The Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor ZD1839 (Iressa) Suppresses c-Src and Pak1 Pathways and Invasiveness of Human Cancer Cells

Zhibo Yang,1 Rozita Bagheri-Yarmand,1 Rui-An Wang,1 Liana Adam,1 Vali Vassiliki Papadimitrakopoulou,4 Gary L. Clayman,2 Adel El-Naggar,3 Reuben Lotan,4 Christopher J. Barnes,1 Waun Ki Hong,4 and Rakesh Kumar1

Departments of 1Molecular and Cellular Oncology, 2Head and Neck Surgery, 3Pathology and 4Thoracic/Head and Neck Medical Oncology, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Purpose: Abnormalities in the expression and signaling pathways downstream of the epidermal growth factor receptor (EGFR) contribute to the progression, invasion, and maintenance of the malignant phenotype in human cancers, including those of the head and neck and breast. Accordingly, agents such as the EGFR tyrosine kinase inhibitor (EGFR-TKI) ZD1839 (Iressa) are promising, biologically based treatments that are in various stages of preclinical and clinical development. The process of tumor progression requires, among other steps, increased transformation, directional migration, and enhanced cell survival; this study explored the effect of ZD1839 on the stimulation of c-Src and p21-activated kinase 1 (Pak1), which are vital for transformation, directional motility, and cell survival of cancer cells.

Experimental Design: We examined the effect of ZD1839 on biochemical and functional assays indicative of directional motility and cell survival, using human head and neck squamous cancer cells and breast cancer cells.

Results: ZD1839 effectively inhibited c-Src activation and Pak1 activity in exponentially growing cancer cells. In addition, ZD1839 suppressed EGF-induced stimulation of EGFR autophosphorylation on Y1086 and Grb2-binding Y1068 sites, c-Src phosphorylation on Y215, and Pak1 activity. ZD1839 also blocked EGF-induced cytoskeleton remodeling, redistribution of activated EGFR, and in vitro invasiveness of cancer cells.

Conclusions: These studies suggest that the EGFR-TKI ZD1839 may cause potent inhibition of the Pak1 and c-Src pathways and, therefore, have potential to affect the invasiveness of human cancer cells deregulated in these growth factor receptor pathways.

INTRODUCTION

Growth factors and their receptors play an essential role in the regulation of epithelial cell proliferation, and abnormalities in their expression and signaling pathways contribute to the progression and maintenance of the malignant phenotype in human cancers. For example, deregulation of epidermal growth factor receptor (EGFR) activation has been shown to be closely associated with head and neck and breast cancers (1–5). The EGFR is one of a family of four closely related receptors: EGFR (erbB1), HER2/neu (erbB2), HER3 (erbB3), and HER4 (erbB4), (Refs. 6, 7). The regulation of these family members is complex, because the receptors can be transactivated by heterodimeric interaction between two family members and, thus, can use multiple pathways to execute their biological functions. High expression of EGFR has been shown to induce transformed properties in recipient cells (8), possibly because of excessive activation of signal transduction pathways. Recent studies have shown the widespread presence of HER2 in addition to EGFR in head and neck tumors (9, 10), which has raised the possibility of EGFR interaction with HER2 in head and neck tumor cells. On binding a ligand, EGFR dimerizes and becomes phosphorylated on multiple tyrosine residues, and stimulation of signaling pathways such as Ras/mitogen-activated protein kinase (MAPK) and Src kinases occurs (11–13). The protein tyrosine kinase c-Src is a major signal transduction element in many growth factor receptor signals for proliferation and transformation (14). Tumors that exhibit enhanced EGFR signaling have been reported to possess constitutively activated Src family kinases such as c-Src. Furthermore, c-Src is an important antiapoptotic signaling molecule downstream of the EGFR, and it up-regulates the expression of the antiapoptotic gene Bcl-xL (15, 16). Thus, c-Src may positively regulate the transformed phenotype of cells expressing high levels of EGFR via not only its mitogenic signaling element, but also as an antiapoptotic signaling protein (17). Both EGFR and Src kinases are activated in head and neck cancer; therefore, anticancer agents that could potentially inhibit these kinases need to be explored.

The exposure of cells to polypeptide growth factors causes reorganization of the cytoskeleton, formation of lamellipodia, membrane ruffling, and changes in cell morphology; accordingly, such exposure is implicated in stimulating cell migration and invasion (18). The leading edge of a motile...
cell contains thin protrusions of membrane that continuously extend and retract, mediating the initial stage of cell movement and determining the direction of advance (18–20). The motility function is normally repressed in many cells, but it can be activated by appropriate stimuli, oncogenic transformation, or both. The small GTPases cdc42 and Rac1 regulate the formation of motile structures via p21-activated kinase 1 (Pak1), which is a serine/threonine kinase. In addition to its effects on the cytoskeleton, Pak1 also activates c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK) kinases and, thus, influences nuclear signaling (19). Several recent studies have suggested that Pak1 activation may be involved, in addition to cell motility, in the progression of breast cancer, because increased Pak1 activity correlates well with the invasiveness of human breast tumor cells (19, 21). Recent studies have shown a mechanistic role for Pak1 activation in the increased invasiveness of breast cancer cells by heregulin (22) and the way it also promotes cell migration and anchorage-independent growth (19–21). Together, these findings suggest a central role of Pak1 in motility, invasion, and cell survival in human cancer.

Because EGFR pathways are commonly deregulated in human epithelial tumors, therapeutic agents directed against the EGFR represent a promising and important group of biologically based treatment strategies that are in various stages of preclinical and clinical development. One such selective inhibitor of the EGFR-tyrosine kinase inhibitor (TKI) is ZD1839 [‘Iressa’ (4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)quinazoline]. ZD1839 markedly inhibits the auto-phosphorylation of EGF-stimulated EGFR in a broad range of cells (23, 25). In Phase I studies, encouraging antitumor activity has been observed in a selected range of tumor types including non-small cell lung and head and neck cancer (26). This study was undertaken to explore the effect of the EGFR-TKI ZD1839 on c-Src and Pak1 pathways that support motility, cell survival, and invasion using head and neck and breast cancer cell models.

MATERIAL AND METHODS

Cell Cultures and Transfections. The 14A, 14B, 183A, 1483 (Ref. 27), provided by Reuben Lotan (M. D. Anderson Cancer Center, Houston, TX), and MDA-MB-231 (21) cell lines were maintained in DMEM/F-12 medium supplemented with 10% FCS. The following antibodies were used: anti-HER1, -HER2, -HER3, HER4 (Neomarkers, Fremont, CA); anti-mouse myelin basic protein (Upstate Biotechnology, Lake Placid, NY), horseradish peroxidase-conjugate (Amersham, Piscataway, NJ), anti-mouse antibodies (Biosource International, Camarillo, CA); anti-mouse phospho-EGFR and phospho-Src antibodies (Neomarkers, Fremont, CA), anti-vinculin (Sigma, St. Louis, MO); phospho-EGFR and phospho-Src antibodies (Biosource International, Camarillo, CA); anti-mouse horseradish peroxidase-conjugate (Amersham, Piscataway, NJ), myelin basic protein (Upstate Biotechnology, Lake Placid, NY), Pak1 (Santa Cruz Biotechnology, Santa Cruz, CA); ZD1839 (AstraZeneca, Macclesfield, United Kingdom). 1483 cells were seeded in six-well plates at a confluency of 60% in the absence of antibiotics 1 day before transfection. Transfection with EGFR small interfering RNAs (siRNAs) (DHARMACON) was carried out in serum-free DMEM/F-12 with LipofectAMINE (Invitrogen) according to the manufacturer’s specifications. EGFR siRNA was transfected at a concentration of 100 nM.

Cell Extracts and Immunoprecipitation. Cells were lysed in radioimmunoprecipitation assay buffer supplemented with 100 mM NaF, 200 mM NaVO₃, and 1× protease cocktail (Boehringer-Mannheim, Indianapolis, IN) on ice for 15 min. Cell lysates containing equal amounts of protein were immunoprecipitated with the desired antibody and were analyzed by SDS-PAGE (19).

Tissue Samples and Western Blotting. Human head and neck tissue samples were obtained from a tissue bank maintained by Dr. Adel El-Naggar (M. D. Anderson Cancer Center, Breast Cancer Core Pathology Laboratory, The University of Texas). Specimens from patients who had undergone surgery were snap-frozen in liquid nitrogen and were stored at −80°C. Thawed tissue samples were homogenized in Triton X-100 lysis buffer (20 mM HEPES, 150 mM NaCl, 1% Triton X-100, 0.1% (v/w) deoxycholate, 2 mM EDTA, 2 mM NaVO₃, and protease inhibitor cocktail), and equal amounts of protein were analyzed by Western blotting. The protein vinculin was used routinely as a loading control.

Pak1 Activity. Lysate (200 μg) was immunoprecipitated with anti-Pak1, and kinase assays were performed in kinase buffer using myelin basic protein as a substrate (19).

Immunofluorescence Studies. For immunofluorescence studies, 1483 or 183A cells were plated on coverslips in regular DMEM/F-12 medium supplemented with 10% FCS. After 1 day, cells were shifted to serum-starved medium for 24 h and treated (or not) with EGF (10 ng/ml) for 20 min with or without pretreatment with 1 μM ZD1839 for 20 min. Cells were fixed in 3.8% paraformaldehyde, blocked in 10% goat serum, and then stained.
processed for indirect immunofluorescence. Primary rabbit antibodies against total EGFR or against phosphorylated residues were visualized by using Alexa488 goat antirabbit antibodies. Monoclonal anti-EGFR was used when costaining with antibodies against phospho-EGFR when needed and was visualized using an antimouse-Alexa633 secondary antibody. To visualize F-actin, Alexa456-labeled phalloidin was added during the incubation with the secondary antibodies. Nuclear staining used Topro-3 (Molecular Probes, Eugene, OR) to stain DNA. All of the secondary antibodies were purchased from Molecular Probes. Cells were analyzed by confocal microscopy using appropriate filters.

Chemoinvasion Assays. To test the invasion behavior of treated cells, 8 μm filters were coated with Matrigel (20 μg/filter) and placed in Boyden chambers. Cells (10⁵), suspended in DMEM containing 0.1% BSA and different treatments, were added to the upper chamber. Conditioned medium from mouse fibroblast NIH3T3 cells was used as a source of chemoattractant and was placed in the lower compartment of the Boyden chambers. After 24 h of incubation at
Fig. 3 Temporospatial distribution of epidermal growth factor receptor (EGFR) in activated cells. Serum-starved 1483 cells treated with or without EGF (10 ng/ml for 30 min) were subjected to triple staining against DNA (blue), F-actin (red) and against total EGFR (A and B), EGFR-Y1086P (C and D), EGFR-Y1068P (E and F), or c-Src-Y215P (G and H; green). Yellow color resulted from overlap of green and red fluorescence. Left panel, control (CON) cells; right panel, EGF-treated cells. Distribution as well as internalization of activated EGFR in EGF-treated cells is visible.
EGFR-TKI Regulation of c-Src and Pak1 Pathways

In an attempt to delineate the role of EGFR signaling in head and neck cancer cells, we first profiled the expression of EGFR family members, the status of EGFR phosphorylation on specific tyrosine residues, and the activation status of signaling components in a panel of head and cancer cell lines. Fig. 1 shows that head and neck cancer cells express easily detectable levels of HER2 and HER3 but not HER4 (not shown), in addition to the expected high expression of EGFR. Use of phospho-specific antibodies against Y1068 (Grb2-binding site) and Y1086 (autophosphorylation site) EGFR revealed a constitutive activation of each of these molecules in the cell lines tested here (Fig. 1). All of the pathways analyzed here were maximally activated in the 1483 cells, which were consequently used as a model system to delineate the EGFR signaling in head and neck cancer cells.

EGFR and c-Src-Y215 Expression in Head and Neck Cancer. To review the significance of our findings, the status of c-Src activation on Y215 residue was examined using phospho-specific antibodies against phosphorylated Y215 of c-Src in a small number of head and neck tumors and adjacent normal-appearing specimens. As shown in Fig. 2A, five of eight tumors showed a significant increase in c-Src-Y215 phosphorylation compared with the level of phosphorylated c-SrcY215 in normal tissues, suggesting a potential close association between high EGFR expression and c-Src signaling in head and neck cancer cells. Immunostaining of paraffin-embedded premalignant and invasive head and neck tumors also showed that phosphorylated c-Src-Y215 activation could be effectively detected in paraffin-embedded tissues from invasive tumors and, to a lesser extent, from dysplasia tissue. As expected, the activated c-Src was predominantly localized in the membranous compartment and in the cytoplasm (Fig. 2B).

Spatial Distribution of Activated EGFR and c-Src in EGF-stimulated Head and Neck Cancer Cells. To better understand the effect of ZD1839 on the motile phenotypes of head and neck cancer cells, the effect of EGF on cytoskeleton remodeling and subcellular distribution of EGFR and activated EGFR in 1483 cells was examined by confocal microscopy. As expected, abundant EGFRs were situated on the membrane, especially at intercellular adhesive sites (shown as arrowheads) and in cellular projections formed occasionally (shown as arrows) in untreated control cells (Fig. 3A). As shown in Fig. 3B, EGF stimulation of cells was accompanied by distinct cytoplasm internalization of a large pool of EGFR (shown as red arrows), as well as a drastic remodeling of the F-actin-containing membranes with motile ruffles that contain EGFR (shown as arrowheads). Staining the cells with antibodies against the tyrosine-phosphorylated forms of EGFR (Y1086 and Y1068) as well as with an antibody against the tyrosine-phosphorylated c-Src (Y215) enabled the identification of the activated EGFR and c-Src pools, either internalized or on the F-actin-containing membrane ruffles (Figs 3, D, F, and H, shown as yellow pixels). None of these antibodies were detectably immunoreactive in serum-starved cells, which suggests a lack of detectable phosphorylation of EGFR or c-Src on the tyrosine residues in the absence of ligand (Figs 3, C, E, and G).

RESULTS AND DISCUSSION

EGFR Family Members in Head and Neck Cancer Cells. In an attempt to delineate the role of EGFR signaling in head and neck cancer cells, we first profiled the expression of EGFR family members, the status of EGFR phosphorylation on specific tyrosine residues, and the activation status of signaling components in a panel of head and cancer cell lines. Fig. 1 shows that head and neck cancer cells express easily detectable levels of HER2 and HER3 but not HER4 (not shown), in addition to the expected high expression of EGFR. Use of phospho-specific antibodies against Y1068 (Grb2-binding site) and Y1086 (autophosphorylation site) EGFR revealed a constitutive activation of each of these molecules in the cell lines tested here (Fig. 1). All of the pathways analyzed here were maximally activated in the 1483 cells, which were consequently used as a model system to delineate the EGFR signaling in head and neck cancer cells.

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Regulation of EGFR and c-Src Phosphorylation by EGF and ZD1839 in Head and Neck Cancer Cells. The growth of both 1483 and 183A cells was inhibited by suboptimal doses of ZD1839 (Fig. 4A). To determine how ZD1839 acts in head and neck cancer cells, its effect on the status of EGF-induced phosphorylation of EGFR and c-Src was examined. ZD1839 was a potent inhibitor of EGF-stimulated phosphorylation of EGFR at the Y1068 and Y1086 sites and of stimulation of phosphorylation of c-Src at the Y215 site (Fig. 4B). These findings reinforce the significance of EGFR signaling in the activation of the c-Src pathway and support the idea that c-Src may be a downstream EGFR target of ZD1839 in head and neck cancer cells.

A more visual aspect of the inhibitory effects of ZD1839 on EGFR activation by EGF is shown in Fig. 4C, wherein cells were costained with antibodies against total EGFR (shown in blue), F-actin (shown in red), and against phosphorylated EGFR-Y1086 (Fig. 4C, upper panel) or c-Src-Y215 (Fig. 4C, lower panel). Confocal analysis of thin optical sections throughout these cells enabled us to closely follow the EGFR-Y1086

Fig. 4 Continued.
and c-Src-Y215 internalization induced by EGF (Fig. 4C, middle panels). Interestingly, not all internalized EGFR (shown in blue) was tyrosine-phosphorylated or contained c-Src. These findings suggest that EGFR might become dephosphorylated during its internalization or might suffer from conformational changes that make it undetectable with the antibodies used here. Interestingly, 183A cells treated with both EGF and ZD1839 exhibited internalization of EGFR that was devoid of phosphorylation on Y1086 (Fig. 4C, shown in blue) and phosphorylated c-Src-Y215 (Fig. 4C). These observations indicate that ZD1839 inhibited EGFR tyrosine phosphorylation and, consequently, stimulation of downstream signaling components and that ZD1839 had no effect on EGFR internalization. In this context, earlier studies have shown that EGFR internalization is not dependent on its phosphorylation. Together, these results show a close relationship between high EGFR expression and c-Src-Y215 activation, and they show that ZD1839 may also inhibit the c-Src pathway under EGFR-activation conditions in head and neck cancer cells.

EGF-induced Cytoskeleton Remodeling and Pak1 Activation in Head and Neck Cancer Cells. Fig. 3 shows that EGF is able to trigger cytoskeleton reorganization. To determine the role of EGFR in cell motility and invasion of head and neck cancer cells, we investigated whether EGF treatment promotes the formation of actin-containing motile structures in cells grown on a thin layer of collagen. As shown in Fig. 4C, EGF stimulation of 183A cells was associated with rapid induction of membrane protrusions such as pseudopodia and ruffles. Furthermore, phase-contrast microscopy of live 183A cells grown on an in vitro reconstituted extracellular matrix (Matrigel) revealed the formation of a vascular-like lattice inside a thick layer of Matrigel rather than the two-dimensional culture seen on a regular plastic surface (Fig. 4D).

Recent studies have shown that Pak1 is one of the major pathways through which actin cytoskeleton reorganization is regulated and motile or invasive phenotypes of breast cancer cells are maintained (19–21). Knowing that Pak1 activation is also involved in directed migration and control of downstream signaling, we investigated whether EGF stimulates Pak1 activity in head and neck tumors (T) and their adjacent normal tissues (N). Tissue lysates were immunoprecipitated with an anti-Pak1 antibody, and an in vitro immune-complex kinase assay was performed using basic myelin protein as a substrate. Subsequently, the blot was immunoblotted with Pak1 antibody. C, immunohistochemical detection of Pak1 in paraffin-embedded expression of Pak1 in dysplasia and tumor tissues using an anti-Pak1 antibody.

![Fig. 5](image_url) Status of p21-activated kinase 1 (Pak1) pathway in head and neck cancer cells. A, epidermal growth factor (EGF) stimulation of Pak1 activity. 183A and 1483 cells were treated with 10 ng/ml EGF for indicated times; cell lysates were immunoprecipitated with an anti-Pak1 antibody; and in vitro immune-complex kinase assay was performed using basic myelin protein as a substrate. B, Pak1 kinase activity in head and neck tumors (T) and their adjacent normal tissues (N). Tissue lysates were immunoprecipitated with an anti-Pak1 antibody, and an in vitro immune-complex kinase assay was performed using basic myelin protein as a substrate. Subsequently, the blot was immunoblotted with Pak1 antibody. C, immunohistochemical detection of Pak1 in paraffin-embedded expression of Pak1 in dysplasia and tumor tissues using an anti-Pak1 antibody.
Pak1 activity was higher in four of five tumors than it was in the corresponding normal tissues. Accordingly, dysplasia tissues had low levels of Pak1 immunoreactivity, and invasive tumors exhibited intense Pak1 staining (Fig. 5C).

**ZD1839 Inhibits Pak1 Activity and Invasiveness of Head and Neck Cancer Cells.** To further explore the role of EGFR in the activation of the Pak1 pathway, we examined the effect of the EGFR-TKI ZD1839 on invasiveness and Pak1
activity in 183A and 1483 cancer cells. Cotreatment of the cells with ZD1839 inhibited the ability of EGF to promote in vitro invasiveness (Fig. 6A). ZD1839 effectively inhibited Pak1 activity in EGF-stimulated head and neck cancer cells (Fig. 6B). However, ZD1839 had no effect on Pak1 activity when we tested the effect on direct addition of ZD1839 in vitro Pak1 kinase assay (data not shown). siRNA-mediated inhibition of the expression of EGFR followed by EGF and/or ZD1839 treatment confirmed that EGF-induced Pak1 activity is EGFR dependent (Fig. 6C).

ZD1839 Inhibits Pak1 Activity and Invasiveness of Breast Cancer Cells. To examine the generality of ZD1839 inhibition of EGFR and its downstream targets, its effect in the highly invasive MDA-MB231 breast cancer cell line was investigated. ZD1839 completely blocked EGF-induced phosphorylation of EGFR and c-Src-Y215 as well as Pak1 activity (Fig. 7A). As was seen in the head and neck cancer cells, ZD1839 inhibited EGF-induced increased invasiveness of MDA-231 cells (Fig. 7B).

Summary. In summary, we have shown that the EGFR-initiated signaling pathway is a potent inducer of c-Src and Pak1 pathways, as well as of reorganization of the cytoskeleton, which allows increased tumor invasiveness. The observed stimulation of c-Src and Pak1 was closely related to high EGFR expression in head and neck cancer cells, and inhibition of EGFR by ZD1839 was accompanied by complete inhibition of Pak1 activation and in vitro invasiveness of cancer cells. Although ZD1839 was not a direct inhibitor of Pak1 activity, it did effectively inhibit Pak1 activity induced by EGF or serum. However, high expression of EGFR was not a prerequisite of the action of ZD1839; it also inhibited both EGFR and Pak1 pathways in EGF-activated breast cancer cells with low levels of EGFR. This emphasizes the possibility that ZD1839 might be useful against cancer cells that express modest levels of EGFR. Because deregulation of EGFR expression, signaling, or both, is commonly associated with stimulation of c-Src and Pak1 pathways, the results presented here suggest that the EGFR-TKI ZD1839 may lead to secondary inhibition of c-Src kinase and Pak1 and invasiveness of human cancer cells. Together, these findings generate a testable hypothesis wherein the use of ZD1839 (Iressa) in cells with activated c-Src or Pak1 pathways may potentially lead to beneficial antitumor activity.

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