only nine produced robust signals in all the samples. By using these nine markers as a subgroup predictor, we calculated LPS for each sample and 14%, 38% and 48% patients were classified into Poor I, Poor II and Good subgroups respectively (Fig. 2E). Due to the small size of the cohort we combined Poor I and Poor II into a single “Poor group”, which showed significantly shorter survival times than the Good prognosis group (log-rank test $p = 0.030$, Fig. 2F and Supplementary Fig. S3). All markers, except PPP1CC, exhibit similar expression profiles in the MUPET subgroups as in the TCGA Poor I, Poor II and Good samples (Supplementary Fig. S4).

To further evaluate the reproducibility of the PSFinder results, we used gene expression data from AOCS (8), Yoshihara (28), Ferriss (29), Crijns (30), Mok (31) and Mateescu (32) cohorts.
containing a total of 558 HGS-OvCa patients. The expression data in these six cohorts are measured using various microarray designs and 38% of Poor I and 100% of Poor II characterization genes identified by PSFinder from the gene-level analysis were available in these validation cohorts. Despite the suboptimal number of Poor I genes available for prognostic type prediction using the LPS method, the groups with similar expression profiles and survival associations as in the TCGA discovery set were still reproduced (Fig. 3).

**Increased promoter hypermethylation is a characteristic of Poor I patients**

While Poor II corresponds to the high stromal response molecular subtype, Poor I type has not been identified earlier and none of the transcripts or genes that characterize Poor I were reported by Tothill et al. or CLOVAR. Even though gene microarrays lack many of the Poor I characterizing genes, the lack of overlap was unexpected because C1-C6 subtypes are defined by approximately 7,700 genes (8), and many of the C1-C6 groups are associated with poor survival. Since TCGA provides also miRNA and DNA methylation data, we investigated whether Poor I can be characterized at epigenetic or miRNA expression level. At the miRNA level, there were no miRNAs that were significantly differentially expressed between Poor I and Poor II (nominal p-value < 0.05, absolute fold-change > 2), which suggests that Poor I is not different from Poor II at the miRNA regulation level.

A possible cause for the distinct gene expression profile in Poor I type is DNA methylation and thus we explored DNA promoter hypermethylation differences between Poor I and other types. The frequency of hypermethylated CpG loci in Poor I samples (mean frequency 5.1% in the discovery set and 4.7% in the validation set) is significantly higher than that in Poor II (mean frequency 4.1% in the discovery set and 4.0% in the validation set) or Good (mean frequency 3.7% in the discovery set and 3.6% in the validation set) tumor samples and normal ovary tissue samples in both TCGA discovery and validation cohorts (Fig. 4A and 4B).
Differential methylation analysis between Poor I vs. Good (Fig. 4C) and Poor I vs. Poor II (Fig. 4D) resulted in 178 and 153 significantly differentially methylated CpG loci (|Δβ| > 0.1 and multiple-hypotheses corrected $p < 0.001$), respectively. The number of common CpG loci identified by both comparisons is 140, of which all except one (cg11484576) exhibit higher hypermethylation in Poor I samples. There were no significant differences at the DNA methylation level between Poor II and Good types (Supplementary Fig. S5). The genes whose expressions were affected by promoter hypermethylation based on the criteria described in Methods include several transcription regulators, such as $TOP3B$ and $RPAP2$ (Supplementary Fig. S6 and Table S3).

DNA methyltransferases (DNMTs) are enzymes that catalyze the addition of a methyl group to cytosine residues at CpG loci and play key roles in establishing the global or regional hypermethylation patterns in cancers (33). Thus, we studied the expressions of $DNMT1$, $DNMT3A$ and $DNMT3B$ in Poor I, Poor II and Good patient groups and observed significant up-regulation of two full length protein-coding isoforms (ENST00000328111 and ENST00000348286) and one shorter form (ENST00000537219) of the de novo methyltransferase $DNMT3B$ in Poor I samples (Supplementary Figure S7). High expression levels of $DNMT3B$ have been reported in several cancers, including breast cancer, endometrial cancers and leukemia (34-36), and DNMT3B has been shown to promote tumorigenesis (37). This suggests that de novo methylation mediated by DNMT3B may contribute to the formation of aberrant hypermethylation patterns in Poor I HGS-OvCa samples.

**Methylation inhibition exhibits strong effects on Poor I characteristic HGS-OvCa cell lines**

As samples in the Poor I group are significantly more hypermethylated than samples in Poor II or Good groups, we tested whether DNA methylation inhibitor 5-Aza-2’-Deoxycytidine (Decitabine) is able to decrease viability of HGS-OvCa cells in samples with Poor I characteristics. Decitabine inhibits the function of DNMTs leading to reduced DNA methylation (38). We used three patient
derived cell lines and one control cell line (Fig. 4E): OC002 and M022i (high expression levels of Poor I genes), and M068i (low expression levels of Poor I genes) and compared the cytotoxic effects to control cell line OVCAR8 (very low expression levels of Poor I genes).

When we tested Decitabine on these four Cisplatin resistant HGS-OvCa cell lines, the greatest effect was seen in OC002 followed by M022i, M068i and OVCAR8 (Fig. 4F, Supplementary Fig. S8). The cytotoxic effects were most significant at the concentrations of 0.1-1 µM (Fig. 4G), and the effects correlate with the overall expression of DNMT3A and DNMT3B (Fig. 4H), which are targets of Decitabine (39). IC50 values are provided in Supplementary Fig. S8. These results are in line with the hypothesis that cancer cells with higher expressions of Poor I characterizing genes are more responsive to Decitabine.

Combining PSFinder expression signature and BRCA1/2 mutation data improves stratification of HGS-OvCa patients

Ovarian cancer patients with dysfunctional DNA repair genes BRCA1 and/or BRCA2 have increased overall survival due to better response to cytotoxic chemotherapy (40). Thus, we examined whether BRCA1/2 dysfunction could explain the three groups identified by PSFinder, and whether integrating PSFinder predictor and BRCA1/2 mutation status is able to improve the classification of platinum-taxane treated individuals into different outcome groups.

BRCA1/2 mutation data were available for 117 samples in the TCGA discovery cohort and 139 samples in the TCGA validation cohort. BRCA1/2 mutation carriers had longer overall survival than BRCA1/2 wild-type patients as expected (log-rank test \( p = 0.0004 \), Supplementary Fig. S9). The mutation status of BRCA1/2 does not show enrichment in any of the PSFinder identified types (Fig. 5A). This suggests that there are other factors involved in predicting outcome of an HGS-OvCa patient to platinum and taxane therapy. Indeed, multivariate Cox regression analysis using age, grade, stage, residual disease, BRCA1/2 mutation status and PSFinder identified types shows
that the strongest predictors of HGS-OvCa patient outcome are the BRCA1/2 mutation status ($p=0.0003$) and the PSFinder identified prognostic types ($p=0.02$) (Table 2). While the low predictive power of grade and stage was not unexpected due to the cohort selection, the importance of PSFinder subgroups over tumor debulking is of interest, as the extent of residual disease after primary cytoreductive surgery is regarded as a significant prognosticator in clinical practice (41).

We combined Poor I and Poor II types and BRCA1/2 mutation status information into the following groups: 1) Good prognosis with BRCA1/2 mutation (“good-mu”), 2) good prognosis with wild-type BRCA1/2 (“good-wt”), 3) poor prognosis with BRCA1/2 mutation (“poor-mu”) and 4) poor prognosis with BRCA1/2 wild-type (“poor-wt”) groups. Strikingly, none of the BRCA1/2 mutation carriers in the good prognostic group of the discovery set died during the 5-year follow-up period, whereas 61% of the “poor-wt” patients died (Fig. 5B). The annotation of prognostic subgroups and BRCA1/2 status for patients from the TCGA discovery and validation sets is available in Supplementary Table S5-6.

The improved prognostic accuracy due to the combination of BRCA1/2 mutation status and PSFinder types were further validated in the TCGA validation cohort (Fig. 5C). Thus, combined PSFinder and BRCA1/2 dysfunction data provides better segregation of responders than either one alone in identifying patient who benefit from platinum and taxane treatment.

**Discussion**

The ability to separate poor and good responders to the HGS-OvCa first-line therapy, platinum and taxane combination regimen is crucial for implementation of precision medicine to ovarian cancer. We have developed a novel computational approach, PSFinder that allows identification of patients with poor and good prognosis after chemotherapy using treatment naïve transcriptomics and clinical survival data. PSFinder uses an iterative rule-based approach to search for co-expressed features,
which enables forming groups that have statistically significant association to a clinical end-point, such as overall survival, and the transcripts or genes characterizing a group are tightly co-expressed. The major findings of this study include: (1) identification of a novel poor outcome group of HGS-OvCa patients treated with surgery and platinum and taxane combination therapy, (2) association of this group with increased DNA hypermethylation and demonstration of the effect of Decitabine in cancer cells belonging to this group, (3) identification of a restricted set of transcriptomic biomarkers that provides a solid basis for further development of a diagnostic test, and (4) demonstration of the additive prognostic value of the combination of PSFinder markers and BRCA1/2 pathogenic mutation over either test alone.

When we applied PSFinder to clinical and expression data measured by exon arrays from platinum-taxane treated HGS-OvCa patients, we identified two poor (Poor I and Poor II) and one significantly better (Good) outcome groups. Poor II type corresponds to the high stromal response molecular subtype (42). Poor overall survival in Poor II or high stromal response type is in line with recent results that highlight the importance of stromal cell types in response to therapy (1). However, the overall survival of the Poor I type is equally poor to the Poor II type, which suggests that there are other factors than stromal response that play a key role in drug resistance. The Poor I type has not been reported earlier and several genes that characterize Poor I type have not been associated with poor survival in ovarian cancer. This is mostly due to the fact that the genes characterizing Poor I type are not present in traditional gene microarrays, which also hindered our efforts to validate the Poor I type in earlier cohorts. A few Poor I characteristic genes, however, have been linked to drug response. For instance, PARG is a DNA repair gene that has been indicated in ovarian cancer tumor growth and drug response (43); BEST1 is a regulator of an ion channel, which is suggested as one of the major mechanisms conveying drug resistance in ovarian cancer (44); and CXCL3, which is a proinflammatory chemokine has been recently suggested as a therapeutic target in ovarian cancer (45).
Samples belonging to the Poor I type have significantly higher rate of promoter hypermethylation than samples belonging to Poor II or Good types, which prompted us to test the effect of DNA methylation inhibitor 5-Aza-2’-Deoxycytidine (Decitabine) in four HGS-OvCa cell lines. Earlier studies have examined the combinatorial effects of Decitabine and platinum, and demonstrated that nontoxic doses of Decitabine sensitize ovarian tumor xenografts to platinum (46) and Decitabine can be combined safely with platinum (47). A recent phase II trial, however, did not find increased efficacy when Decitabine and platinum were combined, most likely because of non-optimal patient-selection strategy (48). Our results suggest that Poor I type samples are more sensitive to Decitabine than other samples. Therefore, it is tempting to speculate that the PSFinder signature could be useful to stratify patients in clinical trials that test the effect of Decitabine alone or in combination with other agents.

PSFinder identified a set of only 61 transcripts as putative prognostic biomarkers to platinum-taxane treated HGS-OvCa patients, which provides a solid basis for further work to develop a diagnostic assay. Indeed, one of the clinically applicable major findings in this study is the combination of PSFinder signature and BRCA1/2 mutation status to predict the effect of platinum and taxane therapy from treatment naïve tumor specimens. The use of PSFinder predicted types and BRCA1/2 mutation status allowed identification of HGS-OvCa patients who truly benefit from platinum and taxane combination therapy. In the discovery and validation sets this group of excellent responders included approximately 8% of the patients in the cohort.

In conclusion, our novel computational approach identified a restricted set of previously unknown isoform-level biomarkers. In combination with the BRCA mutation information this panel may be used for stratification of HGS-OvCa patients on the basis of their response to state-of-the-art platinum-taxane combination therapy. Identification of the extreme responders and primarily chemo-resistant patients can help to develop alternative treatment strategies and to select patients...
for future therapeutic trials.

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References


## Table 1. Clinical features of the cohorts used in this study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
<th>TCGA (discovery)</th>
<th>TCGA (validation)</th>
<th>AOCS</th>
<th>Yoshihara</th>
<th>Ferriss</th>
<th>Crijns</th>
<th>Mok</th>
<th>Mateescu</th>
<th>MUPET</th>
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<td>Number of patients Total</td>
<td>180</td>
<td>327</td>
<td>172</td>
<td>84</td>
<td>44</td>
<td>130</td>
<td>53</td>
<td>75</td>
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<td>Age at diagnosis Median</td>
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<td>59</td>
<td>59</td>
<td>59</td>
<td>62</td>
<td>61</td>
<td>58</td>
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<td>31-86</td>
<td>50-80</td>
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<td>Neoadjuvant therapy Yes</td>
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<td></td>
<td>16</td>
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<td>(100%)</td>
<td>327</td>
<td>172</td>
<td>44</td>
<td>130</td>
<td>53</td>
<td>75</td>
<td>13</td>
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<td>274</td>
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<td>Grade Unknown</td>
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<td>(2.8%)</td>
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<td>Tumor stage I</td>
<td>7</td>
<td>(3.9%)</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>2</td>
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<tr>
<td>Tumor stage II</td>
<td>11</td>
<td>(6.1%)</td>
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<td>13</td>
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<td>130</td>
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<td>257</td>
<td>145</td>
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<td>40</td>
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<td>(17.8%)</td>
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<td>13</td>
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<td>13</td>
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<td>(1.0%)</td>
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<td>(28.9%)</td>
<td>50</td>
<td>(15.3%)</td>
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<td>5</td>
<td>(17.2%)</td>
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<td>Residual disease 1-10 mm</td>
<td>72</td>
<td>(40 %)</td>
<td>157</td>
<td>48.0%</td>
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<td>(55.2%)</td>
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<td>Residual disease 11-20 mm</td>
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<td>(4.4 %)</td>
<td>23</td>
<td>(7.0%)</td>
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<td>4</td>
<td>(13.8%)</td>
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<td>Residual disease &gt;20 mm</td>
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<td>64</td>
<td>(19.6%)</td>
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<td>4</td>
<td>(13.8%)</td>
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<td>Residual disease Unknown</td>
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<td>(12.8%)</td>
<td>33</td>
<td>(10.1%)</td>
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<tr>
<td>Primary therapy outcome Complete response</td>
<td>124</td>
<td>(68.9%)</td>
<td>166</td>
<td>(50.8%)</td>
<td></td>
<td>22</td>
<td>(75.9%)</td>
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<tr>
<td>Primary therapy outcome Partial response</td>
<td>15</td>
<td>(8.3 %)</td>
<td>45</td>
<td>(13.8%)</td>
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<td></td>
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<td>Primary therapy outcome Stable disease</td>
<td>4</td>
<td>(2.2 %)</td>
<td>22</td>
<td>(6.7 %)</td>
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<td>1</td>
<td>(3.4%)</td>
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<tr>
<td>Primary therapy outcome Progressive disease</td>
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<td>(5.6 %)</td>
<td>26</td>
<td>(7.9 %)</td>
<td></td>
<td>6</td>
<td>(20.7%)</td>
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<td></td>
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<tr>
<td>Primary therapy outcome Unknown</td>
<td>27</td>
<td>(15 %)</td>
<td>68</td>
<td>(20.8%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tumor site in analysis Ovary</td>
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<td>(100.0%)</td>
<td>327</td>
<td>172</td>
<td>84</td>
<td>130</td>
<td>53</td>
<td></td>
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22
Table 2. Multivariant Cox regression analysis on PSFinder subgroups and clinical prognostic factors.

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>P-value</th>
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<tr>
<td>Age</td>
<td>1.013</td>
<td>0.997-1.029</td>
<td>0.103</td>
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<tr>
<td>PSFinder Subgroups (Poor vs. Good)</td>
<td>1.542</td>
<td>1.061-2.241</td>
<td>0.023</td>
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<tr>
<td>ResidualDisease</td>
<td>1.179</td>
<td>0.986-1.411</td>
<td>0.071</td>
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<tr>
<td>BRCA1_2 (wt vs. mu)</td>
<td>2.384</td>
<td>1.492-3.811</td>
<td>0.0003</td>
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<tr>
<td>Grade</td>
<td>1.379</td>
<td>0.89-2.137</td>
<td>0.151</td>
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<tr>
<td>Stage</td>
<td>1.351</td>
<td>0.892-2.045</td>
<td>0.156</td>
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</tbody>
</table>

Figure Legends

**Figure 1. The overall workflow of PSFinder.** PSFinder is an iterative rule-based approach to search for co-expressed features, which are able to divide the samples into subgroups that have significant association to the clinical data. Inputs consist of feature (transcript isoforms or genes) expression data from a cohort of samples and survival times of the sample donors. The iterative process starts from all the samples in the cohort proceeding to a subset of samples and is executed until there are no features fulfilling the requirements to further divide the subset of samples into groups (left tree). When given a set of samples, PSFinder feature selection starts with seed search (Step 1), continues with initial clique collection (Step 2) and iterative clique merging (Step 3). In Step 1, PSFinder identifies individual features whose discretized expression values (high/low) are associated with statistically significant survival effect (Kaplan-Meier with log-rank test). These are used as seeds in Step 2, where highly correlated transcripts ($r \geq |0.6|$ in our case study) form initial cliques. In Step 3, PSFinder iteratively merges initial cliques into larger cliques until either 1) the correlation between the expression profiles or 2) the survival association in the merged clique falls under the threshold. From each iteration process, all the features from the merged clique with the maximum score are selected as features to stratify the subset of samples. After iterations the samples at the leaf nodes form subgroups and all the features used in the sample divisions are returned as markers for prognostic type stratification.
Figure 2. Prognostic subgroups identified by PSFinder using isoform-level expression data. (A) Prognostic subgroups identified by PSFinder using isoform-level expression data from 180 high-grade serous ovarian cancer patients treated with platinum and taxane combination therapy. Rows correspond to transcript isoforms and columns to samples. PSFinder identified two poor prognosis types: Poor I (red), Poor II (blue), and one Good prognosis type (green). Poor I and Poor II specific isoform markers are colored in pink and light blue respectively in the row-side color bar. (B) Kaplan-Meier survival curves on overall 5-year survival for Poor I, Poor II and Good type patients identified from the isoform-level data. Patients in Poor I and Poor II types are associated with poor prognosis and patients in Good type are associated with good prognosis with log-rank test \( p=0.007 \). (C) LPS predicted prognostic subgroups by using isoform-level prognostic signatures for the 327 high-grade serous ovarian cancer patients from the TCGA validation set. Poor I and Poor II specific isoform markers are colored in pink and light blue, respectively, in the row-side color bar. (D) Kaplan-Meier survival curves on overall 5-year survival for predicted poor and good prognostic patients from the TCGA validation set (log-rank test \( p=0.014 \)). (E) Prognostic outcome prediction for 29 HGS-OvCa patients from MUPET prospective cohort using expression of nine isoform-specific markers by Taqman qRT-PCR. Poor I and Poor II specific isoform markers are colored in pink and light blue, respectively, in the column-side color bar. Patients predicted to be Poor I, Poor II and Good types are colored as red, blue and green at y-axis. (F) Kaplan-Meier survival curves on time to progression in month for 29 MUPET patients with predicted poor and good prognosis. The significance was measured with the log-rank test \( p = 0.030 \). The number of patients at risk is listed below each survival curve for each time point (x-axis of Kaplan-Meier survival curves).

Figure 3. Prognostic subgroup validation in six independent validation sets at gene-level. (A) PSFinder identified prognostic signatures (gene-level) and predicted prognostic subgroups in six independent validation cohorts (AOCS, Yoshihara, Ferriss, Crijns, Mok and Mateescu). Poor I and Poor II specific gene markers are colored in pink and light blue respectively in the row-side color
bar. (B) Kaplan-Meier survival curves on overall 5-year survival for predicted Poor I, Poor II and Good prognosis patients (log-rank test \( p=0.004 \)). (C) Kaplan-Meier survival curves on overall 5-year survival for merged poor and good prognosis patients (log-rank test \( p=0.001 \)). The number of patients at risk is listed below each survival curve for each time point (x-axis of Kaplan-Meier survival curves).

**Figure 4. Association of DNA methylation with Poor I vs. Poor II or Good types.** (A) Promoter hypermethylation frequency in tumor (Poor I, Poor II and Good from TCGA discovery set) and normal ovary samples. (B) Promoter hypermethylation frequency in tumor (Poor I, Poor II and Good from TCGA validation set) and normal ovary samples. The statistical significance by two-sided \( t \)-test between Poor I, Poor II and Good samples are indicated by asterisks (***** \( p<0.00001 \), **** \( p<0.0001 \), *** \( p<0.001 \), ** \( p<0.01 \), * \( p<0.05 \) or empty \( p \geq 0.05 \)) in Fig. 4A and 4B. (C) Volcano plot of DNA methylation changes between Poor I and Good samples. Blue dots: CpG loci with Benjamini-Hochberg FDR < 0.001 and hypermethylation changes in mean \( |\Delta \beta| > 0.1 \) between Poor I and Good samples. (D) Volcano plot of DNA methylation changes between Poor I and Poor II samples. Blue dots: CpG loci with Benjamini-Hochberg FDR < 0.001 and hypermethylation changes in mean \( |\Delta \beta| > 0.1 \) between Poor I and Poor II samples. (E) Average expression fold change of Poor I marker genes in primary cell lines (OC002, M022i and M068i) to OVCAR8. Mean fold changes and standard errors are illustrated for all these three primary cell lines. (F) Percentage of AA values (mean ± SEM) from Decitabine treatment calculated from drug response curves. (G) Drug response curves for Decitabine treatment in primary (OC002, M022i, M068i) and established (OVCAR-8) HGS-OvCa cell lines. Cell viability is presented as percentage of untreated control (mean ± SEM) in 0.01, 0.1, 1.0 and 10 \( \mu \)mol/L. The response changes between cell lines at each concentration were evaluated by one-way ANOVA test and the concentrations with significant \( p \)-values are shown with asterisks (***** \( p<0.00001 \), **** \( p<0.0001 \), *** \( p<0.001 \), ** \( p<0.01 \), * \( p<0.05 \) or empty \( p \geq 0.05 \)). (H) The expression of \( DNMT3A \) and \( DNMT3B \) in all test...
cell lines.

**Figure 5. Association of BRCA1/2 mutation with PSFinder subgroups.** (A) BRCA1/2 mutation status and PSFinder subgroups in the TCGA discovery and validation sets. (B) Kaplan-Meier survival curves for patients from TCGA discovery set given platinum-taxane combination therapy with 1) poor prognosis and wild-type BRCA1/2, 2) poor prognosis and mutant BRCA1/2, 3) good prognosis and wild-type BRCA1/2, 4) good prognosis and mutant BRCA1/2. The log-rank test \( p \) is 0.0002. (C) Kaplan-Meier survival curves for patients from TCGA validation set with 1) poor prognosis and wild-type BRCA1/2, 2) poor prognosis and mutant BRCA1/2, 3) good prognosis and wild-type BRCA1/2, 4) good prognosis and mutant BRCA1/2. The log-rank test \( p = 0.017 \). The number of patients at risk is listed below each survival curve for each time point (x-axis of Kaplan-Meier survival curves).
Figure 2
Figure 3
Identification of Prognostic Groups in High-Grade Serous Ovarian Cancer Treated with Platinum-Taxane Chemotherapy

Ping Chen, Kaisa Huhtinen, Katja Kaipio, et al.

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