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

Chengyi Jiang1, A, D, F, Lei Zhou2, B, E, F, Hongtao Wang3, B, F, Qiang Zhang4, C, F, Yajia Xu1, C, F

1 Department of Otolaryngology Head and Neck Surgery, The First Affiliated Hospital of Bengbu Medical College, Bengbu, China
2 Department of Pathology, Bengbu Medical College, Bengbu, China
3 Department of Immunology, Bengbu Medical College, Bengbu, China
4 Department of Laboratory, The First Affiliated Hospital of Bengbu Medical College, Bengbu, China

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.
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 significant 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 systematically 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.

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total No. of patients, n = 86</th>
<th>No. of patients</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Axl positive</td>
<td>Axl negative</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>≤ 45</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>&gt; 45</td>
<td>46</td>
<td>34</td>
</tr>
<tr>
<td>Sex</td>
<td>male</td>
<td>64</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>WHO type</td>
<td>1 + II</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>80</td>
<td>54</td>
</tr>
<tr>
<td>TNM stage</td>
<td>1 + II</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>III + IV</td>
<td>60</td>
<td>49</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>yes</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>67</td>
<td>42</td>
</tr>
</tbody>
</table>

NPC – nasopharyngeal carcinoma; TNM – tumor-node-metastasis; WHO – world health organization; * statistically significant.
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 × 10⁵ 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 ± SEM from at least three independent replicates. Where appropriate, a Pearson χ² 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 χ² 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.
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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall survival</th>
<th>Disease-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>TNM stage</td>
<td>2.87</td>
<td>1.42–5.82</td>
</tr>
<tr>
<td>Axl expression</td>
<td>1.85</td>
<td>1.02–3.35</td>
</tr>
</tbody>
</table>

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
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 repression 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 onco-gene 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 acquisition 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.

References


Address for correspondence:
Chengyi Jiang
Department of Otolaryngology Head and Neck Surgery
The First Affiliated Hospital of Bengbu Medical College
Bengbu 233004
China
E-mail: jiangchengyi.2014@163.com

Conflict of interest: None declared

Received: 31.10.2014
Revised: 9.11.2014
Accepted: 24.03.2015