

ARTICLE

PTTG1 Levels Are Predictive of Saracatinib Sensitivity in Ovarian Cancer Cell Lines

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Src kinase is recognized as a key target for molecular cancer therapy. However, methods to efficiently select patients responsive to Src inhibitors are lacking. We explored the sensitivity of ovarian cancer cell lines to the Src kinase inhibitor saracatinib to identify predictive markers of drug sensitivity using gene microarrays. Pituitary tumor transforming gene 1 (PTTG1) was selected as a potential biomarker as mRNA levels were correlated with saracatinib resistance, as well as higher PTTG1 protein expression. PTTG1 expression was correlated with proliferation, cell division, and mitosis in ovarian cancer tissues data sets. In sensitive cell lines, saracatinib treatment decreased PTTG1 and fibroblast growth factor 2 (FGF2) protein levels. Downregulating PTTG1 by siRNAs increased saracatinib sensitivity in two resistant cell lines. Our results indicate PTTG1 may be a valuable biomarker in ovarian cancer to predict sensitivity to saracatinib, and could form the basis of a targeted prospective saracatinib trial for ovarian cancer.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE OF THE TOPIC?

✓ Src kinase is recognized as a key target for cancer therapy; however, methods to efficiently select patients most likely to derive therapeutic benefit from Src inhibitors are lacking.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ We explored the sensitivity of ovarian cancer cell lines to the Src kinase inhibitor, saracatinib, and sought to identify predictive markers related to drug sensitivity.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ Saracatinib's effect on ovarian cancer cell line proliferation was examined. We identified differentially expressed

genes correlated with saracatinib sensitivity by expression microarrays. Pituitary tumor transforming gene 1 (PTTG1) was selected as a potential biomarker for saracatinib sensitivity, and was further validated by an independent panel of cell lines, protein level, and siRNA approaches.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND TRANSLATION SCIENCE

✓ Our results indicate that PTTG1 mRNA or protein level may be a valuable biomarker in ovarian cancer to predict a patient's sensitivity to saracatinib, and could form the basis of a targeted prospective saracatinib trial for ovarian cancer.

Ovarian cancer is the leading cause of death from gynecological malignancy.¹ Standard of care is cytoreductive surgery followed by platinum- or taxane-based chemotherapy with an initial response rate of at least 70% in patients with advanced stage disease.² However, most patients relapse with chemotherapy-resistant disease and overall 5-year survival probability is estimated at 30%.³ Thus, the development of novel targeted agents and drug combinations are needed to improve the outcome of this fatal disease.

The introduction of small molecule inhibitors targeting specific receptor tyrosine kinases (RTKs) and their

downstream kinases has brought a major impact on the management of various malignancies.^{4–6} Among them, Src tyrosine kinase, a “proto-oncogene” identified in the 1970s, has been shown to be one of the most promising targets for anticancer therapy.^{7,8} The aberrant activation of Src signaling contributes to multiple aspects of tumor development such as cell proliferation, invasion, angiogenesis, and motility in a large number of malignancies, including ovarian cancer.^{2,9} The most notable characteristic of Src is its extensive interaction with a variety of transmembrane RTKs such as EGFR, HER2, c-Met, and other molecules.¹⁰ Through these

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interactions, Src regulates RTK signaling as a facilitator and directly transduces survival signals to downstream effectors. Combining a Src inhibitor with other RTK-targeted therapies was demonstrated to enhance the therapeutic efficacy and overcome the therapeutic resistance to anti-RTK drugs based on preclinical studies of breast and pancreatic cancers.^{11,12}

The Src inhibitor saracatinib (AZD0530) in a preclinical study showed significant growth inhibitory, antimigratory, and antiinvasive activities in cell lines of various origins.¹³ *In vitro*, saracatinib showed <20 nM IC₅₀ activity against ubiquitously expressed Src as well as other Src-family members Yes and Fyn. In addition, it has similar activity against immune cell restricted Src-family members Lck, Lyn, and Fgr. Recently, a randomized, placebo-controlled trial of combined paclitaxel and the Src inhibitor saracatinib in platinum-resistant ovarian cancer was reported.¹⁴ The patients enrolled in this trial (35 paclitaxel vs. 69 paclitaxel + saracatinib) had completed at least one round of chemotherapy (maximum = 7) and thus could have developed more difficult to treat cancers. Although the end points (overall survival and progression-free survival) of this study were not statistically significant, the combined treatment group had four patients with progression-free survival at 16 months compared with zero patients in the paclitaxel only group.

The ability of saracatinib to modify fulvestrant (an antiestrogen) sensitivity in estrogen receptor (ER α)-positive ovarian cell lines has also been studied.¹⁵ Combined treatment of ER α -positive ovarian cancer cell lines with fulvestrant and saracatinib increased cell cycle arrest, decreased proliferation, and lowered tumor growth in a xenograft model. In addition, this study examined 338 primary ovarian cancers, of which 67% of the patients had detectable ER α protein, and they found the ER α mRNA and protein levels were highly correlated. The ovarian cancers also exhibited a range of phosphorylated Src (*P*-Src) levels, potentially identifying patients most likely to respond to a combined fulvestrant/saracatinib therapy (high ER α / low *P*-Src phenotype). Both of these studies suggest a molecular-based criteria to predict which patients may benefit the most from a targeted therapy (such as saracatinib) regimen that could lead to significantly better outcomes than randomly enrolled patients.

In this study we sought to identify predictive biomarkers that correlate with saracatinib sensitivity utilizing gene expression profiles from a panel of ovarian cancer cell lines. We identified human pituitary tumor transforming gene 1 (PTTG1) as a candidate biomarker since a significant difference was observed in its expression level between saracatinib-sensitive and -resistant ovarian cancer cell lines. PTTG1 was discovered as an oncogene in pituitary tumors,¹⁶ and it is now recognized as a multifunctional oncogene interacting with a variety of signaling pathways.¹⁷ Other studies have suggested that overexpressed PTTG1 has a significant impact on the development and progression of malignancies including ovarian cancer.^{18–20} However, its potential role as a biological marker for determining the efficacy of a molecularly targeted therapy has never been pursued.

MATERIALS AND METHODS

Cell line tissue culture

Thirteen ovarian cancer cell lines (**Supplementary Table S1**) were obtained from the Gynecologic Tissue and Fluid Bank at the University of Colorado, and authenticated using short tandem repeat (STR) assays.²¹ The obtained data were compared with available databases to confirm the cell lines' identities. Cells grown in complete RPMI medium 1640 (Life Technologies, Bethesda, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies) were exposed to saracatinib (AZD0530), provided by Astra-Zeneca (London, UK) or purchased from ApexBio (Houston, TX).

For cell proliferation measurements, cells were plated into 96-well tissue culture plates at a density of 1,000 to 5,000 cells per well, incubated overnight in complete medium, then exposed to increasing concentrations of saracatinib (ranging from 0–10 μ M). Saracatinib dissolved in DMSO was diluted into the micromolar range with the culture medium. DMSO diluted with the medium was used as a vehicle control. Viable cells were evaluated after exposure for 120 h by MTT reagent (Research Products International, Mt. Prospect, IL). The validation cell line set was assayed using the MTS reagent (Promega, Madison, WI); side-by-side comparison to the MTT assay gave comparable results. A 120-h treatment was chosen to allow three to four cell doublings in the presence of saracatinib. The IC₅₀ values were calculated from the resulting linear regression curve. These experiments were performed three to five independent times in three to six replicates for each cell line.

Gene expression profiling of ovarian cancer cell lines

Expression microarrays were run using RNA isolated from untreated ovarian cancer cell lines growing in complete medium. RNA stabilization, isolation, and microarray sample labeling were carried out as described before.²² Human Gene 1.0 ST microarrays (Affymetrix, Santa Clara, CA) were hybridized with cRNA and processed according to the manufacturer's protocol. Hybridization signals and detection calls were generated and analyzed in Partek Genomics Suite 6.6 (Partek, St. Louis, MO) using the Robust Multi-Array Average (RMA) expression measurement. To statistically identify differentially expressed genes, we first removed genes with low variance (<0.15) of expression across the whole data set, and then used analysis of variance (ANOVA) between the saracatinib sensitive and resistant groups, to calculate the *P*-value for each gene. Genes with limited fold-change differences (<1.5-fold) between sensitive and resistant groups were removed. There were 21 genes remaining with a False Discovery Rate (FDR) *P*-value < 0.05. The cell line gene expression data were deposited at the Gene Expression Omnibus (GEO; accession number GSE55628). Four additional independent data sets (GSE9891, GSE28724, GSE53418, and E-TABM-254) were downloaded from publicly available data repositories (GEO and Array Express, respectively). GSE9891 was used to examine PTTG1 mRNA levels in primary ovarian cancer tissues.²³ The other two data sets were composed of ovarian cancer cell lines and were analyzed using Partek, as described above.

RNA interference

PTTG1 was transiently silenced in the ES2 and MCAS (resistant) or SKOV3 (sensitive) cell lines using a combination of two siRNAs plasmids (GI340161 and GI340164, Origene, Rockville, MD). These two siRNAs plasmids worked individually but gave a stronger silencing effect when cotransfected. The targeted sequence of 29 bp siRNA was constructed in the pGFP-V-RS retroviral vectors (TG310042, Origene). A nontargeting scramble siRNA construct (TR30013, Origene) was used as a negative control. Parental cells were plated in a 6-well plate at 20,000 cells per well. The log-phase cells, at 70–90% confluence after 24-h incubation, were transfected with PTTG1 targeting siRNA or scrambled siRNA sequences. siRNA was transfected into the ES2 and SKOV3 cells with X-tremeGENE 9 and TransIT-LT1 transfection reagents according to the manufacturer's standard protocols, respectively (Roche, Nutley, NJ, and Mirus, Madison, WI). They were allowed to recover from transfection reagent treatment for 6–8 h incubation in complete medium before adding saracatinib. After a 96-h exposure to saracatinib, the cell number was counted using a hemocytometer. Ninety-six-h saracatinib treatment was used to balance cell growth and protein recovery from sensitive cells. These experiments were repeated four times under duplicate conditions.

Western blot assays

Cells were incubated overnight and followed by treatment with 1 μ M saracatinib. After 24- or 72-h exposure, they were washed with phosphate-buffered saline (PBS) three times and lysed in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) with protease and phosphatase inhibitor (Thermo-Scientific, Pittsburgh, PA). Insoluble material was cleared by centrifugation. A 72-h saracatinib treatment was selected to balance cell growth and protein recovery from the sensitive cell lines. The total protein was quantified using Micro BCA Reagents (Thermo-Fisher Scientific). Cell lysates (50 μ g protein per lane) were heated for 10 min at 95°C in SDS-Sample Buffer containing 5% β -mercaptoethanol. Proteins were separated on polyacrylamide gels and semidry transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h in 5% nonfat dry milk (or 2% bovine serum albumin (BSA)) in Tris-buffered saline and 0.05% Tween 20 (TBS-T) buffer, and incubated overnight with primary antibody in TBS-T with 5% nonfat dry milk or 2% BSA. The following primary antibodies were used: Rabbit polyclonal anti-PTTG1 antibody (Life Technologies, 1:100 dilution), rabbit polyclonal antiphosphorylated P-Src (Tyr416) (1:1,000), rabbit polyclonal anti-Src (1:1,000), rabbit monoclonal anti-GAPDH (D16H11, 1:1,000), and mouse anti-beta-actin (8H10D10, 1:1,000), all purchased from Cell Signaling Technology, Beverly, MA. After washing with TBS-T three times, membranes were incubated with secondary antibody (anti-IgG-HRP (Pierce, Rockford, IL; 1:15,000 dilution)) for 1 h at room temperature. Blots were washed three times and detection was carried out using enhanced chemiluminescence (Thermo-Scientific). Western blots were quantitated by densitometry using ImageJ software (NIH, Bethesda, MD).

Fibroblast growth factor 2 (FGF2) measurement by enzyme-linked immunosorbent assay (ELISA)

After 72 h of treatment with saracatinib (0, 0.5, and 1.0 μ M), cells were washed, lysed, and quantified as described above. Cell lysates were diluted and FGF2 concentration was measured in 96-well plate format using Human FGF basic Quantikine HS ELISA kit (R&D Systems, Minneapolis, MN). Cytosolic FGF2 concentration was determined following the manufacturer's protocol.

RESULTS

Antiproliferative effect of saracatinib on ovarian cancer cell lines

We first evaluated the antiproliferative effect of saracatinib treatment on a panel of 13 ovarian cancer cell lines using MTT-based cell proliferation assays. The IC₅₀ values ranged from 0.53–8.22 μ M (**Supplementary Table S1**). This cell line panel included common pathological subtypes of ovarian cancers (serous, clear cell, mucinous, endometrioid, and undifferentiated). The saracatinib dose–response curves for the 13 cell lines are shown in **Figure 1** (listed in order of increasing resistance). Based on the pharmacokinetic information in a clinical trial using saracatinib,²⁴ eight cell lines were designated sensitive at a cutoff IC₅₀ value of ≤ 1.0 μ M, which is a clinically attainable drug concentration.²⁵ The other five cell lines with higher IC₅₀ values (≥ 2.0 μ M) were designated resistant.

Statistical identification of differentially expressed genes

Gene expression microarrays were run for the 13 cell lines and we performed statistical analysis to detect differentially regulated genes between saracatinib-sensitive and -resistant cell lines. After filtering out the genes with low variance (< 0.15), the ANOVA test between the two sensitivity groups with a false discovery rate (FDR) P -value < 0.05 was used to identify 21 genes whose expression levels were significantly associated with sensitivity to saracatinib treatment (**Figure 2, Supplementary Table S2**).

Correlation analysis of the gene expression levels and IC₅₀ values using Spearman's ranking was used as an additional measure for identifying potential genes. This approach identified 21 genes with a correlation value of $|r| \geq 0.80$ with a P -value < 0.001 (**Supplementary Table S3**). Given the limited number of genes identified, pathway analysis using DAVID (<https://david.ncifcrf.gov/home.jsp>) was uninformative. Four genes (PTTG1, FECH, LEPROTL1, and RPP30) were common to both lists. Of these four genes, we selected PTTG1 as a potential biomarker for its known role as a tumor oncogene. PTTG1's expression was +1.79-fold upregulated (ANOVA test, P -value = 4.10×10^{-4}) in resistant cell lines, and it showed a positive correlation with saracatinib IC₅₀ of the ovarian cancer cell lines ($r = +0.819$; P -value = 6.20×10^{-4}).

To explore the reproducibility of the PTTG1 mRNA expression levels found in our study, we downloaded two independent data sets (GSE28724 and E-TABM-254) to see if the same cell lines' PTTG1 mRNA levels followed a similar pattern (**Supplementary Figure S1, Supplementary Table S4**). Each data set had a different number of ovarian

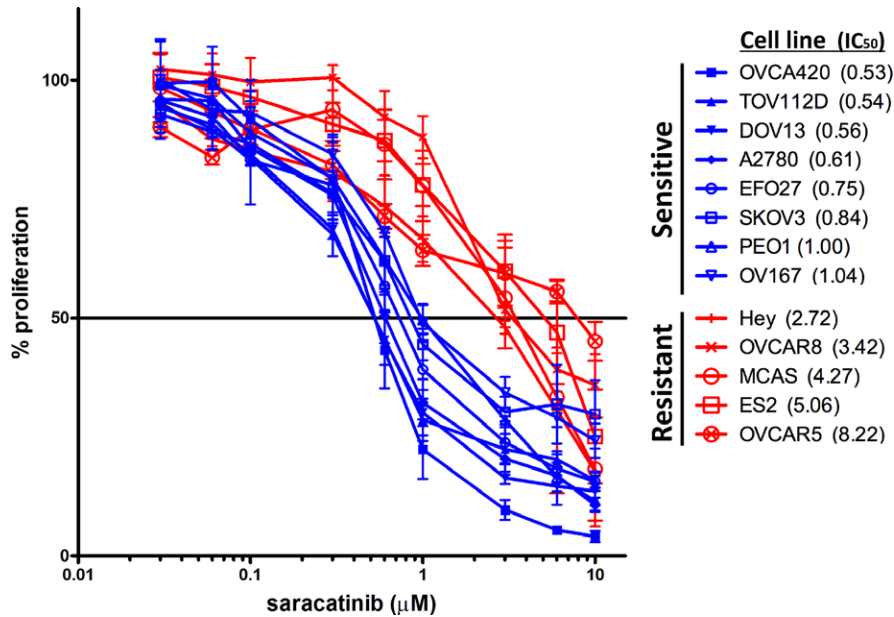


Figure 1 Cell growth inhibition by saracatinib treatment in ovarian cancer cell lines. Ovarian cancer cell lines were exposed to increasing concentrations of saracatinib (0–10 μM). Cell growth was measured by MTT assays after 120 h of treatment. Cell proliferation is shown as percentage of the untreated control. Eight cell lines were designated sensitive at a cutoff IC₅₀ value of ≤1.0 μM, which is a clinically attainable drug concentration. The other five cell lines with high IC₅₀ values (≥2.0 μM) were designated resistant. Blue or red line indicates the assignment of each cell line to sensitive or resistant group, respectively.

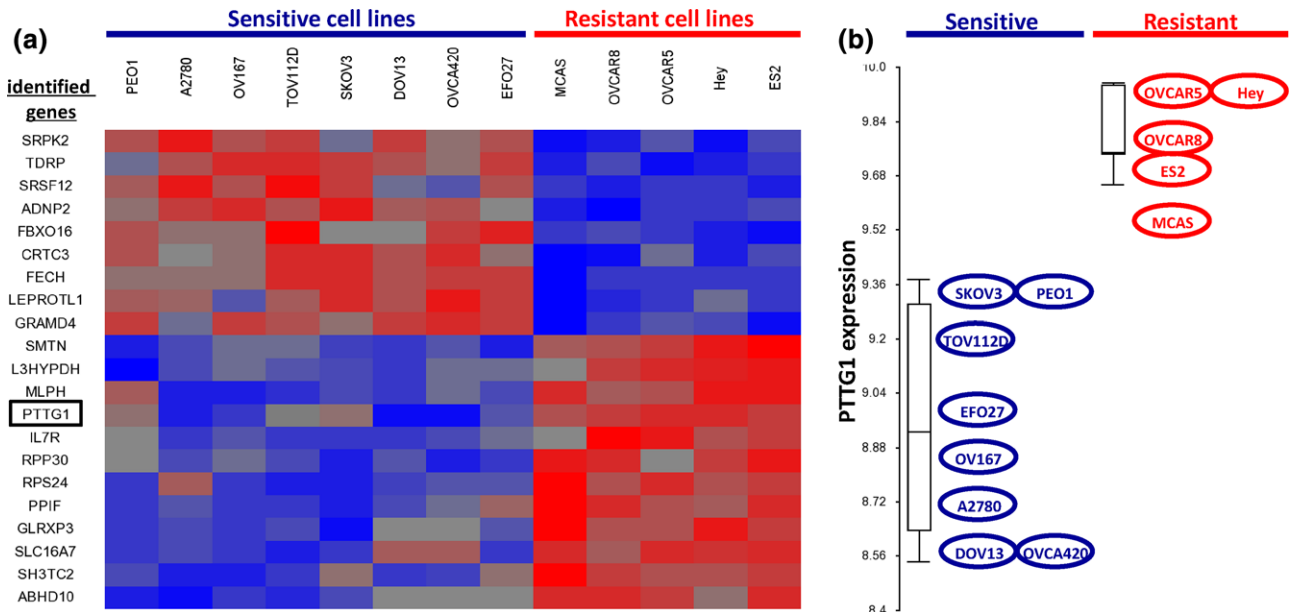


Figure 2 Intensity plot of differentially expressed genes associated with saracatinib sensitivity and the plot of PTTG1 expression. (a) The red and blue colors in the intensity plot are reflective of standardized high and low gene expression, respectively. Gray color represents relatively normal expression of a gene. The intensity of each color indicates amplitude of gene expression. Twenty-one genes that significantly correlate to the saracatinib sensitivity are shown. See **Supplementary Table S3** for more detailed gene information. (b) Differentially expressed PTTG1 is illustrated (P -value = 4.10×10^{-4} in ANOVA test). The circles show each cell line examined. The box-and-whisker plot shows the distribution of PTTG1 mRNA expression for the resistant and sensitive cell lines.

cancer cell lines overlapping the cell lines we tested (8 and 11 cell lines, respectively). Categorizing the cell lines into our defined saracatinib-resistant and -sensitive groups, they showed significant differences in PTTG1 mRNA levels in both data sets (+2.97-fold and +1.54-fold upregulation; t -test,

P -value = 0.025 and 0.0019, respectively). Using the E-TABM-254 data set, which had seven and four sensitive/resistant cell lines, the rank-based Spearman coefficient between PTTG1 expression and saracatinib IC₅₀ ($r = +0.79$; P -value = 0.0037) was similar to what our analysis showed.

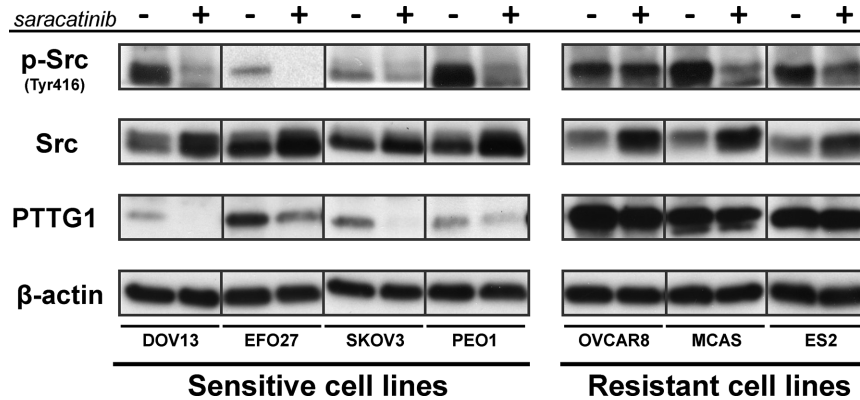


Figure 3 Saracatinib-associated change in protein expression in sensitive and resistant cell lines. Each cell line was treated with 0 or 1 μM saracatinib for 24 h. An equal amount of cell lysate protein (50 μg) was used. P-Src: phosphorylated Src (Tyr416).

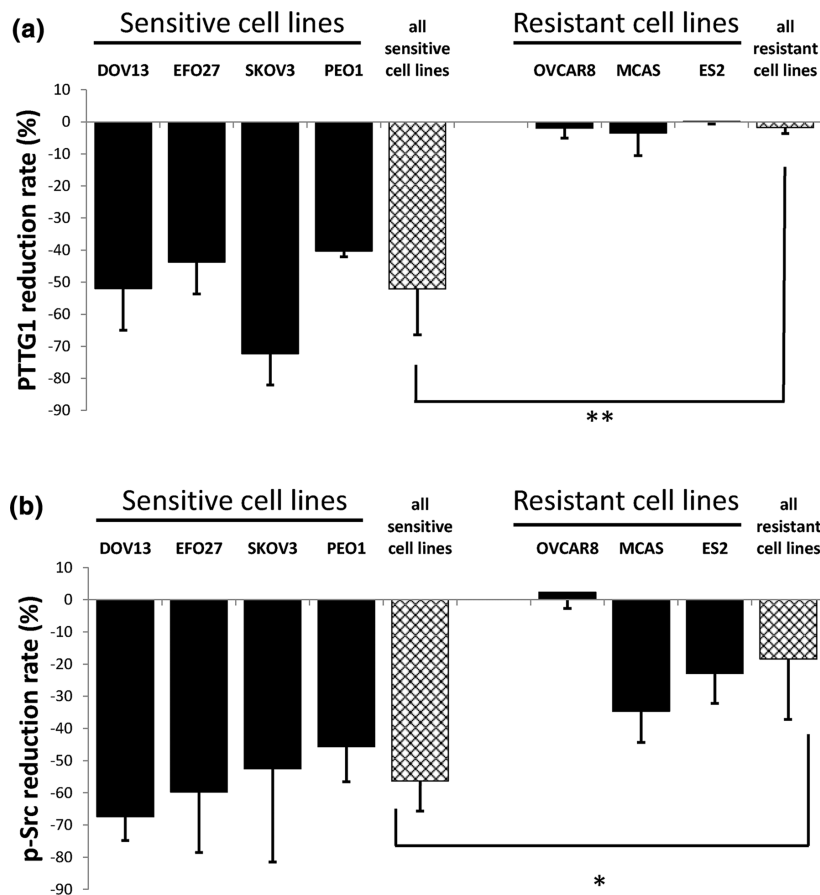


Figure 4 Downregulation of phosphorylated Src and PTTG1 proteins induced by saracatinib treatment. The reduction rates of PTTG1 (a) and P-Src (b) levels were calculated in the comparison of untreated and treated conditions with saracatinib (24 h) in each cell line. One micromolar saracatinib treatment was chosen based on its clinically attainable drug concentration in patients. Quantitative analysis of protein bands was determined using ImageJ software. **PTTG1 levels: resistant $-1.80 \pm 1.81\%$, sensitive $-52.06 \pm 14.34\%$, fold reduction (sensitive/resistant) = 28.9-fold; P -value = 0.0020. *P-Src levels: resistant $-18.42 \pm 18.90\%$, sensitive $-66.37 \pm 9.37\%$, fold reduction (sensitive/resistant) = 3.1-fold; P -value = 0.016, t -tests between sensitive and resistant cell lines. Each bar graph is shown as mean reduction rate \pm standard deviation (%) in replicated experiments.

Independent validation of PTTG1 mRNA expression level and saracatinib sensitivity

The Ovarian Cancer Cell Line Panel²⁶ analyzed 39 ovarian cancer cell lines under uniform growth conditions, complete with DNA fingerprinting, mRNA/miRNA expression, targeted

exon sequencing, and a limited panel of drug sensitivities. An additional seven ovarian cancer cell lines (validation set, **Supplementary Table S5**), chosen from Beaufort *et al.* (GEO accession GSE53418²⁶), were used to independently test PTTG1 level's ability to predict saracatinib sensitivity.

These seven ovarian cell lines had a range in PTTG1 expression and were available from commercial cell repositories. The PTTG1 mRNA levels were measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and found to correspond to the relative ranking predicted by their microarray data (**Supplementary Figure S2**). Using the saracatinib IC₅₀ cutoff of 1–2 μM, five out of the seven cell lines clearly fell into their respective categories. TOV-21G predicted resistant had a IC₅₀ at the borderline (1.14 μM) while OV-7 predicted resistant was sensitive (0.48 μM).

PTTG1 is differentially expressed at the protein level

We selected four and three representative cell lines from the sensitive and resistant groups to investigate PTTG1, Src, and P-Src protein expression levels (sensitive: DOV13, EFO27, SKOV3, PEO1; resistant: OVCAR8, MCAS, ES2, **Figure 3**). We found that the PTTG1 protein level was differentially expressed among the cell lines, and conformed to the same pattern of mRNA expression determined by microarray. Specifically, baseline PTTG1 protein levels were higher in the resistant cell lines, compared with the sensitive lines (4.7-fold difference, *t*-test, *P*-value = 0.0006, **Figure 3**). In the sensitive lines, saracatinib treatment reduced PTTG1 protein expression, while resistant cell lines showed limited changes (over 28-fold difference, *t*-test, *P*-value = 0.0020, **Figure 4a**). Steady-state PTTG1 mRNA levels were not affected by saracatinib treatment, suggesting PTTG1 protein degradation is increased in sensitive cell lines (**Supplementary Figure S3**).

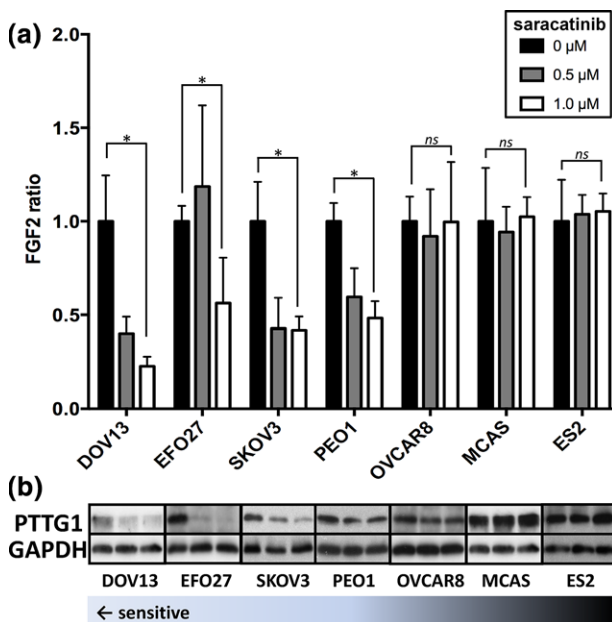


Figure 5 The concordant responses of FGF2 and PTTG1 to saracatinib treatment in ovarian cancer cell lines. **(a)** After exposure to saracatinib for 72 h at 0, 0.5 and 1.0 μM, cytosolic FGF2 concentrations were quantified by ELISA in three experiments. All graphs are shown as mean ± standard deviation relative to each cell line's initial FGF2 level. (**Supplementary Figure S3** shows FGF2 levels plotted as nanogram FGF2 / milligram protein extract). **(b)** PTTG1 and GAPDH protein expression were evaluated by western blot. Cell lines are presented by increasing IC₅₀ values from left to right. **P*-value < 0.05 in *t*-test, ns: not significant.

Saracatinib reduces phosphorylation of the Src protein

Further experiments were completed to explore the relevance of PTTG1 protein level to phosphorylated Src (Tyr416; P-Src) inhibition by saracatinib treatment. Western blot assay revealed that the cell lines had a range of P-Src protein level without saracatinib addition. However, 1 μM saracatinib treatment reduced P-Src in all the cell lines except for the resistant cell line OVCAR8 (**Figures 3, 4b**). By group comparison, there was a 3.1-fold larger reduction of P-Src in the sensitive cell lines than in the resistant lines (*t*-test, *P*-value = 0.016).

PTTG1 expression in ovarian cancer tissue

Three large studies of ovarian cancer tissues were previously published.^{23,27,28} Bowen *et al.* compared microdissected human normal ovarian surface epithelial cells and ovarian cancer cells by gene expression microarrays.²⁸ They identified PTTG1 as significantly upregulated (+2.8-fold change; FDR *P*-value = 0.0001) in ovarian cancer cells.²⁸ Tothill *et al.* compared tumor to neighboring stromal tissue in ovarian cancer tissue by gene expression microarrays.²³ PTTG1 was also upregulated in tumor compared with stromal tissue (+3.7-fold change; FDR *q*-value = 0.0051). Within their study²³ they had low malignant potential (LMP) and malignant ovarian cancer tissue. We found PTTG1 was upregulated in malignant tissue (+2.0-fold change; FDR *q*-value < 0.0001) compared with LMP tissue (**Supplementary Figure S4**).

The Cancer Genome Atlas (TCGA: <https://tcga-data.nci.nih.gov/tcga/>) focused on ovarian serous cystadenocarcinoma tumors (~580 patients) as one of their targeted cancers.²⁷ Using the Web-based interface cBioPortal (<http://www.cbioportal.org/>), we identified and downloaded specific ovarian cancer tissue gene expression files from the TCGA collated database, based on PTTG1 expression. The gene expression data are normalized around the median value found with the ovarian cancer tissue data set. Using a PTTG1 gene expression z-score ≥ |1.5|, 44, and 19 low and high PTTG1-expressing tissues, respectively, were identified (**Supplementary Figure S5a**). Grouping the PTTG1 gene expression z-score ≥ |1.5| ranking into high and low categories (≥ +1.5 vs. ≤ -1.5) gave a 7.4x-fold change in PTTG1 expression (*t*-test, *P*-value < 0.0001), somewhat larger than the range observed within the ovarian cancer cell line data sets. In the ovarian cancer cell lines, SNP-microarrays showed a correlation between higher PTTG1 expression and copy number (**Supplementary Methods**). There was no correlation of PTTG1 expression and copy number alterations in these selected patients (one of the high expression patients had slight amplification, while all the other patients were within the normal diploid value). The proximal portion of 5q (5q11.2/5q13.1) was reported as showing a significant frequency of deletion, while the distal portion containing PTTG1 (5q33.3) was diploid.²⁷ Survival analysis demonstrated no significant difference between the high and low PTTG1-expressing patients (**Supplementary Figure S5b**).

Finally, an analysis of the correlation of gene expression within the TCGA's high and low PTTG1 tissues was completed (**Supplementary Table S6**). There was a striking positive correlation of genes (*n* = 142 genes, leaving out PTTG1's

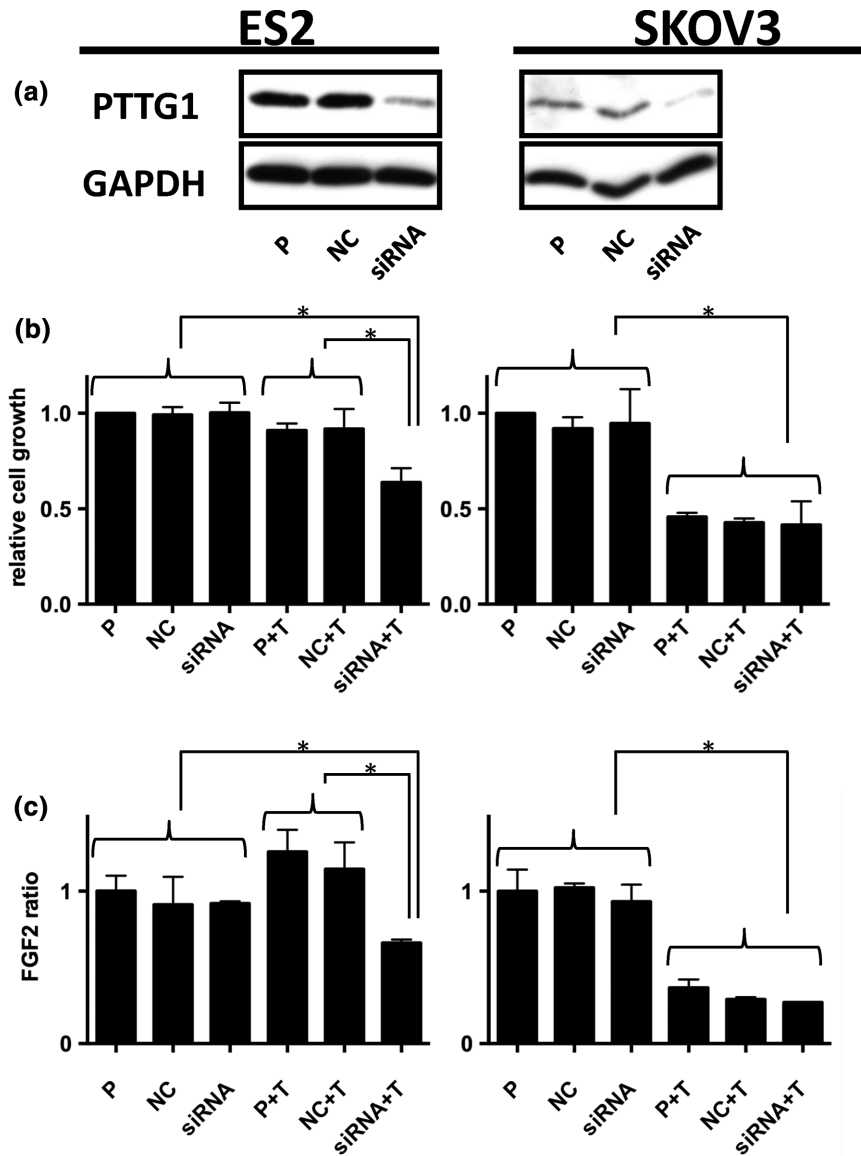


Figure 6 Saracatinib treatment combined with siRNA knockdown of PTTG1 decreases cytosolic FGF2 protein expression and slows cell growth. (a) Efficient PTTG1 knockdown is confirmed by western blot assay. (b) Relative cell growth after 96 h of treatment is shown in the comparison to untreated parental cells ($*P$ -value < 0.05). (c) After siRNA transfection, ES2 cells were treated with 0 or $1 \mu\text{M}$ saracatinib for 72 h. Cytosolic FGF2 concentration was normalized to nanogram FGF2 / milligram protein extract and represented in the comparison to untreated parental cells ($*P$ -value < 0.05). P, parental cells; NC, negative control scrambled siRNA transfection; +T, saracatinib treatment.

related homologs PTTG2 and PTTG3; Pearson's correlation $r = +0.70$ to $+0.91$, P -values $< 2 \times 10^{-10}$ to 4×10^{-23}), which DAVID pathway analysis showed statistically significant involvement in cell proliferation, cycle cell control, and mitosis (GO_Biological Processes; corrected P -value $< 1 \times 10^{-52}$). The quality of negatively correlated genes was significantly worse ($n = 142$ genes; Pearson's correlation $r = -0.48$ to -0.70 , P -values $< 7 \times 10^{-05}$ to 8×10^{-10}) and did not identify any significant pathway enrichment.

PTTG1 cooperates with a proangiogenic factor, FGF2

One of PTTG1's principal functions in the tumor microenvironment is promoting angiogenesis by activating growth factors like FGF2.^{29,30} FGF2 is an also known proliferation

activating molecule in cells expressing FGFRs. Although our gene expression microarrays did not show any correlation between PTTG1 and FGF2 baseline mRNA levels (Spearman's correlation; $r = -0.126$, $P = 0.681$), we sought to determine the interplay of FGF2 and PTTG1 protein levels with saracatinib treatment. Cytosolic FGF2 and PTTG1 total protein levels were evaluated after 72-h saracatinib treatment in the selected cell lines (Figure 5; Supplementary Figure S6). At baseline (no saracatinib treatment), FGF2 levels were highly elevated in all sensitive cell lines except for the EFO27 cell line compared with the resistant cell lines. Saracatinib addition decreased the FGF2 levels prominently in the sensitive lines in dose-dependent manner, which were correlated with PTTG1 inhibition. Even the EFO27 cell line

that had an extremely low intrinsic FGF2 level showed the trend of FGF2 decline after 1 μ M saracatinib treatment. In the resistant cell lines, PTTG1 expression persisted after 72-h treatment and FGF2 concentrations were relatively stable.

PTTG1 gene silencing decreases saracatinib resistance and FGF2 levels in saracatinib-resistant cell lines

We wanted to test if silencing PTTG1 in a saracatinib-resistant cell line affected proliferation and FGF2 expression. To assess these effects, PTTG1 was silenced in the saracatinib-resistant cell lines ES2 (Figure 6) and MCAS (Supplementary Figure S7) using siRNA plasmid constructs. Transfected ES2 was evaluated for PTTG1 expression (Figure 6a), saracatinib sensitivity to proliferation (Figure 6b), and FGF2 expression (Figure 6c). Untransfected parental cell lines and a scrambled siRNA negative control plasmid were used for comparison. The sensitive cell line, SKOV3, showed no added effect of PTTG1 silencing, as its expression level was low, and there was no additional effect on cell proliferation or FGF2 levels (Figure 6). In untransfected ES2 or ES2 transfected with the scrambled siRNA negative control plasmid, saracatinib treatment alone had only limited impact on cell growth or FGF2 levels. However, once PTTG1 protein levels were decreased by targeted PTTG1 siRNA, saracatinib caused significant cell growth inhibition, as well as downregulation of FGF2 levels.

DISCUSSION

PTTG1 was originally identified as a member of the securin family, which controls genomic stability by regulating sister chromatid separation during mitosis.³¹ PTTG1's SH-3 domain may mediate Src kinase activity through signal transduction pathways and could potentially activate a variety of growth factor pathways. Previous studies have demonstrated that higher expression of PTTG1 was strongly associated with tumor progression and worse prognosis in renal, liver, and lung cancer.³²⁻³⁴ In addition, regulating FGF2 is known to be another central function for PTTG1 in pituitary, thyroid, and colon tumors.^{17,35-38} To our knowledge, this is the first report demonstrating gene expression of PTTG1 and its association with sensitivity to a small molecule agent targeting a specific kinase.

Vlotides *et al.* suggested there is a positive feedback regulation between PTTG1 and FGF2.³⁹ Our results suggest sensitive cell lines may be more dependent on FGF2 expression for proliferation than resistant cell lines. Saracatinib treatment of sensitive ovarian cancer cell lines resulted in decreased protein expression of PTTG1 and FGF2 as well as lower phosphorylated Src levels. The analysis of the ovarian cancer data set in TCGA yielded several important insights. PTTG1 mRNA expression was over a sevenfold range, although there was no evidence for a relationship to patient survival. However, there was a strong correlation with genes involved in proliferation, cell division, and mitosis. Saracatinib inhibition of sensitive cell lines may thus interfere with FGF2-dependent proliferation. Two recent articles showed ovarian cancer cell lines, with elevated FGFR2 expression, were sensitive to preclinical FGFR targeting agents PD173074⁴⁰ and RPT835.⁴¹ We speculate that PTTG1 expression level may

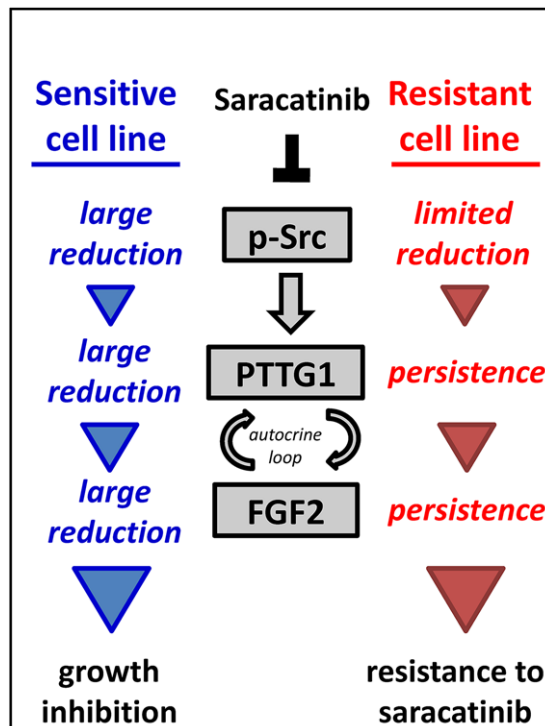


Figure 7 A model for the possible functional relationship between Src, P-Src, PTTG1, and FGF2.

not only be a correlative biomarker for saracatinib sensitivity but also functionally important in regulating FGF2 expression (Figure 7). However, an indirect effect may exist, with PTTG1 serving as a marker for FGF2-dependent proliferation.

In the upcoming era of “personalized medicine,” identifying patients who would benefit from particular targeted therapies is crucially important.⁶ Src kinase is a representative molecular target known to be involved in diverse pathways and molecular interactions. In this study we demonstrate that PTTG1 may play a key role in determining the efficacy of saracatinib, a Src inhibitor, in ovarian cancer cell lines. In contrast to dasatinib (which is FDA-approved), saracatinib is thought to feature fewer off-target effects.²⁴ Our data suggest low expression of PTTG1 (mRNA or protein level) could be a potential clinical biomarker to stratify ovarian cancer patients (and potentially patients with other cancer types) into targeted saracatinib trials. Dividing patients using multiple markers such as PTTG1 and FGFRs expression with a proliferation index may help identify candidates for targeted Src and FGFR combination therapy.

A Src inhibitor alone may have an impact on only a limited number of cancers with low expression of PTTG1. However, our findings suggest tumors refractory to Src-inhibitory therapy could be sensitized when a Src inhibitor is combined with other agents that target additional molecule(s) such as FGF2 and FGF receptors. Continuing *in vitro* studies, as well as validations in mouse models, would be needed to better understand the complicated relationship between PTTG1 and Src-related pathways. In addition, combinatory treatment of saracatinib and a FGFR tyrosine kinase inhibitor in sensitive and resistant ovarian cancer cell lines should be examined.

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