Effect of AXL on the epithelial-to-mesenchymal transition in non-small cell lung cancer

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Abstract. Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-associated mortality in the United States. AXL, which is a member of the receptor tyrosine kinases, has been established as a strong candidate for the targeted therapy of cancer. Therefore, the present study aimed to investigate the role of AXL in NSCLC; in particular the molecular mechanisms underlying the involvement of AXL in the epithelial-to-mesenchymal transition (EMT). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis demonstrated that AXL, EMT-inducing Twist and the mesenchymal marker N-cadherin were upregulated, and the epithelial markers E-cadherin and β-cadherin were downregulated, in the PC9 NSCLC cell line. Furthermore, downregulation of AXL expression by RNA interference was shown to inhibit cell growth by inducing the apoptosis of PC9 cells, as demonstrated by MTT and flow cytometry analyses. Notably, inhibition of AXL attenuated the regulation of EMT-associated genes, specifically downregulating Twist and N-cadherin, and upregulating E-cadherin and β-cadherin. Conversely, downregulation of Twist did not affect the expression levels of AXL. These results suggested that AXL may inhibit the EMT by the regulation of EMT-associated genes in the PC9 cell line. The results of the present study indicated that AXL may have a role in the regulation of EMT and the cell cycle of the PC9 cells; thus suggesting that AXL may have clinical significance in the design of therapeutic strategies targeting NSCLC and EMT signaling pathways.

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Introduction

Lung cancer is one of the leading causes of cancer-associated mortalities worldwide, with ~1 million new cases annually in terms of incidence and mortality (1-4). Lung cancer accounted for 13% (1.6 million) of the total cases and 18% (1.8 million) of mortalities in 2008 (1). Non-small-cell lung cancer (NSCLC) accounts for ~80% of all lung cancer cases (5). The management of patients with NSCLC is based on systemic chemotherapy; the standard treatment for patients with advanced NSCLC includes platinum-based combination chemotherapy, which has demonstrated modest but significant improvements in survival rates over best supportive care (6). However, the survival rates remain low (7). In addition, although chemotherapy may prolong the survival of patients with advanced disease, clinically significant adverse side effects, including excessive toxicity, reduce its effectiveness (8). Therefore, novel agents are urgently required for the treatment of this disease.

The epithelial-to-mesenchymal transition (EMT) has an important role in numerous physiological and pathological processes in the human body, including regulating the transcription of genes involved in embryonic development (6), the inflammatory response (8), tissue regeneration (9), organ fibrosis (10,11), and tumor invasion and metastasis. During EMT, epithelial cells loosen and cell-cell adhesion is lost (12). Furthermore, EMT is characterized by the downregulation of E-cadherin and cytokeratins, and the upregulation of mesenchymal proteins, including vimentin, fibronectin and N-cadherin (13,14). A previous study demonstrated that activation of the epidermal growth factor (EGF) receptor (EGFR) by EGF promoted EMT in hepatocellular carcinoma cell lines by altering the expression levels and molecular structures of EMT-associated markers, including the E-cadherin/β-catenin complex (14).

AXL is a member of the receptor tyrosine kinase family (9). Previous studies demonstrated that downregulation of AXL expression in solid tumors by RNA interference (RNAi) was able to decrease cell invasion and proliferation, and increase the chemosensitivity of cells via the activation of AKT and mitogen-activated protein kinase (MAPK) pathways (10,11,13). In addition, AXL was identified as an oncogene in human leukemia cells (14-16), and the expression levels of AXL were shown to be increased in >50% of NSCLC cell lines (17).

Similarly, a previous study reported that the expression levels of AXL were upregulated in 48.3% of lung adenocarcinoma tissues, in which they were correlated with lymph node metastasis and the stage of the disease (16). These reports highlight the clinical importance of AXL. Targeting of AXL with RNAi or specific monoclonal antibodies has previously been shown to inhibit the proliferation of NSCLC cells and tumor cells in a mouse xenograft model (18). A previous study on NSCLC reported that increased activation of AXL was associated with acquired resistance to EGFR-targeted therapy (19).

The present study aimed to investigate the role of AXL in NSCLC, in particular the molecular mechanism underlying its involvement in EMT. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis demonstrated that AXL was upregulated in NSCLC cells. Subsequently, inhibition of *AXL* by RNAi was conducted in order to investigate the effects of downregulation of AXL on cell viability and the expression levels of EMT-associated genes.

Materials and methods

Agents. Doxorubicin, paclitaxel, vincristine, cisplatin and 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich, Inc. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell lines. The human H1299, A549 and PC9 NSCLC cell lines, and normal lung cells (as the control), were purchased from the Cancer Research Institute of China Medical University (Shenyang, China). The cells ($1x10^5$ cells/ml) were cultured in DMEM supplemented with 10% (m/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Sangong Biotech, Shanghai, China) at 37°C in a 5% CO_2 atmosphere with stable humidity.

MTT assay. Cell viability was assessed using the MTT assay, as described in a previous study (20). Briefly, the cells were plated at a density of 3,000 cells/well into 96-well plates. At the end of treatment, the supernatant was removed using a pipette and 20 μl MTT and 270 ml fresh DMEM were added to the supernatant. Following incubation for 4 h at 37°C, 120 μl dimethyl sulfoxide was placed in each well to dissolve the tetrazolium crystals, after which the absorbance at 570 nm was recorded using a Multi-Well Plate Reader (Tecan Group, Ltd., Männedorf, Switzerland). Each experiment was performed four times. The results are presented as the percentage growth inhibition with respect to the untreated cells.

RNAi procedure. Double-strained small interfering RNA (siRNA) probes targeting the human *AXL* gene (GenBank accession no. NM-021913) and the *Twist* gene (GenBank accession no. NM_000474.3) were synthesized using the Silencer siRNA Construction kit (Ambion; Thermo Fisher Scientific, Inc.), as described in a previous study (21). Briefly, single-stranded gene-specific sense and antisense RNA oligomers were synthesized by *in vitro* transcription using T7 RNA polymerase (Sangong Biotech). In order to promote annealing of the siRNA, sense and antisense single-stranded RNA oligomers were mixed and incubated at 37°C overnight, after which the siRNA was ethanol-precipitated and resuspended in nuclease-free water. The integrity of the siRNA was assessed by gel electrophoresis (Sangong Biotech), and it was quantified of by measuring the absorbance at 260 nm. Subsequently, the cells were transfected with four pooled siRNA duplexes (20 nM; Invitrogen; Thermo Fisher Scientific, Inc.) using the TransIT-TKO® Transfection Reagent (Mirus Bio, LLC, Madison, WI, USA), in order to target the endogenous *AXL* and *Twist* genes. The cells were transfected twice at 3-day intervals, and were then collected at 72 h following the second transfection. Specific silencing of the target genes was confirmed using qPCR and western blot analysis. A mock transfection with the transfection reagent alone served as the control.

Flow cytometry (FCM) analysis. Log phase PC9 cells were collected at a final concentration of $2x10^5$ cells/ml, washed with phosphate-buffered saline (PBS) and fixed with 70% ethanol. The cells were centrifuged at 8,000 x *g* for 5 min at 4°C to remove ethanol, washed with PBS, and stained with propidium iodide (PI; Sangong Biotech) in the dark for 30 min prior to FCM analysis. The FACSCalibur™ platform (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect the cell cycle distribution. The cells were sampled using the CellQuest Pro software, version 3.0 (BD Biosciences), and the proportion of cells in the various stages of the cell cycle were quantified using ModFit LT 3.0 (Verity Software House, Inc., Topsham, ME, USA) (22). Each experiment was performed four times.

Evaluation of apoptosis. PC9 cells ($1x10^6$) were collected by centrifugation at 8,000 x *g* for 5 min at 4°C. The cell pellets were lysed using DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM EDTA and 1% Triton X-100), followed by centrifugation at 6,000 x *g* for 8 min at 4°C. The supernatant was incubated overnight with proteinase K (0.1 mg/ml; Sangong Biotech), then with RNase (0.2 mg/ml; Sangong Biotech) for 2 h at 37°C. DNA was extracted using phenol:chloroform (1:1), separated by 2% agarose gel electrophoresis (Sangong Biotech), and visualized by ultraviolet illumination after staining with 10% ethidium bromide and washing twice with water. Quantitative assessment of apoptotic cells was conducted using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method (BD ApoAlert DNA Fragmentation Assay kit; cat. no. 630107; BD Biosciences), which examines DNA-strand breaks during apoptosis.

RT-qPCR. RNA extraction from the cells was carried out using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. Genomic DNA was eliminated and RT conducted using the PrimeScript RT Reagent kit with gDNA Eraser, and qPCR was conducted using the SYBR Green PCR kit (both Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's recommendations. The process was conducted using a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc. The primers used in the PCR were as follows: *AXL* forward, 5'-TCGCCAGTGGCATGG AGTATCTG-3' and reverse, 5'-GGCGATACGTCCCTGGCG GTA-3'; *Twist* forward, 5'-CATCCACACCGTCCCCTCCC-3'

and reverse, 5'-GACTGGCGAGCTGGACACGTC-3'; and *β-actin* forward, 5'-TGAAGTACCCCATCGAGCAC-3' and reverse, 5'-CTTGGGGTTCAGGGGGGCCT-3' (GenBank accession no. BC004251.1). *β-actin* was used as an internal reference.

The reaction mix consisted of 4 μl cDNA, 0.5 μl forward and reverse primer mix (20 μM of each), 1 μl 50X ROX Reference Dye II and 25 μl 2X SYBR Green PCR mix in a final volume of 50 μl. All reactions were setup in triplicate and every sample was replicated in parallel three times to ensure statistical relevance. A negative control without cDNA template, and RT control without RT transcription were included. The following thermal conditions were used for all PCR reactions: 30 sec at 95°C, followed by 40 cycles of 30 sec at 95°C and 34 sec at 60°C. Primer specificity was confirmed by RT-PCR amplification prior to qPCR, which generated single amplicons of the expected size for each primer set. Furthermore, the amplicons were sequenced by a commercial sequencing service (BGI, Shenzhen, China) in order to validate their specific amplification, and the specificity of qPCR was confirmed by the presence of dissociation curves with single peaks and the sequencing of its products with unique bands of the expected size. Amplicon dissociation curves were obtained after cycle 40 using default settings suggested by the instrument. Data were analyzed using the SDS software (version 2.0.6; Applied Biosystems; Thermo Fisher Scientific, Inc.). All quantifications were normalized against the quantity of β-actin using the $2^{-\Delta\Delta Cq}$ method (23).

Western blot analysis. Cell lysates were prepared in radio-immunoprecipitation assay buffer [50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)], supplemented with 1X Halt protease inhibitor and 1X Halt phosphatase inhibitor cocktails (Pierce Biotechnology, Inc., Rockford, IL, USA). The Bio-Rad protein assay (cat. no. 500-0006; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine protein concentrations. Proteins (100 ng) were separated by 10-12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare Life Sciences, Chalfont, UK). The membranes were blocked for 1 h at room temperature using bovine serum albumin (BSA; Sangong Biotech). Subsequently, the membranes were incubated with specific primary antibodies (1:100 dilution) targeting Axl (cat. no. 4566), Twist (cat. no. 46702) and β-actin (cat. no. 4790; all from Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. After washing the membrane three times with TBST (5 min per wash), it was incubated with the recommended dilution (1:100) of horseradish peroxidase-conjugated secondary antibodies (cat. no. 7071; Cell Signaling Technology, Inc.) at room temperature for 1 h, and then washed as described above. The protein bands were visualized using the Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA). The chemiluminescence of proteins transferred to the PVDF membranes was detected using the ECL Plus Western Blot analysis Reagent (GE Healthcare Life Sciences). Relative protein expression levels were semi-quantified by densitometry using ImageJ software (version 2.1.4.7; National Institutes of Health, Bethesda, MA, USA).
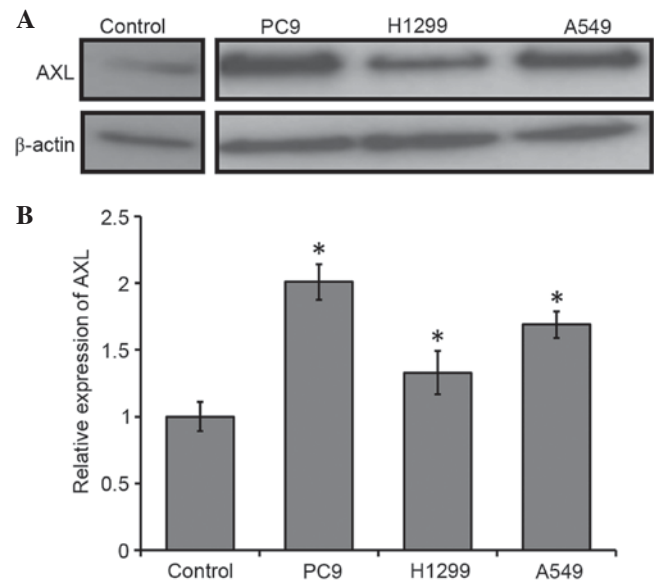

Figure 1. Determination of AXL expression levels in the NSCLC cell lines. (A) Western blot analysis and (B) reverse transcription-quantitative polymerase chain reaction demonstrated that AXL was upregulated in the three NSCLC cell lines, as compared with the control (normal lung cells). The PC9 cell line exhibited the highest expression levels of AXL. *P<0.05 vs. the control by analysis of variance. NSCLC, non-small-cell lung cancer.

Statistical analysis. Data were analyzed using SPSS statistical software, version 16.0 (SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean ± standard deviation. The mean was compared using the Student's t-test or by analysis of variance. The statistical significance of the studies was determined using the parametric unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

AXL is upregulated in NSCLC cells. To evaluate the expression of AXL in the NSCLC cell lines, RT-qPCR and western blot analysis were conducted. AXL protein expression levels were markedly upregulated in the NSCLC cell lines compared with the control (Fig. 1A). Consistent with this, the mRNA expression levels of *AXL* were significantly upregulated in the NSCLC cells, as compared with the control (P<0.05; Fig. 1B). PC9 exhibited the highest level of *AXL* transcripts (~2-fold upregulation), as compared with the control; the *AXL* transcripts were ~1.7-fold upregulated in the A549 cells and 1.3-fold upregulated in the H1299 cells, as compared with the control (Fig. 1B). These results suggest that AXL is upregulated in NSCLC cells.

AXL promotes NSCLC cell survival. To characterize the role of AXL in NSCLC cells, a gene-silencing experiment using double-stranded siRNA to silence *AXL* in the PC9 cells was conducted. PC9 cells were selected for further experimentation, as the greatest upregulation of *AXL* expression levels was observed in this cell line. PC9 cells were transfected with the siRNA and, after 3-7 days, RNA was extracted from control and transfected cell lines. A marked reduction was detected in the mRNA and protein expression levels of AXL in the siRNA-transfected cells, as compared with the control cells (Fig. 2A and B), and this was shown to be significant

Table I. Differences in cell cycle induced by downregulated AXL.

Group	Cell debris (%)	Apoptosis (%)	Diploid (%)	Aneuploid (%)
Control	1.88±0.2167	0.0267±0.0252	94.7867±4.5027	5.2133±4.5027
Axl siRNA	46.9533±5.0537	0.06934±0.0723	99.9101±0.1558	0.0901±0.1558

Data are presented as the mean ± standard deviation. Control, mock transfection samples. $P<0.05$ vs. the control by Student's t-test.
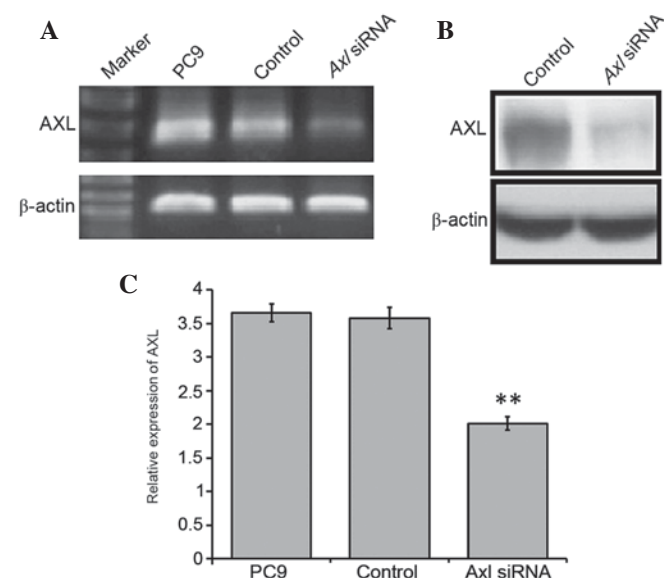

Figure 2. Silencing of AXL in the PC9 cell line was conducted by RNA interference, in which the PC9 NSCLC cell line was transfected with siRNA/AXl. (A) Total RNA was extracted and analyzed using RT-PCR using primers specific to *AXL* and *β-actin*. Cells were collected and lysed, and the expression levels of *AXL* were analyzed by (B) western blot analysis and (C) RT-qPCR. Mock transfection samples were used as the control. Normal PC9 cells were also used in the RT-PCR and RT-qPCR assays. **$P<0.01$ vs. the control by analysis of variance. NSCLC, non-small-cell lung cancer; siRNA, small interfering RNA; RT-PCR, reverse transcription-polymerase chain reaction; qPCR, quantitative-PCR.

Figure 3. Inhibition of AXL in PC9 cells suppressed cell growth. Data are presented as mean ± standard deviation (n=4). Results are expressed as the percentage growth inhibition with respect to the control (mock transfection). *$P<0.05$ vs. control by analysis of variance. siRNA, small interfering RNA.

using RT-qPCR ($P<0.01$; Fig. 2C). In particular, ~1.83-fold downregulation of *AXL* was detected in the transfected cells, as compared with the control (Fig. 2). These results suggested that knockdown of AXL expression in the PC9 cells was successful.

The viability of the PC9 cells was analyzed using the MTT assay. Downregulation of endogenous AXL in PC9 cells significantly reduced cell survival (~29.3%), as compared with the control ($P<0.05$; Fig. 3). These results suggest that inhibition of AXL by RNAi may reduce the survival of NSCLC cells.

AXL is involved in NSCLC cell apoptosis. To further investigate the involvement of AXL in the inhibition of cell proliferation, cell-cycle distribution was evaluated using FCM analysis. As compared with the control group, the number of cells in the G_1/G_0 phase was significantly increased ($P<0.05$), and the number of cells in the S and G_2/M phases was significantly decreased ($P<0.05$), in the PC9 cells treated with AXL siRNA; the majority of cells were in the G_1 phase (Table I). Furthermore, the proportion of diploid cells slightly increased, with a reduction in the number of aneuploid cells, and the cell
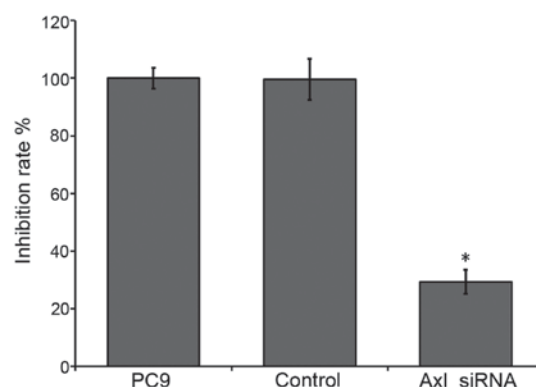
debris was markedly increased in the PC9 cells treated with AXL siRNA, as compared with the control group.

The effect of downregulating AXL expression on the induction of apoptosis in PC9 cells was investigated using a DNA fragmentation assay. Agarose gel electrophoresis demonstrated that knockdown of *AXL* resulted in the formation of DNA fragments in PC9 cells (Fig. 4A). In addition, a quantitative evaluation was conducted using TUNEL to detect DNA-strand breaks. As compared to with the control cells, 30.7% of PC9 G_2 cells treated with *AXL* siRNA were apoptotic cells (Fig. 4B). These results suggest that interfering with AXL expression may inhibit cell-cycle progression in PC9 G_2 cells, thereby resulting in a marked increase in the percentage of cells in the G_1 phase.

Downregulation of AXL inhibits EMT-inducing Twist. 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 (24). EMT-inducing genes that have essential roles in EMT, including *Twist* and *Snail*, are termed master EMT genes (25). These genes function as transcriptional repressors of the cell-cell adhesion glycoprotein, E-cadherin whose functional loss is one of the hallmarks of EMT (26). Therefore, the status of *Twist* expression in PC9 cells was investigated in the present study. PC9 cells that were stably expressing *Twist* exhibited an EMT phenotype characterized by upregulation of the mesenchymal marker N-cadherin (Fig. 5A), and downregulation of the epithelial markers E-cadherin and β-cadherin (Fig. 5B). Notably, Axl was markedly upregulated in the *Twist*-expressing PC9 cells (Fig. 5C). Conversely, in the PC9 cells treated with AXL siRNA, downregulation of Twist
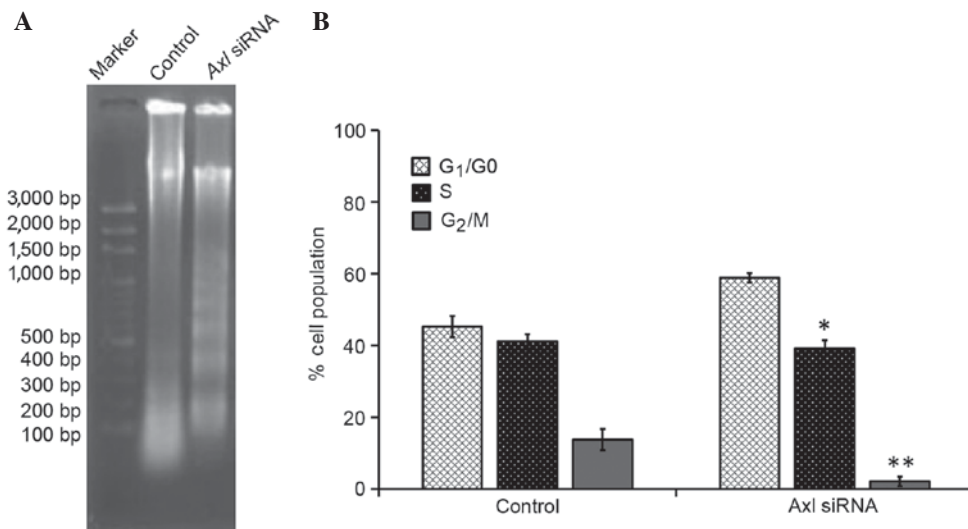
Figure 4. Inhibition of AXL accelerated cell-cycle arrest and apoptosis in PC9 cells. (A) DNA fragmentation was induced following AXL knockdown by siRNA in PC9 cells. (B) Quantitative evaluation of the terminal deoxynucleotidyl transferase dUTP nick end labeling assay by flow cytometry. Mock transfection samples were used as a control. Data are presented as the mean ± standard deviation of three determinations. *P<0.05 and **P<0.01 vs. the control by analysis of variance. siRNA, small interfering RNA.
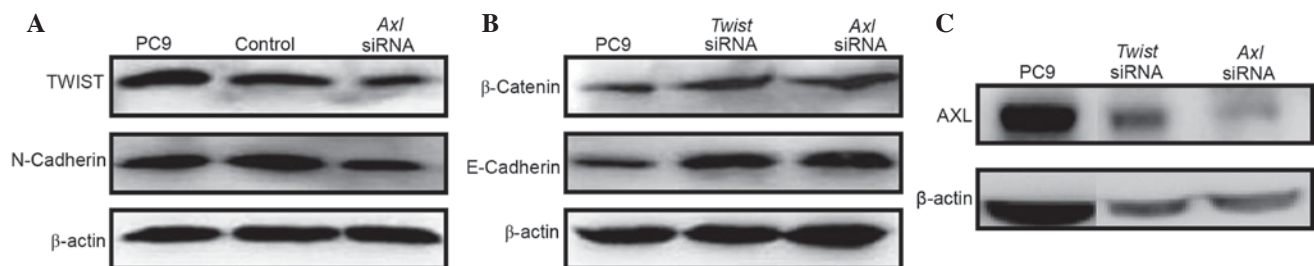

Figure 5. Inhibition of AXL suppressed Twist expression. Upregulation of the master EMT gene Twist was accompanied by (A) upregulation of the mesenchymal marker N-cadherin, (B) downregulation of the epithelial markers E-cadherin and β-cadherin. and (C) upregulation of AXL. (A-C) These expression patterns were reversed by AXL knockdown using siRNA. Mock transfection samples were used as the control. siRNA, small interfering RNA.

and N-cadherin (Fig. 5A), and upregulation of E-cadherin and β-cadherin (Fig. 5B) were observed. However, knockdown of *Twist* did not affect the expression levels of AXL (Fig. 5C), despite the fact that E-cadherin and β-cadherin were upregulated (Fig. 5B). These results suggest that downregulation of AXL may abrogate EMT program induction by inhibiting the expression of *Twist* in NSCLC cells.

Discussion

This study investigated the role of AXL in NSCLC cells, in particular the molecular mechanisms underlying the involvement of AXL in the EMT. AXL was shown to be upregulated in all NSCLC cell lines used in the present study, which is consistent with a previous study (27), suggesting that AXL may be used as a biomarker of NSCLC. Furthermore, in the present study, downregulation of AXL by RNAi was shown to inhibit PC9 cell survival, which may have been due to accelerated cell apoptosis or cell proliferation inhibition at the G_1 stage. A previous study reported that human NSCLC with activating mutations in the EGFR did not respond to treatment with EGFR-targeted tyrosine kinase inhibitors (TKIs), due to the acquisition of resistance (27). Furthermore, activation of the AXL kinase was associated with resistance to EGFR-targeted

therapy in patients with lung cancer (27), suggesting that increased AXL expression levels may contribute to drug resistance in lung cancer. Therefore, downregulation of AXL expression may be considered an effective tool for lung cancer therapy (28). Alterations in AXL-associated signaling pathways have been observed in ~20% of patients with acquired resistance to EGFR-TKI (29), although it remains to be determined whether these patients would benefit from AXL inhibition. In EGFR-TKI resistance, AXL may act as a bypass to activate downstream signals associated with cell survival and growth (30). Therefore, combined treatment with EGFR and AXL inhibitors may effectively abrogate the growth of tumor cells. A similar phenomenon was observed in hepatocyte growth factor receptor-mediated resistance (31).

EMT is an embryonic developmental process involving changes in cell morphology and expression of EMT-associated genes (25). EMT also occurs during the progression of several types of human cancer, in which it confers motility and invasiveness on cancer cells, leading them to acquire the ability to metastasize to distant sites (32). A previous study reported that lung cancer cell lines may be classified into mesenchymal and epithelial phenotypes based on the expression status of E-cadherin and Vimentin, which are markers of epithelial and mesenchymal phenotypes, respectively (33). A genetic study

of early development reported the existence of a number of EMT-inducing genes encoding transcription factors that are capable of inducing EMT when ectopically expressed in epithelial cells (34). Therefore, the present study investigated the expression levels of several master EMT genes, as well as AXL, in order to elucidate the potential role of AXL in EMT. The master EMT gene *Twist* was upregulated in the PC9 NSCLC cells, which was accompanied by upregulation of *AXL*. Conversely, inhibition of *AXL* by RNAi resulted in the downregulation of *Twist* in PC9 cells; thus suggesting that downregulation of AXL inhibits EMT-inducing Twist. Therefore, AXL may regulate EMT via the EMT-associated genes. The results of the present study may have clinical significance in the design of therapeutic strategies targeting AXL and EMT signaling pathways.

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