Upregulation of Periostin and Reactive Stroma Is Associated with Primary Chemoresistance and Predicts Clinical Outcomes in Epithelial Ovarian Cancer

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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 dataset containing 138 ovarian patients from the chemotreatment arm of the ICON7 trial.

Results: We identified a distinct "reactive stroma" gene signature that is specifically associated with primary chemoresistant tumors and was further upregulated in posttreatment recurrent tumors. Immunohistochemistry (IHC) and RNA in situ hybridization (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 a high POSTN expression level predicts shorter progression-free survival following first-line chemotherapy.

Conclusions: 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 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% to 30% of cases exhibit intrinsic chemoresistance or resistance and progress during or within 6 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 chemoresistance, a better understanding of the molecular characteristics associated with such intrinsic chemoresistance is needed.

Activation of the host stromal microenvironment, commonly referred to as "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

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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do: 10.1158/1078-0432.CCR-14-3111
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Translational Relevance
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 that 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.

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 Tables 1 and 2 and Supplementary Table S6, described in the Results and in the Supplementary Materials and Methods. All discovery set tissue samples were obtained from commercial sources and had appropriate Institutional Review Board (IRB) approval. The validation set was from the chemotreatment arm of a phase III trial, ICON7. All tumor tissues were subjected to review by a pathologist to confirm diagnosis and tumor content. Macrodisssection was performed on formalin-fixed, paraffin-embedded (FFPE) tumor tissue to enrich tumor percentage to greater than 70%. Total RNA was purified using High Pure FFPE RNA Micro Kit (Roche Diagnostics). FFPE tumor DNA was prepared by QIAamp DNA FFPE Tissue Kit (Qiagen).

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 chemotolerance, immune, and angiogenesis markers (see Supplementary Table S1 for complete gene list). Two-hundred nanograms of 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
PFS 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 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 upregulated in recurrent tumors after 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 upregulated 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 chemotreatment 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 (PFS) in response to first-line chemotherapy. This study serves as a springboard for identifying primary chemoresistant ovarian cancer patients and developing novel therapeutic strategies to overcome chemoresistance.

| Table 1. Patient clinicopathologic characteristics in the discovery study |
|--------------------------|--------------------------|
|                          | Platinum-resistant       | Platinum-sensitive      |
|                          | (N = 32)                 | (N = 26)                |
| Age, y (median)          | 56 (28–76)               | 47.5 (28–64)            |
| Stage                   |                          |                         |
| I                       | 1 (3.1%)                 | 6 (23.1%)               |
| II                      | –                       | –                       |
| III                     | 31 (96.9%)               | 20 (76.9%)              |
| IV                      | –                       | –                       |
| Histology               |                          |                         |
| Serous                  | 30 (93.8%)               | 25 (96.2%)              |
| Endometrioid            | 2 (6.2%)                 | 1 (3.8%)                |
| PFI (platinum-free interval) from end of primary Tx |
| Median, mo              | 4.4                      | Not reached             |
| 95% CI                  | (3.9–5.0)                | (NA, NA)                |
| Events                  | 32                       | 0                       |
| OS from surgery         |                          |                         |
| Median, mo              | 21.9                     | Not reached             |
| 95% CI                  | (20.0–31.5)              | (NA, NA)                |
| Events                  | 25                       | 0                       |

| Table 2. Patient clinicopathologic characteristics in the validation set from ICON7 chemotreatment arm |
|--------------------------|--------------------------|
|                          | Platinum-resistant       | Platinum-sensitive      |
|                          | (N = 37)                 | (N = 67)                |
| Age, y (median)          | 58 (45–79)               | 58 (37–75)              |
| Stage                   |                          |                         |
| I                       | –                       | 4 (6%)                  |
| II                      | 2 (5.4%)                 | 11 (6.4%)               |
| III                     | 27 (73%)                 | 52 (77.6%)              |
| IV                      | 8 (21.6%)                | –                       |
| Histology               |                          |                         |
| Serous                  | 33 (89.2%)               | 59 (88.3%)              |
| Endometrioid            | 4 (10.8%)                | 8 (11.9%)               |
| PFI from end of primary Tx |
| Median, mo              | 4.6                      | Not reached             |
| 95% CI                  | (4.5–4.8)                | (28.7–NA)               |
| Events                  | 37                       | 19                      |
| OS from surgery         |                          |                         |
| Median, mo              | 24.1                     | Not reached             |
| 95% CI                  | (21.1–NA)                | (NA, NA)                |
| Events                  | 19                       | 0                       |
progression were censored as of the date of their last non-PD tumor assessment. Overall survival (OS) 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 the log-rank test for the difference in the distribution of PFS 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 platinum-sensitive (Plat-S) and platinum-resistant (Plat-R) primary tumors, two-sample t tests were used. 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 assays

Duplex POSTN/LOX and single-plex FAP RNAscope in situ hybridization (ISH) assays were designed, implemented, and scored at Advanced Cell Diagnostics. The single color probe for FAP (NM_004460.2, nt 237–1549) was predesigned 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 4-μm FFPE tissue sections according to the 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 II A (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 ×40 objective. The RNAscope signal is scored on the basis of 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 4-μm thick FFPE tissue sections mounted on glass slides. Primary antibodies against FAP (GNE, clone 10D2.1.1), α-smooth muscle actin (α-SMA; AbCam) and POSTN (BioVendor) were used. FAP staining was performed on the DAKO autostainer, using Trilogy (Cell Marque) antigen retrieval. Detection used horse anti-mouse biotinylated secondary (Vector Labs), followed by streptavidin–HRP with TSA enhancement (PerkinElmer) and DAB visualization (Pierce). SMA and POSTN staining was carried out on the Ventana Discovery XT automated platform (Ventana Medical Systems). 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) followed by ChromoMap DAB (Ventana Medical Systems). The sections were counterstained with hematoxylin, dehydrated, and coverslipped.

H&E assessment of desmoplasia

Representative hematoxylin and eosin (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 nonactivated fibroblasts. The desmoplasia scoring system used is similar to that reported by Tothill and colleagues (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). Five thousand 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 approximately 1,000× 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 (200 ng) was subjected to 17 cycles of preamplification using pooled 35 pairs of gene-specific primers at 50 mmol/L each and TaqMan Preamplification Master Mix (Life Technologies) according to the manufacturer’s protocol. The preamplified samples were diluted and qPCR was performed using Fluidigm 96.96 Dynamic 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 C0 determination and melting curve analyses were carried out by Fluidigm gene analysis software. Relative gene copy numbers were calculated by global Delta Delta C0 method. First, the median C0 of all genes in each sample was used as reference gene to normalize sample DNA input and calculate the Delta C0. The median Delta C0 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 RPMI-1640 medium with 10% FBS and...
2 mmol/L of glutamine and were used in experiments within four passages. Ninety-six-well plates were first coated with recombinant full-length FN1 (Cat# F2006; Sigma-Aldrich), POSTN (Cat# 3548-F2; R&D Systems) or left uncoated at 37°C for 2 hours or 4°C for 16 hours. Cells were then plated in coated plates at 3,000 cells per well. Carboplatin (10 μmol/L) or of pacitaxel (10 μmol/L) were then added to each well on the next day. CellTiter-Glo reagents were added at 72 hours after compound treatment to measure cell viability. The viability in coated wells was then compared with the one in uncoated wells to calculate percentage growth benefit.

**Results**

Identification of a "reactive stroma" gene signature upregulated 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 and Materials and Methods). This discovery set consisted of tumor specimens 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 first-line chemotherapy. Twenty-seven of 32 chemoresistant patients had a patient-matched primary tumors specimen collected prior to chemotherapy along with a recurrent tumor specimen collected at disease progression following chemotherapy (referred to 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 to as Plat-S primary in this report).

We first explored whether we could identify a gene expression signature that was 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 and 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, upregulated genes in the Plat-R tumors represented a distinct "reactive stroma" signature (Fig. 1A), highly enriched in ECM production and remodeling genes (i.e., POSTN, FAP, LOX, TIMP3, and 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 chemoresistant tumors include progesterone receptor (PGR), placental alkaline phosphatase (ALPP), and fibroblast growth factor 4 (FGF4). For the 27 Plat-R patients who had patient-matched primary tumors collected prior to therapy and recurrent tumor collected after therapy at disease progression, we performed further analysis to search for gene signatures characterizing recurrent tumors after 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 upregulated genes in the recurrent tumors (Fig. 1B). These genes included activated fibroblasts marker (ACTA2), ECM production and remodeling enzymes (i.e., POSTN, FAP, FN1, TIMP3, LOX, and MMP11), growth factors (i.e., FGFR1, immune-related genes (i.e., CD36, CDZMK, and CD247), as well as vascular endothelial markers (i.e., PLVAP and PECAM (antigen CD31)) and growth factors (i.e., ANGPI2).

Compared with the primary tumors prior to therapy, the 29 significantly downregulated genes in recurrent Plat-R tumors were estrogen receptors (ESR1 and ESR2) and other differentiated epithelial cell markers (i.e., MUC1, KLK6, KLK7; Fig. 1B). Comparison of the two signatures characterizing primary and recurrent Plat-R tumors identified four common reactive stroma signature genes, POSTN, FAP, TIMP3, and LOX, whose expression levels were highly correlated with each other (Supplementary Fig. S1), significantly upregulated in Plat-R primary tumors comparing with Plat-S primary tumors, and further induced after chemotherapy in Plat-R recurrent tumors (Fig. 1C and D). Together, these results indicated that upregulation 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; refs. 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 (Fig. 1A and 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 and Supplementary Table S5). Nine recurrently amplified genes were identified in this study (Fig. 1A, copy number ≥ 4). Among these, RSF1, AKT1, and AKT3 amplification was only identified in Plat-S tumors, whereas FGFR1 and ZNF703 amplification was 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 Fig. 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 cSMA staining, which is an established marker of an activated myofibroblasts.
Figure 1.

Identification of a “reactive stroma” gene signature upregulated 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 responses to primary chemotherapy, TP53 mutation status, and seven recurrently amplified genes (>4 copies) are 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.
phenotype (18). Consistent with the results from the NanoString gene expression profiling (Fig. 1D), ISH and IHC analysis confirmed expression of reactive stroma genes were significantly higher in Plat-R primary tumors compared with Plat-S primary tumors, and were further upregulated in Plat-R recurrent tumors (Fig. 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 (Fig. 2B).

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 to chemotherapy and recurrent tumors after chemotherapy at disease progression. Images in left two columns: 2-plex chromogenic RNA ISH for detection of POSTN (red) and LOX (green), and singleplex RNA ISH for detection of FAP (brown) mRNA localization. Images in right three columns: IHC staining for POSTN, FAP, and αSMA protein localization. Bar, 100 μm. 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 response group: Plat-S primary, patient-matched Plat-R primary, and recurrent tumors. Both ISH H-score (Materials 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.
Desmoplasia is a common pathologic phenotype found in many types of cancer. Histologic manifestation of desmoplasia include significant overproduction of ECM proteins, and extensive proliferation and disorganization of myofibroblast-like cells (19). Changes in stromal cell proliferation and the deposition of ECM 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 Fig. 3A and 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 with 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 (Fig. 3A).

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 H&E staining for tumor specimens (top) and POSTN ISH images (bottom) 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. DS, desmoplastic stroma; NS, normal stroma; TC, tumor cells. Arrows, examples of tumor cells. Dotted line encircles a region containing tumor cells. Size bars, 100 μm. 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 chemosensitive ES-2 ovarian cells in vitro. Ninety-six-well plates were coated with recombinant protein FNI or POSTN or left uncoated before cells were plated into each well. Then, 10 μmol/L carboplatin or 10 nmol/L Taxol was added to each well on the next day. CellTiter-Glo reagents were added at 72 hours after compound treatment to measure cell viability. The viability in coated wells was then compared with the one in uncoated wells to calculate percentage growth benefit.

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 whether 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 chemosensitive ovarian cancer cell line with no endogenous POSTN expression (Fig. 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 Fig. 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 chemotreatment, its effect on enhancing growth upon chemotreatment 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 used a subset of ovarian tumor tissue samples from the chemotreatment 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 first-line treatment of ovarian cancer (25). Among the 510 patients enrolled in the chemotreatment 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 clinicopathologic 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 ITT population (Supplementary Table S6). We categorized patients from ICON7 chemotreatment 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 $\geq 1.5$; 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 upregulated genes in the primary chemoresistance tumors in this ICON7 dataset (Fig. 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 downregulated 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 chemotherapy 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 first-line carboplatin–paclitaxel chemotherapy in EOC, we performed univariate survival analysis on the ICON7 chemo-control arm patients using each of the four prespecified reactive stroma signature genes, POSTN, FAP, TIMP3, and LOX, as well as PGR. As shown in Fig. 4B, patients with high POSTN expression (median cutoff) had significantly shorter PFS, with median PFS of 12 months, compared with 27 months in patients with low POSTN expression [hazard ratio (HR), 2.4; 95% confidence interval (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 Fig. 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%ile 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 Fig. 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 first-line ovarian cancer survival in response to chemotherapy. None of the signature genes showed significant association with 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 Fig. 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 chemotherapy and matched clinicopathologic characteristics. For the independent validation study, we used 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 upregulated in Plat-R recurrent tumors. More importantly, we further validated this signature in an independent dataset and demonstrated its clinical utility in predicting patient outcome for the first-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 using 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/TGF/b-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 have shown 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 under way using a coculturing 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, upregulation 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 suggest that TGFβ, 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 Fig S5). Therefore, targeting the TGFβ 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.

**Disclosure of Potential Conflicts of Interest**

L.C. Amler has ownership interest (including patents) in Roche/Genentech. C. Bais reports receiving speakers bureau honoraria from Roche/Genentech. Y. Wang reports receiving commercial research grants from Genentech/Roche. No potential conflicts of interest were disclosed by the other authors.

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**Acknowledgments**

The authors 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; Bette Estay for managing the RNA extraction from ICON7 trial; and Luciana Molinero, Marcin Kowaniec, and Priti Hegde for helpful discussion. The authors also 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.

Received December 3, 2013; revised March 2, 2015; accepted March 17, 2015; published OnlineFirst April 2, 2015.

**References**


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