Up-Regulation of Periostin and Reactive Stroma is Associated with Primary Chemoresistance and Predicts Clinical Outcomes in Epithelial Ovarian Cancer

Running title: Reactive stroma signature predicts chemoresistance

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Translational Significance

Primary chemoresistance represents a significant unmet clinical need in ovarian cancer treatment. We discovered and independently validated a reactive stroma signature characterizing primary chemoresistant tumors and demonstrated this signature can predict clinical outcomes of first-line chemotherapy. Our study represents the first report to characterize the reactive stroma as the key component in orchestrating chemoresistance in ovarian cancer. This study sets up the stage for identifying primary chemoresistant ovarian cancer patients and developing novel therapeutic strategies to overcome chemoresistance.
Abstract

**Purpose:** Up to one third of ovarian cancer patients are intrinsically resistant to platinum-based treatment. However, predictive and therapeutic strategies are lacking due to a poor understanding of the underlying molecular mechanisms. This study aimed to identify key molecular characteristics that are associated with primary chemoresistance in epithelial ovarian cancers.

**Experimental Design:** Gene expression profiling was performed on a discovery set of 85 ovarian tumors with clinically well-defined response to chemotherapies as well as on an independent validation data set containing 138 ovarian patients from the chemo-treatment arm of ICON7 trial.

**Results:** We identified a distinct “reactive stroma” gene signature that is specifically associated with primary chemoresistant tumors and was further upregulated in post-treatment recurrent tumors. IHC and RNA ISH analyses on three of the highest-ranked signature genes (POSTN, LOX and FAP) confirmed that modulation of the reactive stroma signature genes within the peritumoral stromal compartments was specifically associated with the clinical chemoresistance. Consistent with these findings, chemosensitive ovarian cells grown in the presence of recombinant POSTN promoted resistance to carboplatin and paclitaxel treatment *in vitro.* Finally, we validated the reactive stroma signature in an independent dataset and demonstrated that high POSTN expression level predicts shorter progression free survival following front line chemotherapy.

**Conclusions:** Our findings highlighted the important interplay between cancer and the tumor microenvironment in ovarian cancer biology and treatment. The identified reactive stromal components in this study provided a molecular basis to the further development of novel diagnostic and therapeutic strategies for overcoming chemoresistance in ovarian cancer.
Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death for gynecologic malignancies and treatment of EOC continues to present as a significant clinical challenge (1). The current standard of care for EOC consists of aggressive surgical cytoreduction followed by adjuvant platinum and taxane-based chemotherapy. Although response rates to the first-line treatment are high, 20-30% of cases exhibit intrinsic chemo-refractory or resistance and progress during or within six months of completion of primary therapy (2). These patients gain little benefit from the standard treatment and represent a significant unmet clinical need in ovarian cancer management. In order to predict response to chemotherapy and develop novel strategies to overcome primary chemo-resistance, a better understanding of the molecular characteristics associated with such intrinsic chemo-resistance is needed.

Activation of the host stromal microenvironment, commonly referred as to “reactive stroma”, has been implicated as a critical component of carcinoma progression in many types of cancers (3-6). Reactive stroma in cancer has similarities to the biology of the wound healing process in normal tissues, where stromal cells exhibit elevated production of extracellular matrix (ECM) components, growth factors and matrix remodeling enzymes to create a tumor microenvironment that promotes cancer cell survival, proliferation, and invasion (7). In particular, the tumor microenvironment has been increasingly recognized to play an important role in the pathogenesis of EOC (8, 9). Unlike many other types of cancers, distant metastasis via hematogenous routes is rare in EOC; instead, EOC cells typically spread within the peritoneal cavity through dissemination of tumor cells into the peritoneal fluid, followed by implantation on the mesothelial linings of the omentum and other peritoneal surfaces that overlie connective and adipose tissues (10). As a result, the growth and spreading of EOC are likely to be orchestrated
by dynamic interplay between tumor cells and the local microenvironment. However, the key regulators of reactive stroma and the specific mechanisms through which reactive stroma affect tumor progression, treatment response and clinical outcomes in EOC are poorly understood.

In this study, we describe the discovery of a distinct reactive stroma signature that is specifically associated with primary chemoresistance in ovarian cancer and further up-regulated in recurrent tumors post progression of chemotherapy. *In situ* analysis of several key components of this signature, including periostin (POSTN), fibroblast activating protein (FAP) and lysyl oxidase (LOX), revealed that they are specifically up-regulated in the tumor-associated fibroblasts in chemoresistant tumors. We further investigated the specific role of POSTN in promoting chemoresistance in ovarian cancer cells *in vitro*. Finally, we validated the reactive stroma signature in an independent dataset from the chemo-treatment arm of ICON7 Phase III trial and demonstrated that high expression of POSTN and other components of the signature were associated with shorter progression free survival in response to front line chemotherapy. This study serves as a springboard for identifying primary chemoresistant ovarian cancer patients and developing novel therapeutic strategies to overcome chemoresistance.
Materials and Methods

Patients and tumor specimens

Clinical characteristics of the discovery set of 85 and validation set of 138 high-grade serous and endometrioid ovarian tumors can be found in Table 1, Table 2, Table S6, described in the Results section and in the Online Materials and Methods. All discovery set tissue samples were obtained from commercial sources and had appropriate IRB approval. The validation set was from the chemo-treatment arm of a phase III trial, ICON7. All tumor tissues were subjected to review by a pathologist to confirm diagnosis and tumor content. Macro-dissection was performed on FFPE tumor tissue to enrich tumor percentage to greater than 70%. Total RNA was purified using High Pure FFPE RNA Micro Kit (Roche Diagnostics, Indianapolis, IN, USA). FFPE tumor DNA was prepared by QIAamp DNA FFPE Tissue Kit (Qiagen, CA).

Gene expression profiling using an Ovarian Cancer Biomarker Nanostring panel

A custom NanoString 800 GX CodeSet was designed to measure gene expression of 800 biomarkers and controls that are associated with ovarian disease biology including subtype and prognosis classifiers, efflux ABC transporters, as well as chemo-tolerance, immune and angiogenesis markers (see Supplemental Table S1 for complete gene list). 200ng RNA was analyzed using the NanoString nCounter Analysis System following the manufacturer’s protocol (NanoString Technologies). Output raw counts were normalized by the median counts of all 800 assays for each sample.

Statistical analysis

Progression-free survival was calculated from the date of randomization to the date of the first indication of disease progression or death, whichever occurred first; the data for patients who
were alive without disease progression were censored as of the date of their last non-PD tumor assessment. Overall survival was calculated from the date of randomization to the date of death from any cause; data for patients still alive were censored at the date the patient was last known to be alive. Survival analysis was carried out using log-rank test for the difference in the distribution of progression-free survival between the biomarker high and low groups. Median survival time was computed using the product-limit estimate by the Kaplan Meier method.

To compare gene expression differences between Plat-S and Plat-R primary tumors, two-sample t tests were employed. To compare gene expression differences between Plat-R matched primary and metastatic tumors, paired t tests were used. Two-sided p values were derived and adjusted for multiple comparisons by controlling for FDR using the Benjamini Hochberg method.

**RNA in situ hybridization (RNA ISH) assays**

Duplex POSTN/LOX and single-plex FAP RNAscope® in situ hybridization (ISH) assays were designed, implemented and scored at Advanced Cell Diagnostics, Hayward, CA. The single color probe for FAP (NM_004460.2, nt 237-1549) was pre-designed and commercially available. Dual color paired double-Z oligonucleotide probes were designed against LOX (GenBank accession number NM_001178102.1, nt 223-1725) and POSTN (NM_006475.2, nt 13-1199) RNA using custom software as described previously (11). RNA ISH was performed using the RNAscope® 2-plex Chromogenic Reagent Kit and RNAscope® 2.0 HD Brown Reagent Kit on 4μm formalin-fixed, paraffin-embedded (FFPE) tissue sections according to manufacturer’s instructions. RNA quality was evaluated for each sample with a dual colored probe specific to the housekeeping gene cyclophilin B (PPIB) and RNA polymerase subunit IIA (PolR2A). Negative control background staining was evaluated using a probe specific to the bacterial dapB
gene. Only samples with an average of >4 dots per cell with the housekeeping gene probe staining and an average of <1 dot per 10 cells with the negative control staining were assayed with target probes. To verify technical and scoring accuracy, references slides consisting of FFPE HeLa cell pellets were tested for PPIB and dapB together with tissue FFPE slides. Bright field images were acquired using a Zeiss Axio Imager M1 microscope using a 40x objective. The RNAscope signal is scored based on number of dots per cell and as follows 0 = 0 dot/cell, 1 = 1-3 dots/cell, 2 = 4-9 dots/cell, 3 = 10-15 dots/cell, and 4 = >15 dots/cell with >10% of dots in clusters). To evaluate heterogeneity in marker expression, H-Score analysis is performed. The H-Score is calculated by adding up the percentage of cells in each scoring category multiplied by the corresponding score, so the scores are on a scale of 0-400.

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed on 4um thick formalin-fixed, paraffin-embedded tissue sections mounted on glass slides. Primary antibodies against FAP (GNE, clone 10D2.1.1), alpha smooth muscle actin (SMA) (AbCam, Cambridge, MA) and POSTN (BioVendor, Asheville, NC) were used. FAP staining was performed on the DAKO autostainer, utilizing Trilogy (Cell Marque, Rocklin, CA) antigen retrieval. Detection employed horse anti-mouse biotinylated secondary (VectorLabs, Burlingame, CA), followed by Strepavidin-HRP with TSA enhancement (PerkinElmer, Waltham, Massachusetts) and DAB visualization (Pierce, Rockford, IL). SMA and POSTIN staining was carried out on the Ventana Discovery XT automated platform (Ventana Medical Systems; Tucson, AZ). Sections were treated with Cell Conditioner 1, standard time. Specifically bound primary antibody was detected by incubating sections in OmniMap anti-Rabbit-HRP (Ventana Medical Systems; Tucson, AZ) followed by ChromoMap
DAB (Ventana Medical Systems; Tucson, AZ). The sections were counterstained with hematoxylin, dehydrated, and coverslipped.

**H&E assessment of desmoplasia**

Representative H&E stained sections of the discovery tumor samples (85 total including primary Plat-S, patient-matched Plat-R primary and recurrent tumors) were examined for evidence of stromal activation associated with tumor insult and a desmoplasia score was assigned. Some cases were deemed too difficult to score due to tissue damage, necrosis, edema or limited stroma present. Desmoplasia was identified as fibrotic regions typified by an increased density and disorganization of myofibroblasts distinct from resident non-activated fibroblasts. The desmoplasia scoring system used is similar to that reported by Tothill et al in 2008 (8).

Desmoplasia Scores were defined as follows: 0 = no desmoplasia, 1 = few scattered desmoplastic foci abutting cancer cells, 2 = several desmoplastic foci abutting cancer cells or moderate confluent (wider) desmoplasia, but not present throughout the section, 3 = desmoplastic reaction throughout section.

**TP53 mutation status**

We performed deep sequencing on all exons and exon-intron junctions of the entire TP53 gene using a previously developed MMP-Seq targeted cancer panel (12). Quality of the FFPE DNA samples was quantified as number of functional copies using a TRAK2 qPCR “ruler assay” (12). 5000 functional copies of DNA from each sample were used as the input for target enrichment and library construction using Fluidigm Access Array followed by deep sequencing on an Illumina MiSeq sequencer. The average coverage of TP53 gene was ~1000x per amplicon. Sequence alignment, primary variant calling and filtering was performed as previously described (12).
Copy number variation analysis by real-time PCR

Genomic FFPE DNA (200ng) was subjected to 17 cycles of pre-amplification using pooled 35 pairs of gene specific primers at 50nM each and Taqman Preamplification Master Mix (Life Technologies) according to the manufacture protocol. The preamplified samples were diluted and qPCR was performed using Fluidigm 96.96 Dyaminc Arrays on BioMark™ system. In brief, sample mix contains DNA, Taqman gene Expression Master Mix (Life Technologies), DNA binding sample loading reagent (Fluidigm) and EvaGreen dye (Biotium). Assay mix contains gene specific primer pairs and sample loading reagent (Fluidigm). The Ct determination and melting curve analyses were carried out by Fluidigm gene analysis software. Relative gene copy numbers were calculated by global Delta Delta Ct method. First, the median Ct of all genes in each sample was used as reference gene to normalize sample DNA input and calculate the Delta Ct. The median delta Ct of all samples for individual gene was then used as 2 copy calibrator sample. Results are the average of three primer pairs for each gene.

Cell-based assays

Ovarian cell line ES-2 was obtained directly from the Genentech Cell Bank where it was authenticated by short tandem repeat profiling prior to banking and SNP fingerprinting after expansion. Cells were cultured in RPMI1640 medium with 10% FBS and 2mM of glutamine and were used in experiments within four passages. 96-well plates were first coated with recombinant full-length FN1 (Cat# F2006, Sigma-Aldrich, St. Louis, MO), POSTN (Cat# 3548-F2, R&D Systems, Minneapolis, MN) or left uncoated at 37°C for 2 hrs or 4°C for 16 hrs. Cells were then plated in coated plates at 3,000 cells/well. 10µM carboplatin or 10nM of paclitaxel were then added to each well on the next day. Cell-Titre Glo® reagents were added at 72hrs after
compound treatment to measure cell viability. The viability in coated wells was then compared with the one in uncoated wells to calculate % growth benefit.
Results

Identification of a “reactive stroma” gene signature up-regulated in primary chemoresistant ovarian tumors

To identify molecular characteristics associated with primary chemoresistance in EOC, we characterized a set of high-grade serous and endometrioid ovarian tumors with clinically well-defined response to primary chemotherapy (Table 1, Materials and Methods). This discovery set consisted of tumor specimen from 32 patients with primary chemoresistance and 26 patients who were sensitive to primary chemotherapy. All patients were treated with a combination of platinum and taxane as the front-line chemotherapy. Twenty seven out of 32 chemoresistant patients had patient-matched primary tumor specimen collected prior to chemotherapy along with a recurrent tumor specimen collected at disease progression following chemotherapy (referred as Plat-R primary and Plat-R recurrent, respectively, in this report). For the 26 chemosensitive patients, only primary tumor specimens prior to therapy were available for analysis in this study (referred as Plat-S primary in this report).

We first explored whether we could identify a gene expression signature that associated with resistance to platinum-based chemotherapy. Gene expression profiling was performed on these samples using an 800-gene ovarian cancer biomarker panel (Materials and Methods, Supplementary Table S1) developed on the Nanostring platform (13). Two-sample t tests comparing 32 Plat-R and 26 Plat-S primary tumors prior to chemotherapy identified 14 genes that are significantly differentially expressed between the two groups (FDR ≤ 10% and fold change ≥1.5, Supplementary Table S2). Interestingly, up-regulated genes in the Plat-R tumors represented a distinct “reactive stroma” signature (Figure 1A), highly enriched in ECM production and remodeling genes (i.e. POSTN, FAP, LOX, TIMP3, COL4A1), genes involved in
cell migration and invasion (i.e. NUAK1), as well as genes involved in immune modulation (i.e. TDO2). On the other hand, key genes associated with chemo-sensitive tumors include progesterone receptor (PGR), placental alkaline phosphatase (ALPP) and fibroblast growth factor 4 (FGFR4). For the 27 Plat-R patients who had patient-matched primary tumor collected prior to therapy and recurrent tumor collected post therapy at disease progression, we performed further analysis to search for gene signatures characterizing recurrent tumors post progression of chemotherapy. Paired t-test identified 65 genes that were significantly differentially expressed between the primary and recurrent tumors (FDR ≤ 10% and fold change ≥1.5, Supplementary Table S3). Consistently, hallmark genes representing several of the same tumor stromal components, along with additional genes of related function were highly enriched among the 36 significantly up-regulated genes in the recurrent tumors (Figure 1B). These genes included activated fibroblasts marker (ACTA2), ECM production and remodeling enzymes (i.e. POSTN, FAP, FN1, TIMP3, LOX, MMP11), growth factors (i.e. FGF1), immune related genes (i.e. CD36, GZMK, CD247), as well as vascular endothelial markers (i.e. PLVAP and PECAM (antigen CD31)) and growth factors (i.e. ANGPL2). Compared to the primary tumors prior to therapy, the 29 significantly down-regulated genes in recurrent Plat-R tumors were estrogen receptors (ESR1 and ESR2) and other differentiated epithelial cell markers (MUC1, KLK6, KLK7) (Figure 1B). Comparison of the two signatures characterizing primary and recurrent Plat-R tumors identified 4 common reactive stroma signature genes, POSTN, FAP, TIMP3 and LOX whose expression levels were highly correlated with each other (Supplementary Figure S1), significantly up-regulated in Plat-R primary tumors comparing to Plat-S primary tumors, and further induced after chemotherapy in Plat-R recurrent tumors (Figure 1C &D). Together, these
results indicated that up-regulation of reactive stroma genes may play an important role in modulating chemoresistance in EOC.

Mutations in the tumor suppressor gene TP53 (14) and amplification of cyclin E1 (CCNE1) (15, 16) have been previously associated with primary chemoresistance in ovarian cancer. We performed deep sequencing on all exons of the entire TP53 gene using the MMP-Seq targeted cancer panel (12). TP53 mutations were found in 32 out of 32 (100%) Plat-R primary tumors and 23 out of 26 (88%) Plat-S primary tumors (Figure 1A, Supplementary Table S4). The observed overall high frequency of TP53 mutation is consistent with TCGA findings in high-grade serous ovarian tumors (17) and indicate that TP53 mutation status is likely not the main driver of chemoresistance. We also performed a qPCR-based copy number analysis on 35 genes that have been reported to be frequently altered in many types of cancer (Materials and Methods, Supplementary Table S5). Nine recurrently amplified genes were identified in this study (Figure 1A, copy number ≥ 4). Among these, RSF1, AKT1 and AKT3 amplification were only identified in Plat-S tumors, while FGFR1 and ZNF703 amplification were only identified in Plat-R tumors. However, no significant association was observed between response to chemotherapy and amplification of any one (including CCNE1) or combination of these genes.

The reactive stroma signature genes are derived and modulated specifically in tumor associated fibroblasts

To distinguish what specific cell types produced the reactive stroma signature genes, we performed POSTN and FAP RNA ISH analysis on whole slides of tumor specimens from the entire set of 85 tumor specimens. In addition, POSTN and FAP IHC, as well as LOX RNA ISH analysis were also performed on 15 representative tumor specimens. Representative images showing ISH and IHC of these markers are shown in Figure 2A. IHC and ISH of Plat-S primary
tumors showed no detectable or very minimal protein or RNA expression for the reactive stroma signature genes products in stromal or tumor cells. In contrast, in Plat-R primary and recurrent tumors, we found that POSTN RNA and protein were exclusively expressed in the tumor-associated fibroblasts, while LOX and FAP were predominantly expressed in tumor-associated fibroblasts and at lower levels in tumor cells. The POSTN/LOX/FAP expressing tumor-associated fibroblasts also showed strong alpha-smooth muscle actin (αSMA) staining, which is an established marker of an activated myofibroblasts phenotype (18). Consistent with the results from the Nanostring gene expression profiling (Figure 1D), ISH and IHC analysis confirmed expression of reactive stroma genes were significantly higher in Plat-R primary tumors compared to Plat-S primary tumors, and were further up-regulated in Plat-R recurrent tumors (Figure 2B). Importantly, the observed modulation of the reactive stroma genes was mostly restricted to the peritumoral stromal compartment in primary and recurrent Plat-R tumors (Figure 2B), highlighting the tumor associated stromal components as a potential specific site of action in mediating chemoresistance in ovarian cancer.

**Stromal expression of POSTN is associated with a desmoplastic phenotype**

Desmoplasia is a common pathological phenotype found in many types of cancer. Histologic manifestation of desmoplasia include significant overproduction of extracellular matrix proteins, and extensive proliferation and disorganization of myofibroblast-like cells (19). Changes in stromal cell proliferation and the deposition of extracellular matrix components result in dramatic changes in overall tissue heterogeneity and elasticity, as well as accompanying interstitial fluid pressure. These changes have been suggested to contribute to chemoresistance in cancer (20, 21). To evaluate potential links between the reactive stroma molecular signature and histologic features of desmoplasia, H&E stained whole tissue sections for all 85 tumor specimens
in our discovery set were quantitated for the degree of desmoplasia. Twenty-six specimens were
deemed too difficult to score due to tissue damage, necrosis, edema or limited stroma present.
The remaining specimens comprised 21 Plat-S primary, 18 Plat-R primary and 21 Plat-R
recurrent tumors. As shown in Figure 3A&B, while no or only a few scattered desmoplastic foci
were observed in the majority of the Plat-S primary tumors, the Plat-R primary and recurrent
tumors were highly enriched for a moderate to high desmoplastic phenotype when compared to
the Plat-S primary tumors. Furthermore, the degree of desmoplasia was highly correlated with
stromal expression level of POSTN, one of the key components of the reactive stroma signature
characterizing primary chemoresistance (Figure 3A).

**POSTN promotes in vitro chemoresistance**

One of the key component of the reactive stroma signature, POSTN, has been previously
reported to interact with multiple cell-surface receptors, most notably integrins, and signals
mainly via the PI3K/Akt and FAK-mediated pathways to promote cancer cell survival,
angiogenesis, epithelial-mesenchymal transition (EMT), invasion, and metastasis (22, 23). We
next investigated if POSTN play a specific role in promoting chemoresistance in ovarian tumor
cells. For this, recombinant human POSTN protein was used to coat tissue culture dishes to
directly test whether it confers resistance to chemotherapeutics in ES-2 cells, a chemo-sensitive
ovarian cancer cell line with no endogenous POSTN expression (Figure 3C). Because fibronectin
(FN), a glycoprotein and key component of ECM, has been shown to modulate docetaxel
resistance in ovarian cancer cells (24), FN protein coating was used as a control in this
experiment. As shown in Figure 3C, ES-2 cells grown on POSTN coated plates were found to be
significantly more resistant to carboplatin or paclitaxel treatment than cells grown on untreated
culture dishes. Although POSTN coating alone also showed a small increase in cell growth in the
absence of chemo-treatment, its effect on enhancing growth upon chemo-treatment was predominant and significant. In contrast, FN coating did not promote drug resistance to carboplatin or paclitaxel treatment in ES-2 cells. This study demonstrated that exogenous POSTN can promote chemoresistance in EOC cells in vitro. Together, these results provided further supporting evidence that POSTN and other reactive stromal components may play a direct role in promoting chemoresistance in vivo.

Independent validation of the reactive stroma signature in association with primary chemoresistance

To further validate the association between the reactive stroma signature and primary chemoresistance in an independent dataset, we utilized a subset of ovarian tumor tissue samples from the chemo-treatment arm of the ICON7 trial. The ICON7 phase III trial showed survival benefit from the addition of bevacizumab to standard doublet carboplatin-paclitaxel chemotherapy in front line treatment of ovarian cancer (25). Among the 510 patients enrolled in the chemo-treatment arm, 138 patients with high-grade serous or endometrioid ovarian tumors had tissue available for gene expression profiling on the Nanostring ovarian cancer biomarker panel (Table 2). No significant biases in terms of the distribution of Plat-R and Plat-S patients, or clinicopathological characteristics were found in the biomarker evaluable population, and PFS in the biomarker evaluable population was similar to that in the overall intent to treat (ITT) population. These findings suggest the biomarker evaluable population is representative of the intention-to-treat (ITT) population (Supplementary Table S6). We categorized patients from ICON7 chemo-treatment arm into Plat-S and Plat-R groups using the same clinical definition as our discovery study. Two sample t-test analysis on 49 Plat-R and 86 Plat-S primary tumors prior to chemotherapy identified 10 genes that are significantly differentially expressed between the
two groups (p ≤ 0.01 and fold change ≥ 1.5, Supplementary Table S7). Comparison of the differentially expressed gene lists from ICON7 dataset and the discovery dataset showed that the four reactive stroma signature genes (POSTN, FAP, TIMP3 and LOX) from our discovery set were also the top four significantly up-regulated genes in the primary chemoresistance tumors in this ICON7 data set (Figure 4A). These results independently confirmed that the reactive stroma signature is a robust and reproducible biomarker of chemoresistance in EOC. Interestingly, expression of PGR was consistently down-regulated at least 2-fold in the chemoresistant group in both the discovery and the ICON7 datasets (p < 0.001 and fold change = 3.3 in the discovery dataset; and p = 0.0058 and fold change = 2 in ICON7), suggesting progesterone signaling may play an important role in mediating sensitivity to chemotherapies in ovarian cancer.

**POSTN predicts clinical outcome of first line platinum-based chemotherapy in EOC**

To examine whether the reactive stroma signature genes can predict clinical outcome of front line carboplatin-paclitaxel chemotherapy in EOC, we performed univariate survival analysis on the ICON7 chemo-control arm patients using each of the four pre-specified reactive stroma signature genes, POSTN, FAP, TIMP3 and LOX, as well as PGR. As shown in Figure 4B, patients with high POSTN expression (median cutoff) had significant shorter progression free survival (PFS) with median PFS of 12 months comparing to 27 months in patients with low POSTN expression (HR = 2.4, 95% CI: 1.6-3.7, p = 0.0001). Although weak associations were observed between POSTN expression level and several known clinical prognostic factors, including debulking status, serum CA125 level and FIGO stages (Supplementary Figure S2), the association between POSTN level and PFS remained significant (HR = 1.76, p = 0.015) after adjusting for these covariates. TIMP3 expression was also found to be significantly associated with PFS (HR = 1.8, 95% CI: 1.2-2.8, p = 0.0073) in the univariate Cox model. On the other
hand, association between FAP or LOX expression and PFS using a median cutoff was not statistically significant, but highly significant when using a 75%tile cutoff (HR = 2.2, 95% CI: 1.4-3.4, p < 0.001 for FAP; HR = 1.9, 95% CI: 1.2-3.0, p = 0.005 for LOX). Next expression of all four genes (POSTN, FAP, LOX and TIMP3) dichotomized using median cutoff was analyzed in a multivariate Cox regression model to assess the strength of association for each gene. Only expression of POSTN was significant in this multivariate analysis, suggesting POSTN is the main driver and provides the predominant power for predicting patient outcome of first line chemotherapy (Supplementary Figure S3). In addition, when expression of the four genes was averaged for each patient, the resulting overall stroma score did not improve association with PFS (HR = 2.0, 95% CI: 1.3-3.1, p = 0.0013), confirming POSTN’s role as the defining stromal factor in predicting front line ovarian cancer survival in response to chemotherapy. None of the signature genes showed significant association with overall survival (OS). To assess whether PGR provides additional predictive power of patient survival, we performed multivariate COX model analysis with dichotomized POSTN and PGR as covariates (Supplementary Figure S4). After adjusting for POSTN expression level, patients with higher PGR expression experienced a 35% decrease in risk of progression of ovarian cancer, however, the effect is only marginal with a p value of 0.055 (HR = 0.65, 95% CI: 0.42-1.01).
Discussion

Despite the fact that a substantial fraction of patients are intrinsically resistant to chemotherapy, nearly all ovarian cancer patients receive first line platinum/taxane-based treatment regimen to date as the standard of care. Ability to identify patients who gain little benefit from primary treatment is one of the highest priorities in ovarian cancer research, and an important step toward achieving improved treatment strategies in EOC patients (26). Several previous studies have reported gene signatures associated with response to platinum-based therapy (27-29); however, validation of many of these signatures across studies has been difficult (30). In addition, the mechanisms and functional characterization of these chemoresistance signatures have also been lacking. To date, no biomarkers for prediction of response to therapy are yet in clinical use.

In this study, we carried out a systematic and in-depth analysis to discover, functionally characterize and independently validate the key molecular characteristics associated with chemoresistance to primary carboplatin-paclitaxel treatments. One of the key strengths of this study is the high quality and clinically well annotated tumor tissue samples used for both the discovery and independent validation studies. For discovery, we explicitly selected a set of patients with clinically well-defined response to primary chemo-treatment and matched clinicopathological characteristics. For the independent validation study, we utilized the tissue samples from patients enrolled in the chemo-control arm of ICON7, a phase III clinical trial with representative ITT patient population and well-balanced clinical characteristics, well-annotated clinical response and patient outcomes. From the discovery study, we identified a reactive stroma signature to be specifically associated with the Plat-R primary tumors and were further up-regulated in Plat-R recurrent tumors. More importantly, we further validated this signature in an independent data set and demonstrated its clinical utility in predicting patient outcome for the
front line platinum-based chemotherapy. Therefore, our findings provided a promising diagnostic strategy for identifying primary chemoresistant ovarian cancer patients. Further validation of the signature in both retrospective and prospective clinical trials will pave the way to utilizing a biomarker-based test for predicting response to primary chemotherapy in clinical care of ovarian cancer patients.

Another key advantage of this study is the in-depth characterization of the underlying biology of the identified reactive stroma signature genes, including characterizing their specific site of action in situ and recapitulating their functional role in vitro for mediating chemoresistance in EOC. Several previous studies have identified prognostic markers associated with survival (PFS and/or OS) in EOC (31-33), including a few recently published studies on POSTN/collagen-remodeling/TGFβ-associated stromal signature predicts debulking status or poor survival in ovarian cancer (34-36). However, none of these studies further characterized the direct role of these potential prognostic markers in modulating chemoresistance. In this study, using in situ analysis including both IHC and RNA ISH, we unambiguously identified that the reactive stroma signature genes were exclusively or predominantly produced by the activated fibroblast cells adjacent to the tumor cells. More importantly, we showed that modulation of the reactive stroma signature genes within the stromal components but not tumor cells were strongly correlated with the clinical chemoresistance. Importantly, we further demonstrated that chemosensitive ovarian cells grown in the presence of recombinant POSTN promoted resistance to carboplatin and paclitaxel treatment in vitro. To our best knowledge, our study provided the first strong evidence that POSTN, a key component of the reactive stroma, may play a specific role in orchestrating chemoresistance in ovarian cancer. Further dissection of molecular mechanism of POSTN in promoting chemoresistance is underway using a co-culturing system of
ovarian cancer cell lines with either primary tumor associated fibroblast cells derived from ovarian cancer patients or conditioned media.

In addition to providing potential diagnostic strategy for identifying primary chemoresistant ovarian cancer patients, this study also provided important clues for further developing novel therapeutic strategies to overcome chemoresistance. For example, the specific association between reactive stroma, chemoresistance and poor clinical outcome identified from this study, highlighted the important interplay between cancer and the tumor microenvironment in ovarian cancer biology and treatment. Thus, targeting components of the tumor stroma in combination with agents directly targeting the tumor cells may provide a potential novel approach for overcoming resistance and improving efficacy. Indeed, up-regulation of POSTN has been observed in many cancer types, such as breast, lung, colon, pancreatic, and ovarian cancers (23). A recent study has demonstrated that stromal POSTN is crucial for metastatic colonization by regulating the interactions between breast cancer stem cells (37). Furthermore, targeting endogenous POSTN with a neutralizing antibody in an ovarian cancer cell line inhibited ovarian tumor growth and metastasis in animal models (38). Taken together, the important roles of POSTN in cancer development, progression and treatment response make it a promising novel therapeutic target for overcoming chemoresistance. In addition to individual stromal components, our study has revealed that the reactive stroma signature characterizing chemoresistance is highly enriched in genes involved in the normal process of wound healing. Consistent with previous experimental evidence, our data has suggests that TGF-β, a key mediator of the stromal response in wound repair, is likely to play an important role in regulating extensive cross-talks between tumor cells and their associated stroma (Supplementary Figure
S5). Therefore, targeting TGF-β signaling pathway may be another potential promising therapeutic strategy for overcoming chemoresistance.

In conclusion, our findings highlight the important interplay between cancer and the tumor microenvironment in ovarian cancer biology and treatment. The identified reactive stromal components in this study provide a molecular basis to guide development of novel diagnostic and therapeutic strategies for overcoming chemoresistance in ovarian cancer.
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We thank Ashi Malekafzali for helping with the acquisition of clinical tumor tissue samples; Erica Schleifman and Teiko Sumiyoshi for assistance with the FFPE tissue processing and RNA and DNA preparation; Ivette Estay for managing the RNA extraction from ICON7 trial; Luciana Molinero, Macin Kawanetz and Priti Hedge for helpful discussion. We would also like to acknowledge the work and contribution of the ICON7 trial team, MRC CTU at UCL, the ICON7 Translational Research Sub-group, the University of Leeds for their work on the coordination of samples from the ICON7 trial, and all the patients who participated in the trial.
Author Contributions

YW conceived the study, contributed to data analysis, and wrote the manuscript. LR, YG and YW developed the ovarian cancer biomarker Nanostring panel. LR performed the Nanostring analysis, IHC analysis and prepared figures and tables. YG analyzed the RNA ISH data and performed and analyzed the cell-based assays. RF performed and analyzed the IHC data. EF performed the independent pathological review of the acquired tumor tissue specimen and scored the desmoplasia level. YX, YJC and CR performed statistical analyses, prepared figures and tables. SL performed the TP53 sequencing. LH and ML performed the CNV analysis. LF contributed the tissue processing and RNA/DNA extraction. LA and CB contributed to study design and interpretation of the data.
References


Tables
Table 1. Patient clinicopathological characteristics in the discovery study.

<table>
<thead>
<tr>
<th></th>
<th>Platinum-Resistant (N = 32)</th>
<th>Platinum-Sensitive (N = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>56 (28 – 76)</td>
<td>47.5 (28 – 64)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1 (3.1%)</td>
<td>6 (23.1%)</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>31 (96.9%)</td>
<td>20 (76.9%)</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>30 (93.8%)</td>
<td>25 (96.2%)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>2 (6.2%)</td>
<td>1 (3.8%)</td>
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<tr>
<td><strong>PFI (Platinum-Free Interval)</strong></td>
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<tr>
<td>From End of Primary Tx</td>
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<tr>
<td>Median (months)</td>
<td>4.4</td>
<td>Not Reached</td>
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<tr>
<td>95% Confidence Interval</td>
<td>(3.9, 5.0)</td>
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<tr>
<td>Events</td>
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<td>0</td>
</tr>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From Surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (months)</td>
<td>21.9</td>
<td>Not Reached</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>(20.0, 31.5)</td>
<td>(NA, NA)</td>
</tr>
<tr>
<td>Events</td>
<td>25</td>
<td>0</td>
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Table 2. Patient clinicopathological characteristics in the validation set from ICON7 chemo-treatment arm.

<table>
<thead>
<tr>
<th></th>
<th>Platinum-Resistant (N = 37)</th>
<th>Platinum-Sensitive (N = 67)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
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<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>58 (43 – 79)</td>
<td>58 (37 – 75)</td>
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<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (5.4%)</td>
<td>11 (16.4%)</td>
</tr>
<tr>
<td>II</td>
<td>27 (73%)</td>
<td>52 (77.6%)</td>
</tr>
<tr>
<td>III</td>
<td>8 (21.6%)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>33 (89.2%)</td>
<td>59 (88.1%)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>4 (10.8%)</td>
<td>8 (11.9%)</td>
</tr>
<tr>
<td><strong>PFI</strong></td>
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<td></td>
</tr>
<tr>
<td>From End of Primary Tx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (months)</td>
<td>4.6</td>
<td>Not Reached</td>
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<tr>
<td>95% Confidence Interval</td>
<td>(4.5, 4.8)</td>
<td>(28.7, NA)</td>
</tr>
<tr>
<td>Events</td>
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<td>19</td>
</tr>
<tr>
<td><strong>OS</strong></td>
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<tr>
<td>From Surgery</td>
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<tr>
<td>Median (months)</td>
<td>24.1</td>
<td>Not Reached</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
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<td>(NA, NA)</td>
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<tr>
<td>Events</td>
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<td>0</td>
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Figures Legends

Figure 1. Identification of a “reactive stroma” gene signature up-regulated in primary chemoresistant ovarian tumors. (A). Hierarchical clustering of the top 14 most differentially expressed genes (FDR ≤ 10%, fold change ≥ 1.5) between 32 Plat-R primary and 26 Plat-S primary ovarian tumors. Clinically defined response to primary chemotherapy, TP53 mutation status and 7 recurrently amplified genes (≥ 4 copies) were annotated at the bottom; (B). Hierarchical clustering of the top 65 most differentially expressed genes (FDR ≤ 10%, fold change ≥ 1.5) between 27 patient-matched Plat-R primary and Plat-R recurrent ovarian tumors; (C) Venn diagram of common signature genes significantly differentially expressed in Plat-R primary and recurrent tumors; (D). Gene expression of the four reactive stroma signature genes in 26 Plat-S primary, 32 Plat-R primary and 27 Plat-R recurrent tumors.

Figure 2. In situ analysis of the reactive stroma signature genes POSTN, LOX, and FAP by RNA ISH and IHC. (A). Representative ISH and IHC images from a Plat-S primary tumor, and a patient-matched Plat-R primary tumor prior chemotherapy and recurrent tumors post chemotherapy at disease progression. Images in left two columns: 2-plex chromogenic RNA ISH for detection of POSTN (red), LOX (green) and singleplex RNA ISH for detection of FAP (brown) mRNA localization. Images in right three columns: IHC staining for POSTN, FAP and aSMA protein localization. Bar = 100um. S: peritumoral stroma, T: tumor cells. (B). Summary of ISH scores and IHC scores in all 85 samples (POSTN and FAP ISH) or five representative tumor specimens (LOX ISH, POSTN and FAP IHC) from each of the response group: Plat-S primary, patient-matched Plat-R primary and recurrent tumors. Both ISH H-score (Material and
Methods, plotted with means and standard deviations) and IHC overall score were determined in tumor and stromal cells respectively. * p < 0.05, *** p < 0.001

**Figure 3.** POSTN correlates desmoplasia phenotype *in vivo* and promotes chemoresistance *in EOC cells in vitro.* (A). Increased desmoplasia is correlated with POSTN expression and primary chemoresistance. Representative high magnification images of hematoxylin and eosin (H&E) staining for tumor specimens (upper panels) and POSTN ISH images (lower panel) are shown. Desmoplasia scores were defined as follows: 0= no desmoplasia, 1= few scattered desmoplastic foci abutting cancer cells, 2= several desmoplastic foci abutting cancer cells or moderate confluent (wider) desmoplasia, but not present throughout the section, 3= desmoplastic reaction throughout section, associated with most cancer cells. Labels, DS = desmoplastic stroma, NS = normal stroma, TC = tumor cells. Arrows point to examples of tumor cells. Dotted line encircles a region containing tumor cells. Size bars, 100um. (B). Summary of desmoplasia scores in 21 Plat-S primary, 18 Plat-R primary and 21 Plat-R recurrent tumor specimens; (C). POSTN promotes chemoresistance in chemo-sensitive ES2 ovarian cells *in vitro.* 96-well plates were coated with recombinant protein FN1 or POSTN or left uncoated before cells were plated into each well. 10uM carboplatin or 10nM of taxol were then added to each well on the next day. Cell-Titre Glo® reagents were added at 72hrs after compound treatment to measure cell viability. The viability in coated wells was then compared with the one in uncoated wells to calculate % growth benefit.

**Figure 4.** Expression of reactive stromal genes predicts clinical outcome of first line chemotherapy in ICON7 chemo-treatment arm (A) Correlation of fold changes (Plat-R vs. Plat-S) between the discovery dataset (x-axis) and the independent validation set ICON7 control
arm (y-axis). Red: genes are significantly differentially expressed in both datasets (p ≤ 0.01 and fold change ≥ 1.5); (B). Association of expression of reactive stroma signature genes (median cutoff) with patient outcome (PFS) from primary chemotherapy in an independent dataset (ICON7 chemo-treatment arm).
Figure 1.

A. Heatmap of gene expression levels for various genes, including POSTN, FAP, TIMP3, LOX, TDO2, NUAK1, COL4A1, ABCB9, FGFR4, RB1, ANXA1, FOXO1, PGR, GPR, and ALPP.

- Plat-S Primary
- Plat-R Primary
- Plat-R Recurrent
- TP53 Mutation
- Amplification

B. Heatmap of gene expression levels for genes such as LOX, BGN, FGF1, TIMP3, FN1, FAP, ANGPTL2, POSTN, ACTA2, MMP11, RBP4, CD36, PLVAP, PECAM1, ESR1, ESR2, KLK7, KLK6, MUC1, DTX4, FGFR4, and TSPAN8.

C. Venn diagram comparing Plat-R vs. Plat-S Primary Tumors and Plat-R Recurrent vs. Primary Tumors.

D. Scatter plots showing gene expression levels for POSTN, FAP, TIMP3, and LOX in Plat-S Pri, Plat-R Pri, Plat-R Recur conditions.

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 2.

A.

<table>
<thead>
<tr>
<th></th>
<th>POSTN/LOX (ISH)</th>
<th>FAP (ISH)</th>
<th>POSTN (IHC)</th>
<th>FAP (IHC)</th>
<th>SMA (IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Plat-S</td>
<td>S</td>
<td>T</td>
<td>S</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>Primary Plat-R</td>
<td>T</td>
<td>S</td>
<td>S</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>Recurrent Plat-R</td>
<td>S</td>
<td>T</td>
<td>S</td>
<td>T</td>
<td></td>
</tr>
</tbody>
</table>

B.

- **POSTN (ISH)**
  - Stroma: H-Score
  - Tumor: H-Score

- **FAP (ISH)**
  - Stroma: H-Score
  - Tumor: H-Score

- **LOX (ISH)**
  - Stroma: H-Score
  - Tumor: H-Score

- **POSTN (IHC)**
  - Overall IHC Score

- **FAP (IHC)**
  - Overall IHC Score

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Figure 3.

A. Plat-S Desmoplasia = 0  Plat-R Desmoplasia = 1  Plat-R-R Desmoplasia = 3

H&E

POSTN (ISH)

B. Percent (%)

C. Plate coating POSTN/FN  Culture EOC cells  Chemo-treatment Carboplatin/Paclitaxel

Assay EOC cell growth

% Growth benefit vs. uncoated

DMSO  10μM Carboplatin  10N M Paclitaxel

p=0.005  p=0.05
Figure 4. Authors manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

A.

B.

PostN

HR = 2.4
95% CI: 1.6, 3.7
P = 0.0001

TimP3

HR = 1.8
95% CI: 1.2, 2.8
P = 0.0073
Clinical Cancer Research

Up-Regulation of Periostin and Reactive Stroma is Associated with Primary Chemoresistance and Predicts Clinical Outcomes in Epithelial Ovarian Cancer

Lisa Ryner, Yinghui Guan, Ron Firestein, et al.

Clin Cancer Res Published OnlineFirst April 2, 2015.

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