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Expression of Axl in Lung Adenocarcinoma and Correlation with Tumor Progression[1]

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Abstract

We used the Transwell system to select highly invasive cell lines from minimally invasive parent cells, and we compared gene expression in paired cell lines with high and low invasive potentials. Axl was relatively overexpressed in the highly invasive cell lines when compared with their minimally invasive counterparts. However, there is only limited information about the role of Axl in cancer invasion. The biologic function of Axl in tumor invasion was investigated by overexpression of full-length *Axl* in minimally invasive cells and by siRNA knockdown of *Axl* expression in highly invasive cells. Overexpression of Axl in minimally invasive cells increased their invasiveness. siRNA reduced cell invasiveness as Axl was downregulated in highly invasive cells. We further investigated the protein expression of Axl by immunohistochemistry and its correlation with clinicopathologic features. Data from a study of 58 patient specimens showed that Axl immunoreactivity was statistically significant with respect to lymph node status ($P < .0001$) and the patient's clinical stage ($P < .0001$). Our results demonstrate that Axl protein kinase seems to play an important role in the invasion and progression of lung cancer.

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Introduction

Tumor invasion and metastasis are the most challenging problems faced by oncologists. Although studies have focused on these issues, no major breakthrough has been made in preventing tumor invasion and metastasis. In order to achieve invasion and metastasis, a cancer cell must break away from its tumor and invade tissues in either the circulatory system or the lymphoid system, which then carries it to a new location. These processes require a cadre of molecular and cellular alterations that enable cells to circumvent normal growth control mechanisms and to manipulate their local environment [1]. To elucidate the mechanisms required for the invasion and metastasis of human cancers, comparative studies of biologic behavior and genetic status among clonally related cell lines with different metastatic potentials may prove helpful [2].

Axl, also called UFO, ARK, and Tyro7, was originally identified as a transforming gene in human leukemia [3–5]. The 894–amino-acid human protein has a mass of 140 kDa, with roughly equal distribution of amino acids on either side of the plasma membrane. The ligand of Axl, Gas6 protein, is so named by virtue of the initial finding that the gene (*growth arrest–specific gene 6*) that encodes the protein is highly expressed in growth-arrested cells [6,7]. The biologic function of Axl/Gas6 in normal and malignant cell biology has not been completely elucidated, but appears to be complex. For example, in noncancer cells, Axl prevented serum deprivation–induced cell death and played a role in cell proliferation [8–11]. Axl overexpression has been demonstrated in breast, colon, and thyroid cancer cell lines and in melanoma cells [12–15], and the function of Axl in tumors was suggested to be involved in cell cycle reentry, survival, and cell transformation (tumorigenesis) [16–18]. Nonetheless, there is limited information about the role of Axl in cancer invasion and metastasis.

Recent studies have provided some information on Axl in tumor invasion and metastasis. Jacob et al. [19] analyzed the expression of Axl in prostate cancer cells and found that it was highly expressed in metastatic DU145 cells compared to normal prostate cells and the prostatic carcinoma cell line. Furthermore, Axl was expressed at higher levels in metastatic tumors than in normal tissues or primary tumors [20]. Thus, Axl may be implicated in cancer invasion and metastasis. The present study was undertaken to isolate panels of cell lines with different invasion potentials and to compare their gene expression. The involvement of Axl in cancer invasion and progression was examined.

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Materials and Methods

Cell Culture, Patients, and Tissues

Human lung adenocarcinoma cell lines CL1-0 and CL1-5 are minimally and highly invasive sublines that were selected from the clonal cell line of human lung adenocarcinoma [2]. The other human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured as previously described [21]. The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. The media were changed three times per week. The cells were free of *Mycoplasma* contamination during the study period. Specimens were obtained from 50 paraffin-embedded lung adenocarcinomas that had been collected in the Department of Pathology, Tri-Service General Hospital (Taipei, Taiwan). For each case, a representative paraffin block that contained both tumor and benign tissues was selected. Serial sections were then cut for hematoxylin and eosin (H&E) staining and experimental studies.

Selection Procedure

Selection of highly invasive subpopulations from parent cell lines HT1197 and A59 was performed using a Transwell system (6.5 mm diameter, 8 μm pore size with polycarbonate membrane; Corning Costar, Acton, MA). Briefly, Transwell inserts were coated with Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA). Cells were resuspended in RPMI 1640 containing 10% NuSerum (BD Biosciences) and seeded into the wells. Following incubation for 24 to 72 hours at 37°C, the inserts were removed. The cells that invaded through the membranes and attached to the lower well were harvested and expanded for second-round selection. As the serial selection of higher invasiveness cells continued up to the fourth generation, the sublines of cells were designated as HT1197-4 and A59-4.

Microarray Analysis

Microarray membranes containing 9600 cDNA sequences were generously provided by Dr. Konan Peck (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). Single-color detection and image analysis were performed as previously described [22]. The microarray image was digitized using a drum scanner (ScanView, Foster City, CA); image analysis and spot quantification were performed as described previously [23]. Program isolates differentially expressed genes by measuring the integrated density of each spot, performing regression analysis on integrated density data, and locating statistical outliers as differentially expressed genes. Genes whose expression profiles correlated either positively or negatively with the invasiveness of cell lines were identified.

In Vitro *Invasion Assay*

The invasion ability of tumor cells was examined using 24-well culture insert-based assays (BD Biosciences, Franklin Lakes, NJ). The culture insert, with 8 μm pore size, was precoated to a density of 30 μg/insert of Matrigel Basement Membrane Matrix (BD Biosciences). Cells were suspended in medium containing 10% NuSerum, and 2.5×10^4 cells were added onto the insert. After incubating for 24 hours at 37°C, the cells that invaded or migrated through the FluoroBlok membrane were stained with propidium iodine, and fluorescence images were taken. The invasive cells were then counted with Analytical Imaging Station software package (Imaging Research, Ontario, Canada). The samples were plated in triplicate or quadruplicate, and the experiment was repeated at least twice.

RNA Extraction, Reverse Transcription Polymerase Chain Reaction (RT-PCR), and Real-Time PCR

Total cellular RNA was extracted, purified, and converted to cDNA with the use of oligo $d(T)_{12-18}$ primers to preserve the relative mRNA profile and to produce a template suitable for PCR. The PCR primers used were: *Axl* sense 5′-AACCT TCAAC TCCTG CCTTC TCG-3′ and antisense 5′-CAGCT TCTCC TTCAG CTCTT CAC-3′. Primers specific for a segment of GAPDH (sense 5′-AGGGG TCTAC ATGGC AACTG-3′ and antisense 5′-CGACC ACTTT GTCAA GCTCA-3′) were used to confirm cDNA integrity and normalization of cDNA yields. In the PCR step, 50 pmol each of sense and antisense primer was used. Standard PCR amplification conditions consisted of a hot start at 94°C for 5 minutes, followed by 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 30 cycles, with a final amplification at 72°C for 10 minutes. The DNA was visualized using ethidium bromide staining, and band density was determined with a densitometer. Real-time PCR from this cDNA product was performed with the use of a thermocycler with continuous fluorescence-monitoring capabilities (Roche, Mannheim, Germany). The SYBR Green 1 LightCycler reverse transcriptase (RT) PCR kit (Roche) was used for PCR amplification under conditions recommended by the manufacturer.

Protein Extraction and Western Blot Analysis

Cells were lysed in a lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1 mg/ml each of aprotinin and leupeptin, pH 7.4) for 20 minutes at 4°C, and the concentration of protein in each cell lysate was measured using a commercial bicinchoninic acid kit (Pierce, Rockford, IL). A 50-ìg sample of each lysate was subjected to electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gel. Proteins were then transferred to nitrocellulose membrane and immunoblotted with antibodies. Detection was performed using the Western blot reagent ECL, and chemiluminescence was exposed onto Kodak X-Omat film (Kodak, Chalon/Paris, France).

Construction of Full-Length Human Axl and Cell Transfection

Human *Axl* cDNA was amplified by RT-PCR with sense 5′-CCCAA GCTTG GAAAG TTTGG CACCC ATG-3′ and antisense 5′-CCCAA GCTTG GTTGT CTCAG GCACC ATC-3′. The resulting 2698-bp PCR product was digested with *Hin*dIII restriction enzymes and subcloned into the mammalian

expression vector pCMV-Tag 2A (Stratagene, La Jolla, CA). Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and subcultured into selective media containing 1 mg/ml G418 (Invitrogen).

siRNA

Double-stranded siRNA targeting *Axl* (accession no. NM-021913) was synthesized using Silencer siRNA Construction Kit (Ambion, Austin, TX), as recommended by the manufacturer. Briefly, single-stranded gene-specific sense and antisense RNA oligomers were synthesized through *in vitro* transcription using T7 RNA polymerase. For annealing of siRNA, sense and antisense single-stranded RNA oligomers were mixed and incubated at 37°C overnight. siRNA was ethanol-precipitated and resuspended in nuclease-free water. The integrity and quantification of the siRNA were confirmed by gel electrophoresis and measured at 260-nm absorbance. Transfection of four pooled siRNA duplexes for the targeting of any endogenous *Axl* gene was performed using TransIT-TKO Transfection Reagent (Mirus, Madison, WI) at a final concentration of 20 nM. Cells were transfected twice at 3-day intervals and collected 72 hours after the second transfection. Specific silencing of the targeted gene was confirmed by RT-PCR and Western blot analysis. Mock transfection with transfection reagent alone served as the control.

Immunohistochemistry and Assessment of Axl *Expression*

Specimens from the paraffin blocks were cut into 5-μm sections. The sections were routinely stained with H&E for histologic diagnosis, and additional sequential sections were selected for immunohistochemical studies. Sections were dewaxed and subjected to microwave antigen retrieval. Endogenous peroxidase activity and nonspecific binding were blocked by incubation with 3% hydrogen peroxide and nonimmune serum, respectively. Slides were then incubated sequentially with rabbit anti–human Axl antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 for 60 minutes and peroxidase-labeled secondary antibody for 10 minutes. Diaminobenzidine hydrochloride (Dako, Carpinteria, CA) was then added in order to localize positive staining sequentially by light microscopy. For negative control experiments, primary antibodies were omitted or replaced with an irrelevant antibody, mouse immunoglobulin G1 (Dako). Sections were counterstained with hematoxylin and coverslipped. Using a semiquantitative scale described previously [24], staining results were classified into positive (>25%) and negative (<25%) categories according to the percentage of immunostaining-positive cells. Immunostaining results were evaluated by two investigators (Y.S.S. and H.S.L.) without prior knowledge of the tumor's histopathologic features and the patient's clinical status. TNM classification and staging were in accordance with the designated system of the World Health Organization. For further analysis, tumor stage was subdivided into two groups: 1) localized tumors: stages 0, IA, and IB; and 2) advanced tumors: stages IIA, IIB, IIIA, IIIB, and IV.

Statistical Analysis

Immunohistochemistry results were analyzed using chi-square analysis. The correlation between Axl and various disease parameters was determined using Fisher's exact test. Differences were considered significant when $P < .05$.

Results

Selection of Highly Invasive Cancer Cell Lines from Minimally Invasive Parent Cells

CL1-0 and CL1-5 were previously established with minimally and highly invasive abilities, respectively, in our laboratory [2].

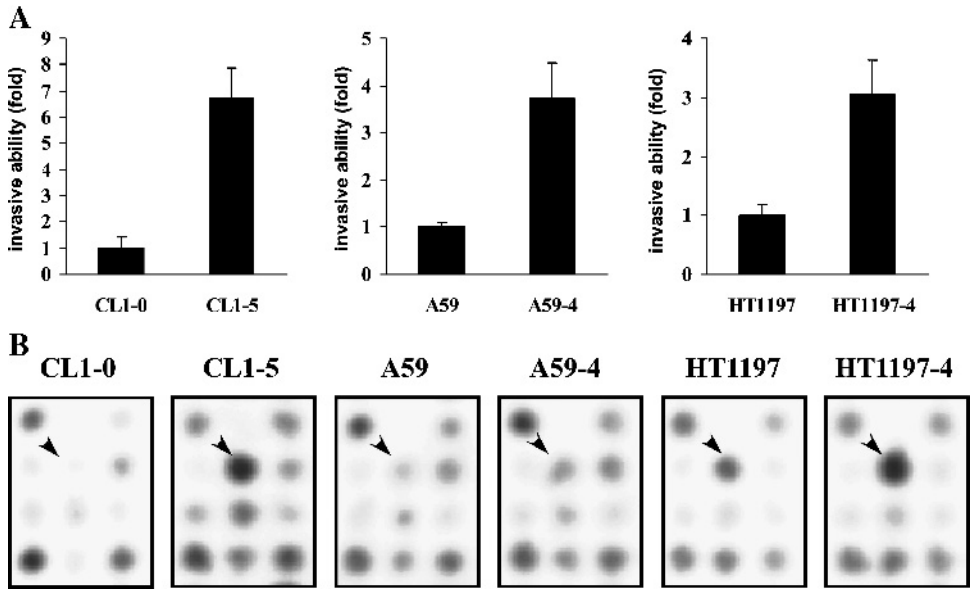

Figure 1. *Establishment of cell lines with high and low invasion ability. (A) Lung cancer CL1-0, bladder cancer HT1197, and ovary cancer A59 cells were subjected to repeated selection procedures and denoted CL1-5, A59-4, and HT1197-4, respectively. Their invasion abilities were evaluated using Matrigel-coated Transwell membranes. (B) Gene expression analysis revealed that* Axl *expression was significantly different in highly and minimally invasive cell lines.*

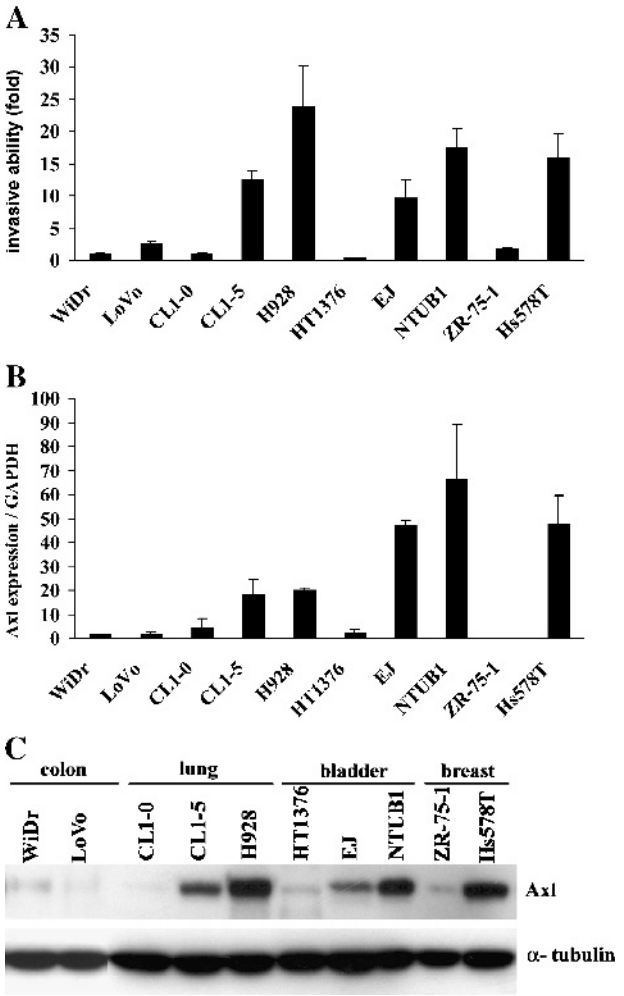

Figure 2. Axl *expression is relevant to cell invasion ability. Several cell lines from different tissues (colon, lung, bladder, and breast) were investigated for their invasion abilities (A).* Axl *RNA expression (B) and Axl protein levels (C) were also analyzed relative to their invasiveness.*

We used parental cells A59 and HT1197 to select highly invasive cancer cell lines A59-4 and HT1197-4 as the cells that invaded the Matrigel four times. Both cell lines showed morphologic characteristics of their epithelial cell origin, with positive staining of cytokeratin (data not shown). We further measured the difference in invasiveness associated with these cell lines. As shown in Figure 1*A*, the invasive ability of the sublines A59-4 and HT1197-4 increased three- to four-fold compared to their parent cell lines. The invasive potentials of these two cell lines were continuously maintained in culture throughout this study.

Axl *Expression in Highly and Minimally Invasive Cell Lines*

To compare gene expression differences in highly and minimally invasive cell lines, cDNA microarrays were used to examine their gene expression profiles between paired cell lines. Among these genes that displayed more than a two-fold expression change, we found that *Axl* was relatively overexpressed in the three highly invasive cell lines compared to their minimally invasive counterparts (Figure 1*B*). In order to confirm this finding, the association of *Axl* expression and invasive potential was examined in additional human cancer cell lines. Regardless of tissue origin, *Axl* mRNA expression was relevant to cell-invasive ability (Figure 2, *A* and *B*). Similar findings were seen when protein expression was evaluated (Figure 2, *A* and *C*).

Inhibition of Invasiveness by Axl siRNA

If Axl is important in regulating cell invasion, then changes in Axl expression should affect cell invasiveness. We performed a gene-silencing experiment utilizing double-stranded siRNA to silence the *Axl* gene in the highly invasive cell lines. Cancer cells were transfected with the siRNA and, after 3 to 7 days of transfection, RNA was extracted from both control and transfected cell lines. RT-PCR and Western blot analysis revealed a significant decrease in *Axl* gene expression in the siRNA-transfected cells compared to the control cells (Figure 3, *A* and *B*). Then, we evaluated the effect of siRNA/*Axl* in cell invasion. Concomitantly, transfection of siRNA/*Axl* reduced cell invasion ability (Figure 3*C*).

Axl *Transfection Enhances Cell-Invasive Ability*

A full-length *Axl* expression vector was constructed and transfected to the minimally invasive cancer cell line CL1-0. The vector-alone transfectant CL1-0/neo and the *Axl* transfectant CL1-0/*Axl*-p were obtained. Stable *Axl*-overexpressing single cell clones CL1-0/*Axl*-10 and CL1-0/*Axl*-15 selected from CL1-0/*Axl*-p were subjected to further investigation. Axl expression was increased in overexpression clones relative to wild type and vector control cells both in RNA and protein

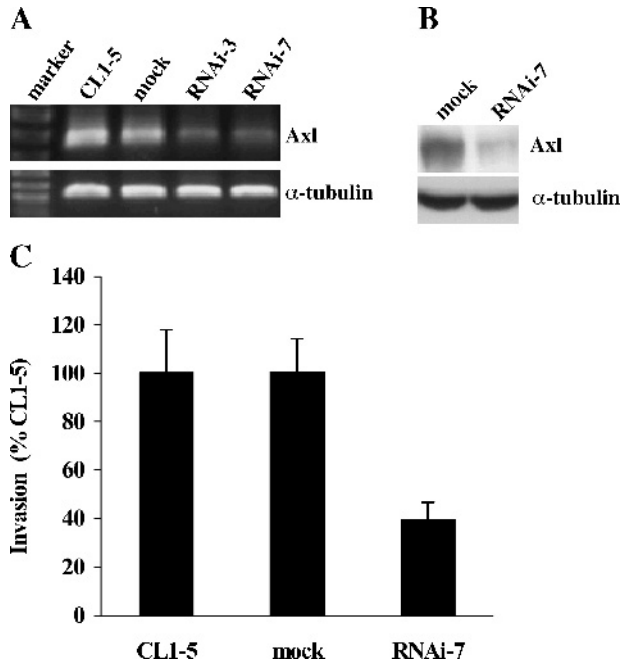

Figure 3. *Double-stranded siRNA/*Axl *downregulation of* Axl *expression accompanied the reduction of cell invasion ability. The highly invasive cell line CL1-5 was transfected with siRNA/*Axl*. Total RNA was extracted and analyzed by RT-PCR using primers specific to* Axl *and GAPDH (A). Cells collected on the indicated day (RNAi-3, 3 days; RNAi-7, 7 days) were lysed, and the expression of* Axl *was analyzed by Western blot analysis using anti-*Axl *or anti-tubulin antibodies (B). Invasion assays showed reduced cell invasiveness due to* Axl *knockdown compared to control (mock) cells (C).*

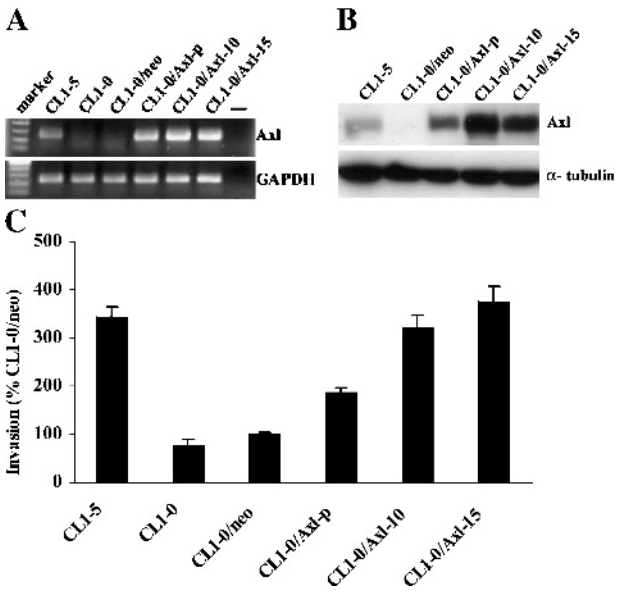

Figure 4. Axl *transfection of minimally invasive cell lines resulted in increased cell invasion in minimally invasive cell lines. CL1-0/*Axl-*p (a mixed population and stable clone), CL1-0-*Axl-*10, and CL1-0-*Axl-*15 showed increased* Axl *RNA (A) and protein expressions (B) compared to control cells CL1-0 and CL1-0-neo (vector alone). Cell invasiveness was also increased in* Axl-*transfected cells compared to the control cells (C).*

levels (Figure 4, *A* and *B*). When cell invasiveness was examined using the standard Transwell assay, more Axl-overexpressing cells invaded the bottom chamber than did the control-vector-alone–expressing cells (CL1-0/neo) and original cells (CL1-0) (Figure 4*C*). As Axl is proposed to be involved in cell proliferation, it is important to ensure that increased cell invasion in these assays is not due to higher growth rates of Axl-overexpressing cells. To exclude this possibility, the growth rate of Axl-overexpressing cell lines was measured, and no significant difference to that of the controls was found (data not shown).

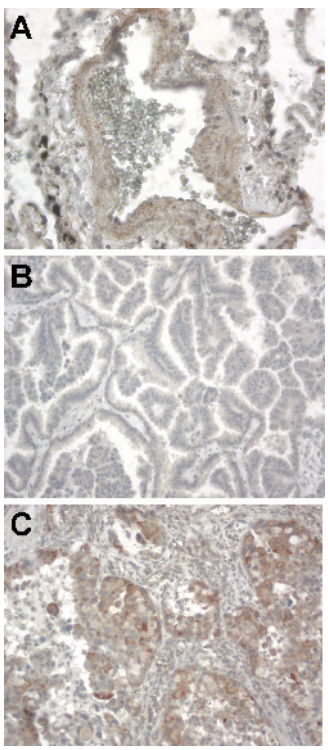

Figure 5. Axl *expression in vascular (A) and cancer tissues with negative (B) and positive staining (C).*

Table 1. Correlation of *Axl* Expression with Clinicopathologic Features of Patients with Lung Cancer.

Clinicopathologic Features	*Axl* Expression		
	Negative (n = 30)*	Positive (n = 28)*	P value
Gender			
Male (n = 31)*	15	16	.389
Female (n = 27)	15	12	
Size (cm)			
≤3.0 (n = 21)	12	9	.364
>3.0 (n = 37)	18	19	
Lymph node involvement			
No (n = 29)	23	6	<.0001
Yes (n = 29)	7	22	
Differentiation			
Well (n = 13)	8	5	.380
Moderate (n = 29)	16	13	
Poor (n = 16)	6	10	
Staging			
Localized (n = 20)	17	3	<.0001
Advanced (n = 38)	13	25	

*Data are numbers of cases. The expression of *Axl* is classified as positive if >25% of tumor cells are stained and as negative if <25% of cancer cells are stained.

Immunohistochemistry in Tumor Tissues

To further examine whether Axl expression relates to disease features of cancer, we compared Axl expression in 58 lung adenocarcinoma patients by immunostaining. Immunoreactivity of Axl was detected in vascular structures (which acted as positive internal control for analysis), as well as in lung cancer cells (Figure 5). In cancer tissue, positive and negative Axl protein expressions were observed in 28 of 58 (48.3%) and in 30 of 58 (51.7%) cases, respectively. Correlations between the incidence of positive immunoreactivity for Axl and the clinicopathologic features of lung adenocarcinoma are shown in Table 1. Axl protein expression was significantly associated with lymph node involvement (P < .0001) and clinical stage (P < .0001).

Discussion

Tumor invasion is a complex process [25] by which malignantly transformed cells detach from the primary tumor, migrate, and cross structural barriers, including basement membranes and surrounding stromal collagenous extracellular matrix [25]. However, our knowledge regarding the mechanism by which tumorigenic cells initiate further invasive and metastatic processes is limited. In an effort to identify genes that are relevant to invasive and metastatic spread, it is advantageous to study variants of the same tumor that differ in their invasion and metastasis patterns. Comparison of phenotypic differences associated with the differential invasive and metastatic abilities of tumor cells may enhance our understanding of the mechanisms underlying

cancer metastasis. Therefore, the ultimate goals of the present study were to identify potential markers that could predict the possibility of tumor invasion and metastasis. Previously, our laboratory isolated the highly invasive cell line CL1-5 from the lung cancer parent cell line CL1-0 using a Transwell chamber [2]. This *in vitro* selection process provides a useful approach to isolating cell variants with different degrees of malignancy, which can be used to assess properties associated specifically with tumor invasion and metastasis. By similar application with the selection procedure in a Transwell chamber, the present study further established sets of cell lines from HT1197 and A59 with different invasive capacities. Using these materials, the mechanisms of invasion and metastasis of cancer could be further investigated.

We were interested in identifying common genes contributing to differences in tumor invasion and metastasis. Therefore, we compared gene expressions among these highly and minimally invasive cell lines, and *Axl* was identified. *Axl* is a known proto-oncogene in the tyrosine kinase family whose exact role in the neoplastic process has not been fully elucidated. The suggestion that Gas6/Axl may be important for invasion and metastasis came from clinical studies. Craven et al. [20] isolated a series of DNA fragments coding for tyrosine kinases that were expressed in a metastatic human colon tumor and subsequently analyzed for their expression patterns at the protein level in human tumors. Axl receptor tyrosine kinase was expressed at levels in a peritoneal metastatic nodule that were 10-fold higher than those in other normal and malignant tissues. Jacob et al. [19] used a modified differential display PCR protocol for isolating 3′ restriction fragments of cDNA specifically expressed or overexpressed in the metastatic prostate carcinoma cell line DU145. In the present study, we further demonstrated that the expression of Axl is associated with lung tumor invasion ability. Taken together, these results suggest that Axl plays an important in tumor invasion and progression.

The involvement of Axl in tumor invasion was observed using overexpression and siRNA knockdown expression. Interestingly, growth kinetics of *Axl*-transfected cells and growth rates showed no differences between *Axl*-transfected and control cells. Similar results also were demonstrated in the study of Wimmel et al. [26] on transfected Axl-negative lung cell lines with expression vectors containing wild-type *Axl*, and they found that no growth occurred in any of the transfected cell lines. Thus, it is possible that the function of Axl is cell- and tissue-specific and may depend on the biologic context in which it is expressed. In addition, our staining results in cancer tissues demonstrated that upregulation of Axl significantly correlated with tumor progression. Although the expression of Axl was not associated with tumor size and differentiation, it significantly correlated with cancer lymph node metastases (Table 1). These results were consistent with previous findings that Axl expression was associated with malignant phenotypes from patients with myeloid leukogenesis [16,20]. Patients with acute myeloid leukemia who had high Axl levels were found to have a worse prognosis for progression-free and overall survival [27]. Thus, tumor staining data confirmed our observations in cell cultures and further extended the role of Axl in tumor invasion and metastasis.

In conclusion, our study demonstrates that Axl expression appears to be involved in cancer invasion. This result shows that Axl expression is involved in tumor progression and that protein expression analysis of Axl may be a biologic marker of tumor progression for patients with lung cancer. Further investigations will be required to determine the intrinsic signaling mechanisms by which Axl modulates cancer cell invasion and cancer progression.

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