

# Activation of Abl Tyrosine Kinases Promotes Invasion of Aggressive Breast Cancer Cells

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## Abstract

**The Abl family of nonreceptor tyrosine kinases consists of two related proteins, c-Abl and Abl-related gene (Arg). Activated forms of the Abl kinases (BCR-Abl, Tel-Abl, and Tel-Arg) induce the development of human leukemia; it is not known, however, whether Abl kinases are activated in solid tumors or whether they contribute to tumor development or progression. Previously, we showed that Abl kinases are activated downstream of growth factor receptors, Src family kinases, and phospholipase C $\gamma$ 1 (PLC $\gamma$ 1) in fibroblasts and influence growth factor-mediated proliferation, membrane ruffling, and migration. Growth factor receptors, Src kinases, and PLC $\gamma$ 1 are deregulated in many solid tumors and drive tumor invasion and metastasis. In this study, we found that Abl kinases are constitutively activated, in highly invasive breast cancer cell lines, downstream of deregulated ErbB receptors and Src kinases. Furthermore, activation of Abl kinases promotes breast cancer cell invasion, as treatment of cells with the Abl kinase inhibitor, STI571, or silencing c-Abl and Arg expression with RNA interference dramatically inhibits Matrigel invasion. This is the first evidence that (a) Abl kinases are deregulated and activated in a nonhematopoietic cancer, (b) activation of Abl kinases in breast cancer cells occurs via a novel mechanism, and (c) constitutive activation of Abl kinases promotes invasion of breast cancer cells. These data suggest that pharmacologic inhibitors targeted against Abl kinases could potentially be useful in preventing breast cancer progression in tumors harboring activated Abl kinases.** (Cancer Res 2006; 66(11): 5648-55)

## Introduction

Breast cancer is the most common malignancy in U.S. women, and 40,000 women die from the disease each year (1). Most cancer deaths result from metastasis of tumor cells to distant organ sites rather than from the primary tumor (2). The ability of epithelial-derived cancers to progress to an invasive, metastatic state correlates with a shift from an adherent, epithelial shape to a motile, fibroblast-like morphology [epithelial-mesenchymal transition (EMT); ref. 3]. During EMT, stable cell-cell contacts and cell-extracellular matrix (ECM) contacts are disassembled, the actin cytoskeleton is remodeled, and cells have an increased ability to migrate and degrade the ECM (3). Activation of Ras, Src kinases, transforming growth factor- $\beta$ , and growth factor receptors, such

as c-Met and members of the ErbB family [epidermal growth factor (EGF) receptor (EGFR)/ErbB1 and HER-2/ErbB2], induces EMT (4, 5). Proteins that regulate migration in fibroblasts [i.e., RhoGTPases and phospholipase C $\gamma$ 1 (PLC $\gamma$ 1)] also influence invasion of epithelial-derived cancer cells (2).

The mammalian Abl family of nonreceptor tyrosine kinases (also called Abelson kinases) includes two homologous proteins, c-Abl and Abl-related gene (Arg), which are encoded by *Abl1* and *Abl2* genes, respectively. Abl kinases have highly homologous NH<sub>2</sub> termini (SH3, SH2, and kinase domains) but are more divergent in their COOH termini (6). c-Abl is negatively regulated by intramolecular interactions: the kinase domain binds a NH<sub>2</sub>-terminal myristoyl residue (7), and the SH3 domain interacts with proline residues in the interlinker region (between SH2 and kinase domains; ref. 6). Mutations that disrupt these interactions activate the kinases, which produces oncogenic proteins that transform a variety of cell types (6). In 95% of patients with chronic myelogenous leukemia (CML), *Abl1* is translocated next to the *BCR* gene [t(9;22)], generating a BCR-Abl fusion protein that has constitutively active tyrosine kinase activity (8). *Abl1* and *Abl2* also are translocated next to the *Tel* gene (Ets family transcription factor) in leukemia and myeloproliferative diseases, and *Abl1* is amplified in T-cell acute lymphocytic leukemia (8, 9). Hematopoietic cells expressing BCR-Abl display decreased adhesiveness to bone marrow stroma and an increased ability to survive, proliferate, migrate, and invade (8, 10). Gleevec [signal transduction inhibitor 571 (STI571), imatinib mesylate], an inhibitor of the Abl kinases, which binds the ATP-binding pocket, induces remission in patients with early-stage CML (11).

Although the role of BCR-Abl in the development of CML has been well studied, the normal function of the Abl kinases has remained elusive. Previously, we showed that Abl kinases are transiently activated by platelet-derived growth factor (PDGF) and EGF stimulation in fibroblasts (12). Activation of c-Abl by PDGF occurs in a Src-dependent manner, as Src kinases directly phosphorylate c-Abl at Y412 and Y245, residues required for full activity (12, 13). In addition to Src, PLC $\gamma$ 1 also is required for activation of c-Abl downstream of PDGF receptor (PDGFR)- $\beta$  (14). Following activation by PDGF, c-Abl activity is rapidly down-regulated by the PTP-PEST phosphatase (6). Significantly, we showed that c-Abl activation is required for PDGF-mediated proliferation, membrane ruffling, and PLC $\gamma$ 1-mediated migration in fibroblasts (12, 14).

Although Abl kinases play key roles in the development of human leukemia, it is not known whether their kinase activities are increased in solid tumors, because translocations involving c-Abl and Arg have not been identified. Upstream regulators of the Abl kinases (growth factor receptors, Src kinases, and PLC $\gamma$ 1; refs. 12, 14) are frequently deregulated in solid tumors, such as breast cancer, and their activation increases tumor invasiveness and is associated with a poor clinical outcome (2, 15-17). Constitutive activation of EGFR family members (i.e., EGFR/ErbB1 and

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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HER-2/ErbB2) by receptor overexpression, presence of an autocrine growth loop, or mutation promotes breast cancer cell proliferation, survival, migration, invasion, and metastasis (17, 18). Src kinases also are frequently activated in breast cancer and cooperate with EGFR to promote the malignant process (18). Because Abl kinases are involved in cytoskeletal reorganization and migration and are activated downstream of EGFR and Src kinases in fibroblasts (12), we reasoned that Abl kinases may be activated in solid tumors containing constitutively active growth factor receptors and/or Src kinases and may increase tumor cell migration and/or invasion.

c-Abl protein levels, as assessed by immunohistochemistry, are increased in many solid tumors, but increased expression is not consistently correlated with disease grade (19, 20). Arg expression is increased in high-grade colon tumors but not in adenomas or adjacent normal tissue, suggesting that Arg expression may correlate with disease progression (21). However, low-level overexpression of the Abl kinases does not activate their kinase activities due to tight regulation (6). To date, Abl kinase activities have not been assessed in solid tumors, and a direct relationship between Abl kinases and the development or progression of solid tumors has not been established. Here, we show that Abl kinases are constitutively activated in highly invasive breast cancer cells by a mechanism that does not involve chromosomal translocation. Rather, Abl kinases are activated downstream of deregulated EGFR, HER-2, and Src kinases. Significantly, we show that Abl kinases play a functional role in breast cancer progression, as activation of Abl kinases potently drives breast cancer cell invasion.

## Materials and Methods

**Reagents.** Antibodies directed against phosphotyrosine (PY99),  $\alpha$ -tubulin, c-Kit, insulin-like growth factor-I receptor (IGF-IR), glutathione *S*-transferase (GST), and c-Abl (K12; immunoprecipitation) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to phosphorylated EGFR (Y1173), Src (GD11), HER-2/ErbB2, and phosphotyrosine (4G10) were obtained from Upstate Biotechnology (Lake Placid, NY); antibodies to phosphorylated Src (Y416), phosphorylated Abl (Y245), and phosphorylated Crk/CrkL (Y221/Y207) were purchased from Cell Signaling Technology (Danvers, MA); antibodies to EGFR, PLC $\gamma$ 1, and c-Abl (8E9; Western blotting) were procured from BD Biosciences (Chicago, IL); PLC $\gamma$ 1 (Y783) antibody was obtained from Biosource (Camarillo, CA). The Arg antibody was described previously (22). Antibodies to PDGFR were provided by Dr. Andrius Kazlauskas (Harvard University, Cambridge, MA). STI571 was a gift of Novartis Pharmaceuticals (Basel, Switzerland). Pharmacologic inhibitors of Src (SU6656), EGFR (PD153035), and EGFR/HER-2 (PD158780) kinases were obtained from Calbiochem (La Jolla, CA). EGF and IGF-I were obtained from Roche Diagnostics Corp. (Indianapolis, IN), and Upstate Biotechnology (Charlottesville, VA), respectively.

**Cell lines.** 10T-1/2 cells overexpressing EGFR (12) and MCF-7 cells were gifts of Dr. Sally Parsons (University of Virginia, Charlottesville, VA) and Dr. Vivek Rangnekar (University of Kentucky, Lexington, KY), respectively. Fibroblasts lacking c-Abl and Arg and subsequently reconstituted with c-Abl and Arg (4-79-AA) were described previously (22). Fibroblasts lacking Src, Fyn, and Yes kinases (SYF) were obtained from American Type Culture Collection (Manassas, VA). Breast cancer cell lines were obtained from University of North Carolina (Chapel Hill, NC), and Cos-7 cells were from Duke University (Durham, NC) tissue culture facilities. Human mammary epithelial cells (HMEC) were purchased from Cambrex (Baltimore, MD). Cells were maintained as suggested by suppliers and serum starved for 24 hours in 0.1% fetal bovine serum for kinase assays and in basal medium without serum for invasion assays.

**Immunoprecipitation, kinase assays, Western blotting, GST pull-down, and far Western analyses.** Procedures were done as described previously (12, 22). Immunoblotting with phosphospecific antibodies was done according to the manufacturer's protocols.

**RNA interference.** MDA-MB-435S cells were transfected with validated small interfering RNAs (siRNA; 20 nmol/L; Abl-1336, Arg-1478, scrambled control; Ambion, Austin, TX) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Transfection complexes were removed after 24 hours and cells were retransfected for 24 hours and serum starved overnight.

**Semiquantitative reverse transcription-PCR.** Total RNA was isolated from siRNA-transfected cells using a RNeasy kit (Qiagen, Valencia, CA) and digested with DNase I (Invitrogen). RNA was reverse transcribed using SuperScript reverse transcriptase and random primers (Invitrogen) and subjected to PCR using primers specific for the COOH termini of c-Abl and Arg (20  $\mu$ mol/L; *Abl1*: forward primer 5'-CCTTCATCCCTCATAT-CAACC-3' and reverse primer 5'-TGGACCACTGCCTGTGTCGC-3' and *Abl2*: forward primer 5'-CATCCGTCCATCTGCTCAGAC-3' and reverse primer 5'-GGACAGTAGGTCAGCACATTC-3') together with internal  $\beta$ -actin control primers (7.5  $\mu$ mol/L; forward primer 5'-CCTTCCTGGGCATGGAG-TCCCT-3' and reverse primer 5'-GGAGCAATGTCTTTGATCTTC-3'), MgCl<sub>2</sub> (1.5 mmol/L), deoxynucleotide triphosphates, and Taq DNA polymerase (Invitrogen). PCR cycling variables involved 31 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. Aliquots were taken at cycles 23, 27, and 34 to check for linearity. Scanned photographs were quantified using ImageQuant (GE Healthcare Life Sciences, Piscataway, NJ), and *Abl1*- or *Abl2*-specific bands were normalized to  $\beta$ -actin internal controls.

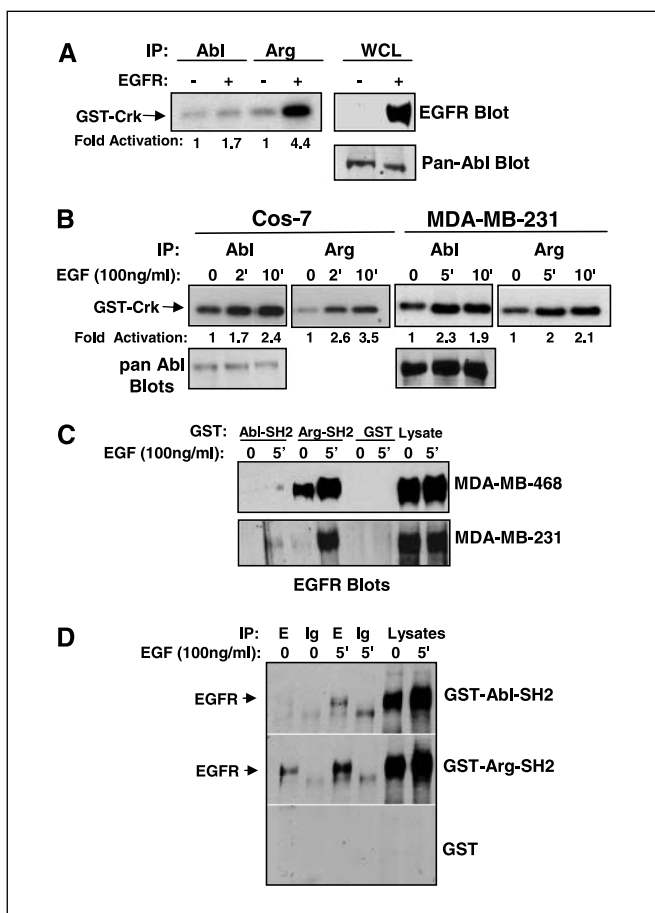
**Invasion assays.** Serum-starved siRNA-transfected cells or serum-starved cells treated with STI571 (10  $\mu$ mol/L for 24 hours) were suspended in migration medium (basal medium containing 1% bovine serum albumin) and placed in the top well of invasion chambers (BD Biosciences). Chemoattractant (IGF-I, 5 nmol/L) was placed in the lower chamber in migration medium. Cells were allowed to invade for 48 hours at 37°C. Cells on the upper surface of the membrane were removed, and cells on the undersurface were fixed, stained (Difco kit, Fisher Biosciences, Pittsburgh, PA), and counted.

## Results

**Abl kinases bind EGFR and are activated by EGF in breast cancer cells.** Previously, we showed that Abl kinases are activated by EGF in fibroblasts engineered to overexpress EGFR (12). To determine whether c-Abl and Arg are activated by EGFR overexpression, we assayed c-Abl and Arg activities in serum-starved 10T-1/2 fibroblasts transfected with either vector or EGFR. c-Abl and particularly Arg kinase activities were significantly elevated in cells overexpressing EGFR in the absence of EGF or serum (Fig. 1A). These data show that constitutive activation of EGFR induced by overexpression activates Abl kinases. Next, we tested whether EGF stimulation activates Abl kinases in cells that express endogenous EGFR. Indeed, EGF stimulation of Cos-7 cells and MDA-MB-231 breast cancer cells activates c-Abl and Arg (Fig. 1B).

Abl kinases bind to and are phosphorylated by PDGFR- $\beta$  (22). To determine whether Abl kinases bind EGFR in breast cancer cells, we did GST pull-down and far Western analyses. We found that the SH2 domains of c-Abl and Arg bind EGFR in breast cancer cell lines (Fig. 1C), and far Western analyses show that the binding is direct and does not involve a protein bridge (Fig. 1D). In MDA-MB-231 cells, binding of c-Abl and Arg SH2 domains to EGFR is EGF inducible, whereas in MDA-MB-468 cells, which express extremely high levels of EGFR, binding of the Arg SH2 domain to EGFR is constitutive (Fig. 1C and D). Taken together, these data suggest that the Abl kinases may act downstream of EGFR in breast cancer cells.

**Abl kinases are constitutively active in highly aggressive breast cancer cell lines.** Fibroblasts expressing oncogenic forms of the Src kinases have dramatically elevated c-Abl and Arg kinase activities (12), and Arg activity is significantly elevated in fibroblasts overexpressing EGFR (Fig. 1A). These data indicate



**Figure 1.** Abl kinases bind EGFR and are activated by EGFR overexpression and EGF stimulation in breast cancer cells. *A*, c-Abl and Arg were immunoprecipitated from serum-starved 10T-1/2 fibroblasts stably overexpressing either vector (-) or EGFR (+) and incubated in an *in vitro* kinase assay using GST-Crk as substrate (*left*). Whole-cell lysates (WCL) were blotted with antibodies directed against EGFR (*top right*) and Abl kinases (Pan-Abl) (*bottom*). *B*, c-Abl and Arg kinase activities were assessed in lysates from serum-starved, EGF-stimulated Cos-7 cells and MDA-MB-231 cells by *in vitro* kinase assay (*top*) and blotted with Pan-Abl antibody (*bottom*). *C*, lysates from serum-starved, unstimulated (0), or EGF-stimulated MDA-MB-468 and MDA-MB-231 breast cancer cells were incubated with 1  $\mu$ g of the indicated GST-fusion proteins and glutathione-sepharose. Precipitates were probed with an EGFR antibody. Lysates are 5% and 1% of input for MDA-MB-468 and MDA-MB-231, respectively. *D*, lysates from serum-starved, unstimulated, or EGF-stimulated MDA-MB-468 cells were subjected to immunoprecipitation with EGFR (E) or control (IgG) antibody, and the blots were incubated with the indicated GST-fusion proteins. Binding was assessed by Western blotting with anti-GST antibody. Arrow, location of EGFR. Representative of at least three independent experiments.

that deregulation of proteins that lie upstream of the Abl kinases (growth factor receptors and Src kinases) activates Abl kinases in fibroblasts. Hence, we tested whether Abl kinases are activated in breast cancer cells that express deregulated growth factor receptors and/or Src kinases. Most of the breast cancer cell lines that we analyzed expressed higher levels of Abl protein compared with HMECs (Fig. 2A, *middle*). Considering that Abl kinases are tightly regulated and that low-level overexpression does not usually activate their kinase activities (6), we assessed the activities of c-Abl and Arg under serum-starved conditions. Basal c-Abl and/or Arg kinase activities were dramatically elevated in several poorly differentiated, highly invasive, estrogen receptor (ER)-negative breast cancer cell lines (BT-549, MDA-MB-231, MDA-MB-468, and

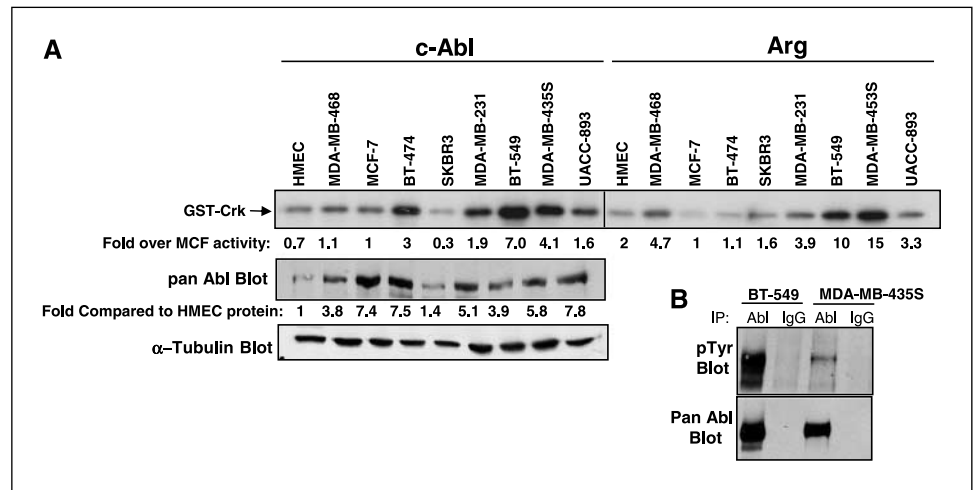
MDA-MB-435S; refs. 23, 24) grown in the absence of serum or growth factors (Fig. 2A, *top*). In contrast, highly differentiated, noninvasive, ER-positive MCF-7 cells expressed the most Abl protein but had low endogenous c-Abl and Arg kinase activities (Fig. 2A). To confirm that MCF-7 cells were noninvasive, whereas the ER-negative cell lines were highly invasive, we did Matrigel invasion assays. MCF-7 cells, which have low c-Abl and Arg activities, were unable to invade a Matrigel matrix, whereas BT-549, MDA-MB-231, and MDA-MB-435S cells, which contain highly active c-Abl and Arg kinases (Fig. 2A), were extremely invasive (Supplementary Fig. S1). MDA-MB-468 cells, which only have increased Arg activity (Fig. 2A), invaded the Matrigel more slowly, and incubation for 72 hours was required to observe appreciable invasion (data not shown).

It is not usually possible to detect tyrosine phosphorylation of endogenous Abl proteins following treatment of cells with extracellular stimuli (6) either because only a small pool of Abl molecules are activated by the stimulus (12) or because wild-type Abl proteins are tightly regulated, and their activities are rapidly returned to basal levels via dephosphorylation and ubiquitin-mediated degradation (6, 25). Significantly, we found that endogenous c-Abl was constitutively tyrosine phosphorylated in serum-starved BT-549 and MDA-MB-435S cells (Fig. 2B), which suggests that c-Abl is unregulated in these cell lines.

**Growth factor receptors, Src kinases, and PLC $\gamma$ 1 are constitutively active in breast cancer cell lines.** To dissect the mechanism of activation of Abl kinases in breast cancer cell lines, we first assessed the expression level and activity status of EGFR, HER-2, Src, and PLC $\gamma$ 1 in lysates from serum-starved breast cancer cells. Most cell lines express an EGFR family member, except MDA-MB-435S cells, which overexpress IGF-IR (Fig. 3A; ref. 26). EGFR, HER-2, and Src kinases are constitutively activated/phosphorylated in many of the cell lines (Fig. 3A; refs. 27, 28). Because Src kinases and PLC $\gamma$ 1 bind to and are activated by growth factor receptors, we tested whether the activities of Src and PLC $\gamma$ 1 were dependent on constitutive activation of EGFR. Treatment of cells with a pharmacologic agent that inhibits the EGFR (PD153035) blocked the activation of PLC $\gamma$ 1 in MDA-MB-468 cells but did not affect constitutive Src activity in MDA-MB-468, MDA-MB-231, or BT-549 cells (Fig. 3B), suggesting that Src activation occurs via an EGFR-independent manner in these cell lines.

**Abl kinases are activated downstream of deregulated EGFR, HER-2/ErbB2, and Src kinases in breast cancer cells.** To identify the mechanism(s) by which the Abl kinases are dramatically activated in breast cancer cell lines, we tested whether constitutive activation of EGFR induces activation of the Abl kinases. Treatment of serum-starved cells with the EGFR inhibitor, PD153035, decreased the activities of c-Abl and Arg (Fig. 4A, *left*). PD153035 did not directly inhibit c-Abl and Arg activities because, in fibroblasts that do not express activated EGFR, c-Abl and Arg were not affected by PD153035 treatment (Fig. 4A, *right*). To determine whether deregulation of another EGFR family member induces activation of the Abl kinases, we inhibited constitutive activation of HER-2/ErbB2 in BT-474 and UACC-893 cells with PD158780, a pharmacologic agent that inhibits both EGFR and HER-2 kinases, and assessed the effect on c-Abl and Arg kinase activities. PD158780 treatment of serum-starved BT-474 and UACC-893 cells inhibited activation of c-Abl (BT-474) or Arg (UACC-893; Fig. 4B), consistent with the activation status of c-Abl and Arg in the two cell lines. PD158780 did not have a direct effect on the Abl kinases, because c-Abl and Arg activities were not

**Figure 2.** Abl kinases are overexpressed and constitutively activated in many breast cancer cell lines. *A*, c-Abl and Arg protein expression (*middle*) and kinase activities (*top*) were assessed in serum-starved HMECs and in breast cancer cell lines by Western blotting and *in vitro* kinase assay (*top*). Kinase activities were quantitated on a PhosphorImager (GE Healthcare). Relative protein levels were quantitated by analyzing scanned Pan-Abl blots with ImageQuant software. *B*, c-Abl was immunoprecipitated from serum-starved breast cancer cell lysates. Blots were probed with phosphotyrosine antibody (PY99/4G10; *top*), stripped, and re probed with anti-Abl antibody (*bottom*). Representative of at least three independent experiments.



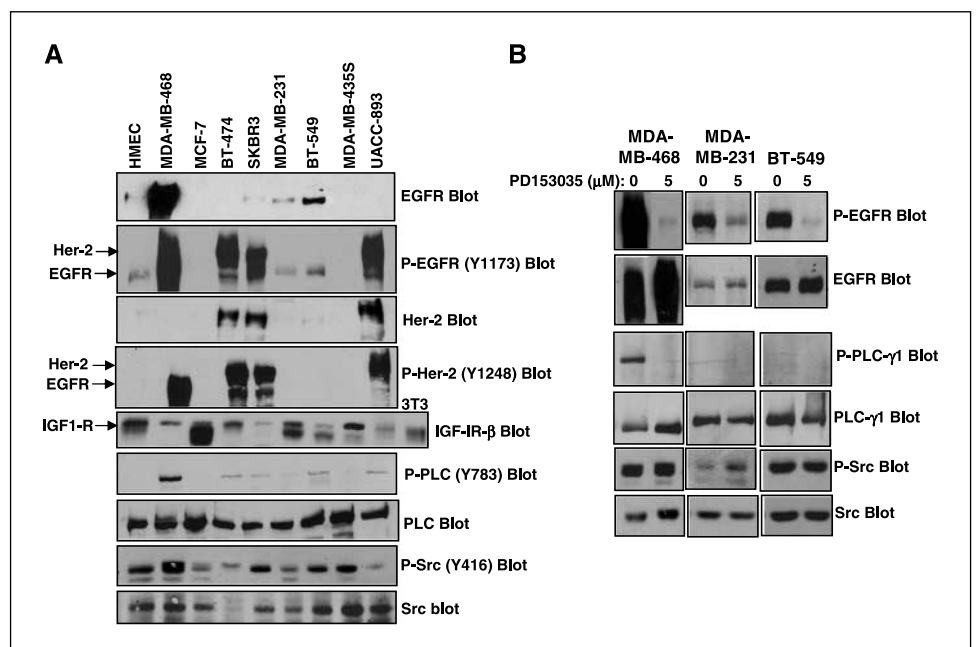
inhibited in PD158780-treated fibroblasts, which do not express HER-2 (Fig. 4B, right). Taken together, these data show that Abl kinases are constitutively activated downstream of deregulated ErbB receptors in breast cancer cells.

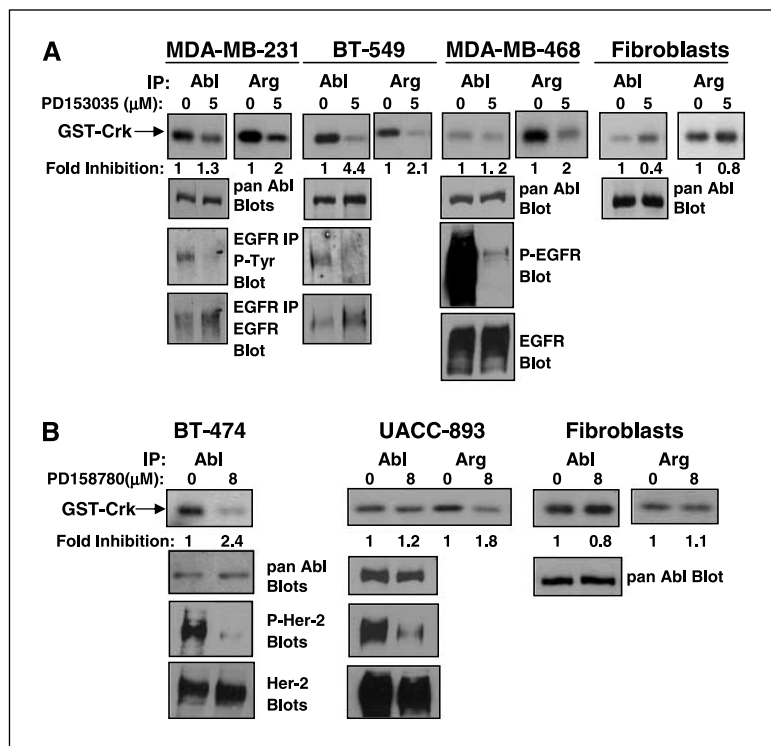
We and others showed that Src kinases directly phosphorylate and activate c-Abl (12, 13). Src kinases are constitutively active in many of the breast cancer cell lines that we analyzed, and activation occurs in an EGFR-independent manner (Fig. 3). To determine whether constitutive activation of the Src kinases contributes to activation of Abl kinases in breast cancer cells, c-Abl and Arg activities were assessed in cells treated with SU6656, a pharmacologic inhibitor of the Src family kinases. SU6656 treatment reduced the constitutive activities of c-Abl and/or Arg in MDA-MB-468, MDA-MB-435S, and BT-549 cells (Fig. 5, left) but did not inhibit c-Abl or Arg activities in fibroblasts lacking expression of the Src kinases (SYF; Fig. 5, right). These data show that the effect of SU6656 on the Abl kinases is due to its inhibitory

effect on the Src family kinases. Thus, in addition to EGFR and HER-2, Src kinases also activate Abl kinases in breast cancer cells.

**Abl kinases are required for breast cancer cell invasion.** Abl kinases are constitutively activated in highly aggressive, invasive breast cancer cells, and the extent of c-Abl and Arg activation correlates with the degree of invasiveness of the cells (Fig. 2A; Supplementary Fig. S1; refs. 23, 24). These data suggest that Abl kinases may be activated as cells transition to an invasive, metastatic state. To determine whether activation of Abl kinases contributes to the invasiveness of breast cancer cells, we examined whether blocking c-Abl and Arg activation using the c-Abl/Arg inhibitor, STI571, or silencing c-Abl and Arg expression with RNA interference (RNAi) affects breast cancer invasion. We chose to study MDA-MB-435S cells because they are highly invasive and both c-Abl and Arg activities are dramatically elevated (Fig. 2A; Supplementary Fig. S1; ref. 24). First, we determined the concentration of STI571 that effectively inhibits the constitutive

**Figure 3.** Constitutive activation of PLC $\gamma$ 1 is mediated by deregulated EGFR, whereas Src activation occurs independent of activated EGFR. *A*, lysates from serum-starved breast cancer cells were probed with various antibodies and phospho-specific antibodies. Phospho-specific antibodies to HER-2 and EGFR cross-react with phosphorylated EGFR and HER-2, respectively. NIH3T3 cell lysate is a positive control for the IGF-IR $\beta$  blot. *B*, serum-starved breast cancer cell lines were treated with the EGFR inhibitor, PD153035, or DMSO (0) for 24 hours, and lysates were blotted with the indicated antibodies. Representative of three independent experiments.





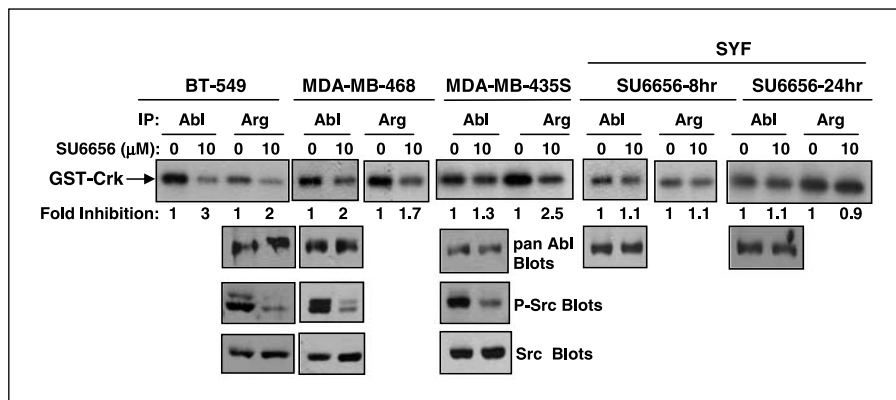
**Figure 4.** Deregulation of EGFR and HER-2/ErbB2 kinases contributes to activation of the Abl kinases in breast cancer cells. *A*, serum-starved breast cancer cells that express activated EGFR (left) and fibroblasts that do not express activated EGFR (4-79-AA; right) were treated with the EGFR inhibitor, PD153035, or DMSO (0) for 24 hours. c-Abl and Arg kinase activities were assessed by *in vitro* kinase assay (top). As a control, EGFR was immunoprecipitated from the lysates, blotted with phosphotyrosine antibody, stripped, and reprobed with EGFR antibody (MDA-MB-231 and BT-549); alternatively, lysates were blotted with antibody to phosphorylated EGFR (MDA-MB-468). *B*, serum-starved BT-474 and UACC-893 breast cancer cells that overexpress HER-2 (left) or fibroblasts that do not express HER-2 (4-79-AA; right) were treated for 8 hours with the dual EGFR/HER-2 inhibitor, PD158780, or DMSO (0), and c-Abl and Arg kinase activities were assessed in the lysates. Representative of three independent experiments.

activities of c-Abl and Arg in MDA-MB-435S cells by testing the effect of STI571 on c-Abl autophosphorylation (Y245) and phosphorylation of Abl/Arg substrates, Crk/CrkL (29). A concentration of 10 μmol/L STI571 was required to effectively decrease (by 65-75%) c-Abl phosphorylation and c-Abl/Arg activity (assessed by phosphorylated Crk/CrkL blots; Fig. 6A, top). This concentration of STI571 had no effect on the phosphorylation/activity of the highly related Src kinases (Fig. 6A, top).

Next, MDA-MB-435S cells were treated with STI571, and Matrigel invasion assays were done. STI571-treated MDA-MB-435S cells were dramatically less invasive (4.4-fold) than vehicle-treated cells (Fig. 6A, bottom). Consistent with this result, another group showed that STI571 treatment also inhibits the invasiveness of MDA-MB-231 cells (30). In addition to the Abl kinases, STI571 also inhibits c-Kit and PDGFR tyrosine kinases but has little effect on other tyrosine kinases (Fig. 6A; ref. 31). To determine whether the effect of STI571 on the invasiveness of MDA-MB-435S cells is due to inhibition of c-Kit or PDGFR rather than an effect on c-Abl and

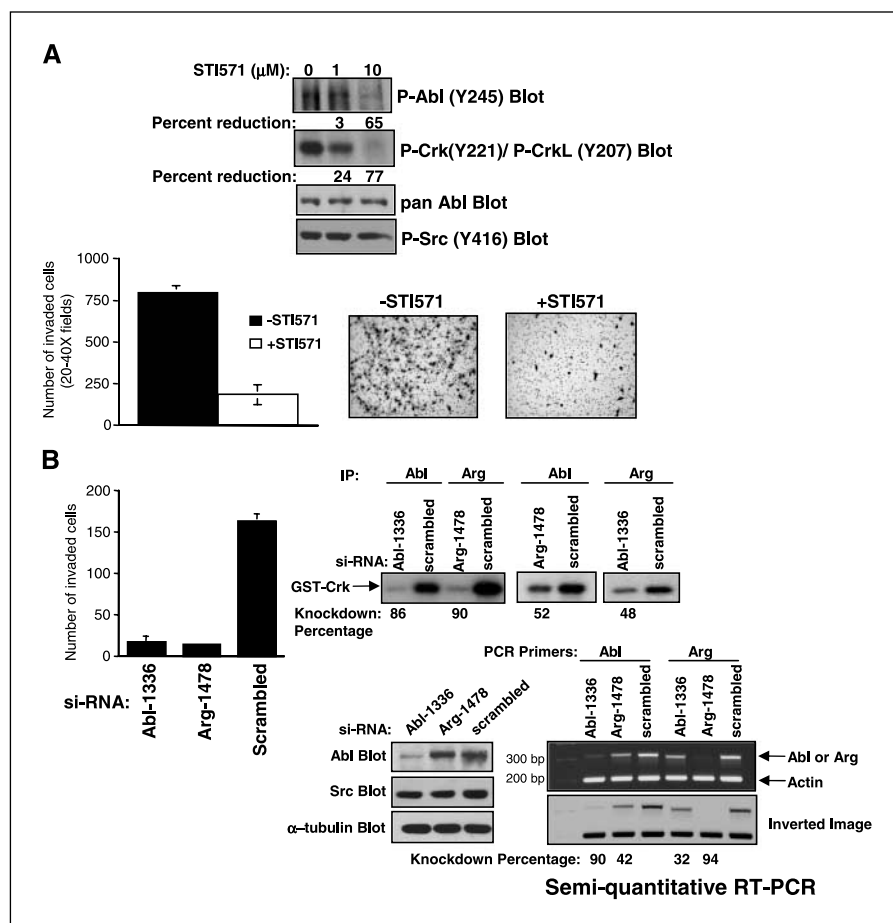
Arg kinase activities, we assayed c-Kit and PDGFR expression by Western blotting. MDA-MB-435S cells did not express PDGFR or c-Kit (Supplementary Fig. S2), which suggests that the effect of STI571 on the invasiveness of MDA-MB-435S cells is likely due to inhibition of c-Abl and Arg kinase activities.

To definitively confirm that Abl kinases promote breast cancer invasion, we assessed the effect of silencing c-Abl and Arg expression on invasion of MDA-MB-435S cells. MDA-MB-435S cells were transfected with siRNAs directed at c-Abl, Arg, or a scrambled control. Semiquantitative reverse transcription-PCR (RT-PCR) analysis showed that c-Abl and Arg were efficiently silenced (90-95%) by the appropriate siRNA (Fig. 6B, bottom right). Western blotting showed that c-Abl protein expression also was dramatically decreased (Fig. 6B, bottom left). Sensitive antibodies that recognize endogenous Arg by Western blotting are not commercially available. Therefore, we tested whether kinase assays could be used to determine protein knockdown efficiency of Arg siRNAs. As shown in Supplementary Fig. S3, expression of a c-Abl siRNA



**Figure 5.** Constitutively active Src kinases induce activation of Abl kinases in breast cancer cells. Serum-starved cells were treated with the Src inhibitor, SU6656, or DMSO (0) for 8 hours (BT-549 and MDA-MB-435S) or 24 hours (MDA-MB-468). c-Abl and Arg activities were assessed by *in vitro* kinase assay (top). Lysates were blotted with antibodies to c-Abl and Arg (Pan-Abl), Src, and phosphorylated Src (Y416; bottom). c-Abl and Arg activities also were assessed in SU6656-treated SYF fibroblasts (lack expression of Src family kinases; right). Representative of three independent experiments.

**Figure 6.** Abl kinases promote breast cancer cell invasion. **A**, serum-starved MDA-MB-435S cells were treated with STI571 (10  $\mu\text{mol/L}$ ) or vehicle (water) for 24 hours and plated ( $2.5 \times 10^5$ ) in the upper well of invasion chambers in the presence or absence of STI571. IGF-I (5 nmol/L) was placed in the lower chambers. After 48-hour incubation, cells on the undersurface of the membrane were stained and counted. Representative fields were taken using a Nikon (Melville, NY) Microphot microscope ( $\times 10$  objective). **B**, serum-starved, siRNA-transfected MDA-MB-435S cells were used in invasion assays as described above. Aliquots of the cells were lysed and subjected to kinase assay, Western blotting (left), and semiquantitative RT-PCR (right). All experiments were done in triplicate. Representative of three independent experiments.



reduced c-Abl expression (Western blot) and activity (kinase assay) by exactly the same percentage. Kinase assays were consistent with RT-PCR analyses, showing 85% to 90% knockdown of c-Abl and Arg kinase activities (Fig. 6B, top right). Because c-Abl and Arg are highly homologous, we tested whether the Arg siRNA affects c-Abl expression/activity and vice versa by performing semiquantitative RT-PCR, kinase assays, and Western blots. Expression of the Arg siRNA inhibited c-Abl mRNA expression and c-Abl kinase activity by 40% to 50%, and the c-Abl siRNA reduced Arg mRNA and kinase activity by 30% to 40% (Fig. 6B, top right). Therefore, the two siRNAs effectively reduce both c-Abl and Arg protein levels and activities. Expression of the highly related Src family kinases was unaffected by expression of either siRNA (Fig. 6B, bottom left).

To determine whether silencing c-Abl and Arg expression affects the invasiveness of MDA-MB-435S cells, siRNA-transfected cells were used in Matrigel invasion assays. Silencing the Abl kinases with either of two independent siRNAs dramatically inhibited invasion >10-fold (Fig. 6B, left). These data are very significant, as they show that activation of the Abl kinases is required for invasion of MDA-MB-435S cells. Similar results also were obtained with MDA-MB-231 cells (data not shown).

## Discussion

This study provides the first evidence that Abl kinases are constitutively activated in breast cancer cells. Most of the breast cancer cell lines that we examined express high levels of Abl

protein, but only highly invasive cell lines have constitutively active Abl kinases, which indicates that activation of the Abl kinases is likely to be a late event in tumor progression. We identify a novel mechanism by which Abl kinases are constitutively activated in breast cancer cells. Unlike in leukemia where Abl kinases are activated by translocation or gene amplification (8, 9), in breast cancer cells, Abl kinases are activated downstream of deregulated EGFR, HER-2, and Src kinases. Taken together, our findings are highly significant because they indicate that Abl kinases are likely to act downstream of ErbB and Src kinase signaling pathways to drive breast cancer cell invasion and metastasis. Finally, using two different approaches (STI571 and RNAi), we show that constitutive activation of Abl kinases promotes breast cancer cell invasion. These data indicate that Abl kinase activation is not merely an irrelevant consequence of breast cancer progression, but rather the kinases are involved in conversion to an invasive state.

Our results showing that Abl kinases promote breast cancer cell invasion contrast with two previous reports, which suggest that Abl kinases inhibit cancer cell migration and/or invasion. STI571 enhances the cellular response of thyroid cancer cells to hepatocyte growth factor (HGF) and increases HGF-induced migration and morphogenesis (32). However, the STI571 target in thyroid cancer cells was not identified using RNAi or similar experiments; therefore, the involvement of Abl kinases in the STI571-dependent effect was not established (32). Using Cos-7 cells and knockout fibroblasts, Kain et al. suggest that activation of cytoplasmic c-Abl inhibits migration and invasion and promotes apoptosis in

cancer cells, thereby acting as a “molecular rheostat.” However, the supporting experiments for this conclusion were not done in cancer cells (33).

Existing evidence regarding the role of Abl kinases in cell migration is conflicting. We and others showed that Abl kinases promote cellular migration and/or invasion in a variety of cell types and organisms: (a) Abl kinases are required for PDGF-induced membrane ruffling, an early event in cell migration (12, 34); (b) overexpression of PLC $\gamma$ 1 promotes PDGF-induced chemotaxis in an Abl-dependent manner (14); (c) hematopoietic cells transformed by BCR-Abl display increased membrane ruffling, spontaneous motility, and invasion of stromal bone marrow fibroblast monolayers (8, 10); (d) c-Abl induces neurite extensions and filopodia-like microspikes, early migratory events in neuronal cells (6, 35); (e) *Drosophila* Abl (D-Abl) transmits signals from cell surface receptors to the actin cytoskeleton, positively regulating axon guidance and neuronal migration (6); (f) loss of D-Abl disrupts migration and cell shape changes during dorsal closure in *Drosophila* (36); and (g) overexpression of the Pyk2 tyrosine kinase promotes heregulin-induced invasion of human T47D breast cancer cells in an Abl-dependent manner (37).

Interestingly, Abl kinases can also inhibit cell migration in some contexts. In fibroblasts for instance, Abl kinases inhibit migration toward collagen and insulin and inhibit wound-healing migration (38). Abl kinases may have different effects on motility depending on the signal, cell type, ECM surface, or migration stimulus. Opposing effects on motility are not unique to Abl kinases and are observed for PDGFR- $\alpha$ , Rac, and IGF-IR (39–42). It is also possible that in cells containing tightly regulated Abl kinases (low c-Abl and Arg kinase activities) Abl kinases restrict cellular migration (32, 38), but when the activities of the Abl kinases are unregulated and dramatically increased either by mutation (v-Abl and BCR-Abl; refs. 10, 14) or by activation of upstream regulators (i.e., PLC $\gamma$ 1; ref. 14) Abl kinases promote migration. Abl kinases clearly are critical regulators of migration and can act in either a stimulatory or inhibitory capacity. Our data show that, in breast cancer, constitutive activation of Abl kinases dramatically promotes invasion. Ongoing experiments are aimed at defining the molecular mechanism by which the Abl kinases promote invasion of breast cancer cells.

The data presented here suggest that pharmacologic inhibitors of Abl kinases may potentially be useful in preventing breast cancer

progression in tumors harboring constitutively active Abl kinases. In addition to CML, the Abl kinase inhibitor Gleevec also is effective for treating patients with gastrointestinal stromal tumors that overexpress c-Kit and/or PDGFR, two other Gleevec targets (43). Gleevec also has been tested in patients with small cell lung cancer, bone sarcoma, and metastatic breast cancer (44–46). Most of these trials were not successful. However, patients in these studies were not selected based on having tumors with constitutively activated PDGFR, c-Kit, or Abl kinases (44–46). Lessons have been learned from clinical trials involving Herceptin/trastuzumab (ErbB2 monoclonal antibody) and Iressa (EGFR inhibitor); i.e., tumors whose transformed and/or invasive phenotype are dependent on increased receptor activity are more likely to respond to drugs that target the receptor. This understates the importance of targeting in the design of clinical trials, as potentially useful agents may be disregarded based on negative results from untargeted trials (47). Our data indicate that constitutively active Abl kinases may drive progression of some breast cancers. Hence, Abl kinase inhibitors may prevent breast cancer metastasis in a targeted population (those containing constitutively active Abl kinases).

Drugs designed to target abnormally regulated proteins in breast cancer are currently in clinical trials. Herceptin trials were very successful, showing a 23% response rate in a targeted population (48). EGFR inhibitor (i.e., Iressa and Tarceva) trials have not been as successful either due to lack of targeting or because some tumors are resistant to the drugs despite expressing activated EGFR (49). Drug resistance may develop by several different mechanisms, including acquired mutations in the receptor, such that it cannot bind the drug or due to constitutive activation of downstream signaling proteins (50). Use of drugs targeting the same pathway (such as the use of Abl kinase inhibitors) may be effective in overcoming EGFR drug resistance (50).

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## References

- Chang JC, Hilsenbeck SG, Fuqua SA. Genomic approaches in the management and treatment of breast cancer. *Br J Cancer* 2005;92:618–24.
- Wells A, Kassis J, Solava J, Turner T, Lauffenburger DA. Growth factor-induced cell motility in tumor invasion. *Acta Oncol* 2002;41:124–30.
- Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;2:442–54.
- Thant AA, Sein TT, Liu E, et al. Ras pathway is required for the activation of MMP-2 secretion and for the invasion of src-transformed 3Y1. *Oncogene* 1999;18:655–63.
- Wells A, Grandis JR. Phospholipase C- $\gamma$ 1 in tumor progression. *Clin Exp Metastasis* 2003;20:285–90.
- Pendergast AM. The Abl family kinases: mechanisms of regulation and signaling. *Adv Cancer Res* 2002;85:51–100.
- Nagar B, Hantschel O, Young M, et al. Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell* 2003;112:859–71.
- Pendergast AM. BCR-ABL protein domain, function, and signaling. In: Carella AM, Daley GQ, Eaves CJ, Goldman JM, Helmanns R, editors. *Chronic myeloid leukaemia: biology and treatment*. London: Martin Dunitz Ltd.; 2001. p. 19–39.
- Kim HJ, Woo HY, Koo HH, Tak EY, Kim SH. ABL oncogene amplification with p16(INK4a) gene deletion in precursor T-cell acute lymphoblastic leukemia/lymphoma: report of the first case. *Am J Hematol* 2004;76:360–3.
- Skorski T, Nieborowska-Skorska M, Wlodarski P, et al. The SH3 domain contributes to BCR/ABL-dependent leukemogenesis *in vivo*: role in adhesion, invasion and homing. *Blood* 1998;91:406–18.
- Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001;344:1031–7.
- Plattner R, Kadlec L, DeMali KA, Kazlauskas A, Pendergast AM. c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. *Genes Dev* 1999;13:2400–11.
- Brasher BB, Van Etten RA. c-Abl has high intrinsic tyrosine kinase activity that is stimulated by mutation of the Src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines. *J Biol Chem* 2000;275:35631–7.
- Plattner R, Irvin BJ, Guo S, et al. A new link between the c-Abl tyrosine kinase and phosphoinositide signaling via PLC- $\gamma$ 1. *Nat Cell Biol* 2003;5:309–19.
- Kassis J, Moellinger J, Lo H, et al. A role for phospholipase C $\gamma$ -mediated signaling in tumor cell invasion. *Clin Cancer Res* 1999;5:2251–60.
- Recchia I, Rucci N, Festuccia C, et al. Pyrolypyrimidine c-Src inhibitors reduce growth, adhesion, motility and invasion of prostate cancer cells *in vitro*. *Eur J Cancer* 2003;39:1927–35.
- Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. *Annu Rev Pharmacol Toxicol* 2004;44:195–217.
- Hynes NE. Tyrosine kinase signalling in breast cancer. *Breast Cancer Res* 2000;2:154–7.
- O'Donovan M, Russell JM, O'Leary JJ, et al. Abl

- expression, tumour grade, and apoptosis in chondrosarcoma. *Mol Pathol* 1999;52:341-4.
20. Singer CF, Hudelist G, Lamm W, et al. Expression of tyrosine kinases in human malignancies as potential targets for kinase-specific inhibitors. *Endocr Relat Cancer* 2004;11:861-9.
  21. Chen WS, Kung HJ, Yang WK, Lin WC. Comparative tyrosine kinase profile in colorectal cancers: enhanced Arg expression in carcinoma as compared with adenoma and normal mucosa. *Int J Cancer* 1999;83:579-84.
  22. Plattner R, Koleske AJ, Kazlauskas A, Pendergast AM. Bidirectional signaling links the Abelson kinases to the platelet-derived growth factor receptor. *Mol Cell Biol* 2004;24:2573-83.
  23. Huang S, New L, Pan Z, Han J, Nemerow GR. Urokinase plasminogen activator/urokinase-specific surface receptor expression and matrix invasion by breast cancer cells requires constitutive p38 $\alpha$  mitogen-activated protein kinase activity. *J Biol Chem* 2000;275:12266-72.
  24. Nawrocki Raby B, Polette M, Gilles C, et al. Quantitative cell dispersion analysis: new test to measure tumor cell aggressiveness. *Int J Cancer* 2001;93:644-52.
  25. Echarri A, Pendergast AM. Activated c-Abl is degraded by the ubiquitin-dependent proteasome pathway. *Curr Biol* 2001;11:1759-65.
  26. Arteaga CL, Osborne CK. Growth inhibition of human breast cancer cells *in vitro* with an antibody against the type I somatomedin receptor. *Cancer Res* 1989;49:6237-41.
  27. Oude Weernink PA, Ottenhoff-Kalff AE, Vendrig MP, et al. Functional interaction between the epidermal growth factor receptor and c-Src kinase activity. *FEBS Lett* 1994;352:296-300.
  28. Belsches-Jablonski AP, Biscardi JS, Peavy DR, et al. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* 2001;20:1465-75.
  29. Burton EA, Plattner R, Pendergast AM. Abl tyrosine kinases are required for infection by *Shigella flexneri*. *EMBO J* 2003;22:5471-9.
  30. Roussidis AE, Mitropoulou TN, Theocharis AD, et al. STI571 as a potent inhibitor of growth and invasiveness of human epithelial breast cancer cells. *Anticancer Res* 2004;24:1445-7.
  31. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 1996;2:561-6.
  32. Frasca F, Vigneri P, Vella V, Vigneri R, Wang JY. Tyrosine kinase inhibitor STI571 enhances thyroid cancer cell motile response to hepatocyte growth factor. *Oncogene* 2001;20:3845-56.
  33. Kain KH, Gooch S, Klemke RL. Cytoplasmic c-Abl provides a molecular "rheostat" controlling carcinoma cell survival and invasion. *Oncogene* 2003;22:6071-80.
  34. Sini P, Cannas A, Koleske AJ, Di Fiore PP, Scita G. Abl-dependent tyrosine phosphorylation of Sos-1 mediates growth-factor-induced Rac activation. *Nat Cell Biol* 2004;6:268-74.
  35. Woodring PJ, Litwack ED, O'Leary DD, et al. Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. *J Cell Biol* 2002;156:879-92.
  36. Grevengoed EE, Loureiro JJ, Jesse TL, Peifer M. Abelson kinase regulates epithelial morphogenesis in *Drosophila*. *J Cell Biol* 2001;155:1185-98.
  37. Zrihan-Licht S, Avraham S, Jiang S, Fu Y, Avraham HK. Coupling of RAFTK/Pyk2 kinase with c-Abl and their role in the migration of breast cancer cells. *Int J Oncol* 2004;24:153-9.
  38. Kain KH, Klemke RL. Inhibition of cell migration by Abl family tyrosine kinases through uncoupling of Crk-CAS complexes. *J Biol Chem* 2001;276:16185-92.
  39. Ronnstrand L, Heldin CH. Mechanisms of platelet-derived growth factor-induced chemotaxis. *Int J Cancer* 2001;91:757-62.
  40. Ridley AJ. Rho GTPases and cell migration. *J Cell Sci* 2001;114:2713-22.
  41. Chernicky CL, Yi L, Tan H, Gan SU, Ilan J. Treatment of human breast cancer cells with antisense RNA to the type I insulin-like growth factor receptor inhibits cell growth, suppresses tumorigenesis, alters the metastatic potential, and prolongs survival *in vivo*. *Cancer Gene Ther* 2000;7:384-95.
  42. Pennisi PA, Barr V, Nunez NP, Stannard B, Le Roith D. Reduced expression of insulin-like growth factor I receptors in MCF-7 breast cancer cells leads to a more metastatic phenotype. *Cancer Res* 2002;62:6529-37.
  43. Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002;347:472-80.
  44. Krug LM, Crapanzano JP, Azzoli CG, et al. Imatinib mesylate lacks activity in small cell lung carcinoma expressing c-kit protein. *Cancer* 2005;103:2128-31.
  45. Verweij J, van Oosterom A, Blay JY, et al. Imatinib mesylate (STI-571 Glivec, Gleevec) is an active agent for gastrointestinal stromal tumours, but does not yield responses in other soft-tissue sarcomas that are unselected for a molecular target. Results from an EORTC Soft Tissue and Bone Sarcoma Group phase II study. *Eur J Cancer* 2003;39:2006-11.
  46. Modi S, Seidman AD, Dickler M, et al. A phase II trial of imatinib mesylate monotherapy in patients with metastatic breast cancer. *Breast Cancer Res Treat* 2005;90:157-63.
  47. Sledge GW, Jr., Miller KD. Exploiting the hallmarks of cancer: the future conquest of breast cancer. *Eur J Cancer* 2003;39:1668-75.
  48. Marty M, Cognetti F, Maraninchi D, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *J Clin Oncol* 2005;23:4265-74.
  49. Arteaga CL, Baselga J. Tyrosine kinase inhibitors: why does the current process of clinical development not apply to them? *Cancer Cell* 2004;5:525-31.
  50. Vilorio-Petit AM, Kerbel RS. Acquired resistance to EGFR inhibitors: mechanisms and prevention strategies. *Int J Radiat Oncol Biol Phys* 2004;58:914-26.