Original Article
The role of Axl in drug resistance and epithelial-to-mesenchymal transition of non-small cell lung carcinoma

Fang Wu, Junhe Li, Changchen Jang, Jianfeng Wang, Jianping Xiong

Department of Oncology, The First Affiliated Hospital of Nanchang University, Nan Chang 330006, China

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Abstract: Axl, a member of receptor tyrosine kinases (RTKs), has been established as a strong candidate for targeted therapy of cancer. Some reports showed that Axl is a promising therapeutic target to enhance EGFR TKI response in selected EGFR WT NSCLC patients. The present study was aimed to investigate the role of Axl in non-small cell lung carcinoma (NSCLC) drug resistance and the progress of epithelial-to-mesenchymal transition (EMT). MTT was used to detect the cytotoxicity of chemotherapeutic drugs in NSCLC cells, and Western blot to detect the expression of Axl in EGFR wild type NSCLC cell lines. The EMT markers were also determined by Western blot. We found that when downregulating Axl in EGFR WT NSCLC cells, the cells showed a more sensitive response to erlotinib than those overexpressed Axl. The further study showed that when downregulating Axl, the EMT markers E-cadherin was increased while N-cadherin and vimentin were decreased. Those data showed that the inhibition of Axl could reverse the EMT. Combined therapeutic strategies of the inhibitor of Axl and EGFR TKI could be more effective in the treatment of NSCLC drug resistance patients. The EMT signature and Axl might be predictive biomarkers of drug response and therapeutic targets in patients with NSCLC.

Keywords: Axl, E-cadherin, N-cadherin, vimentin, EMT, MDR

Introduction
Lung cancer is the leading cause of cancer morbidity and mortality worldwide. Non-small cell lung carcinoma (NSCLC) is a highly malignant and aggressive neoplasm accounting for approximately 75-80% of all lung cancers reported [1]. The NSCLC is classified as squamous cell carcinoma, large cell carcinoma and adenocarcinoma and characterized by high mortality and poor prognosis [2]. Even though the treatments of NSCLC has been progressed in recent years, the 5-year survival rate of lung cancer is still lowest (15%) [3]. Although surgery is regarded as the best possible treatment for NSCLC, only 20-25% of tumors are suitable for potentially curative resection and despite complete surgical resection, the recurrence rate remains high (30-70%) [4]. Adjuvant chemotherapy may reduce recurrences by eradicating the subclinical and micro-metastatic disease [4]. However, the heterogeneity of NSCLC tumors and resistance to multiple drugs results in a poor response to chemotherapy and reduced survival [4]. Multidrug resistance (MDR) is a multifactorial process and a major obstacle to treatment for NSCLC patients [4].

Epithelial-mesenchymal transition (EMT) plays an important role in multiple physiological and pathological processes of human body. EMT pathway was found to play important roles in regulating the transcription of genes involved in embryonic development [5], inflammatory response [6], tissue regeneration [7], organ fibrosis [8, 9], tumor invasion and metastasis. The morphology of EMT was characterized the loose of epithelial cells and loss of cell-cell adhesion. The down-regulation of E-cadherin and cytokeratins, with the up-regulation of mesenchymal proteins like vimentin, fibronectin and N-cadherin were the markers of EMT [10, 11]. The previous reports had shown that EGF and resultant EGFR activation could promote
EMT by altered the expression and morphology of EMT-associated markers (E-cadherin/β-catenin complex) [11].

Axl is a member of receptor tyrosine kinases (RTK) family [12]. Some research showed that blocking Axl expression by RNAi could lead to a decrease of cells invasion, proliferation and increased cells chemosensitivity through activation of AKT and mitogen-activated protein kinases (MAPK) pathways in solid tumors [13-15]. Besides, over-expression of Axl was initially identified as an oncogene of human leukemia cells [16-18]. The expression of Axl was found to be increased in more than half of NSCLC cell lines [19]. A clinical importance of Axl was also suggested by the data that in 48.3% of lung adenocarcinoma tissues, Axl level was high and proportional to lymph node metastasis as well as disease stage [18]. Furthermore, targeting of Axl with RNA interference or specific monoclonal antibodies has been shown to inhibit proliferation of cancer cells and even tumor growth in mouse xenograft model. Recent studies on NSCLC indicated that increased activation of Axl-induced acquired resistance to epidermal growth factor receptor (EGFR)-targeted therapy [20].

The aim of this study was to investigate the role of Axl in NSCLC drug resistance and the molecular mechanism that was associated with EMT. These novel findings provide strong evidence that Axl may be used as a potential therapeutic target to sensitize cancer cells to chemotherapeutic drugs in NSCLC and provide more evidence for making a new strategy in the treatment of NSCLC patients.

Material and methods

Cell lines

The human NSCLC cancer cell lines, H460, A549 were obtained from ATCC (Manassas, VA). These cells were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS (Invitrogen Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (Gibco). All cells were grown in drug-free culture medium for more than 2 weeks before assay.

Chemicals and reagents

Erlotinib and 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyllapatinibrazolium bromide (MTT) were products of Sigma Chemical Co. from Genewindows Co. (Guangzhou, China). Dulbecco’s modified Eagle’s medium (DMEM) and RPMI medium 1640 were products of Gibco BRL from Genewindows Co. (Guangzhou, China). Axl, E-cadherin, N-cadherin, Vimentin, p-Akt, Akt, p-Erk, Erk and GAPDH antibodies were purchased from Santa Cruz Biotechnology Inc from Genetime Co. (Guangzhou, China). Other routine laboratory reagents were obtained from commercial sources of analytical (Guangzhou, China).

MTT assay

Cell viability was assessed by MTT assay as described previously [13]. Briefly, cells were plated at a density of 3000 cells per well into 96-well plates. At the end of treatment, the supernatant was removed, and 20 µl of the tetrazolium compound, MTT, and 270 ml of fresh DMEM medium were added. After incubation for 4 h at 37°C, 120 µl of DMSO was placed in each well to dissolve the tetrazolium crystals. Finally, the absorbance at a wavelength of 570 nm was recorded using a multi-well plate reader (Tecan, Maennendorf, Switzerland).

Plasmids and siRNAs

Multiple short hairpin RNA (siRNA) probes targeting the human Axl gene and a probe containing a scrambled sequence (control) were designed and constructed into a pU6-mRFP expression vector as described previously [26]. Two siRNA Axl probes, one containing the sequence 5’-AACCTTCAACTCCTGCCTTCTCG-3’ in the coding region, and another with the sequence 5’-CAGCTTCTCCTGAGCTTCTAC-3’ in the 3’-UTR region, were verified by Western blot analysis to be effective in silencing the Axl gene.

Western blot analysis

Cell lysates were prepared in RIPA buffer (50 mmol/l Tris-HCl buffer, pH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with 1×Halt protease inhibitor cocktail and 1×Halt phosphatase inhibitor cocktail (Pierce, Rockford, IL). A Bio-Rad protein assay (Bio-Rad) was used to determine protein concentrations. Proteins were separated on 10-12% sodium dodecyl sulfate-polyacrylamide
gel electrophoresis and transferred to PVDF membranes (Whatman, Boston, MA). Membranes were first hybridized with specific primary antibodies and then with HRP-conjugated secondary antibodies (Cell Signaling Technology). Protein bands were visualized using a commercial Immobilon Western Chemiluminescent HRP Substrate detection reagent (Millipore, Billerica, MA). The chemiluminescence of proteins transferred to PVDF membranes was detected with ECL Plus (GE Healthcare Amersham, Piscataway, NJ). Relative protein expression values were quantitatively determined via densitometry with ImageJ software.

**Statistical analysis**

All findings were analyzed with statistical software package SPSS 16.0 and Graphpad Prism.
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Table 1. Effect of chemotherapeutic agents on the growth of NSCLC cells transfected with Axl siRNA

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC(_{50}) ± SDs μM (fold-reverse)</th>
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<tbody>
<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td></td>
<td>H460</td>
</tr>
<tr>
<td></td>
<td>A549/siAxl</td>
</tr>
<tr>
<td></td>
<td>H460/siAxl</td>
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<tr>
<td>Doxorubicin</td>
<td>1.1004 ± 0.1022</td>
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<tr>
<td>Vincristine</td>
<td>0.0066 ± 0.0053</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.0017 ± 0.0134</td>
</tr>
<tr>
<td></td>
<td>12.0003 ± 1.0243</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.0014 ± 0.0091</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.0030 ± 0.0230</td>
</tr>
</tbody>
</table>

**Cell survival was determined by MTT assays as described in “Materials and Methods”. Data are the means ± SDs of at least three independent experiments. ‘’ and ‘‘ represent \(P < 0.05\) and \(P < 0.01\), respectively, for values versus that obtained in the parent cells.**

5. The results were expressed as means ± SD. The statistical significance of the studies was determined by the parametric unpaired Student’s t test. Differences with \(P < 0.05\) are considered significant.

**Results**

*Downregulation of Axl suppresses cell viability of NSCLC*

To investigate the role of Axl in NSCLC cell survival, we chose two NSCLC cells for our research: the EGFR wild type A549 cell and H460 cell. We employed A549 and H460 cells with high endogenous Axl expression to stably or transiently down-regulation Axl, respectively (Figure 1A and 1B). Western blot analysis data confirmed the down-regulation of exogenous and endogenous Axl proteins as indicated by the full-length protein (138 kDa) and the short fragment (55 kDa; Figure 1A and 1B). The cell viability assay results demonstrated that the downregulation of Axl expression in A549 and H460 cells significantly inhibited cell viability compared to control cells (Figure 1C and 1D). The data indicated that knockdown of endogenous Axl significantly slow down the cell survival. To assess the effect of Axl inhibition on long-term cellular colony formation potential, control and knockdown shAxl clones were plated in soft agar for 3 weeks. The colony formation data showed that the cell growth in shAxl cells was less than 20% of control in both A549 and H460 cells. These data indicated that inhibition of Axl significantly block cell survival and colony formation in NSCLC cells.

*Downregulation of Axl abrogates drug resistance in NSCLC cells*

To identify the relationship of Axl overexpression in NSCLC cells drug resistance, we knockdown the Axl expression in A549 and H460 cells. The concentrations of chemotherapeutic agents (such as vincristin, doxorubicin and paclitaxel) required for the IC\(_{50}\) values in A549/siRNA and H460/siRNA cells were higher than those in their parental sensitive A549, H460 cells (Table 1). Furthermore, down-regulating Axl could recover the erlotinib sensitivity to A549 and H460 cells (Figure 2). The data unequivocally indicated that knockdown of endogenous Axl significantly enhanced the sensitivity of cells to chemotherapeutic drugs. In addition, we demonstrated that the pro-survival function of Axl was dependent on its kinase activity. These results indicate that knockdown Axl expression can strongly enhance the efficacy of conventional chemotherapeutic agents in NSCLC cells.

**Inhibition of Axl blocks the phosphorylation of AKT and ERK1/2**

Previous studies have shown that the inhibition of the AKT and ERK1/2 pathways may decrease the resistance to antineoplastic drugs in cancer cells. Consequently, we determined the effect of Axl inhibitor and siRNA on the levels of total and phosphorylated forms of AKT and ERK1/2 in all cell lines. As shown in Figure 3, the incubation of cells with Axl siRNA for 48 hours did significantly alter the total and phosphorylated forms of AKT and ERK1/2. This suggested that the reversal effect of Axl inhibition in NSCLC cells is dependent on the inhibition of AKT and ERK1/2 phosphorylation.

*Down-regulation of Axl could inhibit the progress of EMT*

The acquisition of mesenchymal cell characteristics by epithelial cells, in particular the ability to migrate as single cells and invade the extracellular matrix, is the functional hallmark of EMT (3). Therefore, we investigated whether EMT markers expression is associated with Axl in human lung cancer cells. After treated the cells with Axl siRNA for 48 hours, we observed...
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Figure 2. Down-regulation Axl could reverse drug resistance in A549 and H460 cells. Down-regulation Axl could significantly reverse erlotinib resistance in A549 and H460 cells (A, B). The MTT assays showed that knockdown Axl could decreased cell viabilities in NSCLC cells (C, D).

Figure 3. Effect of Axl on blockage of AKT and ERK1/2 phosphorylation. A549 and H460 cells were treated with Axl siRNA for 48 h. Equal amounts of protein were loaded for Western blot analysis as described in “Materials and Methods”. Independent experiments were performed at least three times and results from a representative experiment were shown.

Discussion
In this study, we explored the role of Axl in drug resistance and EMT progress of NSCLC cells. Our data was similar to the data published in previous reports, we observed that downregulation of Axl could abrogate EMT program induction in NSCLC cells.
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strated that increased Axl expression could contribute to drug resistance in cell lines. Altered Axl-related signaling was also observed in approximately 20% of patients with acquired resistance to EGFR-TKI [21], although it remains unknown whether these patients could benefit from Axl inhibition. Axl could activate the downstream signals such as Akt and Erk to motivate cell survival and growth in EGFR-TKI resistance cells. Therefore, combined treatment with EGFR and Axl inhibitors might effectively abrogate the growth of tumor cells. A similar phenomenon can be observed in MET-mediated resistance, as shown in a previous report [22].

NSCLC could be classified into 3 groups in the terms of EGFR gene status: EGFR wild type, EGFR mutant and UN-EGFR-GS [23]. The patients with EGFR mutations in NSCLC can benefit from EGFR tyrosine kinase inhibitor (TKI) therapy and several TKIs was used as a first-line treatment for patients with EGFR mutations. Compared with the traditional chemotherapy, the EGFR TKI can make longer progression-free survival (PFS) and fewer side effects but the overall survival (OS) was not extended [24]. The EGFR TKI gefitinib and erlotinib had been approved for the treatment of advanced NSCLC in 2007 [25-27]. However, the drug resistance to TKI is an obstacle for the treatment in NSCLC patients [28]. The mutations in exon 21 missense point mutation L858R and frame deletions exon 19 deletion are highly response to EGFR-TKI [29]. In addition, the EGFR T790M mutation and MET amplification are responsible for the acquired resistance in most patients [30, 31]. The challenge of tumor drug resistance therefore represents a barrier that confounds the ultimate goal of cure or long-term control of NSCLC [32].

We tested whether the down-regulation of Axl can inhibit the phosphorylation of Akt or Erk1/2. As shown in Figure 3A, inhibiting Axl significantly blocked the phosphorylation of Akt and Erk1/2 in any of the two cell lines. This result suggested that Axl induced the resistance of chemotherapeutic agents in EGFR WT cells may be due to its activation of EGFR receptors. In addition, the EMT markers E-cadherin was increased while N-cadherin and vimentin were decreased after downregulated Axl. These results indicated clinical significance in the design of therapies targeting Axl and EMT signaling. Some reports pointed that the effect of dual treatment with Axl and EGFR inhibitors may be more reliant on Axl or EMT for downstream signaling than EGFR itself, and that the effects of increased EGFR abundance may be in large part manifested by activation of Axl or EMT. Consequently, blocking Axl or EMT signal may be more effective that blocking signal from EGFR and that inhibitors of their kinase activity or treatments that reduce receptor abundance may be more effective [33-36]. In some carcinomas, EMT and the expression of Axl and c-MET amplification have been identified as a mechanism of acquired resistance to EGFR-TKI therapies [37, 38]. After EGFR-TKI treatment, the cross talk between cells is halted due to RTK-mediated secondary resistance and may provide qualitatively distinct signaling through new receptors, though in response to the original activating ligand. Consequently, upon withdrawal of treatment, cells could respond to the original ligand with a more invasive or aggressive phenotype as a result of the dynamic network rewiring that created resistance [39, 40].
Our work more broadly raises the implication that modulation of RTK expression may benefit the treatment of NSCLC. Thus, cancer therapies targeting Axl may be circumvented by activation in inhibitor resistance that may take place by amplification of signaling through other receptors.

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Disclosure of conflict of interest

None.

Address correspondence to: Jianping Xiong, Department of Oncology, The First Affiliated Hospital of Nanchang University, 17 Yong Wai Zheng Street, Nan Chang 330006, China. Tel: +86-(20)-8869-2559; Fax: +86-(20)-8869-2559; E-mail: jianpingxiongmed@163.com

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