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Anexelekto (AXL) Increases Resistance to EGFR-TKI and Activation of AKT and ERK1/2 in Non-Small Cell Lung Cancer Cells

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Recently, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have revolutionized non-small cell lung cancer (NSCLC) treatment. However, resistance remains a major obstacle. Anexelekto (AXL) is a member of receptor tyrosine kinases (RTKs) and shares the same downstream signaling pathways with EGFR, such as PI3K/AKT and MAPK/ERK. AXL overexpression in resistant tumors has been implicated in many previous studies in vitro and in vivo. In this study, we further examined whether expression of AXL and its downstream targets increased in gefitinib-resistant PC9 cells (PC9GR). In addition, we hypothesize that knocking down AXL in PC9GR and overexpressing AXL in PC9 using genetic tools can restore and decrease the sensitivity to gefitinib, respectively. We found that silencing AXL could sensitize the resistance to gefitinib, and the downstream pathways were significantly inhibited. Interestingly, we also discovered that increased AXL expression did promote the resistance, and its downstream targets were activated accordingly. Then 69 NSCLC patients who harbored EGFR mutation were recruited to analyze the expression of AXL and the association between AXL expression and clinical characteristics. We found that 5 of the 69 patients were AXL positive (about 7%), and AXL was related to tumor differentiation and tumor size. In this study, we concluded that the molecular mechanisms of AXL mediated resistance involved in the increased activity of the PI3K/AKT and MAPK/ERK1/2 pathways, and AXL overexpression could promote resistance, but it can be weakened when AXL expression is silenced.

Key words: Anexelekto (AXL); Non-small cell lung cancer (NSCLC); Epidermal growth factor receptor (EGFR); Resistance

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Even though some progress has been made in its treatment, the average overall 5-year survival rate is still as low as 17.1% (2). The discovery of epidermal growth factor receptor (EGFR) mutation is a milestone in the treatment of lung cancer. Patients who harbor the EGFR-activated mutation can be targeted by the EGFR-tyrosine kinase inhibitor (TKI) such as classical gefitinib and erlotinib, which can prolong progression-free survival (PFS) of the patients. The mutation rate is approximately 20% in Caucasians, but for Asians, their mutation rate can reach 30% or more (3). Despite the fact that EGFR-TKI is significantly effective at the beginning of treatment, almost all patients develop resistance after 10 to 14 months of treatment. However, there are still 20–30% of patients who have a poor response because of primary or intrinsic resistance to EGFR-TKIs (4,5). Moreover, the current standard practice is to switch to traditional cytotoxic chemotherapy when the patients develop resistance. At present, the elucidated mechanisms of resistance include secondary T790M mutation in 20 exons and amplification of c-met (6–8), but approximately 30% of patients who obtain resistance cannot be explained through the mechanisms above.

In 2012, Zhang et al. demonstrated that the activation of anexelekto (AXL) kinase, one member of the receptor tyrosine kinases (RTKs), can cause resistance to

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EGFR-TKI in lung cancer (9). Before this discovery in lung cancer, AXL-mediated resistance had been reported in many other kinds of cancers, such as a tyrosine kinase switch from KIT to AXL mediated imatinib resistance in gastrointestinal stromal tumor (GIST) cells (10), lapatinib resistance in HER2-positive breast cancer (11), erlotinib resistance in head and neck cancer (HNC) cells (12), and nilotinib resistance in chronic myelocytic leukemia (CML) (13). A study from Korea observed that increased AXL expression was approximately 19% in the patients who had a good initial response and then developed resistance (14). Another Korean study revealed that AXL can also mediate primary resistance in non-small cell lung cancer (NSCLC) (15). However, the particular molecular mechanisms of the resistance have not been stated clearly, so the aim of this study is to investigate the specific mechanisms.

AXL was originally isolated from patients with CML in 1988 (16), and it is a member of the TAM RTK family, which includes Tyro3, AXL, and Mer. AXL is a transmembrane protein with a molecular weight of about 140 kDa. A unique extracellular domain that consists of two N-terminal immunoglobulin (Ig)-like domains and dual fibronectin type III (FNIII) repeats is responsible for the interaction between the cells. Growth arrest-specific 6 (Gas6), a vitamin K-dependent protein, is the ligand of AXL. AXL activation through Gas6 binding or independent of Gas6 both can activate its downstream targets, mainly including tyrosine phosphorylation of PI3K/AKT and MAPK/ERK1/2 pathways (17,18). The downstream signaling cascades of AXL are closely related to anti-apoptotic effect (19), and progression and development of tumors (20,21). AXL overexpression has been found in many kinds of cancers such as breast cancer, gastric cancer, prostate cancer, ovarian cancer, and lung cancer. The purpose of this study is to clarify the specific mechanism of AXL-mediated resistance to overcome the resistance in NSCLC.

MATERIALS AND METHODS

Cell Lines and Cell Culture

PC9 [EGFR exon 19 deletion (delE746-A750)] and its gefitinib-resistant cell line PC9GR were gifts from the Shanghai Pulmonary Hospital, Tongji University, China. Both were cultured in DMEM growth medium (Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco) and 1× penicillin-streptomycin solution (Thermo) at 37°C in a humidified 5% CO_2 incubator.

Reagents and Antibodies

Gefitinib was purchased from Risheng Technology Co. (Wuhan, China). It was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at −20°C. All antibodies were purchased from Cell Signaling Technology, except the antibody to AXL, which was purchased from R&D Systems.

Cell Viability Assay

To perform the MTT assay, 3,000 to 3,500 cells per well were plated in 96-well sterile plastic plates and allowed to attach overnight; the cells were then exposed to different concentrations of gefitinib for 72 h, and 20 µl thiazolyl blue (MTT) was added in each well. After incubation for 4 h at 37°C, the supernatant was removed and 150 µl DMSO was placed in each well to dissolve formazan for 10 min with gentle shaking at room temperature. Absorbance at 490 nm was determined on a microplate reader (Model 680; Bio-Rad), and percentage of inhibition of cell population growth was calculated relative to untreated controls: Percentage of inhibition = 100% × [OD490 (untreated controls) − OD490 (treated cells)]/OD490 (untreated controls). Six duplications were set at each drug concentration, the average value after removing the maximum and minimum was used for analysis.

Western Blotting

For Western blotting, whole-cell lysates were extracted. Total proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto polyvinylidene difluoride (PVDF) membranes, blocked with 5% nonfat milk in TBST, and incubated with primary antibody at room temperature for 4 h or overnight at 4°C. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 1.5 h, protein expression was detected using ECL (Millipore).

Quantitative RT-PCR

Total RNA was extracted with TRIzol reagent (Takara). For quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of mRNA expression, 0.5 µg of total RNA was reverse transcribed into cDNA in 10 µl of reaction system using a reverse transcription kit (#A036; Takara). Real-time PCR was conducted on ABI StepOnePlus using SYBR mix agents (#420; Takara). Expression of AXL was relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA calculated by the $2^{-\Delta\Delta Ct}$ method.

Transfection

For AXL stable silencing and upregulation, PC9GR and PC9 parental cells were transfected with lentivirus-based gene transduction method to knock out and overexpress AXL, respectively. After transfection for 72 h, the green fluorescence was observed to roughly judge the transfection efficiency, and the proteins and total RNA were extracted from the cells for further analysis. The

lentivirus vector of targeted gene and nontargeting control was constructed by GenChem (Shanghai, China).

Immunohistochemical Staining

Formalin-fixed, paraffin-embedded blocks of tumors between 2013 and 2014 were obtained from the pathology archives of Drum Tower Hospital, Affiliated to the Medical School of Nanjing University. Informed consent was obtained from all patients, and this study was approved by the ethics committee of the Medical School of Nanjing University. Immunohistochemical staining for AXL and hematoxylin-eosin staining were performed. Sections were cut at 4 μm and deparaffinized in xylene, and then rehydrated by using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% hydrogen peroxide for 15 min, and then antigen retrieval was performed in a pressure cooker. The primary antibody was incubated overnight at 4°C. A PBST wash was followed by the incubation of HRP-conjugated secondary antibody. Finally, 3,3′-diaminobenzidine (DAB) tetrahydrochloride was applied as a chromogen to visualize positive tissues. All specimens were scored independently by two board-certified pathologists. The average score of two pathologists was used for analysis.

Statistical Analysis

Differences between two groups were assessed by Student's *t*-test. The association between AXL expression

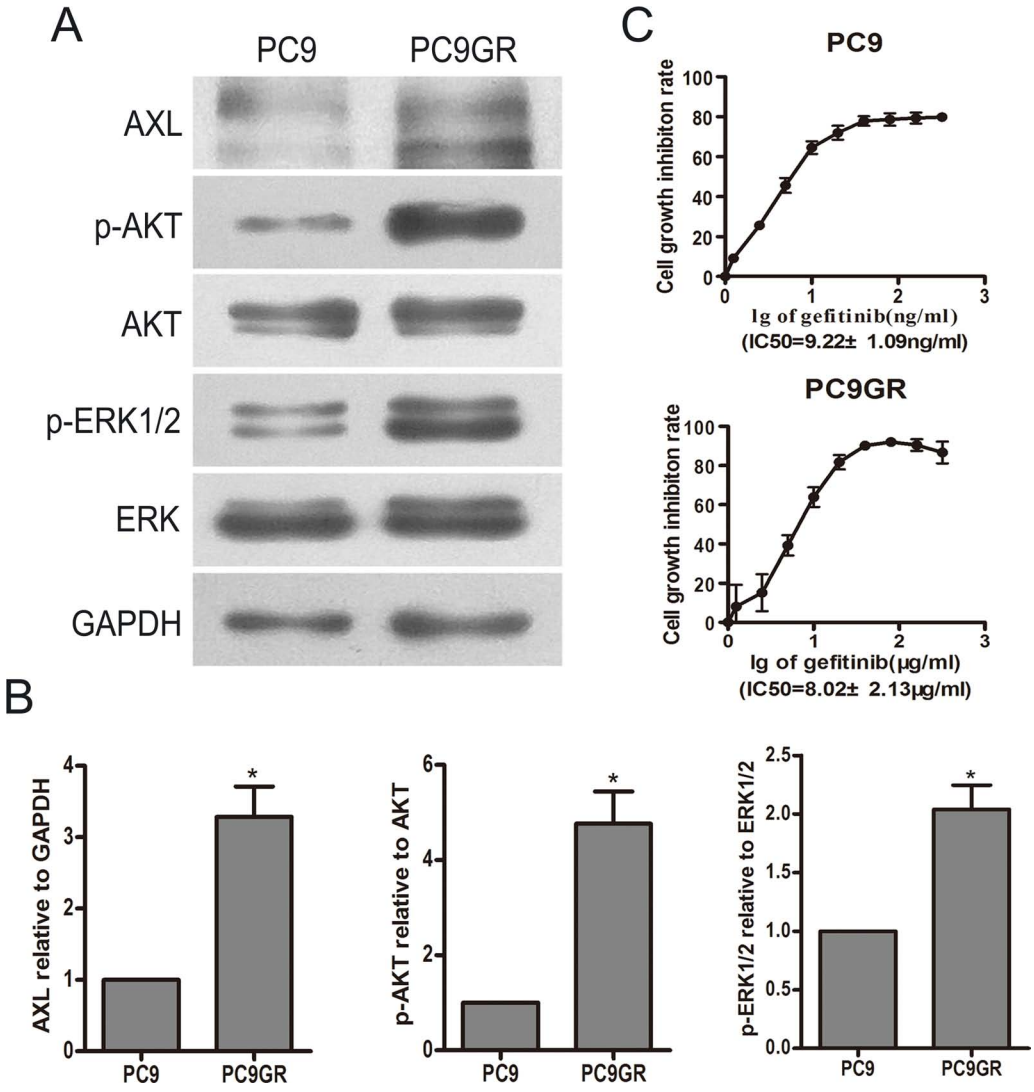

Figure 1. AXL and its downstream targets were overexpressed in PC9GR. (A, B) Whole-cell lysates were harvested from the PC9 and PC9GR cells followed by immunoblotting for the indicated proteins; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (C) Cell growth inhibition rate was determined by the MTT assay, and IC_{50} was calculated by SPSS. Data are presented as mean ± SD. *$p < 0.05$.

and clinical factors was tested using chi-square or Fisher's exact tests. Statistical analysis was performed using SPSS version 16.0 for Windows. The data are presented as mean ± SD for the indicated number of independently performed experiments. The result was considered to be statistically significant with a value of $p < 0.05$. All statistical tests were two sided.

RESULTS

Increased expression of AXL was accompanied by activation of downstream signaling pathways in resistant cells. PC9GR cells were established through chronic exposure to gefitinib in vitro, and the resistance was confirmed before being used in the experiment. The median inhibitory concentration (IC_{50}) of PC9GR was 8.02 ± 2.13 μg/ml, about 870-fold higher than the IC_{50} of parental PC9 (9.22 ± 1.09 ng/ml) (Fig. 1C), which is sensitive to gefitinib. The phenomenon proved that PC9GR cells used in this experiment did develop resistance to gefitinib. Then we found that AXL expression was higher in PC9GR cells than in parental PC9, and the phosphorylation levels of its downstream signaling targets AKT and ERK were elevated correspondingly (Fig. 1A and B). The results demonstrated that AXL-mediated acquired resistance to EGFR-TKI may be associated with activation of PI3K/AKT and MAPK/ERK1/2 pathways.

Silencing of AXL Weakens the Resistance to Gefitinib

Because AXL was overexpressed in the resistant cells, we hypothesized that knocking out AXL gene in PC9GR

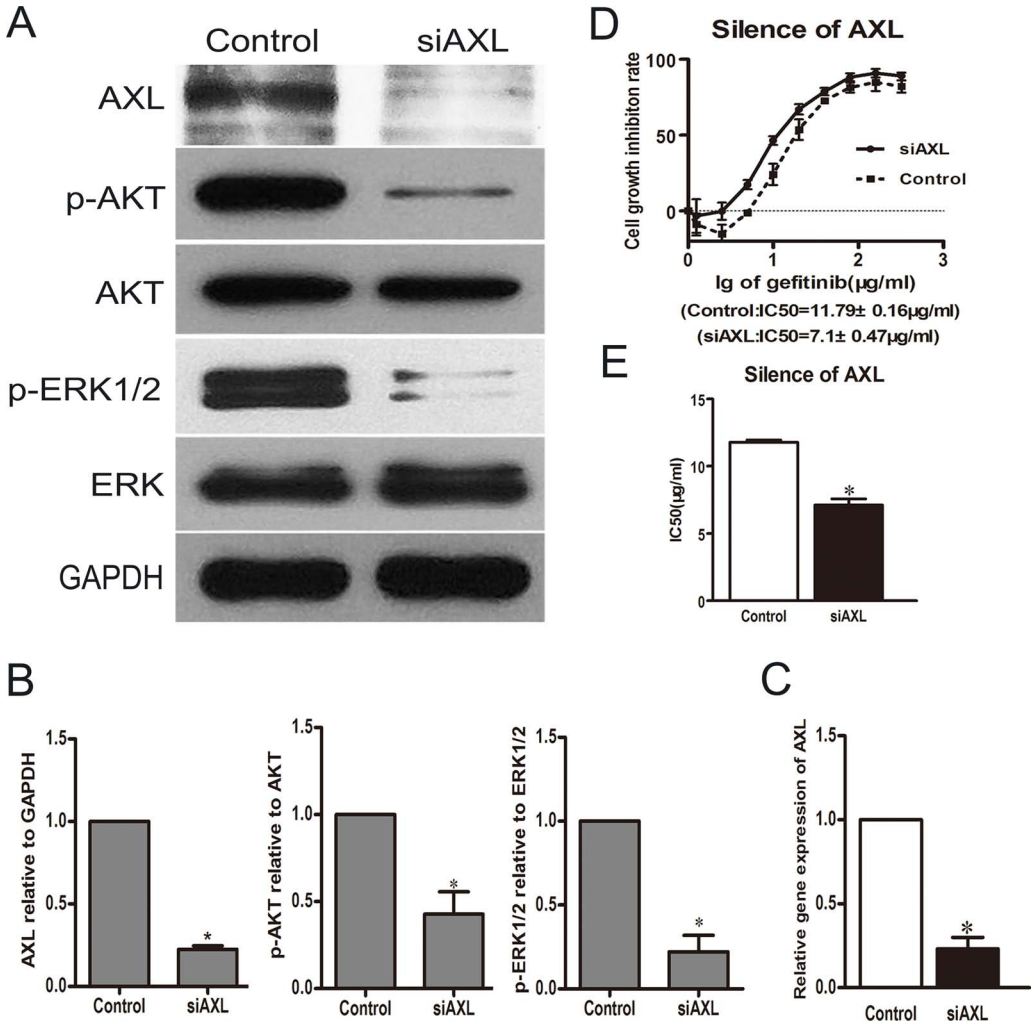

Figure 2. Silencing of AXL can weaken the resistance to gefitinib. (A, B) PC9GR cells were separately transfected with siAXL and nontargeting control for 72 h before whole-cell lysates were harvested and immunoblotting was performed. (C) The efficiency of transfection was verified by reverse transcription polymerase chain reaction (RT-PCR). (D, E) The transfected PC9GR cells were incubated with gefitinib for 72 h before MTT assay was performed. IC_{50} was calculated by SPSS, and data are presented as mean ± SD. *$p < 0.05$.

by small interfering RNA would reestablish the sensitivity to gefitinib. We used the lentivirus-based gene silencing method to stably knock down the gene expression of AXL (siAXL), and a nontargeting control was used. After transfection for 72 h according to the instructions, the knockout efficiency confirmed by RT-PCR reached at least 70% (Fig. 2C). In accordance with our hypothesis, AXL and its downstream targets, phospho-AKT (p-AKT) and phospho-ERK1/2 (p-ERK1/2), were significantly decreased after knocking out AXL (Fig. 2A and B). We expectedly found that the sensitivity of the siAXL group cells to gefitinib was improved relative to control group cells (Fig. 2D). Furthermore, the IC_{50} of the siAXL group was also lower than that of the control group (Fig. 2E). These results indicated that silencing AXL sensitizes the resistance to the specific TKI mainly through downregulating the phosphorylation levels of its downstream signaling targets.

Overexpression of AXL Triggered the Formation of Acquired Resistance

In order to more fully understand the influence of AXL to acquired resistance, we constructed a lentivirus-based expression vector to upregulate AXL expression in sensitive PC9 cells (upAXL), and also a nontargeting control (control) was used. Lentivirus and PC9 were incubated together for 72 h followed by RT-PCR, which was performed to verify the transfection efficiency (Fig. 3C). Our results displayed that the phosphorylation levels of AXL downstream targets were obviously upregulated accordingly after AXL was stably overexpressed (Fig. 3A and B). Interestingly, the drug sensitivity to

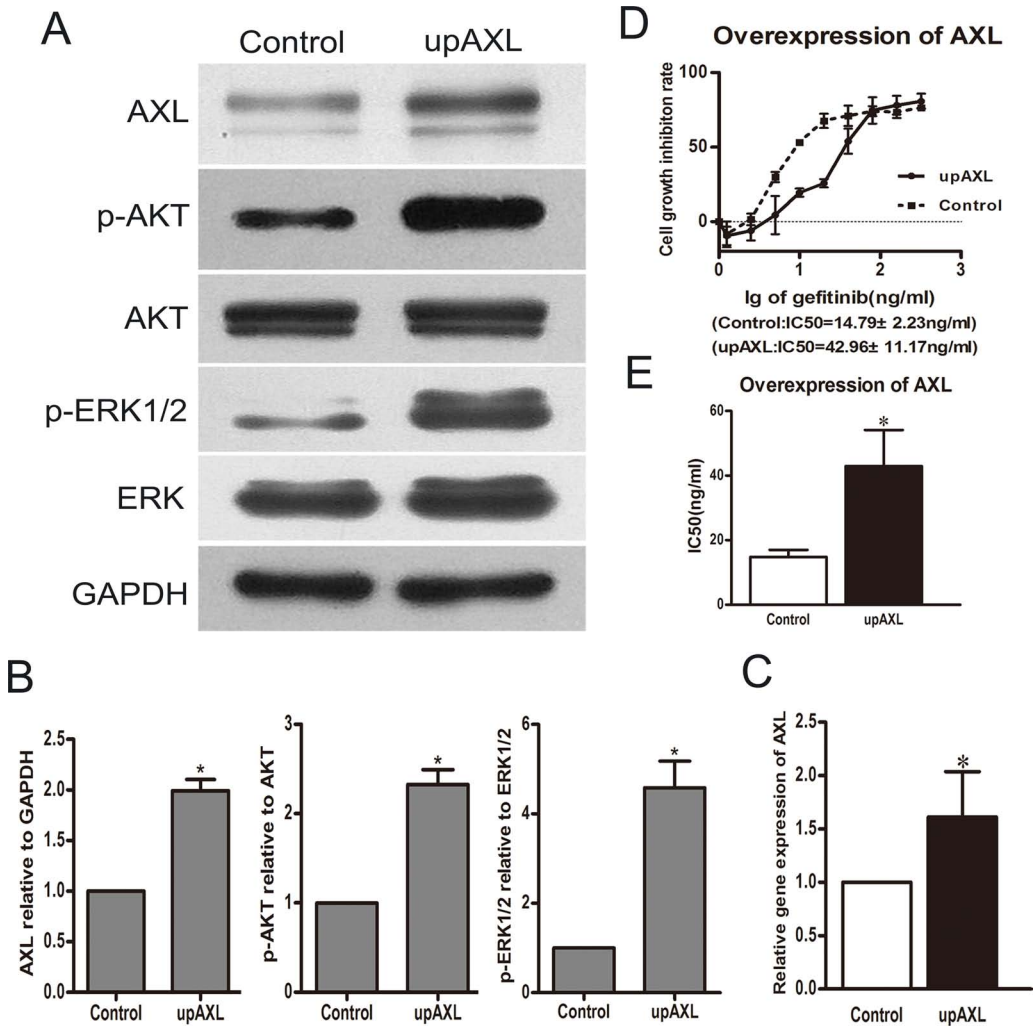

Figure 3. AXL can drive the acquired resistance. (A, B) Prior to immunoblotting for indicated proteins, PC9 cells were transfected for 72 h to upregulate AXL. (C) RT-PCR was conducted to verify the transfection efficiency. (D, E) MTT assay was performed to detect the cell viability. IC_{50} was calculated by SPSS, and data are presented as mean ± SD. $^*p < 0.05$.

Table 1. AXL Is Related to Tumor Differentiation

Patient Characteristics	Negative (%)	Positive (%)	*p* Value
Age			0.763
<65	40 (62.5)	4 (80)	
≥65	24 (37.5)	1 (20)	
Gender			0.763
Female	40 (62.5)	4 (80)	
Male	24 (37.5)	1 (20)	
Smoking history			1.000
Never smoker	48 (75.0)	4 (80)	
Former/current smoker	16 (25.0)	1 (20)	
Mutation type			1.000
19Del	31 (48.4)	2 (40)	
L858R	33 (51.6)	3 (60)	
Tissue differentiation			<0.05
Poor	9 (14.1)	5 (100)	
Moderate	42 (65.6)	0	
Well	13 (20.3)	0	
Stage			0.122
I–II	42 (65.6)	1 (20)	
III–IV	22 (34.4)	4 (80)	

Patients' clinical characteristics and AXL expression status are presented. The difference between patients' characteristics and AXL expression was evaluated by chi-square test ($p < 0.05$).

gefitinib determined by MTT detected that the IC_{50} of upAXL cells (42.96 ± 11.17 ng/ml) increased nearly threefold relative to the control group (14.79 ± 2.23 ng/ml) (Fig. 3D and E). These results confirmed that AXL can promote acquired resistance to gefitinib via the activation of PI3K/AKT and MAPK/ERK1/2 pathways in NSCLC.

AXL Was Related to Tumor Differentiation and Volume in Patients

Sixty-nine patients who harbored the EGFR mutation were enrolled to evaluate the association between AXL expression decided by immunohistochemistry and clinical characteristics. The characteristics of the patients and statistical results are shown in Table 1. Observation under a microscope found that AXL was densely expressed on cell membrane and but scarcely in the cytoplasm (Fig. 4A and B). Roughly consistent with some previous studies (15), the positive rate of AXL in our study was approximately 7%. We found that the status of AXL had no relation to patients' age, gender, smoking history, mutation type of EGFR, or tumor stage. However, positive AXL expression was strongly associated with poor tissue differentiation of tumors in this study. In addition, we

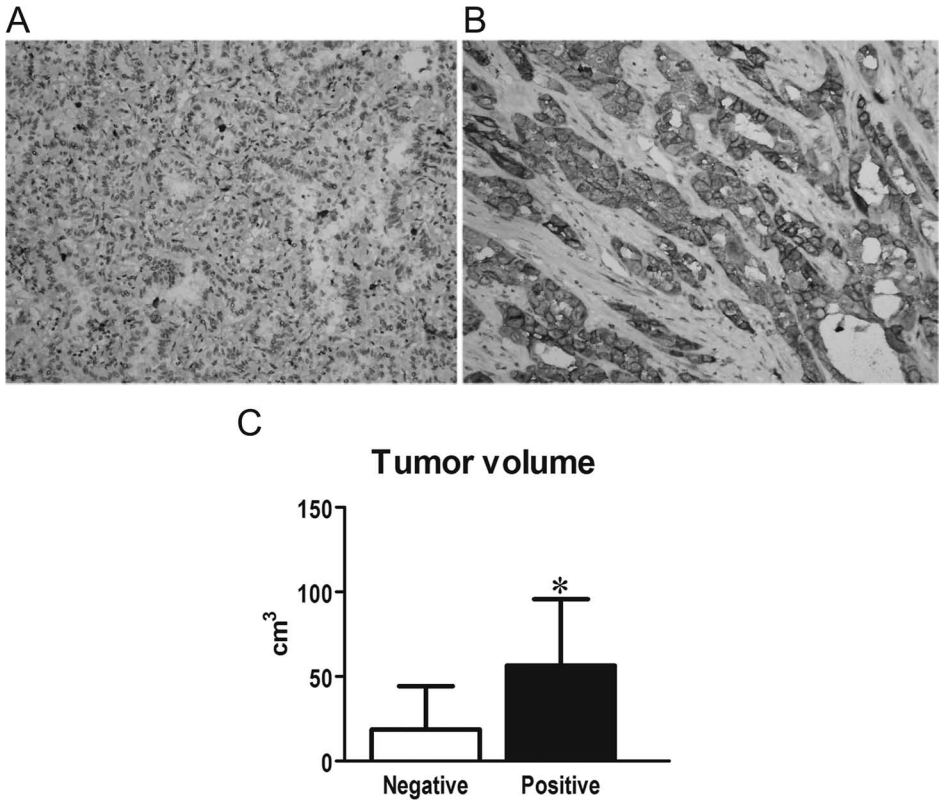

Figure 4. AXL was related to tumor volume. (A, B) Negative and positive staining examples for AXL are exhibited, respectively. Pictures were taken under 200-fold amplification. (C) Tumor volume (cm^3) = length × width × height. The difference between negative and positive was evaluated by Student's *t*-test. *$p < 0.05$.

discovered that the average tumor size of patients who harbored positive AXL expression (56.48 ± 39.15 cm^3) was about threefold larger than patients with negative AXL expression (18.66 ± 25.28 cm^3) (Fig. 4C). The small sample size was a limitation of this study; therefore, further work needs to be done to confirm the above results.

DISCUSSION

The results indicated that AXL can promote acquired resistance to EGFR-TKI in NSCLC, and suppression of AXL can weaken the resistance. In addition, AXL expression was related to tumor differentiation and tumor size in NSCLC patients who harbored the EGFR mutation. Several previous studies had confirmed that inhibition of AXL through small interfering RNA or specific inhibitors can restore the sensitivity to EGFR-TKI in vitro and in vivo (9,22). On the contrary, a previous study considered that AXL inhibition alone cannot overcome acquired resistance to EGFR-TKIs, but AXL inhibition did sensitize resistant mesenchymal cancer cells to antimitotic drugs, such as docetaxel (23). At the same time, there are many studies that indicated a combined therapy with AXL inhibition may be an effective way to overcome the resistance in NSCLC, such as a combination with Mer or MET inhibition (24,25). Not only can AXL regulate the resistance to EGFR-TKIs, but it can also mediate resistance to cetuximab, an EGFR antibody, in NSCLC and head neck squamous cell carcinoma (HNSC) (26). These findings revealed that a combined treatment strategy is an option to conquer resistance.

To our knowledge, the mechanism of AXL activation in resistant tumors remains unknown. A study indicated that after the suppression of EGFR by EGFR-TKI, AXL was compensatorily activated to drive cancer growth since they share the same downstream pathways (27). This study is consistent with a review about the signaling cross-talk between EGFR/HER2-mediated pathways and pathways triggered by other receptors (28). Furthermore, another study indicated that EGFR can transactivate AXL because of their colocalization in spatial position. The activated AXL can diversify EGFR signaling beyond those triggered by EGFR alone and limit the response to TKI in triple-negative breast cancer cells (29). This phenomenon did not happen just in AXL, but also in other RTKs (10). The results in our study also support the opinion that signaling cross-talk exit between EGFR and AXL, when the resistance to EGFR-TKI happened, inhibited the activation of EGFR and may trigger the tyrosine kinase switch to transactivation of AXL or other RTKs and their downstream pathways to maintain tumor growth. PI3K/AKT and MAPK/ERK1/2 are two main downstream signaling pathways of both AXL and EGFR. Our study confirmed again that the activation of these pathways changed along with AXL expression.

In 2013, the first AXL inhibitor, BGB324, entered clinical trials (30). It predicted that AXL may be a promising and important target to overcome resistance. Many researchers also found that they could achieve the same goal to inhibit AXL in aspects other than specific AXL inhibition. For example, our previous study proved that targeting AXL altered microRNAs, such as miR-374a and miR-548b, and may also provide a therapeutic method to overcome gefitinib resistance in NSCLC (31). AUY92, a newly developed non-geldanamycin class HSP90 inhibitor, can effectively overcome MET- and AXL-mediated EGFR-TKI resistance in lung cancer cells (32). We can also accelerate the degradation of AXL to reverse the resistance in NSCLC (33).

The results of the case analysis in our study are barely satisfactory. The limitations mainly lie in the small sample size and the difficulty in obtaining the tissues again when patients develop resistance. For these reasons, much more clinical research needs to be done to explore the role of AXL to resistance. Our study concluded that AXL can trigger the generation of resistance, and inhibition of AXL did weaken the resistance. The effect of AXL-mediated resistance was mainly determined by the activation of its downstream signaling pathways, PI3K/AKT and MAPK/ERK1/2. From what is mentioned above, we concluded that AXL is a promising target to reverse resistance to EGFR-TKI in NSCLC.

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