

Imatinib Mesylate (Gleevec) Inhibits Ovarian Cancer Cell Growth through a Mechanism Dependent on Platelet-Derived Growth Factor Receptor α and Akt Inactivation

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ABSTRACT

Purpose: We identified the platelet-derived growth factor receptor α (PDGFR α) as an ovarian cancer-specific gene by microarray hybridization using primary cultures. The purpose of this study is to evaluate whether disruption of the platelet-derived growth factor-regulated growth pathway by Imatinib mesylate (Gleevec), a partially selective PDGFR inhibitor, inhibits growth of ovarian cancer cells expressing PDGFR.

Experimental Design: To investigate the effects of Imatinib mesylate in ovarian cancer, we established an *in vitro* model by immortalizing primary ovarian cells, which express endogenous PDGFR, and we evaluated the effects of Imatinib on cell proliferation. In addition, we investigated the involvement of Akt in mediating Imatinib-inhibited cell growth inhibition.

Results: We found that 39% of ovarian tumors express PDGFR by immunohistochemistry. We showed that Imatinib inhibits the growth of ovarian cancer cells in a PDGFR-specific manner, at clinically relevant concentrations ($IC_{50} < 1 \mu M$). Imatinib inhibits the growth of three primary ovarian cultures and two immortalized cultures (PDGFR positive), but has no effects on SkOv3 and CaOv3 cell lines (PDGFR negative). Imatinib exerts antiproliferative effects by arresting cells at G₀-G₁ and preventing progression through S phase. Imatinib inhibits both PDGFR α

and Akt phosphorylation at a concentration of 1 μM . Stable expression of constitutively active Akt induces partial resistance to PDGFR inhibition in ovarian cancer cells, as demonstrated by cell proliferation assay and cell cycle analysis.

Conclusions: Our data indicate that Imatinib mesylate inhibits the growth of ovarian cancer cells through PDGFR inactivation. In addition, our results suggest that constitutive Akt activation modulates sensitivity to Imatinib in ovarian cancer cells.

INTRODUCTION

Epithelial ovarian cancer (EOC) is the leading cause of mortality among gynecological malignancies and the fifth leading cause of cancer-related death in women (1). Most patients present with advanced stage ovarian tumors, and treatment is based on extensive surgery followed by chemotherapy. The backbone of chemotherapeutic regimens remains a platinum derivative, to which taxanes have been added in recent years. However, mortality due to advanced ovarian cancer has largely remained unchanged over the past 2 decades. Incomplete understanding of the pathways driving neoplastic transformation and ovarian tumor growth has limited the development of efficient targeted therapies for patients with EOC.

On the basis of microarray gene expression analysis using primary ovarian cells, we identified differential expression of the platelet-derived growth factor receptor α (PDGFR α) between primary cells derived from epithelial ovarian tumors and primary cells derived from normal ovarian epithelium. The level of PDGFR α expression is 6-fold higher in tumor-derived cells than in normal ovarian epithelial cells ($P = 0.00137$; Ref. 2). PDGFR is normally expressed in mesenchymal and in glial tissues, and is absent in epithelia. The ovarian epithelium that originates in the coelomic embryonic layer has dual epithelial and mesenchymal characteristics (3), and undergoes an epithelio-mesenchymal transition during neoplastic transformation (4). These specific characteristics of the ovarian epithelium may explain the aberrant PDGFR α expression in EOC-derived cells. In a previous report, immunohistochemical staining demonstrated PDGFR α expression in 16 of 45 ovarian tumors (5). The level of PDGFR α expression correlated with higher histological grade and advanced surgical stages of ovarian tumors. Furthermore, stage for stage, patients with PDGFR α -positive tumors had shorter survival times than those with negative tumors. These observations suggested that the platelet-derived growth factor (PDGF)-PDGFR system can play a functional role in the progression of EOC and that autocrine or paracrine activation of PDGFR within the tumor tissue contributes to tumor expansion.

PDGFR activation by the PDGF induces receptor dimerization and autophosphorylation of tyrosine residues in the intracellular kinase domain (6). Receptor phosphorylation makes possible the subsequent engagement of several proteins

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with Src homology domains, including phosphatidylinositol 3'-kinase (PI3k), phospholipase γ C, GTP-ase activating protein, Grb, and Nck, that convey mitogenic signals to the nucleus. Interruption of this growth pathway could be instrumental for inhibiting ovarian tumor growth. Imatinib mesylate (STI 571 or Gleevec; Novartis, East Hanover, NJ) is a 2-phenylaminopyrimidine derivative that selectively inhibits the abl, c-kit, and PDGFR tyrosine kinases, but does not affect signal transduction through related receptors such as c-FMS, Tek, or the Flt family of receptors (7, 8).

There is ample evidence in the literature supporting the critical role played by the PI3k/Akt pathway in ovarian oncogenesis (9, 10). This pathway can be activated in ovarian tumors through Akt (protein kinase B; Ref. 11) or PI3k gene amplification (12, 13), through activating mutations of the p85 α regulatory subunit of PI3k (13) or by down-regulation of the phosphatase, PTEN (14). In addition, PI3k and downstream Akt can be activated by various growth factors (15), including by PDGF (16). Paracrine or autocrine engagement of growth pathways within the tumor bed activates Akt. Phosphorylated Akt (pAkt) modifies the activity of survival-related proteins and proliferation factors. Akt promotes cell survival by inhibiting proapoptotic proteins, such as BAD (17), caspase 9 (18), apoptosis-signal regulating kinase, forkhead transcription factors (19), and the growth-inhibitory protein glycogen synthase kinase-3 (20). On the other hand, Akt promotes cell proliferation by up-regulating the translation of proteins required for cell cycle progression. For instance, Akt phosphorylates through an m-TOR/FRAP-dependent mechanism (21, 22), the p70 S6 kinase, and inactivates the translational repressor, 4E-BP1 (23). The phosphatase PTEN, which has tumor suppressor properties, inactivates Akt (24–26).

In this study, we confirmed by immunohistochemical staining that PDGFR α is expressed in 39% of ovarian tumors. We showed that Imatinib mesylate inhibits PDGFR α phosphorylation and the growth of ovarian cancer cells harboring PDGFR α . This conclusion was reached by using primary ovarian cells, immortalized primary tumor-derived ovarian cells, and two ovarian cancer cell lines, with different levels of baseline endogenous PDGFR α expression. The effects of Imatinib mesylate are restricted to PDGFR-expressing cells, as ovarian cancer cell lines lacking PDGFR α expression are insensitive to the drug. Inhibition of the PDGFR-PDGF pathway by Imatinib mesylate inactivates Akt, and loss of Akt phosphorylation parallels growth inhibition. Furthermore, stable expression of constitutively active Akt in ovarian cancer cells harboring PDGFR α renders these cells resistant to the growth-inhibitory properties of Imatinib mesylate.

MATERIALS AND METHODS

Immunohistochemistry. Forty-one paraffin-embedded tumor specimens obtained from the Cooperative Human Tissue Collection, two normal ovarian specimens, and one ovarian tumor of low malignant potential from the Indiana University Tissue Bank Collection were immunostained using anti-PDGFR α antibody (R&D Systems, Minneapolis, MN) at a concentration of 20 μ g/ml, following the manufacturer's instructions. In brief, the slides were deparaffinized in xylene, treated with proteinase K (Dako, Carpinteria, CA) for 5 min, and

incubated with primary antibody overnight at 4°. Secondary labeling was based on the avidin/biotin system (Dako; LSAB2 kit). The slides were stained with 3–3' diaminobenzidine and counterstained with hematoxylin. Negative controls were run in parallel, with omission of the primary antibody. All of the slides were read by a board-certified pathologist.

Cell Lines. SKOV3 and CaOv3 cells (American Type Culture Collection, Manassas, VA) were grown according to the manufacturer's instructions. CSOC848, CSOC908, and CSOC918 (generous gift from the Cedars Sinai Ovarian Cancer repository, Dr. Rae Lynn Baldwin, UCLA, Los Angeles, CA) are primary ovarian cancer cultures established as described previously (27, 28). In culture, ovarian epithelial cells tend to assume atypical fibroblast-like morphology and dual epithelio-mesenchymal phenotype, characterized by the expression of both keratin (an epithelial marker) and vimentin (a mesenchymal marker; Ref. 29). We have relied on homogenous cytokeratin and vimentin staining and the absence of factor VIII staining (an endothelial cell marker) to ensure purity of primary cultures (2, 27). All of the primary cell cultures represent early passage cells (<10 passages for tumor-derived cells and <4 passages for primary cultures derived from normal ovarian epithelium). Three immortal ovarian cell lines C272/hTert/E7, C889/hTert, and H281/hTert/E7 were obtained by transducing the catalytic unit of human telomerase and the papilloma virus subunit E7 in primary cancer cell cultures through retroviral infection, as described previously (30). C272/hTert/E7 and C889/hTert are derived from two tumor-derived primary cultures (CSOC272 and CSC889), and H281/hTert/E7 was derived from a normal primary ovarian culture (HOSE281). The primary and immortal ovarian cells were grown at 37°C, 4% CO₂ in 1:1 growth medium containing MCDB 105 (Sigma, St. Louis, MO) and M199 (Cellgro, Herndon, VA) supplemented with 10% FCS and 1% penicillin/streptomycin. All of the other cells were grown in the presence of 10% fetal bovine serum, unless otherwise specified, and were harvested during the log growth phase, when reaching 70–80% confluence.

Cell Growth Assay. Cells were plated in equal numbers in serum-containing culture medium. Imatinib mesylate (Gleevec, STI571), synthesized by Novartis, solubilized in water, was added to the culture medium at different concentrations and was renewed every 72 h. All of the experiments were performed in triplicates and were repeated twice. Cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) colorimetric dye reduction method (31). Results are presented as the average of at least three experiments performed in identical conditions.

Apoptosis and Cell Cycle Analysis. Apoptosis was determined in cells incubated with Imatinib mesylate for 72 h, by staining with FITC annexin V antibody and propidium iodide (Molecular Probes, Eugene, OR) followed by flow cytometry analysis, measuring emission at 530 nm (FL1) and 575 nm (FL3). Cell cycle analysis was performed by DNA staining with propidium iodide in cells, after stimulation with 20% fetal bovine serum and treatment with Imatinib mesylate. Cell cycle profile was determined by flow cytometry with FACScan/CellQuest system (Becton-Dickinson, San Jose, CA). The size of sub-G₁, G₀/G₁, S, and G₂-M were determined by analyzing

the histograms using ModFitLT software (Verity Software, Topsham, ME). All of the experiments were performed in duplicate.

PDGFR Immunoblotting. Cells actively growing were lysed using lysis buffer containing leupeptin 1 $\mu\text{g/ml}$, aprotinin 1 $\mu\text{g/ml}$, phenylmethylsulfonyl fluoride 400 μM , and 1 mM Na_3VO_4 . Cell lysates were sonicated and incubated on ice for 30 min. Cellular debris was removed by centrifugation for 15 min. Cell lysates were separated by SDS electrophoresis, transferred to nitrocellulose membrane, and immunoblotted with anti-PDGFR α antibody (Upstate Technology, Waltham, MA) at 1 $\mu\text{g/ml}$ concentration overnight at 4 $^\circ$.

Phospho-AKT Immunoblotting. Cells in log phase of growth were serum starved for 48 h, treated with various concentrations of Imatinib mesylate, and stimulated with 20% serum or PDGF BB (Sigma) at a concentration of 5 ng/ml or 25 ng/ml. They were lysed in 1 \times SDS buffer, containing 62.5 mM Tris (pH 6.8), 2% SDS, 50 mM DTT, and 10% glycerol, and boiled for 10 min. They were then separated by SDS gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with 1 $\mu\text{g/ml}$ polyclonal anti-phospho Akt rabbit antibody (Cell Signaling) able to recognize Akt phosphorylation at position S⁴⁷³. Total anti-Akt antibody (Cell Signaling) was used as a control.

Mitogen-Activated Protein Kinase (MAPK) Immunoblotting. Equal amount of total protein separated by SDS gel electrophoresis were immunoblotted with anti-phospho-MAPK antibody (Zymed, San Francisco, CA) at 1:2000 dilution and with anti-total MAPK antibody (Cell Signaling) as control.

PDGFR α Immunoprecipitation. Cells were lysed on ice in a buffer containing 20 mM HEPES (pH 7.4), 50 mM β glycerophosphate, 2 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, leupeptin 1 $\mu\text{g/ml}$, aprotinin 1 $\mu\text{g/ml}$, and phenylmethylsulfonyl fluoride 400 μM , as described previously (32). After incubation on ice for 15 min, they were centrifuged at 13,000 rpm for 30 min to remove cellular debris. Five hundred μg of protein was incubated for 90 min at 4 $^\circ\text{C}$ with 5 μg of anti-PDGFR α polyclonal rabbit antibody (Upstate Technology, Waltham, MA), then incubated with 50 μg of Protein G Plus-Agarose beads slurry (Oncogene, Boston, MA) for an additional 90 min at 4 $^\circ\text{C}$. The protein-antibody bead complexes were washed twice with a wash buffer containing 0.2% Triton, then boiled for 5 min in 1 \times SDS protein loading dye. PY20 Antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to recognize phosphorylated tyrosine residues. As control, input protein for immunoprecipitation reaction was immunoblotted with anti-PDGFR α antibody.

Stable Transfection with Myristoylated Akt. Actively growing C272-hTERT/E7 ovarian cells were transfected with myristoylated Akt1 cloned downstream of cytomegalovirus promoter in pLNCX eukaryotic expression vector (BD Biosciences, Palo Alto, CA), which contains a neomycin resistance marker (generous gift of Prof. William R. Sellers, Dana-Farber Cancer Institute, Boston, MA; Ref. 33). Empty vector transfection and selection were performed in parallel. Stable clones were selected with neomycin, and Akt expression was verified by immunoblotting with total Akt antibody (Cell Signaling). Akt phosphorylation was confirmed by immunoblotting with phosphorylated Akt antibody (Cell Signaling). Two stable clones expressing Akt (Akt#9 and Akt#16) and a control stable clone (empty vector)

were used for cell growth and Akt phosphorylation experiments in the presence of various Imatinib mesylate concentrations, as described. All of the growth experiments were performed in triplicate in serum and were repeated twice. A two-tailed *t* test was performed for both pairs (Akt stable clone #9 and #16 versus control) at each drug concentration level, and *P*-value was calculated. Cell cycle profiles after treatment with Imatinib (5 μM) were determined in cells expressing stable myrAkt and compared with cells transfected with empty vector.

RESULTS

PDGFR α Expression in Ovarian Tumors. To determine PDGFR α expression in ovarian tumors, we used immunohistochemical staining with a specific anti-PDGFR α antibody. We identified intense membrane staining in 16 of 41 tumors (39% of tumors, Fig. 1). When evaluated by histological subtype, membrane staining was noted in 4 of 5 clear cell carcinomas, 3 of 8 endometrioid carcinomas, and 8 of 26 papillary serous tumors (Table 1). Patchy cytoplasmic staining was identified in 11 additional tumors (1 clear cell, 7 serous, and 3 endometrioid tumors). In contrast, normal ovarian epithelium did not stain (2 normal ovaries and 1 borderline tumor). The staining is significant in clear cell carcinomas and in invasive, poorly differentiated foci of other ovarian specimens. These data are consistent with a previous report, which records positive PDGFR α staining in 16 of 45 ovarian tumors, but no staining in normal ovaries or in benign tumors (5). Pericytes and endothelial cells within vessel walls in the tumor bed react with anti-PDGFR α antibody (internal positive control). Control staining (without primary antibody) was consistently negative.

Imatinib Mesylate Inhibits the Growth of Ovarian Cancer Cells Expressing PDGFR α at Clinically Relevant Concentrations. We first determined PDGFR expression in several primary ovarian cultures derived from tumors or from normal ovarian epithelium, in immortalized ovarian cells and in ovarian cancer cell lines. Primary ovarian cancer cells (CSOC848, CSOC908, and CSOC918) and the immortalized C272-hTert/E7 and C889-hTert cells express high levels of PDGFR α (Fig. 2, A and B). In contrast, normal primary ovarian cells (HOSE302, HOSE305, and HOSE326, generous gift of Dr. Robert Bigsby, Indiana University, Indianapolis, IN) and immortalized cell line H281-hTert/E7 (derived from a normal ovarian primary culture HOSE281) do not express PDGFR α . Three ovarian cancer cell lines (Ov90, CaOv3, and SkOv3) from the American Type Culture Collection have no detectable PDGFR α expression. In parallel, we determined levels of Akt activation in these cell lines or cell cultures. CaOv3 and SkOv3 (American Type Culture Collection) express high endogenous levels of phospho-Akt, whereas in C272-hTert/E7 and H281-hTert/E7, Akt is minimally activated under basal conditions (10% FCS growth conditions). Constitutive activation of Akt in SkOv3 cells and CaOv3 cells was reported previously (34). Activation of Akt is less evident in primary cell cultures (Fig. 2, A and B), in basal growth conditions.

To determine the effects of Imatinib mesylate in ovarian cultures, we treated several cell lines with clinically relevant concentrations of Imatinib (1–10 μM) in the presence of serum-containing culture media. We used four ovarian cancer cell lines

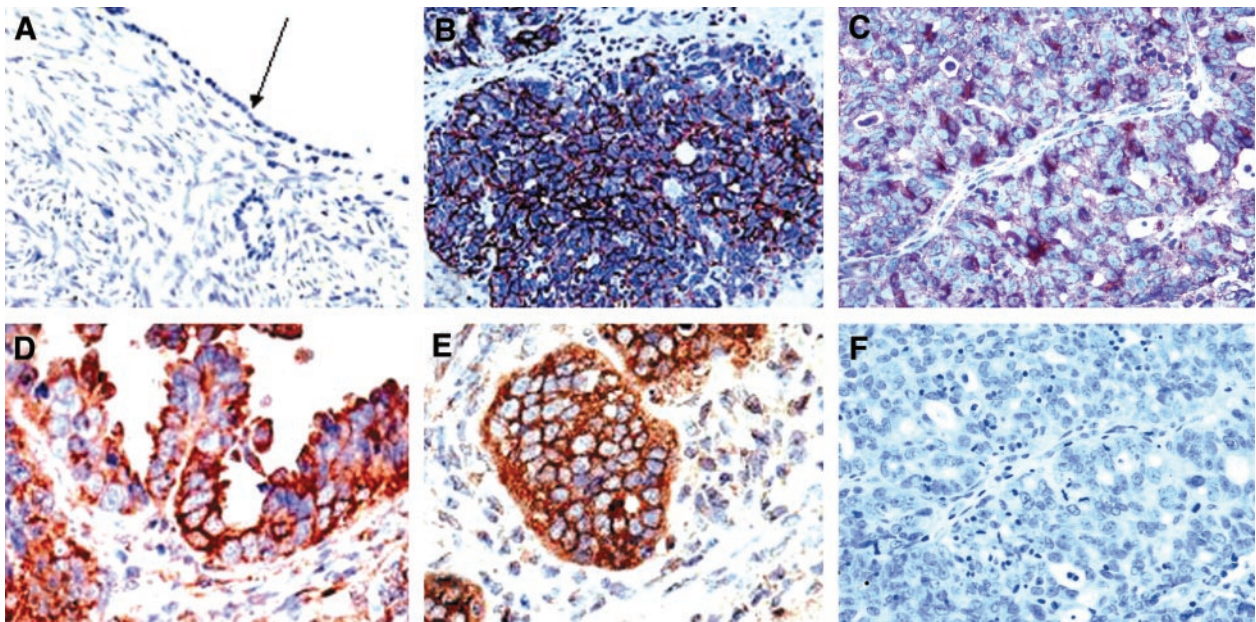


Fig. 1 Immunohistochemical staining for platelet-derived growth factor receptor α . A, normal ovarian epithelium, $\times 200$ magnification. B, ovarian carcinoma endometrioid subtype, $\times 200$ magnification. C and F, ovarian adenocarcinoma, positive (C) and negative control (no primary antibody, F), $\times 200$ magnification. D and E, clear cell ovarian carcinoma, $\times 200$ and $\times 400$ magnification.

Table 1 Immunohistochemical staining for platelet-derived growth factor receptor α in ovarian tumors

Histological type	Number	Membrane staining	Patchy	
			cytoplasmatic staining	Negative
Serous papillary	26	8	7	11
Clear cell	5	4	1	0
Endometrioid	8	3	3	2
Poorly differentiated adenocarcinomas	2	1	1	0
Total	41	16	12	13

with different basal levels of PDGFR α expression and three primary ovarian cancer cultures expressing PDGFR α . The growth of C272-hTert/E7 cells and C889/hTert cells, which express high levels of PDGFR α and of primary CSOC848, CSOC908, and CSOC918 cells, was inhibited at drug concentrations $\geq 1 \mu\text{M}$ (Fig. 3, A and B). This concentration can be achieved in the clinical setting, being tolerated with minimal toxicity (35, 36). By contrast, SkOv3 and CaOv3 cells that do not express PDGFR α are insensitive to the effects of Imatinib up to $10 \mu\text{M}$ concentration (Fig. 3C). Drug concentrations $> 10 \mu\text{M}$ are toxic to all ovarian cells, regardless of their PDGFR status. These data suggest that Imatinib mesylate inhibits the growth of ovarian cancer cells in a PDGFR-specific manner.

Imatinib Mesylate Inhibits Cell Proliferation. To clarify the mechanism underlying cell growth inhibition in the presence of Imatinib, cell cycle profiles 18 h after treatment with Imatinib were analyzed. Representative histograms are shown in Fig. 4. After treatment with Imatinib mesylate, PDGFR-expressing cells (C272hTert/E7) are arrested at G₀-G₁, and serum-

induced S phase progression is prevented. In contrast, when apoptosis was analyzed by propidium iodide/Annexin V staining, we noted that the fraction of apoptotic cells is only minimally increased by treatment with Imatinib (5.5%) versus control (3%) at 72 h, suggesting that apoptosis is not the main mechanism of growth inhibition in this model (data not shown).

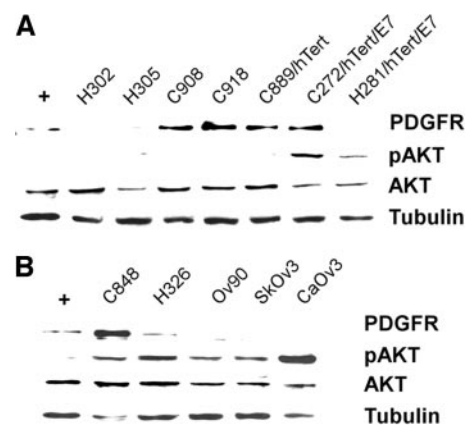


Fig. 2 Platelet-derived growth factor receptor α (PDGFR α), phosphorylated Akt, and total Akt expression in primary ovarian cells and in ovarian cancer cell lines. C848, C908, and C918 are primary ovarian cell cultures derived from ovarian tumors. H302, H305, and H326 are primary ovarian cell cultures derived from normal surface ovarian epithelia. C889-hTert, C272-hTert/E7, and H281-hTert/E7 are immortalized ovarian cells derived from primary ovarian cells. Ov90, SkOv3, and CaOv3 are ovarian cancer cell lines. Levels of PDGFR α , total, and phospho-Akt were determined by immunoblotting. Tubulin was used as a control. Cell lysate from NIH3T3 cells was used as a positive control for PDGFR α (Upstate Technologies).

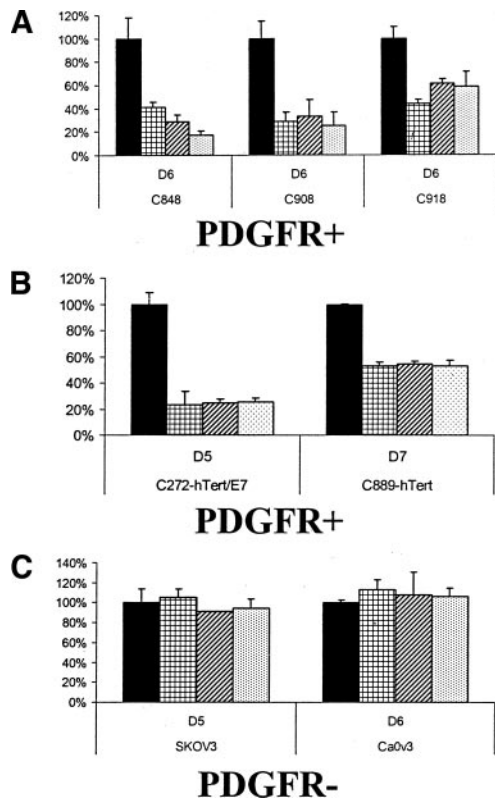


Fig. 3 Imatinib mesylate inhibits the growth of ovarian cancer cells in a platelet-derived growth factor receptor (*PDGFR*)-specific manner. Ovarian cancer cells grown in the presence of serum (10% FCS) were treated with Imatinib mesylate (0, 1, 5, and 10 μM). Cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data presented represent average of three measurements. **A**, effects of Imatinib mesylate on primary tumor-derived ovarian cells (C848, C908, and C918). **B**, effects of Imatinib mesylate on immortalized ovarian cancer cells (C272-hTert/E7 and C889-hTert). **C**, effects of Imatinib mesylate on *PDGFR*-negative ovarian cancer cell lines (CaOv3 and SKOV3). ■, untreated (control), ▨, 1 μM Imatinib, ▩, 5 μM Imatinib, ▪, 10 μM Imatinib mesylate; bars, \pm SD.

Imatinib Mesylate Inhibits *PDGFR* α and Akt Phosphorylation in Ovarian Cancer Cells. C272-hTert/E7 is an immortalized cell line derived from CSOC-272, a primary cell culture established from a serous papillary ovarian tumor (30),

which expresses *PDGFR* α endogenously. In conditions of serum starvation, the receptor is minimally phosphorylated, but after *PDGF* stimulation, the receptor is rapidly activated. Serum stimulation modestly increases the phosphorylation of *PDGFR* over the basal level (Fig. 5A). Serum-starved C272-hTert/E7 cells have low or undetectable levels of phospho-Akt, but Akt activation in response to *PDGF* becomes apparent in 30 min and remains stable for at least 90 min after stimulation of the cells (Fig. 5B). Serum and *PDGF* BB induce rapidly Akt phosphorylation, in a dose-dependent manner (Fig. 5C). In this cell line we verified the effects of Imatinib mesylate on inactivation of *PDGFR* and downstream substrates Akt and MAPK. Imatinib mesylate inhibits tyrosine phosphorylation of *PDGFR* (7, 37) and inactivates the receptor at a concentration of 1 μM , which corresponds to the inhibitory concentration (IC_{50}) in proliferative assays (Fig. 6A). In parallel, 1 μM of Imatinib mesylate

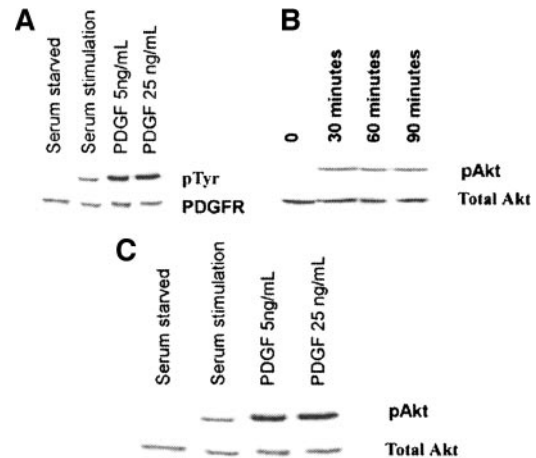


Fig. 5 Platelet-derived growth factor receptor α (*PDGFR* α) and Akt activation in C272hTert/E7 ovarian cancer cells. **A**, immunoprecipitation followed by Western blotting with antiphosphotyrosine antibody (PY20). *PDGFR* is phosphorylated within 30 min in response to serum (20% FCS) or *PDGF* BB (5 and 25 ng/ml). **B**, time course: Akt is activated in 30 min and remains phosphorylated for at least 90 min after stimulation with *PDGF* BB at 25 ng/ml in serum starved C272-hTert/E7 ovarian cancer cells. **C**, serum (20% FCS) and *PDGF* BB (25 ng/ml) induce rapid Akt phosphorylation in *PDGFR* harboring C272-hTert/E7 ovarian cancer cells.

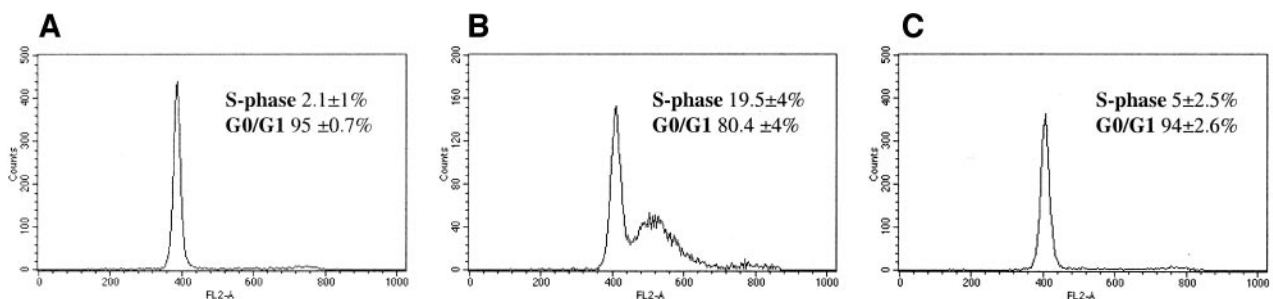


Fig. 4 Cell cycle analysis in C272-hTert/E7 cells. **A**, cells that have been serum starved for 24 h are arrested in G_0/G_1 . **B**, serum stimulation (20% fetal bovine serum) promotes cell entry in S phase. **C**, cells stimulated with serum (20% fetal bovine serum) in the presence of Imatinib mesylate (5 μM) are arrested in G_0/G_1 , similar to basal conditions. G_0/G_1 and S phase subfractions have been calculated as averages of duplicate experiments.

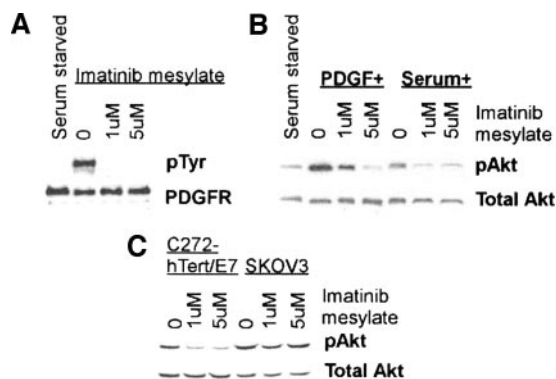


Fig. 6 Platelet-derived growth factor receptor α ($PDGFR\alpha$) and Akt inactivation in response to Imatinib mesylate. **A**, C272-hTert/E7 cells, stimulated with PDGF BB (25 ng/ml) after starvation were treated with Imatinib mesylate at 1 and 5 μM . PDGFR phosphorylation is demonstrated by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine (PY20) antibody. **B**, After serum starvation, cells were stimulated with serum (20% FCS) and PDGF BB (25 ng/ml), and were treated with Imatinib mesylate at 1 and 5 μM . Imatinib mesylate inactivates phosphorylated Akt at concentrations ≥ 1 μM . Total Akt was used as control. **C**, effects of Imatinib mesylate on phosphorylated Akt levels in C272-Htert/E7 cells (PDGFR positive) and SkOv3 cells (PDGFR negative). Cells were grown in basal conditions (10% FCS-containing medium).

inactivates Akt, reducing its level of phosphorylation to basal state in serum-stimulated cells. In PDGF-stimulated ovarian cells, partial Akt inactivation occurs at 1 μM of Imatinib mesylate, and complete Akt inactivation is noted at 5 μM (Fig. 6B). In contrast, Imatinib mesylate does not affect phospho-Akt levels in ovarian cancer cells that do not express $PDGFR\alpha$ (SkOv3; Fig. 6C). In contrast to complete Akt inactivation, the phosphorylation levels of MAPK in serum-stimulated C272/hTERT/E7 cells treated with Imatinib mesylate at concentrations up to 5 μM are only partially inhibited (data not shown), suggesting that the MAPK pathway remains partially “turned on,” despite PDGFR blockade.

Expression of Constitutively Active Akt in Ovarian Cancer Cells Induces Partial Resistance to PDGFR Inhibition by Imatinib Mesylate. After transfection with myristoylated Akt1 cloned into eukaryotic expression vector pLNCX and selection with neomycin, several stable clones with variable Akt expression levels were isolated. In these clones, Akt is constitutively phosphorylated (Fig. 7A). Similar to parental C272-hTert/E7 cells, clones isolated after transfection with empty vector express low levels of phospho-Akt. In Akt-overexpressing stable clones, phospho-Akt inactivation occurs only at high Imatinib concentrations (>10 μM ; Fig. 7B) and is only partial. In contrast, phospho-Akt in cells transfected with empty vector is rapidly inactivated at concentrations of Imatinib as low as 1 μM (Fig. 7C).

To verify whether Akt transfectants are less sensitive to inhibition exerted by Imatinib mesylate, we performed cell growth experiments comparing Akt-expressing cells to cells transfected with empty vector. We demonstrated that stable expression of myristoylated Akt1 in ovarian cells renders these cells less sensitive to the effects of Imatinib, although partial

growth inhibition in these stable transfectants is still observed (Fig. 8; $P < 0.05$). Furthermore, profiling of cell cycle indicates that whereas Imatinib blocks vector-transfected cells at G_0 - G_1 ($82\% \pm 2.8\%$), stable Akt expressors are able to progress through S phase after serum stimulation (Fig. 9).

DISCUSSION

In this study, we demonstrate that inhibition of the PDGF-PDGFR axis by the tyrosine kinase inhibitor Imatinib mesylate curtails *in vitro* the growth of ovarian cells harboring $PDGFR\alpha$. We show that $PDGFR\alpha$ expression occurs in 39% of ovarian tumors, suggesting that a subgroup of ovarian carcinomas is amenable to therapeutic PDGFR targeting. Furthermore, our data demonstrate that Imatinib mesylate inactivates Akt in ovarian cancer cells and that expression of constitutively active Akt induces partial resistance to growth inhibition by Imatinib. These results are consistent with two reports indicating the critical role of Akt as a resistance marker to cytokine-induced growth arrest in breast cancer cells (38, 39).

Imatinib mesylate was studied extensively in chronic myelogenous leukemia, which is characterized by the (9, 22) chromosomal translocation giving rise to a fusion protein with constitutive tyrosine kinase activity. This protein drives the uncontrolled growth of the chronic myelogenous leukemia cells (40), which ultimately overwhelm normal hematopoiesis. Ima-

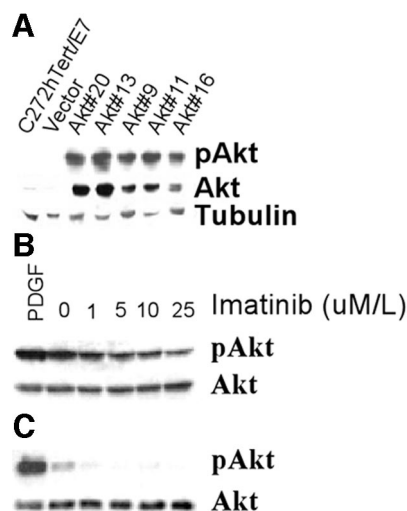


Fig. 7 Stable transfection with myristoylated Akt1 in ovarian cancer cells. **A**, Western blot shows (total) Akt and phosphorylated (pAkt) overexpression in several selected clones (#13, #11, #20, #9, and #16). Transfection with empty vector (pLNCX) and neomycin selection were done in parallel. Vector transfected stable clone (control) does not express Akt. In the overexpressing stable Akt1 clones, Akt is functional (constitutively phosphorylated). **B**, Western blotting for pAkt: stable Akt-transfected cells grown in the presence of 10% FCS were incubated with Imatinib mesylate at various concentrations (1–25 μM) for 1 h. Akt remains phosphorylated even at high concentrations of Imatinib mesylate. **C**, Western blotting for pAkt. Control cells (empty vector) grown in the presence of 10% FCS were incubated with Imatinib mesylate in various concentrations (1–25 μM) for 1 h. The level of pAkt in these cells is very low in basal state and is augmented by platelet-derived growth factor stimulation. Low concentrations of Imatinib mesylate (≥ 1 μM) completely inactivate pAkt.

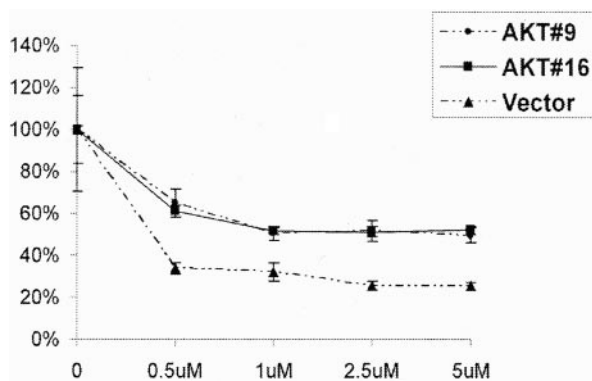


Fig. 8 Stable expression of constitutively active Akt1 induces resistance to growth inhibition by Imatinib mesylate. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based proliferation assay demonstrates that two stable Akt overexpressing clones (#9 and #16) are less sensitive to growth inhibition by Imatinib mesylate, compared with control (empty vector). All experiments were performed in triplicate and were repeated twice. Experiments were performed in 10% FCS-containing media at various concentrations of Imatinib mesylate (from 0.5 to 5 μM). Data shown represent average of three measurements; bars, \pm SD.

tinib mesylate blocks the kinase activity of abl and inhibits the growth of bcr-abl driven cell lines *in vitro* (IC_{50} of 1 μM ; Refs. 41, 42). This concentration is readily achieved *in vivo*, with minimal host toxicity, and accounts for the remarkable activity of the drug in patients with chronic and accelerated phase chronic myelogenous leukemia (35, 36, 43). Outside this setting, Imatinib mesylate inhibits the growth of c-kit-positive small cell lung cancer cell lines (44, 45), PDGFR-expressing glioblastoma cells (46), and c-kit positive gastrointestinal stromal tumor cell lines (47). This inhibitory *in vitro* effect translates into therapeutic efficacy for patients with gastrointestinal stromal tumor tumors (48, 49) or with myeloproliferative disorders characterized by activation of PDGFR β (50) and makes Imatinib mesylate therapeutically interesting for other tumors governed by activated PDGFR or c-Kit pathways (51). In solid malignancies, these pathways can be activated through autocrine or paracrine interactions, or through activating mutations. Such PDGFR α mutations have been described recently in c-kit negative gastrointestinal stromal tumor tumors (52), but not in other malignancies.

We (2) and others (5) have shown that PDGFR α is up-regulated in ovarian tumors and in primary ovarian cancer cells. In addition, it is known that ovarian tumor cells and cell lines express PDGF (53). These reports suggest a functional role for PDGF-PDGFR paracrine or autocrine interactions in the growth of ovarian tumors. Interestingly, ovarian cancer cell lines fail to express PDGFR (53), a finding confirmed in our study for three American Type Culture Collection cell lines tested (SkOv3, CaOv3, and Ov90), whereas primary ovarian cancer cells express high levels of PDGFR α . It is likely that cancer cell lines acquire additional chromosomal aberrations and/or mutations during continuous subculturing, which confer growth factor independence, whereas primary cells more closely resemble the tissues from which they originate. The immortalized ovarian

cancer cell lines (C272-hTert/E7 and C889-hTert) used here preserve endogenous PDGFR α expression.

In this study we show that Imatinib mesylate inhibits the growth of PDGFR α -expressing ovarian cancer cells in the presence of serum and that the inhibitory concentration is $\leq 1 \mu\text{M}$, which is achievable clinically. In contrast, ovarian cancer cells that do not express PDGFR (SkOV3 and CaOv3) are insensitive to this drug at clinically relevant concentrations. In ovarian cancer cells, Imatinib has predominantly antiproliferative effects, arresting cells at G₀/G₁ (Fig. 4), mechanism previously recorded for bcr-abl-positive hematopoietic cells and for PDGFR-expressing glioblastoma cells (46, 54, 55). Other reports have noted that Imatinib induces a significant increase in apoptosis in different tumor models. For instance, proapoptotic effects after treatment with Imatinib have been recorded in leukemic, gastrointestinal stromal tumor, and in dermatofibroma cell lines (44, 47, 56, 57). We have not observed an increased apoptotic fraction by Imatinib in ovarian cancer cells. Similarly, no increase in apoptosis has been recorded in glioblastoma and small cell lung cancer cells (45, 46), suggesting that Imatinib inhibits cell growth through different mechanisms in different cell lines.

Our results indicate that Imatinib inactivates Akt in a dose-responsive manner and to a greater degree than MAPK in PDGFR α -expressing cells. Overexpression of myristoylated Akt1 in ovarian cancer cells induces partial resistance to growth inhibition by Imatinib, implicating Akt as an important effector of growth promotion downstream of PDGFR. Very interestingly, constitutively active Akt1 prevents the growth arrest induced by Imatinib, by allowing cells to progress through S phase. The role of Akt in regulating cell cycle progression by interference with p27 intracellular trafficking (38) and by regulation of protein translation via p70-S6 kinase and 4E-BP1 phosphorylation could account for these antiproliferative effects. The partial growth inhibition by Imatinib noted in Akt transfectants probably reflects the role of other signaling pathways downstream of PDGFR (ras-MAPK, phospholipase C γ , and so forth) that regulate cell growth and proliferation.

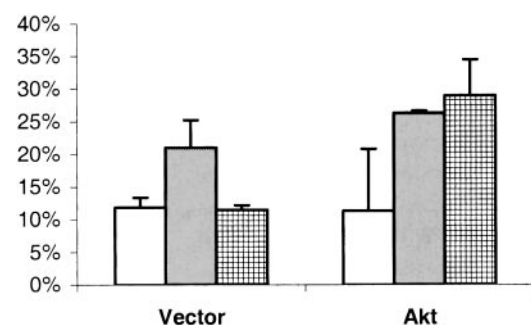


Fig. 9 S-phase distribution in vector and Akt-transfected cells. Cell cycle profiles in stable Akt clones and cells stably transfected with empty vector have been analyzed with propidium iodide staining and fluorescence-activated cell sorter analysis. Data represents average of two independent experiments. Vector-transfected cells are inhibited by Imatinib at G₀/G₁ (82% \pm 2.8%) compared with Akt subclones (67% \pm 0.7%). \square , serum starved; \blacksquare , serum stimulation; ▨ , serum stimulation plus Imatinib 5 μM ; bars, \pm SD.

Activation of the PI3k-Akt pathway through mechanisms dependent or independent of growth factor stimulation plays a central role in ovarian cancer (9, 11, 15). It is not known whether the level of baseline Akt activation in ovarian tumors impacts prognosis and/or response to therapy. Our data show that Akt activation induces partial resistance to Imatinib mesylate *in vitro*. We recognize that myrAkt (used in our experiments) may be more oncogenic than the wild-type Akt, because the myristoylation signal amplifies the oncogenic potential of Akt. Thus, overexpression of myrAkt may not entirely reflect the effects exerted by overexpressed endogenous Akt (58, 59), because overexpression of the membrane-bound form of Akt can nonspecifically activate other colocalized proteins (60). We note that ovarian cancer cell lines CaOv3 and SkOv3 that have high levels of endogenous activated Akt (Fig. 2) are also resistant to Imatinib mesylate, although these cells do not express PDGFR.

The Akt isoform used for transfection in these experiments is Akt1, rather than the ovarian cancer-related isoform, Akt2. Although different Akt isoforms have different tissue distribution (*e.g.*, Akt2 is the isoform overexpressed in ovarian carcinomas; Ref. 61), they share common downstream substrates that mediate their oncogenic signals (62). Therefore, we believe that the effects noted as a consequence of constitutively active Akt1 expression are likely to be very similar to those obtained by activation of Akt2 or Akt3.

In conclusion, our data strongly suggest that PDGFR can be targeted therapeutically in EOC by Imatinib mesylate. *In vitro*, the inhibitory effects of Imatinib are restricted to PDGFR-expressing cells. We show that cancer cells with high basal levels of Akt (either endogenous or induced) are less sensitive to manipulation of the PDGF-regulated growth pathway. Apart from providing a rationale to further study PDGFR inhibition as a novel therapy for patients with EOC, our findings implicate Akt as a potential indicator of sensitivity to treatment.

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