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Axl Is a Potential Cancer Prognostic Marker for the Migration and Invasion of Nasopharyngeal Carcinoma

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Abstract

Background. The Axl receptor tyrosine kinase has been demonstrated to be elevated and activated in many human cancers including liver, lung, breast, and pancreatic cancer. Its high expression has been considered as a cancer biomarker for predicting poor prognosis and increased invasiveness/metastasis.

Objectives. The aim of the study was to investigate the clinical significance of Axl in nasopharyngeal carcinoma (NPC) and its role in cell migration and invasion.

Material and Methods. We detected Axl expression in 86 collected NPC tissues and 20 collected normal nasopharyngeal epithelial tissues using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemical staining. Axl was knocked down by a specific shRNA in NPC cell lines, 5-8F and 6-10B. Transwell assays were used to determine NPC cell migration and invasion.

Results. The expressions of Axl mRNA and protein in NPC tissues were significantly higher than those in normal nasopharyngeal epithelial tissues ($p < 0.05$, respectively). The positive expression of Axl was significantly correlated with distant metastasis and high TNM stage in NPC ($p < 0.05$, respectively). Furthermore, Axl positive expression was correlated with a worse overall survival of NPC patients ($p < 0.05$). Multivariate Cox regression analysis indicated that Axl was an independent factor for predicting overall survival of NPC patients ($p < 0.05$). *In vitro* studies found that Axl knockdown significantly reduced the number of migrated and invaded 5-8F and 6-10B cells ($p < 0.05$, respectively).

Conclusions. The positive expression of Axl is correlated with the poor clinicopathological features in NPC. Furthermore, Axl is an independent prognostic marker for predicting overall survival of NPC patients. Functionally, Axl may facilitate tumour progression by promoting NPC cell migration and invasion (*Adv Clin Exp Med* 2016, 25, 3, 531–537).

Key words: prognosis, migration, invasion, Axl, nasopharyngeal carcinoma.

Nasopharyngeal carcinoma (NPC) is an epithelial malignant tumor in nasopharynx, the global statistics of NPC reveal that the highest incidence rates is observed in Southern China. Although the increased prevalence of intensity-modulated radiation therapy with concurrent chemo-radiation therapy has improved the local and regional control of NPC for patients with locoregionally ad-

vanced disease, their prognosis is still poor due to recurrence and/or distant metastasis. Thus, it is important to develop a potent prognostic marker and therapeutic target for human NPC.

Overexpression of Axl has been reported in a multitude of human cancers. Axl is significantly over-expressed in primary lung cancers, and Axl knockdown reduces cell growth and mobili-

Table 1. Correlation between the clinicopathologic characteristics and expression of Axl protein in NPC.

Characteristics		Total No. of patients, n = 86	No. of patients		p-value
			Axl positive	Axl negative	
Age (y)	≤ 45	40	25	15	0.255
	> 45	46	34	12	
Sex	male	64	46	18	0.265
	female	22	13	9	
WHO type	I + II	6	5	1	0.660
	III	80	54	26	
TNM stage	I + II	26	10	16	< 0.001*
	III + IV	60	49	11	
Distant metastasis	yes	19	17	2	0.026*
	no	67	42	25	

NPC – nasopharyngeal carcinoma; TNM – tumor-node-metastasis; WHO – world health organization; * statistically significant.

ty. The expressions of Axl in highly invasive breast cancer cell lines are higher than those in weakly invasive breast cancer cell lines, suggesting that Axl expression is correlated with mobility and invasiveness of breast cancer cells. Otherwise, Axl has been considered as an independent prognostic marker and potential therapeutic target in acute myeloid leukemia (AML). Elevated expression of Axl has been demonstrated to promote migration and invasion of prostate cancer cells *in vitro*. Furthermore, Axl is also associated with a higher frequency of distant metastasis after surgical treatment in patients with pancreatic adenocarcinoma. However, the clinical significance of Axl and its role in NPC metastasis are poorly investigated.

In this study, the expression status of Axl in human NPC was tested by immunostaining and qRT-PCR. The correlation between Axl expression and clinicopathologic features of NPC were systemically analyzed. Furthermore, we explored the role of Axl in tumor cell migration and invasion to confirm the effect of Axl on the initiation and development of human NPC.

Material and Methods

Clinical Samples

A total of 86 paraffin-embedded NPC and 20 normal nasopharyngeal epithelial tissue samples were obtained from the Department of Otolaryngology Head and Neck Surgery, the 1st Affiliated Hospital of Bengbu Medical College during January 2008 to December 2010. All samples were used

after having obtained informed consent. The demographic and clinicopathological parameters are shown in Table 1. All specimens had confirmed pathological diagnosis and were classified according to the World Health Organization (WHO) criteria. The Bengbu Medical College Ethics Committee approved all protocols according to the Helsinki Declaration (as revised in Edinburgh 2000).

Immunohistochemical Staining

Immunohistochemistry with streptavidin peroxidase conjugated (SP-IHC) method was performed on formalin-fixed paraffin sections. Sections that underwent dewaxed, rehydration, antigen retrieval; endogenous peroxidase activity blocking and goat serum blocking were incubated with anti-Axl (R&D Systems, Minneapolis, USA) antibody at 4°C overnight. SP conjugated secondary antibody (ZSGB-BIO, Beijing, China) and diaminobenzidine were used for staining of sections. Axl expression was classified as negative expression (less than 10%) and positive expression (equal and more than 10%) in accordance with the percentage of positive cells.

Real Time Quantitative Reverse Transcription-PCR (qRT-PCR)

Axl primers (forward: 5'- CCGGCTGGC-GTATCAAGGCC -3'; reverse: 5'- TGGCTGT-GCTTGCCCTGGG -3'). RNA was extracted, treated with DNaseI and reverse transcribed using a RNA isolation and cDNA synthesis kit (Quia-

gen, Hilden, Germany) as recommended by the manufacturer. A SYBR[®] Premix Ex Taq[™] ii (Perfect Real Time) Kit (Takara Bio, Shiga, Japan) was used to quantify the Axl mRNA and the GAPDH mRNA via the PCR amplification, as previously described.

Cell Line and Transfection

Two human NPC cell lines, 5-8F and 6-10B were purchased from Chinese Academy of Sciences, Shanghai, China. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Gibco) with 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma, St-Louis, USA) at 37°C with 5% CO₂.

SiRNAs, including Axl siRNA (sc-29769) and scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA) and were transfected into NPC cells using Lipofectamine 2000 in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, USA).

Western Blot

Immunoblotting assays were performed using Axl (R&D Systems) and GAPDH (G8140, US Biological, Salem, USA) antibodies, which followed by Horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, USA) at a dilution from 1:1000–1:5000 and detected by a Western Blotting Luminol Reagent (sc-2048, Santa Cruz, USA).

Transwell Cell Migration and Invasion Assays

Transwell cell migration assays were done in 12 well plates with 8-µm BioCoat control inserts (Becton Dickinson Labware, Bedford, MA). $1-2 \times 10^5$ Axl siRNA or scrambled siRNA transfected 5-8F or 6-10B cells that were suspended in 500 µL serum free DMEM were seeded in the upper well and DMEM medium with 10% FBS, as indicated, in the lower well. Membranes were removed after completion and were wiped on the side facing the upper well. Then, crystal violet was used for staining. At least 6 representative images of each well were taken and cell numbers were counted using ImageJ. BioCoat Matrigel invasion chamber (Becton Dickinson Labware) was used for transwell cell invasion assays and the following protocols were the same as transwell cell migration assays. The experiments were performed in triplicate.

Statistical Analysis

All data is presented as the mean $\pm$ SEM from at least three independent replicates. Where appropriate, a Pearson χ^2 test, a Kaplan-Meier plot, a log-rank test, the multi-variant Cox regression analysis and a two-tailed Student's *t* test were used with the SPSS statistical package for Windows v. 13 (SPSS, Chicago, USA) or GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, USA). A *p* value of 0.05 or less was considered significant.

Results

Expression of Axl in NPC Tissues

To determine the expression of Axl in NPC specimens, we detected the levels of Axl expression in 86 collected NPC tissues and 20 collected normal nasopharyngeal epithelial tissues using immunohistochemical staining. The Axl expression was considered as either negative or positive. Our data indicated that Axl positive expression was observed in 68.6% (59/86) of NPC tissues, while only 20.0% (4/20) of normal nasopharyngeal epithelial tissues show a positive Axl signal ($p < 0.001$, Fig. 1A). Furthermore, we performed real-time PCR to determine the levels of Axl mRNA in NPC tissues ($n = 20$) and normal nasopharyngeal epithelial tissues ($n = 20$). Quantitative analysis indicated that the level of Axl mRNA in NPC tissues was significantly higher than that in normal nasopharyngeal epithelial tissues ($p < 0.001$, Fig. 1B).

Clinical Significance of Axl in NPC Cases

To explore the clinicopathologic significance and prognostic value of Axl in NPC, we analyzed the correlation between Axl expression and clinicopathological parameters in NPC. As shown in Table 1, Clinical association analysis using a Pearson χ^2 test indicated that Axl positive expression was evidently correlated with high TNM stage ($p < 0.001$) and distant metastasis ($p = 0.026$). Furthermore, Kaplan Meier estimation indicated that tumors with the positive expression of Axl indeed associated with poor overall and disease-free survival of NPC patients ($p = 0.047$ and 0.039 , respectively, Fig. 2). Importantly, Axl expression was an independent prognostic marker for indicating overall and disease-free survival of NPC patients ($p = 0.042$ and 0.039 , respectively, Table 2). Thus, Axl may function as a potent biomarker for indicating prognosis of NPC patients.

Fig. 1. Expression of Axl in NPC and normal nasopharyngeal epithelial tissues. A) Negative (I), low (II), medium (III) and high (IV) expression of Axl in the tumor cells of NPC tissue. The expression of Axl protein in NPC tissues was significantly higher than that in normal nasopharyngeal epithelial tissues. Original magnification $\times 400$; B) Quantitative analysis using qRT-PCR indicated that the levels of Axl mRNA in NPC tissues were significantly higher as compared with that in normal nasopharyngeal epithelial tissues. * $p < 0.05$.

Fig. 2. Kaplan-Meier survival analysis of 86 NPC patients according to Axl protein expression. The positive expression of Axl conferred an unfavorable overall and disease-free survival for NPC patients.

Table 2. Multivariate Cox regression analysis of overall survival duration

Variables	Overall survival			Disease-free survival		
	HR	95% CI	p-value	HR	95% CI	p-value
TNM stage	2.87	1.42–5.82	0.003*	2.59	1.43–4.69	0.002*
Axl expression	1.85	1.02–3.35	0.042*	1.70	1.03–2.82	0.039*

TNM – tumor-node-metastasis; HR – hazard ratio; CI – confidence interval; * statistically significant.

Axl Knockdown Inhibits NPC Cell Migration and Invasion

To confirm the role of Axl in NPC, 5-8F and 6-10B cells that were transduced with scrambled siRNA or Axl siRNA were subjected to Transwell assays for cell migration and invasion. As determined by WB, the level of Axl protein was obviously down-regulated by Axl siRNA in both 5-8F and 6-10B cells (Fig. 3). Transwell cell migration assays were performed to determine the effect of altering Axl levels on tumor cell migration. We found that Axl knockdown resulted in a significant reduction of both 5-8F and 6-10B cell migration ($p < 0.001$, respectively; Fig. 4A). Moreover, as measured by Transwell cell invasion assays, the

Fig. 3. Axl is knocked down by Axl siRNA in NPC cells. NPC 5-8F and 6-10 cells that were transduced with scrambled siRNA or Axl siRNA were subjected to WB for Axl. Representative WB showed that Axl was significantly knocked down by Axl siRNA. Data is representative of multiple repeats with similar results

Fig. 4. Axl knockdown suppresses NPC cell migration and invasion. A) Cell migration as measured by Transwell assays was inhibited by Axl knockdown in 5-8F and 6-10 cells as compared with cells with scrambled siRNA transfection. $n = 6$, $*p < 0.05$; B) Cell invasion was suppressed by Axl knockdown in 5-8F and 6-10 cells; $n = 6$; $*p < 0.05$

amount of invaded 5-8F and 6-10B cells was prominently decreased after Axl knockdown ($p < 0.001$, respectively; Fig. 4B). Thus, Axl may exert a pro-metastatic effect by promoting migration and invasion of NPC cells.

Discussion

Overexpression of Axl correlates with the poor survival of tumor patients and was detected in various types of cancer. Activation of Axl signaling by its ligand Gas6 leads to enhanced proliferation, survival, invasion and metastasis of cancer cells. However, clinical and mechanistic evidences for crucial functions of Axl in NPC progression are very poor. In the present study, Axl expression was detected in 86 NPC tissues and 20 normal nasopharyngeal epithelial tissues using immunohistochemical staining. We found that Axl protein expression in the NPC tissues was prominently higher than that in the normal nasopharyngeal epithelial tissues. Moreover, qRT-PCR results indicated that the difference of Axl mRNA expression between NPC tissues and normal nasopharyngeal epithelial tissues was consistent with Axl protein expression. Clinical analysis showed that the positive expression of Axl was obviously correlated with high TNM stage and distant metastasis in NPC. Importantly, our data showed that Axl positive expression conferred a significant

worse overall survival and disease-free survival for NPC patients. Multivariate Cox regression analysis showed that Axl was an independent biomarker for indicating the overall survival and disease-free survival of NPC patients. Taken together, our results indicate that Axl may be a potential oncogene and act as a prognostic biomarker for predicting survival of NPC patients.

Axl has recently been considered as a crucial factor in tumorigenesis and tumour progression. However, what is the role of Axl in NPC? Here, we disclosed new role of Axl in NPC. Axl was knocked down in 5-8F and 6-10B cells *via* exogenous siRNAs transfection. Transwell cell migration assays found that Axl knockdown led to a significant reduction of cell migration in both 5-8F and 6-10B cells. Otherwise, Transwell cell invasion assays showed that Axl knockdown decreased the number of invaded 5-8F and 6-10B cells. Our data indicates that IF1 may promote tumour progression by promoting cell migration and invasion in NPC. Several recent studies have shown that Axl expression is associated with epithelial-mesenchymal transition (EMT). EMT, a dynamic and reversible cellular process, is characterized by the loss of cell polarity and intracellular junctions and acquirement of mesenchymal features, resulting in increased the migration and invasion of cancer cells. Cancer cells that were underwent EMT lead to tumor metastasis and poor survivals for patients. Several studies have reported that NPC cells with EMT exhibit

enhanced invasion and metastatic potential. Furthermore, tumor tissues that were collected from NPC patients were used for molecular subtyping, the data indicated that tumors with mesenchymal gene characteristics conferred a worse overall survival and treatment-resistant in patients, suggesting that EMT plays a key role in the development of NPC. Thus, we hypothesized that Axl may facilitate cell migration and invasion by promoting EMT in NPC.

In conclusion, our present study disclosed that the expression of Axl is elevated in NPC tissues and its positive expression is associated with poor prognostic parameters. Furthermore, Axl is an independent prognostic biomarker for indicating the overall survival and disease-free survival of NPC patients. Axl knockdown decreases the amount of migrated and invaded NPC cells. Altogether, Axl may be a potential valuable prognostic marker and therapeutic target for human NPC.

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