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Molecular Mechanisms of Action of Imatinib Mesylate in Human Ovarian Cancer: A Proteomic Analysis

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Abstract. *Background: Imatinib mesylate (Gleevec®, Novartis, Basel, Switzerland) is a small-molecule tyrosine kinase inhibitor with activity against ABL, BCR-ABL, c-KIT, and PDGFRα. Several clinical trials have evaluated the efficacy and safety of imatinib in patients with ovarian carcinoma who have persistent or recurrent disease following front-line platinum/taxane based chemotherapy. However, there is limited pre-clinical and clinical data on the molecular targets and action of imatinib in ovarian cancer. Materials and Methods: Human ovarian cancer cells (A2780) were treated with imatinib mesylate for either 6 or 24 h. We employed a 2D (two-dimensional) gel electrophoresis and mass spectrometry-based proteomics approach to identify protein expression patterns and signaling pathways that were altered in response to imatinib. Cells were analyzed for PDGFRα and AKT expression, which were then correlated with imatinib sensitivity. Results: Using 2D gel electrophoresis of overlapping pH ranges from pH 4 to 11, about 4,000 protein spots could be analyzed reproducibly. Proteins whose levels changed between two fold to 30 fold were grouped according to whether changes were in the same direction at both time points of treatment with respect to the control, or changed their levels only at one of the time points. Conclusion: Differentially regulated proteins following imatinib treatment of A2780 cells involved the regulation of actin cytoskeleton, metabolic pathways, cell cycle, cell proliferation, apoptosis, cell junctions, and signal transduction. Thus, exposure of cells to imatinib produces complex changes in the cell that require further investigation.*

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Ovarian carcinoma is the fifth leading cause of cancer death among women in the United States and the most common cause of death among gynecologic malignancies (1). Ovarian cancer affects about 15 women for every 100,000 women under the age of 40 and over 50 women for every 100,000 women above the age of 70 (1). The five-year survival rate for patients with advanced stage ovarian cancer is only 29% which is in contrast to the women with tumors confined to the ovaries exceeding 90% (1). The cornerstone of management for advanced ovarian cancer involves cytoreductive surgery followed by standard adjuvant chemotherapy that consists of the combination of a taxane with a platinum-based drug (2). Despite this initially effective combination therapy, a majority of the advanced ovarian cancer patients will relapse (3). Therapeutic options for relapsed ovarian cancer patients are limited. While a number of agents have demonstrated activity in second-line treatment of recurrent ovarian carcinoma, response rates are low and usually of short duration (3). Molecular targeting approaches manipulating the biology of the disease may hold promise for the future. Therefore, the development of novel treatment strategies in advanced disease is critical to improve patient survival.

Platelet-derived growth factor (PDGF) is a potent mitogen with two isoforms PDGF-A and PDGF-B, and differentially binds to two structurally related receptor tyrosine kinases (RTKs), PDGFRα and PDGFRβ. Ligand-activated receptors trigger downstream signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K)/ AKT and, extracellular signal-regulated kinase 1/2 (ERK1/2) which promote cell proliferation and survival. Several groups reported the differential expression of PDGF, PDGFRα, and PDGFRβ in ovarian cancer compared to normal ovarian epithelium (4-7). Matei *et al*. found that about 39% of ovarian tumors express PDGFRα by immunohistochemistry (8). Another study showed that approximately 58% of epithelial ovarian cancers

(EOC) express PDGFRα and 28% of those express PDGFRβ as determined by immunohistochemistry (7). Patients with PDGFRα positive ovarian cancers have demonstrated an overall shorter survival compared to those whose tumors were PDGFRα negative (5).

Imatinib (Gleevec, Novartis, Basel, Switzerland), is a 2-phenylaminopyrimidine derivative that is a RTK inhibitor with potent activity against ABL (including the BCR-ABL fusion protein found in chronic myelogenous leukemia (CML)), PDGFRα, PDGFRβ, and c-KIT (9, 10). It is approved for the treatment of CML and gastrointestinal stromal tumor (GIST) (11), and is under evaluation in clinical trials for ovarian cancer (http://clinicaltrials.gov/ct2/home, NCT00510653, NCT00041041, NCT00036751), malignant gliomas, prostate cancer, and carcinoid tumor (12-17). About 80-90% patients with metastatic gastrointestinal stromal tumor respond, or achieve stabilization of tumor growth, to continuous imatinib therapy with daily dose of 400 mg to 600 mg (18-20). Matei *et al*. showed that imatinib inhibits the growth of ovarian cancer cells in a PDGFRα positive cell culture but has no effect on PDGFR negative cell culture, at clinically relevant concentrations (8).

In this study, we utilized a proteomic approach to test the cellular response of imatinib to obtain some insights into how this drug might influence the proteome of ovarian cancer cells. Two-dimensional (2D) gel electrophoresis-based approach is a powerful and practical proteomics tool for qualitative and quantitative comparisons of proteomes under different conditions to unravel dynamic biological processes (21). By comparing spot intensities from different samples, changes in the level of individual proteins expression can be quantified enabling the visualization and identification of several thousand proteins on a single gel (22). We thus employed a 2D gel electrophoresis and MALDI-TOF peptide mass fingerprinting proteomic approach to identify the protein expression profile in the A2780 human ovarian cancer cell line treated and untreated with imatinib. The combination of 2D gel proteomics and mass spectrometry resulted in the identification of 1010 proteins, with 501 non-redundant entities and about 509 isoforms. All data are provided at our web site (http://yeung.fccc.edu).

Materials and Methods

Cell lines and response to imatinib treatment. The ovarian cancer cell lines, A2780, OVCAR3, and OVCAR10, were cultured as previously described (23). For growth analysis, cells were seeded at $6.5x10^5$ cells per 60 mm dish. Imatinib (dissolved in water to a stock concentration of 10 mM) was added directly to the media to achieve the final concentration of 1 or 10 μM. Cells were refed with conditioned media with or without drug every 12 hours. Cells were then harvested and stained for the cell number and cell viability using Guava ViaCount reagents (Guava Technology Inc., Burlingame, CA, USA). The cells were counted using a Guava Personal Cytometer and the data analyzed using the Guava CytoSoft software package (Guava Technology Inc., Burlingame, CA, USA). For cell cycle analysis, cells were trypsinized, centrifuged, and fixed in 70% ethanol at 4˚C. Cell pellets were re-suspended in 50 μg/ml propidium iodide in PBS for 30 min at 4˚C. The stained cells were analyzed by flow cytometry performed on a FACScan and the data analyzed with Cell Quest software (Becton Dickinson). Each experiment was performed in triplicates and repeated at least 2 times.

Western blot analysis. Cell lysate preparation and western blot analysis was performed as previously described (24). Anti-β-actin monoclonal antibodies (Sigma, St. Louis, MO, USA) were used at a dilution of 1:5,000 in 5% dried milk. Anti Phospho-PDGFRα (Tyr 754) rabbit polyclonal antibody (Santa Cruz Biotechnology) was used at 1:500 dilution in 5% BSA. Anti-AKT, anti-AKT/phosphoThr^{308} and anti-AKT/phosphoSer^{473} polyclonal antibodies (Cell Signaling) were each used at a dilution of 1:1000 in 5% BSA. Quantification of Western blots were performed using the "NIH image" software as described by the manufacturer.

Preparation of protein sample for 2-DE electrophoresis. A2780 cell cultures were divided into: a) control untreated for 24 hours; b) cultured without imatinib for 12 hours then with 10 μM imatinib for 6 hours; c) imatinib (10 μM) treated for 24 hours. All cell pellets were washed 3 times with cold 1x PBS before storage in –80˚C. Protein extracts from these stored cell pellets were obtained by using 2D-protein extraction buffer (7 M urea, 2 M thiourea, 65 mM CHAPS, 8 mM PMSF, 97.4 mM hydroxyethyldisulfide). For 200 mg wet weight cell pellet, 2x500 μl extraction buffer was used. The detailed protocol for protein extraction with acetone precipitation was described previously (25). Protein concentration was determined by a modified Bradford assay, using a standard curve based on BSA dissolved in the same 2D sample buffer.

Two-dimensional gel electrophoresis. We used established standard operating procedures (SOP) for 2D gel electrophoresis and their analyses (25, 26). All imatinib treated and untreated protein samples were resolved on 2D gels with 3 overlapped pH gradients in the first dimension: pH 4-7, pH 5-8 and pH 6-11. The first dimension was carried out with analytical loading of 100 μg protein using in-sample rehydration method for pH 4-7 (17 cm IPGs) and pH 5-8 strips (17 cm IPGs), and Cup-loading method for pH 6-11 strips (18 cm IPGs). The second dimension was performed using 12% polyacrylamide SDS gel (20 cm x 20 cm x 1 mm). Proteins in the gels were visualized with Sypro Ruby fluorescence stain (Bio-Rad) and scanned with a Perkin Elmer ProXPRESS (Perkin Elmer) scanner. All gels in this study were run in triplicates per pH range, and the two best gels of each sample were used for further image analysis.

Image analyses and protein spot identification. The detail methods for image analysis and protein identification by mass spectrometry were described previously (25, 26). Briefly, 2D gel image analysis was done by Progenesis Discovery workstation software (v2003.02, Nonlinear Dynamics Ltd., Newcastle, UK) assisted by manual editing. The protein spot picking list was generated through image analysis and spot cutting for multiples of 96 spots performed by ProPic Robot (Genomic Solutions, MI, USA). The automated in-gel trypsin digestion of protein spots was facilitated by the robot Tecan Genesis (Tecan US, Durham, NC). Proteins were identified using MALDI-TOF mass spectrometry peptide mass fingerprinting

(PMF) on a Bruker Reflex IV (Bruker Daltonics, MA, USA) using 384 well format AnchorChip™ target plates, automated mass calibration of each sample with internal standards by XMASS software, and protein identification from the Swiss-Prot database by MASCOT software (www.matrixscience.com). We used high loading 2D gels (300 μg) in order to identify low abundance protein spots. By confirming protein identifications at least twice from replicated gels, we validate the fidelity of our electrophoresis, image analysis, robotic spot excision, protein digestion, and protein identification by mass spectrometry.

Gene ontology and pathway analysis. The FatiGO+ tool (http://babelomics2.bioinfo.cipf.es/fatigoplus/cgi-bin/fatigoplus.cgi) was used for the Gene Ontology (GO) classification and KEGG pathway analysis of differentially expressed protein spots identified by mass spectrometry (27-29).

Results

Effect of imatinib on cell growth and viability in human ovarian cancer cells. To test the effect of imatinib on ovarian carcinoma cells we treated OVCAR3, OVCAR10, and A2780 cells with imatinib at a clinically relevant 1 and 10 μM concentrations. Imatinib significantly decreased the growth of A2780 cells at 10 μM, but had no effect on the growth and proliferation of OVCAR3 and OVCAR10 cells at either concentration (Figure 1A and data not shown). The difference in the number of A2780 cells was significantly decreased (five-fold less accumulation of A2780 cells exposed to 10 μM imatinib for 96 hrs than the untreated cells); however, the morphology of the cells was not dramatically changed. We performed a FACS analysis of A2780 cells untreated or treated with 10 μM imatinib for 96 hours. Imatinib did not cause any significant changes in the cell cycle distribution but the total number of cells decreased ~30% after treatment (Figure 1B). These data strongly suggest that imatinib has a cytostatic effect on A2780 cells and does not cause apoptosis.

Effect of imatinib on PDGFRα activity. To identify the potential target of the drug in A2780 cells we assessed the expression and activation of three known targets of this RTK inhibitor by Western blot analysis. KIT was not detected in OVCAR3, OVCAR10, and A2780 cells, while c-ABL was highly expressed but the receptor was not constitutively activated (data not shown). Analysis of PDGFRα revealed expression of this receptor only in A2780 cells (Figure 1C). We also demonstrated that the receptor was constitutively activated as assessed by phosphorylation at Tyr754 (Figure 1C). Mutational analysis of the *PDGFRA* gene in A2780 cells failed to uncover a gain-of-function mutation.

We next examined the effect of imatinib on PDGFRα signaling. As shown in Figure 1D, treatment of A2780 cells with PDGF could significantly induce phosphorylation of the receptor suggesting an autocrine loop. The phospho-PDGFRα expression was inhibited after imatinib treatment in presence or absence of PDGF (Figure 1D). To further elucidate the importance of PDGFRα signaling in sensitization of A2780 cells, we evaluated downstream mediators of PDGFRα signaling, *e.g.*, AKT upon stimulation with its cognate ligand and after imatinib treatment. Interestingly, the constitutively active form of AKT (Ser473) was observed with or without imatinib treatment as detected by Western blot (Figure 1D). This is consistent with previous studies by our group demonstrating AKT2 independent activity of imatinib in GISTs (20).

Proteome analysis and protein identification. Differential protein expression in the A2780 cell line with imatinib treatment for 6 hrs or 24 hrs compared to no treatment was evaluated using 2D gel analyses of total protein extracts. Representative gel images for pH 4-7, pH 5-8 and pH 6-11 IPG strips are shown in Figure 2. Gel reproducibility was assessed by running triplicate gels of each protein extract for each pH range IPG strips, and two gels for each time point were then subjected to analysis by Progenesis Discovery v2003.02 image analysis software. The number of SyproRuby-stained protein spots across pH 4 to 11 consistently displayed approximately 4,000 unique protein spots. The spot intensities were normalized to a virtual reference gel value of 100,000 units where 20 units represented ~10 ng of protein and the quantitative analysis was based on a two-fold cutoff. The average-image of no imatinib treatment was used as the master gel, and the intensities of all matched spots on the gels were compared with 6 and 24 h average-gels to derive differential expression values for each spot. Spots showing at least two-fold intensity differences were considered for further Gene Ontology (GO) classification and signaling pathway analysis.

The numbers of protein spots identified by mass spectrometry with high confidence in the pH 4-7, pH 5-8, and pH 6-11 were 401, 359, and 250, respectively. Thus the total number of identified protein spots in this study was 1,010, including 501 non-redundant protein name entries and about 509 of their isoforms. The files for pH 4-7, pH 5-8 and pH 6-11 searchable point-and-click proteome maps of the human ovarian cancer cell (A2780) with imatinib treatment representing the 1,010 proteins are provided in the Supplemental data files 1-3 and also at our web site: http://yeung.fccc.edu. In each searchable proteome map, the name of each protein will appear when the mouse pointer is rested on top of the spot circled in red, which is also hyperlinked to Human Protein Reference Database web site (http://www.HPRD.org) (30). The protein spots can be located by using the control-F command. Spot identification number are pH range specific (supplemental data files 1-3 and also at our web site: http://yeung.fccc.edu).

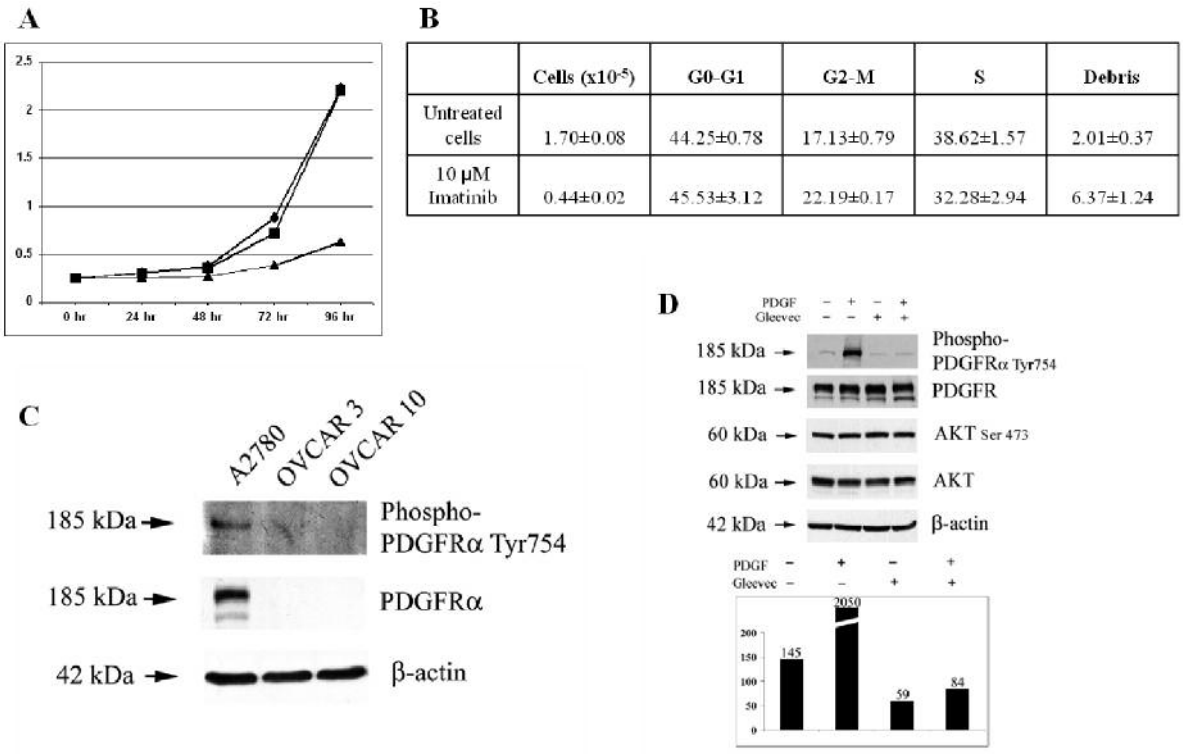

Figure 1. *(A) Growth curves of A2780 cells treated with 1 μM imatinib (■), 10 μM imatinib (▲) and control untreated cells (◆). Cells were treated by adding the drug directly to culture media for 24, 48, 72, and 96 hrs respectively. (B) Cell cycle phase distribution and cell number of A2780 cells with or without 10 μM imatinib for 48 hours as determined by FACS analysis. (C) Western blot analysis of total and phosphorylated PDGFRα receptor in A2780, OVCAR3, and OVCAR10 cells. (D) pPDGFRα levels in A2780 cells following stimulation with PDGF in the presence or absence of imatinib. Bottom panel shows quantification of the above Western blot in relative intensity units for pPDGFRα.*

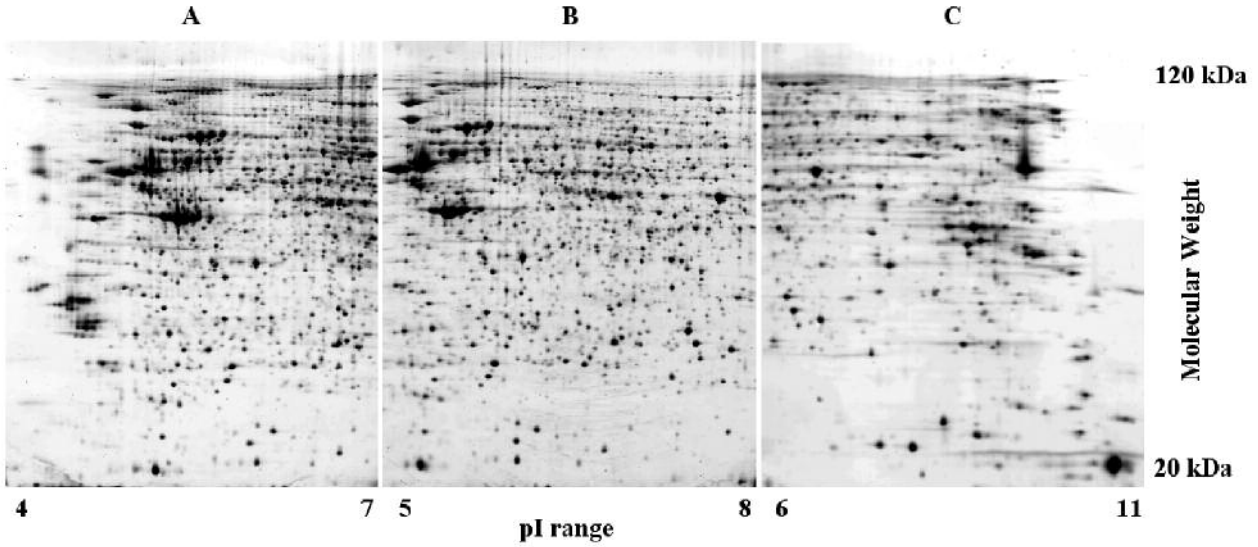

Figure 2. *Three overlapping pH range 2D gels across pI 4-11 resolved more than 4000 spots of human ovarian cancer cells A2780. Whole cell lysates from no imatinib treated A2780 cells were separated on a pH 4-7 (A), pH 5-8 (B), and pH 6-11 (C) range 2D gel and visualized by SyproRuby staining. High resolution figures and original image files are available in the Supplemental data files 1-3 and also at our Web site (http://yeung.fccc.edu) and are searchable and hyperlink enabled to Human Protein Reference Database (www.HPRD.org).*

Gene ontology classification of differentially expressed proteins. 2D gel proteomics studies generate large amounts of data that needs to be grouped into functional categories for easier interpretation and to evaluate biological relevance. In this present study, 379 differentially expressed proteins with more than two-fold changes in protein quantity compared to no imatinib treatment were grouped as early response changes at 6 h, sustained changes at 6 hrs and 24 hrs, and late response changes at 24 h after imatinib treatment. For individual group with up- and down-regulated proteins, the Entrez gene symbols were generated from Swiss-Prot accession numbers using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Gene ID Conversion tool (http://david.abcc.ncifcrf.gov/) and subjected to comparative gene ontology analysis using FatiGO+ tool (27). The clustering of biological processes (levels 3-9) based on the Gene Ontology (GO) Consortium annotations for individual groups were summarized in Table I. The over-expression of several proteins as sustained changes after imatinib treatment were observed for biological processes like protein folding, in response to stress, purine nucleotide metabolic process, amino acid and derivative metabolic process, carbohydrate metabolic process, negative regulation of cell organization and biogenesis, and proteolysis. Notably, many proteins involved in processes like cell cycle arrest, mitotic cell cycle, signal transduction, intracellular signaling cascade, secretory pathway, small GTPase mediated signal transduction, negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, response to DNA damage stimulus were observed to be down-regulated across three groups of differentially expressed proteins after imatinib treatment. Six proteins involved in cell proliferation process were found to be decreased more than two fold in protein quantity after 6 hrs of imatinib treatment.

Pathway analysis of differentially expressed proteins. We subsequently analyzed the 379 differentially expressed proteins from three groups after imatinib treatment in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using FatiGO+ tool (27). The analysis of KEGG pathways revealed changes in different metabolic and cell signaling pathways for individual groups (Table II). The down regulation of several proteins were observed in Insulin signaling pathway, MAPK signaling pathway, T cell receptor signaling pathway after imatinib treatment (Table II). Imatinib treatment resulted in over-expression of many proteins as sustained or late response changes. Pathways involved include glycolysis/gluconeogenesis, purine metabolism, pyrimidine metabolism, pyruvate metabolism, oxidative phosphorylation, Wnt signaling pathway, TGF-beta signaling pathway, and tight junction (Table II).

Imatinib induced differential expressions in serine/threonine protein phosphatases subunits. Imatinib treatment resulted in changes in protein phosphatases type 1 and type 2 subunits as summarized in Table III. The most significant changes in the protein phosphatase 2 family were observed as three-fold and two-fold elevations of the two isoforms of protein phosphatase 2A catalytic subunit beta isoform (PPP2CB), and increase in protein levels of three isoforms of the protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform (PPP2R1A). Protein phosphatase 2A is a major serine/threonine specific phosphatases, and it is implicated in the negative control of cell growth and cell division (31, 32). In addition, protein phosphatase type 2A (PP2A) has a positive regulatory function in apoptosis, by activation of pro-apoptotic and inhibition of anti-apoptotic proteins of the BCL-2 family (33). We also observed down regulations in serine/threonine specific protein phosphatase 1 (PP1) catalytic subunits alpha (PPP1CA) and beta (PPP1CB). After imatinib treatment, three isoforms of PP1-alpha catalytic subunit and one isoform of beta catalytic subunit were decreased more than two fold in protein quantity (Table III). Protein phosphatase 1 (PP1) is known to be involved in the regulation of a variety of cellular processes, such as cell division, protein synthesis, glycogen metabolism, and muscle contractility (34).

Imatinib induced differential expressions in MAPK signaling pathway. Two isoforms of dual-specificity mitogen-activated protein kinase kinase 2 (MAP2K2) were detected with opposing changes in expression (Table III). About 5.4-fold decrease in spot 955 and 3.6-fold increase in spot 2030 were observed after 24 hrs imatinib treatment, suggesting their regulation by post-translational modification. MAP2K2 belongs to the MAP kinase kinase family, and play a critical role in the regulation of normal cell proliferation, survival and differentiation (35). Two isoforms of cAMP-dependent protein kinase (PKA) type I-alpha regulatory chain (PRKAR1A, spot 573 and 576, pH 4-7) protein were consistently up-regulated after imatinib treatment (Table III). Partial down-regulation in PRKAR1A was correlated with increased cell cycle progression, proliferation, survival, and changes in mitogen-activated protein kinase (MAPK) signaling (36, 37). Five other proteins involved in MAPK pathway showed differential changes in one or more isoforms of the same protein (Table III). About 2.4-fold decrease in serine/threonine-protein kinase PAK2 (PAK2), and proto-oncogene c-CRK were observed. Three isoforms of UNR protein (CSDE1), four isoforms of heat shock cognate 71 kDa (HSPA8), and three isoforms of 78 kDa glucose-regulated protein (HSPA5) were consistently down-regulated more than two-fold after imatinib treatment.

Imatinib induced changes in cell cycle, cell proliferation and programmed cell death. Imatinib induced down-regulation of proteins involved in cell proliferation, cell cycle progression

Table I. *Gene ontology classification (biological process).*

Gene ontology classification: biological process (Level 3-9)[a]	Early responses	Sustained changes	Late responses
Response to stress	UCHL1 DDB1 RUVBL2 HSP90B1 MSH2 HSPD1 XRCC5 HSPA8 EIF2B2 EIF2S1 TXNDC4 HSPA1A HSP90AB1 SOD2 XRCC6 RFC5 ANXA5	MSH2 HSPA8 RAD23B EIF2B2 PRDX2 UCHL1 STIP1 APOE ERO1L GSS ABHD2 PCNA HSPH1 AHSA1 XRCC6 CIRBP ANXA5 SERPINH1 VCP	HSPA8 PARP2 DNAJA1 PCNA PPP1CB NPM1 ANXA5 MRPS26 APOE ALB HSPD1 AKR1B1 MYH10
Protein folding	PPIB PPID RUVBL2 HSP90B1 HSPD1 HSPA8 CCT5 TXNDC4 HSPA1A HSP90AB1 FKBP4 PPIA CCT4 TTC1	HSPA8 GRPEL1 ERO1L CCT3 HSPH1 AHSA1 FKBP5 CCT5 CKAP1 CCT6A FKBP10 SERPINH1	CCT7 HSPA8 DNAJA1 CALR CCT5 BAG5 HSPD1 FKBP5 TBCC PPIA
Purine nucleotide metabolic process	MSH2 GMPS PAICS ATP5B	MSH2 ATP5A1 BAT1 GART PPAT IMPDH2 PFAS MTHFD1	GART ATP6V1B2 HPRT1
Amino acid and derivative metabolic process	SMS GMPS PPP2R1A ASNS GLUD1 YARS GFPT1	CLIC1 KARS NARS PPAT GSS PFAS PSAT1 QARS DARS MTHFD1 GFPT1	GARS FARSLB GOT1 DDAH2 KARS PSPH PPP2R1A CTBP2 SHMT2 MARS PSAT1 HPRT1 P4HA1
Carbohydrate metabolic process	GAPDH ALDOA TSTA3 NANS PGD PKM2 SDHB GNPDA1 GFPT1	PDHB PPP1CA ME1 UGDH PGAM1 GAPDH G6PD LDHA PKM2 GFPT1 GANAB	DDX1 TALDO1 PGAM1 SDHA PPP1CB GLO1 PPP1CA UAP1 AKR1B1 LDHA PKM2
Cofactor biosynthetic process	PBEF1 ATP5B	ATP5A1 BAT1 ME1 GSS COQ6 MTHFD1	PBEF1 UROD ATP6V1B2
Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	PA2G4 MSH2 STRAP PSMC5 RBBP7	TSG101 MSH2 RBBP7 PA2G4	STRAP CTBP2
Regulation of cell organization and biogenesis	ARPC2 GSN	ARPC2 CAPZA2 CAPZA1 SPTAN1	RDX
Negative regulation of cell organization and biogenesis	GSN	CAPZA2 CAPZA1 SPTAN1	RDX
Cell cycle	PA2G4 MSH2 CUL3 PPP2R1A CSK SEPT2 TUBB YWHAG RUVBL1 MCM3 YWHAQ CCT4 TUBG1	TSG101 MSH2 GAS2 CSDE1 TUBB PPP1CA DCTN2 CLK1 PA2G4 YWHAG ACTN4 MCM3 CSK PCNA DST GSPT1	CCT7 YWHAG CSDE1 SUGT1 PCNA MCM6 TUBB PPP1CB CALR NPM1 PPP1CA AKAP8 PPP2R1A BUB3 MCM7 PSMD8 DCTN2
Cell cycle arrest	PA2G4 MSH2 CUL3	TSG101 MSH2 GAS2 PA2G4 DST	
DNA replication	MSH2 DUT MCM4 PPP2R1A RBBP7 MCM3 RFC5	MSH2 RBBP7 DUT NARS MCM3 PCNA	RPA2 PCNA MCM6 PPP2R1A MCM7
Mitotic cell cycle	CUL3 TUBB YWHAG RUVBL1 TUBG1	TUBB DCTN2 YWHAG GSPT1	YWHAG SUGT1 TUBB PPP1CB AKAP8 BUB3 DCTN2
Programmed cell death	ARHGDIA HSP90B1 MSH2 CUL3 HSPD1 PSMC5 PPP2R1A HSPA1A TUBB YWHAG NUDT2 ANXA5 YARS	MSH2 GAS2 TUBB GSTP1 CLIC1 DIABLO PDIA3 PRDX2 APOE YWHAG ACTN4 HSPA5 ANXA5 VCP	PDCD6IP YWHAG TXNL1 TUBB CALR DDAH2 GLO1 NPM1 ANXA5 PPP2R1A PDIA3 APOE BAG5 ALB HSPD1 HDAC1

Table I. *continued*

Table I. *continued*

Gene ontology classification: biological process (Level 3-9)[a]	Early responses	Sustained changes	Late responses
Negative regulation of programmed cell death	ARHGDIA HSP90B1 MSH2 PSMC5 HSPA1A YWHAG ANXA5	MSH2 GSTP1 CLIC1 PRDX2 YWHAG HSPA5 ANXA5	YWHAG DDAH2 GLO1 NPM1 ANXA5 ALB HDAC1
Positive regulation of programmed cell death	CUL3 PPP2R1A TUBB NUDT2	TUBB DIABLO PDIA3 APOE	TUBB PPP2R1A PDIA3 APOE
Secretory pathway	SAR1A TXNDC4 SCRN1 YWHAQ	GOSR1 ARCN1	GARS COPE SRP54 EXOC5 MYH10
Signal transduction	ARHGDIA PDE9A CRTAP STRAP GDI2 SAR1A PBEF1 HDGF PPP2R1A CSK PRKAR1A THOP1 RPSA GLUD1 YWHAG YWHAQ CORO1C EEF1D ANXA5	ARFIP2 HOMER1 CRK ZBTB9 PRKAR2A CSDE1 CLIC1 DIABLO PRDX4 HDGF ITGB4BP PDIA3 PRKAR1A PDE9A YWHAG ERO1L TXNRD1 CSK PCNA DST HSPA5 RSU1 ANXA5 VCP	ARHGAP1 YWHAG GDI1 CSDE1 CRKL TXNL1 PBEF1 PRKCSH PAK2 PCNA STRAP DDAH2 NPM1 AKAP8 ANXA5 PPP2R1A PDIA3 DPYSL2 PSCD3 HDGF MPP2 ITGAV
Small GTPase mediated signal transduction	ARHGDIA GDI2 SAR1A YWHAQ	ARFIP2 ZBTB9 CSDE1 RSU1	ARHGAP1 CSDE1 CRKL PSCD3
Cell proliferation	PRDX1 UCHL1 PA2G4 CUL3 PBEF1 HDGF CSK RBBP7	TSG101 RBBP7 DCTN2 HDGF CLK1 UCHL1 PA2G4 CSK PCNA	PBEF1 PCNA FSCN1 ANXA7 PPP1CB NPM1 CTBP2 BUB3 MCM7 PRDX1 FTH1 DCTN2 HPRT1 HDGF
Proteolysis	UCHL1 PSMA7 PA2G4 PSMC5 THOP1 PSMC6 PMPCB SCRN1 PSMC4	CLPP UCHL1 PA2G4 PSMA4 VCP	PSMC2 PREP PSMD8 METAP2 UFD1L UQCRC2 LAP3 PSMA3
DNA repair	DDB1 RUVBL2 MSH2 XRCC5 XRCC6 RFC5	MSH2 RAD23B PCNA XRCC6 VCP	PARP2 PCNA

Gene Ontology classification of 379 differentially expressed proteins after imatinib treatment. The gene names are Entrez gene names. Some gene names appear in more than one pathway description. The complete functional table is available as Supplemental data file 4. Table I and also at our web site http://yeung.fccc.edu. [a]The underlined gene names are more than two-fold decrease in protein quantity compared to no imatinib treatment. The gene names without underlines are more than two-fold increase in protein quantity compared to no treatment.

and apoptosis were observed as sustained changes at 6 hrs and 24 hrs, and as late responses at 24 hrs time point (Table III). Some of the most significant late response changes are down-regulation of Nucleophosmin (NPM1, 3.8-fold and 4.4-fold decrease in spots 2,357 pH 4-7 and 2,100 pH 5-8, respectively), Proliferating Cell Nuclear Antigen (PCNA, more than 2-fold decrease in spots 972, 974, 975), Suppressor of G2 allele of SKP1 homolog (Sgt1) (SUGT1, 2-fold decrease in spot 792), and DNA replication licensing factor MCM6 (MCM6 2.1-fold decrease in spot 623). Down regulation of NPM1 was shown to be correlated with the delays in cell-cycle progression or undergoing apoptosis (38, 39). PCNA is an essential protein for DNA replication, damage repair, cell cycle progression, and a useful marker in cell proliferation study because its expression correlates with the proliferative state of the cell (40). SUGT1 is a critical assembly factor for the mammalian kinetochore, and required for both the G1/S and G2/M transitions in the cell cycle (41).

Consistent with this anti-proliferation and apoptotic induction after imatinib treatment was a 2.2-fold and 4.2-fold decrease in Tumor Susceptibility Gene 101 protein (TSG101, spot 843 pH 5-8), a 3.5-fold and 8.3-fold decrease in dual-specificity protein kinase CLK1 (CLK1, spot 1337 pH 5-8), more than 2-fold decrease in DNA mismatch repair protein MSH2 isoforms, as a sustained changes at 6 hrs and 24 hrs. TSG101 is an essential protein involved in cell cycle control,

Table II. *KEGG pathway analysis*.

KEGG pathways[a,b]	Early responses	Sustained changes	Late responses
Cellular Processes			
Regulation of actin cytoskeleton	*ACTN4 ACTB* CSK ARPC2 ACTG1 VCL GSN	*ACTB VIL2 MAP2K2* CRK CSDE1 PPP1CA CSK ACTN4 ARPC2	*ACTB* CSDE1 CRKL PPP1CB PPP1CA PAK2 VIL2 MYH10 RDX ITGAV
Cell communication	*ACTB* LMNB1 LMNA KRT6A ACTG1	*ACTB* KRT8 LMNB2 VIM	*ACTB VIM* LMNB2 LMNA KRT7 KRT8
Adherens junction	*ACTB ACTN4* ACTG1 VCL	*ACTB* ACTN4	*ACTB*
Tight junction	*ACTB ACTN4* PPP2R1A ACTG1	*ACTB* CSDE1 ACTN4 PPP2CB SPTAN1	*ACTB* CTTN CSDE1 MYH10 PPP2CB PPP2R1A
Focal adhesion	*ACTB ACTN4* ACTG1 VCL	*ACTB* CRK PPP1CA ACTN4	*ACTB* ITGAV CRKL PPP1CB PPP1CA PAK2
Gap junction	TUBB	*MAP2K2* CSDE1 TUBB	CSDE1 TUBB
Cell cycle	MCM4 YWHAG MCM3 YWHAQ	YWHAE YWHAZ YWHAG MCM3 PCNA	YWHAZ PCNA MCM6 YWHAG BUB3 MCM7 HDAC1
Apoptosis	PRKAR1A	PRKAR2A PRKAR1A	
Cell Signaling Pathways			
Insulin signaling pathway	PRKAR1A PKM2	*MAP2K2* CRK PRKAR2A CSDE1 PPP1CA PKM2 PRKAR1A	CSDE1 CRKL PPP1CB PPP1CA PKM2
MAPK signaling pathway	*HSPA5* HSPA8 HSPA1A	*MAP2K2* HSPA8 CRK CSDE1 HSPA5	CSDE1 CRKL PAK2 HSPA8
Wnt signaling pathway	PPP2R1A RUVBL1	PPP2CB	CTBP2 PPP2CB PPP2R1A
TGF-beta Signaling Pathway		PPP2CB	PPP2CB
B cell receptor signaling pathway		CSDE1	CSDE1
Notch signaling pathway			CTBP2 HDAC1
Natural killer cell mediated cytotoxicity		*MAP2K2* CSDE1	CSDE1
mTOR signaling pathway		*EIF4B*	
T cell receptor signaling pathway		CSDE1	CSDE1 PAK2
Antigen processing and presentation	*HSPA5* HSPA8 HSP90AB1 HSPA1A	*HSP90AB1* HSPA8 HSPA5 PDIA3	*HSP90AB1* CALR HSPA8 PDIA3
Metabolic Pathways			
Glycolysis/Gluconeogenesis	*ALDH7A1 ENO1* GAPDH ALDOA PKM2	*ENO1 TPI1* PDHB PGAM1 GAPDH LDHA PKM2	*ALDH7A1 ENO1 TPI1* PGAM1 LDHA PKM2
Purine metabolism	PDE9A GMPS PKM2 POLR1C PAICS NUDT2 RFC5 POLR2E	PDE9A GART PPAT IMPDH2 PFAS ITPA PKM2	GART PKM2 HPRT1

Table II. *continued*

Table II. *continued*

KEGG pathways[a,b]	Early responses	Sustained changes	Late responses
Pyruvate metabolism	*ALDH7A1* PKM2	PDHB ME1 LDHA PKM2	*ALDH7A1* GLO1 AKR1B1 LDHA PKM2
Oxidative phosphorylation	*ATP6V1B2* ATP5B SDHB	UQCRFS1 ATP5A1 BAT1 NDUFS1	*UQCRC1* SDHA PPA1 UQCRC2 NDUFS3 NDUFS1 ATP6V1B2
Aminoacyl-tRNA biosynthesis	*GARS* YARS	KARS NARS QARS DARS	GARS KARS GARS FARSLB MARS
Pyrimidine metabolism	DUT POLR1C NUDT2 RFC5 POLR2E	DUT TXNRD1 ITPA	UCK2
Carbon fixation	ALDOA PKM2	*TPI1* ME1 PKM2	*TPI1* GOT1 PKM2
Alanine and aspartate metabolism	ASNS	PDHB NARS DARS	GOT1
Genetic Information Processing			
Proteasome	PSMA7 PSMC5 PSMC6 PSMC1 PSMC4 PSMC3	PSMA4 PSMC1	PSMC2 PSMA3 PSMD8
Ubiquitin mediated proteolysis	CUL3		
Ribosome	RPS17 RPSA RPS7		RPS5
RNA polymerase	POLR1C POLR2E		
SNARE interactions in vesicular transport		GOSR1	

KEGG pathway classification of 379 differentially expressed proteins after imatinib treatment. The gene names are Entrez gene names. Some gene names appear in more than one pathway description. The complete table functional with hyperlinks is available as Supplemental data file 5. Table II and also available at our Web site http://yeung.fccc.edu. [a]The gene names with underlines are more than two fold decrease in protein quantity compared to no treatment. The gene names without underlines are more than two fold increase in protein quantity compared to no treatment. [b]The gene names highlighted in Italic type are identified as more than one isoform of same protein, and their protein quantities are decreased or increased more than two fold after imatinib treatment compared to no treatment.

and is crucial for cell proliferation and cell survival. Recently, Young and colleagues demonstrated that TSG101 is a direct downstream target of RAS. Silencing of TSG101 in RAS^{V12}-transformed human ovarian epithelial cells by siRNA in SKOV3 ovarian cancer cells led to growth inhibition and cell death (42).

Discussion

We sought to test whether imatinib, a tyrosine kinase receptor inhibitor, could inhibit PDGFRα signaling activity, and hence suppress ovarian cancer cell proliferation. During the course of these studies, Schilder and colleagues reported the results of a Phase II trial of imatinib in patients with recurrent ovarian or primary peritoneal carcinoma (43). In this study, the authors reported that KIT, PDGFRα, and PDGFRβ, were detected in the majority of cases, with the percentage of tumor cells staining positive for each protein being generally greater than 85%. At least one target of imatinib (KIT, PDGFRα, PDGFRβ) was expressed in the tumors of all patients, and the majority of tumors expressed all three. However, there was no association between expression of these proteins and overall survival, and no impact on the probability of having a complete response or stable disease *versus* expression of these targets. What was lacking from these studies was evidence of constitutively activated levels on any of the receptor targets. As shown in our *in vitro* model, only ovarian tumor cells expressing the activated form of the receptor, *i.e.*, PDGFRα, showed sensitivity to imatinib.

Table III. *Imatinib induced significant changes in protein expression*.

pH range specific unique ID	2D gel pH range	Imatinib treatment for 6 h (Fold change, No treatment =1)	Imatinib treatment for 24 h (Fold change, No treatment =1)	Swiss-Prot accession No.	Entrez gene symbol	Protein name	Theoretical pI	Theoretical molecular weight (Da)	Mascot score	Sequence coverage (%)
Serine/Threonine protein phosphatases subunits										
896	4-7	3.4	2.9	P62714	PPP2CB	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	5.2	35575	104	37
2110	5-8	1.2	2.1	P62714	PPP2CB	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	5.2	35575	113	37
1359	4-7	–1.7	3.7	P30153	PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	5.0	65177	132	33
1360	4-7	2.1	-	P30153	PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	5.0	65177	140	35
1371	4-7	1.4	1.3	P30153	PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	5.0	65177	175	46
1428	4-7	2.2	1.8	P30153	PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	5.0	65177	140	35
1164	5-8	–2.7	–3.4	P62136	PPP1CA	Serine/threonine protein phosphatase PP1-alpha catalytic subunit (PP-1A)	5.9	37512	64	19
852	4-7	1.6	–2.0	P62136	PPP1CA	Serine/threonine protein phosphatase PP1-alpha catalytic subunit (PP-1A)	5.9	37512	92	28
2017	5-8	–1.7	–2.2	P62136	PPP1CA	Serine/threonine protein phosphatase PP1-alpha catalytic subunit (PP-1A)	5.9	37512	55	23
860	4-7	–1.0	–4.5	P62140	PPP1CB	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	5.9	37056	108	26
MAPK signaling pathway										
955	5-8	–3.7	–5.4	P36507	MAP2K2	Dual specificity mitogen-activated protein kinase kinase 2	6.1	44424	95	26
2030	4-7	2.4	3.6	P36507	MAP2K2	Dual specificity mitogen-activated protein kinase kinase 2	6.1	44424	71	21
576	4-7	2.2	2.3	P10644	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory chain	5.3	42982	86	31
573	4-7	2.4	1.4	P10644	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory chain	5.3	42982	74	34
541	4-7	–3.4	–4.2	P13861	PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory chain	5.0	45387	105	43
422	5-8	–3.0	–2.1	P11142	HSPA8	Heat shock cognate 71 kDa protein	5.4	70898	99	31
432	4-7	–2.1	–1.0	P11142	HSPA8	Heat shock cognate 71 kDa protein	5.4	70898	113	26
1187	4-7	–2.0	–1.5	P11142	HSPA8	Heat shock cognate 71 kDa protein	5.4	70898	70	24
1197	4-7	–2.6	2.2	P11142	HSPA8	Heat shock cognate 71 kDa protein	5.4	70898	128	26
299	4-7	–6.5	–2.6	P11142	HSPA8	Heat shock cognate 71 kDa protein	5.4	70898	120	34
388	6-11	1.6	–3.9	P11142	HSPA8	Heat shock cognate 71 kDa protein	5.4	70898	116	37
153	5-8	–16	–12	O75534	CSDE1	UNR protein	5.9	88885	233	37
175	5-8	1.5	–2.0	O75534	CSDE1	UNR protein	5.9	88885	152	23
836	4-7	–1.3	–2.0	O75534	CSDE1	UNR protein	5.9	88885	132	21
395	4-7	1.1	–2.4	Q13177	PAK2	Serine/threonine-protein kinase PAK 2 (p21-activated kinase 2) (PAK-2)	5.7	58043	139	44
2000	5-8	–2.7	–2.4	P46108	CRK	Proto-oncogene C-crk (P38) (Adapter molecule crk)	5.4	33831	143	43
1043	4-7	2.3	1.9	P11021	HSPA5	78 kDa glucose-regulated protein precursor (GRP 78)	5.0	70479	234	46
113	4-7	–2.3	–1.2	P11021	HSPA5	78 kDa glucose-regulated protein precursor (GRP 78)	5.0	70479	175	35
268	4-7	3.5	3.6	P11021	HSPA5	78 kDa glucose-regulated protein precursor (GRP 78)	5.0	70479	295	45

Table III. *continued*

Table III. *continued*

pH range specific unique ID	2D gel pH range	Imatinib treatment for 6 h (Fold change, No treatment =1)	Imatinib treatment for 24 h (Fold change, No treatment =1)	Swiss-Prot accession No.	Entrez gene symbol	Protein name	Theoretical pI	Theoretical molecular weight (Da)	Mascot score	Sequence coverage (%)
Cell proliferation, cell cycle and programmed cell death										
2357	4-7		–3.8	P06748	NPM1	Nucleophosmin (NPM)	4.6	32575	73	28
2100	5-8	1.7	–4.4	P06748	NPM1	Nucleophosmin (NPM)	4.6	32575	63	25
2570	4-7	4.4		P12004	PCNA	Proliferating cell nuclear antigen (PCNA)	4.6	28769	55	29
975	4-7	–1.2	–2.5	P12004	PCNA	Proliferating cell nuclear antigen (PCNA)	4.6	28769	77	37
974	4-7	–1.4	–2.0	P12004	PCNA	Proliferating cell nuclear antigen (PCNA)	4.6	28769	84	38
972	4-7	1.3	–4.8	P12004	PCNA	Proliferating cell nuclear antigen (PCNA)	4.6	28769	50	25
997	4-7	4.0	3.1	P12004	PCNA	Proliferating cell nuclear antigen (PCNA)	4.6	28769	64	40
792	4-7	–1.0	–2.0	Q9Y2Z0	SUGT1	Suppressor of G2 allele of SKP1 homolog (Sgt1)	5.1	40893	100	38
623	4-7	–1.2	–2.1	Q14566	MCM6	DNA replication licensing factor MCM6 (P105MCM)	5.3	92889	88	21
843	5-8	–2.2	–4.2	Q99816	TSG101	Tumor susceptibility gene 101 protein	6.1	43944	73	17
1337	5-8	–3.5	–8.3	P49759	CLK1	Dual specificity protein kinase CLK1 (CDC like kinase 1)	9.0	57205	56	15
135	5-8	–2.0	–3.5	P43246	MSH2	DNA mismatch repair protein Msh2	5.6	104743	138	29
685	4-7	–2.0	–1.4	P43246	MSH2	DNA mismatch repair protein Msh2	5.6	104743	86	16
1391	6-11	6.1	2.7	Q15404	RSU1	Ras suppressor protein 1 (Rsu-1)	8.6	31409	112	44

Protein expression differences in human ovarian cancer cells A2780 after imatinib treatment. Only selected proteins of cellular processes and signaling pathways of interest are shown. The combined 1,010 total protein identification list across 3 pH range is available as Supplemental data file 6 and also available at our Web site http://yeung.fccc.edu. In the Progenesis software output, fold change in protein expression refers to the amount of increase or decrease.

In addition, our proteomic analysis of the anti-proliferation result of imatinib on ovarian cancer cells revealed that the anti-proliferation effect of imatinib was not accompanied by changes in the activation status the PI3K/AKT pathways which was observed in the therapeutic treatment of gastrointestinal stromal tumors (24). These results are consistent with a more recent study by Tarn and colleagues demonstrating that exogenous expression of constitutively active AKT1 or AKT2 in GIST cells could not rescue these cells from the imatinib-mediated apoptosis, suggesting that the potential therapeutic effect of imatinib is independent of AKT activity (20). While targeting imatinib at PDGFRα of A2780 ovarian cancer cells and anticipating inhibition of AKT, our large scale 2D gel proteomic studies found that the anticipated causative AKT pathway was not affected while the protein levels of protein phosphatase PP2A complex and PP1C complex were regulated. In this comprehensive proteomic analysis, we also discovered differentially regulated proteins involved in cell cycle, cell proliferation, apoptosis, MAPK pathway, and small GTPase mediated signaling pathway. Protein phosphatase 2A (PP2A) plays an essential role in cell cycle regulation and induction of G2 arrest by a mechanism of phosphorylation/ dephosphorylation with a variety of protein kinases, many of the key components of signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade (32). The MAPK pathway is composed of multiple and interacting signaling cascades that regulate various functions, such as cell proliferation, differentiation, survival, and apoptosis (44). The extracellular signal-regulated kinase (ERK) 1/2 cascade of MAPK is activated by a receptor tyrosine kinase that stimulates the small G-protein Ras, with the sequential phosphorylation/ activation of c-RAF-1 or B-RAF through RAP-1 followed by the activation of MAPK/ERK kinase (MEK) 1/2 and ERK1/2. Phosphorylated ERK1/2 then dimerizes, translocates to the nucleus, and enhances cell proliferation by phosphorylating transcription factors, such as c-Myc, that in turn,

induce the expression of cell cycle-regulating genes, such as *cyclin-dependent kinases* and *cyclin D1* and others that may promote cell cycle progression (44).

In summary, the cellular response to imatinib treatment leading to growth arrest of A2780 ovarian cancer cell line is complex, many of the changes in the proteome occur at the level of individual isoforms of each protein. Thus a focus on post-translational modifications will be important to future studies of the anti-cancer mechanisms of imatinib. Furthermore, these studies emphasize that despite expression of the receptors targeted by imatinib other downstream pathways are likely to be co-activated in these tumors and that redundant inputs drive and maintain downstream signaling, thereby limiting the efficacy of therapies targeting a single or a few RTKs (45). This does not mean that agents with minimal single activity may not be useful, but that they will need to be combined in rationale approaches with other drugs. Thus, effective therapy of ovarian cancer will undoubtedly require combined regimens targeting multiple RTKs. Our proteomic studies begin to provide such insights in pathways that could be targeted in combination with imatinib to ultimately improve the treatment of patients with ovarian cancer.

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