Expression of Axl and its prognostic significance in human breast cancer

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Abstract. Breast cancer is the most common malignant cancer and second leading cause of cancer-related death among women, and its prevalence continues to increase. Axl overexpression has been identified in the many types of human cancer, and it has been demonstrated to participate in signaling pathways related to carcinogenesis and cancer development. In the present study, Axl expression was examined by performing immunohistochemical staining in 60 breast cancer tumors and 40 benign breast lesions (25 mammary dysplasia and 15 breast fibroadenoma). In total, 34 (56.67%) cancer tissues and 13 (32.5%) benign breast lesions were classified as exhibiting high levels of Axl expression, indicating a significant association between malignancy and high Axl expression. High Axl expression was also associated with estrogen receptor (ER) positivity (P=0.028), progesterone receptor (PR) positivity (P=0.007), and poor tumor differentiation (P=0.033). No significant associations were observed between Axl expression and age, tumor size, lymph node metastasis, tumor node metastasis staging, human epidermal growth factor receptor 2 and Ki67 antigen. The Kaplan-Meier survival analysis and Cox proportional hazard model both demonstrated that there was no statistical difference between Axl expression and breast cancer prognosis. However, it remains unclear whether the expression of Axl is correlated with the prognosis of luminal type breast cancer patients.

Introduction

Breast cancer is one of the most common malignant cancers among women, and in some cases, is life threatening (1). The American Cancer Society has reported that new cases of invasive breast cancer in the USA will reach 246,660 by 2016, accounting for 29% of de novo malignancies among women, and breast cancer-related mortality is estimated to reach 40,450 in 2016 (2). In China, breast cancer is the most prevalent cancer in women, and the number of new cases diagnosed is increasing every year (3). Developing novel biomarkers of breast cancer may improve knowledge of how breast cancer develops, and potentially allow more efficient therapeutic strategies to be implemented.

Tyrosine-protein kinase receptor UFO (Axl), belongs to the TAM family of receptor tyrosine kinases, which consists of Axl, tyrosine-protein kinase receptor Tyro3, and tyrosin protein kinase Mer. Axl is a transforming gene that was originally isolated from patients with chronic myelogenous leukemia (4). The growth arrest specific gene 6 (Gas6) is a ligand of Axl, and the binding of Gas6 to Axl results in the dimerization of Gas6/Axl complexes, activating intracellular tyrosine kinase, and triggering a series of signal transduction and biological effects (5). It has previously been reported that Axl participates in signaling pathways related to carcinogenesis and cancer development, such as the phosphatidylinositol 3-kinase/protein kinase B pathway, the Janus kinase/signal transducers and activators of transcription pathway, and nuclear factor-κB signaling pathway (6-8). It has also been demonstrated that Axl is an underlying oncogenic factor involved in the epithelial-mesenchymal transition, which allows epithelial cells to undergo cell migration and invasion, and thus contribute to tumor metastasis (9). Axl has been implicated in different types of human cancer and is related to their development and clinical prognosis. These include: Gastric (10), lung (11), bladder (12), thyroid (13), pancreatic (14) and colon (15) cancer.

Methods of blocking Axl expression by RNA silencing or Axl inhibition have been studied in vivo and in vitro. The results of these studies suggest that Axl is a promising target in cancer therapy. For example, LY2801653, a multi kinase inhibitor of the hepatocyte growth factor receptor (MET) and Axl, is capable of inhibiting cell migration, proliferation and anti-tumor activities (16). The effects of inhibiting Axl expression by Amuvatinib (MP470) have been studied in gastrointestinal stromal tumors and breast cancer (17). Furthermore, Foretinib, an experimental multi kinase inhibitor

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targeting MET, vascular endothelial growth factor receptor 2, and Axl, may inhibit the proliferation and survival of colorectal cancer cells (18).

The association between Axl and breast cancer has been widely researched. However, the clinicopathological significance and the prognostic role of Axl in breast cancer remains controversial, and there have been few studies investigating the exact role of Axl in breast cancer. The present study aimed to measure Axl expression in breast cancer tissue and noncancerous lesions by immunohistochemical staining, and to explore the prognostic value of Axl in patients with breast cancer.

Materials and methods

Patients and surgical specimens. Specimens were obtained from 60 female patients (average age, 50.60±1.36) undergoing surgery at the Department of Breast Surgery, the First Affiliated Hospital, Henan University of Science and Technology, between January and August 2010. Informed consent was obtained from all the patients to use the surgical specimens for scientific research. The specimens included 60 cases of breast infiltrative ductal cancer tissues and 40 cases of benign breast lesions (25 mammary dysplasia and 15 breast fibroadenoma). The median age of patients at breast cancer diagnosis was 48 years old (range, 28-80 years). None of the patients received cheioradiotherapy or endocrine therapy before surgery, and there were no obvious abnormalities in the preoperative function of the heart, lungs, liver, or kidney of any patients. There was also no distant metastasis detected in any patients before operations were performed. The clinicopathological characteristics of the patients with breast cancer are presented in Table I.

Postoperative treatment was performed according to the National Comprehensive Cancer Network (NCCN) guidelines. The tissue specimens were fixed in 10% formaldehyde solution and embedded in paraffin blocks. Paraffin blocks were cut into sections 3 µm thick. All cases of breast cancer were confirmed by professional pathologists by hematoxylin-eosin (HE) staining, and were categorized according to the 7th edition of the American Joint Committee on Cancer Tumor-Node-Metastasis (TNM) stage classification (19). The molecular subtyping of breast cancer followed the guidelines issued at the St. Gallen International Breast Cancer Conference 2013 (20); the luminal type included luminal A and luminal B type breast cancer, the non-luminal type included Erb-B2 overexpression and basal-like type breast cancer.

Immunohistochemistry. The paraffin sections of cancerous and benign lesions were baked for 2 h at 60°C, deparaffinized with dimethylbenzene and hydrated in gradient ethanol. Tissue antigen retrieval was performed using citrate sodium buffer (pH 6.0) at 95°C for 15 min and cooled at room temperature for 30 min. Endogenous peroxidase was blocked with methanol containing 3% H₂O₂ for 10 min, then the slides were treated with 5% normal goat serum (SL2; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min to limit non-specific binding. The anti-Axl rabbit polyclonal antibody (1:100; ab37861; Abcam, Cambridge, MA, USA) was overlaid on the sections and incubated overnight at 4°C. Following re-warming at room temperature for 1 h and three washes in PBS (AR0030; Wuhan Boster Biological Technology, Ltd., Wuhan, China), sections were incubated with horseradish peroxidase-labeled secondary antibody (1:500; SA00001-2; Wuhan Sanying Biotechnology, Wuhan, China) for 30 min at room temperature. Diaminobenzidine (DAB-0031; Fuzhou Maixin Biotech Co., Fuzhou, China) was used for antigen detection. Subsequently, the slides were counterstained with hematoxylin for 10 sec, dehydrated in gradient ethanol and mounted, and then viewed under an optical microscope. Negative controls used for comparison were incubated with phosphate buffer saline instead of the primary antibody.

Evaluation of immunohistochemical staining. Sections were evaluated independently by two different investigators with no prior knowledge of patient clinical information, and disagreements were resolved through discussion. Human epidermal growth factor receptor 2 (HER-2) staining was carried out and evaluated according to the standards provided by NCCN: +, ±, + were all defined as negative; +++ as positive, and FISH quantitative determination was conducted when the staining score ≥++ (21).

Axl expression score was based on an intensity score (IS) and a proportion score (PS). IS was graded on the following scale: No staining, 0; mild staining, 1; moderate staining, 2; intense staining, 3; extremely intense staining, 4. The PS was graded as follows: ≤10%, 0; 11-25%, 1; 26-50%, 2; 51-75%, 3; >75%, 4. Therefore, the range of the combined staining score (IS+PS) was 0-8. A total score ≤1 was considered to be negative (-); a score of 2-3 was considered to be weakly positive (+); a score of 4-6 was considered to be positive (++); a score of 7-8 was considered to be strong positive (+++). Scores of (-) and (+) were regarded as low levels of expression, whereas scores of (++) and (+++) were regarded as high levels of expression.

Follow-up information. The 60 breast cancer patients were followed up by telephone conversation, although home visits were also undertaken if required. Telephone numbers and addresses were acquired from patient records. The follow-up deadline was September 2015. Patient survival time was calculated from the date of operation until the date of cancer-related mortality. Disease-free survival time (DFS) and overall survival time (OS) were adopted as prognostic indexes. The DFS events included locoregional recurrence, distant metastasis and contralateral breast cancer. OS events included all cancer-related mortality. Recurrence, distant metastasis and contralateral metastasis of patients were confirmed by X-ray plain film, ultrasonography, computed tomography and pathological examination.

Statistical analysis. The association between Axl expression and clinicopathological features were analyzed by χ² or Fisher’s exact tests. Correlational analyses were conducted using the analysis of Spearman rank correlation. Patient survival was estimated using the Kaplan-Meier method and compared with the log rank test. Prognostic factors were analysed by the Cox proportional hazard model, and the assignment of risk factors is presented in Table II. All statistical analyses were performed using the SPSS 21.0 program (IBM SPSS, Inc., Armonk, NY, NY, USA).
USA). P<0.05 was considered to indicate a statistically significant difference.

Results

**Axl is highly expressed in malignant breast cancer tissue compared with benign breast lesions.** Positive immunoreactivity for Axl was mainly observed in the cell cytoplasm and membrane; the positive cells were stained brown and diffusely distributed (Fig. 1). In total, 34 (56.67%) patients with breast cancer were classified as ++ or ++++, indicating high levels of Axl expression. Regarding Axl immunoreactive intensity, 16 patients were classified as negative, 10 as weak, 18 as moderate and 16 as intense. Furthermore, there were 13 patients with benign breast lesions exhibiting high expression of Axl, which accounted for 32.50% of all benign breast lesion cases (13/40). There was a significant difference in Axl expression between malignant breast cancer and benign breast lesions (P=0.018; Table III).

**Correlation between Axl expression and clinicopathological characteristics.** There was no significant correlation between Axl expression and age, tumor size, lymph node metastasis, TNM staging, HER-2, and the expression of Ki67 protein, which is associated with cell proliferation. However, a significant correlation was observed between Axl expression and tumor histologic grade, estrogen receptor (ER) expression and progesterone receptor (PR) expression (P=0.033, P=0.028, P=0.007; Table IV). Increased expression of Axl in luminal breast cancer was observed in 69.44% (25/36) of cases, and was significantly higher than positive Axl expression in non-luminal breast cancer which was 37.50% (9/24), P=0.014; Table IV). Moreover, Spearman rank correlation demonstrated that Axl expression was positively correlated with histologic grade (r=0.275, P=0.034), ER (r=0.284, P=0.028) and PR (r=0.364, P=0.007; all Table V).

**Correlation between Axl expression and prognosis of patients with breast cancer.** In the present study, 5 patients were lost to follow-up after surgery. In total, 12 cases of
recurrence/metastasis and 10 cases (one patient died in an accident) of mortality were observed in the follow-up. Kaplan-Meier survival analysis (Fig. 2) demonstrated that there were no significant differences between Axl expression level and DFS/OS (P\textsubscript{log-rank}>0.05). Therefore, it remains unclear whether there is an association between

| Table III. Difference of Axl expression between malignant breast cancer and benign breast lesions. |
|--------------------------------------------------|
| Axl expression | Breast cancer (n=60) | Benign breast lesion (n=40) | $\chi^2$ | P-value |
|----------------|---------------------|-----------------------------|---------|---------|
| High expression | 34                  | 13                          | 5.627   | 0.018*  |
| Low expression  | 26                  | 27                          |         |         |

*aStatistical significance (P<0.05). Axl, tyrosine-protein kinase receptor UFO.

| Table IV. Association between Axl expression and clinicopathological characteristics in breast cancer tissues. |
|--------------------------------------------------|
| Variables | Low expression | High expression | $\chi^2$ | P-value |
|-----------|----------------|----------------|---------|---------|
| Age, years |                |                | 0.134   | 0.714   |
| ≤50       | 15             | 18             |         |         |
| >50       | 11             | 16             |         |         |
| Histological grade |        |                | 4.533   | 0.033*  |
| G1        | 9              | 4              |         |         |
| G2/G3     | 17             | 30             |         |         |
| Tumor size, cm |            |                | 1.629   | 0.202   |
| <2        | 8              | 16             |         |         |
| ≥2        | 18             | 18             |         |         |
| Lymph node status |        |                | 0.008   | 0.930   |
| N0        | 15             | 20             |         |         |
| N+        | 11             | 14             |         |         |
| TNM stage |                |                | 2.267   | 0.322   |
| I         | 7              | 12             |         |         |
| II        | 11             | 17             |         |         |
| III       | 8              | 5              |         |         |
| ER        |                |                | 4.848   | 0.028*  |
| Negative  | 15             | 10             |         |         |
| Positive  | 11             | 24             |         |         |
| PR        |                |                | 7.186   | 0.007*  |
| Negative  | 19             | 13             |         |         |
| Positive  | 7              | 21             |         |         |
| HER-2     |                |                | 0.979   | 0.322   |
| Negative  | 16             | 25             |         |         |
| Positive  | 10             | 9              |         |         |
| Ki-67, %  |                |                | 3.279   | 0.070   |
| ≤14       | 5              | 14             |         |         |
| >14       | 21             | 20             |         |         |
| Molecular subtype |        |                | 5.984   | 0.014*  |
| Luminal type | 11           | 25             |         |         |
| Non-luminal type | 15           | 9              |         |         |

*aStatistical significance (P<0.05). Axl, tyrosine-protein kinase receptor UFO; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Ki-67, antigen Ki-67.
Axl expression and the OS/DFS of the breast cancer patients.

Cox regression analysis results for the prognostic factors of the 60 patients with breast cancer. Potential prognostic factors of 60 cases breast cancer patients were analyzed by the Cox proportional hazard model to explore the relationship between patient survival and several explanatory factors. The results demonstrated that HER-2 was the main factor associated with the DFS of patients with breast cancer (P=0.013; Table VI). Age and HER-2 were the factors associated with the OS of breast cancer patients (P=0.037, P=0.017; Table VI). However there was no significant association between increased Axl expression and DFS/OS.

Discussion

The enhanced expression of Axl has been reported in different types of cancer, indicating that Axl may be important in the onset and progression of malignant tumors. Axl is a member of the TAM receptor tyrosine kinase subfamily, which regulates a series of pathophysiological processes including...
cell proliferation, migration, invasion, cytokine release and apoptosis (22-24). Furthermore, Holland et al (25) have demonstrated that Axl is essential for endothelial cell proliferation, migration, lumen formation, and angiogenesis regulation in tumor formation, which supports tumor progression.

In the present study, Axl expression was assessed by analysing samples taken from patients with breast cancer. The results indicated that Axl expression was significantly upregulated in breast cancer cells compared with benign breast lesions (mammary dysplasia and breast fibroadenoma), which is consistent with the results from a previous study by Berclaz et al (26). Therefore, increased Axl expression may facilitate tumorigenesis and the progression of breast cancer. Furthermore, it may be developed as a method of distinguishing between malignant and benign breast lesions.

The current study demonstrates that Axl overexpression correlates with histological grade in breast cancer. Ahmed et al (27) previously suggested that increased Axl expression was associated with the malignant grade of breast cancer. However, the current study did not find any association between Axl overexpression and other invasive indicators, such as tumor size, lymph node metastasis, TNM staging, HER-2, and Ki-67.

The results of previous studies are conflicting regarding the association between Axl expression and ER status in breast cancer. D’Alfonso et al (28) investigated 569 cases of breast cancer and suggested that there was no relationship between Axl expression and ER status. Ahmed et al (27) reported that high expression of Axl was associated with ER negativity, whereas, Berclaz et al (26) suggested that Axl expression was significantly associated with ER status; Axl expression was confined to ER positive tumors, however, not all ER positive tumors expressed the Axl protein. It was therefore hypothesized that ER mediates the upregulation and activation of Axl, and inhibits cell apoptosis by overexpressing Bcl-2 (an anti-apoptotic gene), leading to the malignant characteristics observed in tumor cells (26).

The results of the current study were similar to those obtained by Berclaz et al (26), however, a minority of ER negative tumors were also identified as overexpressing Axl. The link between Axl expression and ER status remains contentious, and further research investigating larger samples are warranted.

The current study demonstrated that Axl expression is positively correlated with PR status. This differs from the results of previous studies (26,27), and suggests that there is a relationship between Axl expression and PR status. However, the mechanism behind this relationship remains unclear. Furthermore, it was demonstrated that Axl expression was higher in cases of luminal breast cancer than non-luminal
cases, contrary to previous reports by Ahmed et al (27) and Neve et al (29). This may be due to the association between luminal subtype and ER status in breast cancer.

Patient follow-up demonstrated that there was no correlation between Axl expression and the prognosis of breast cancer, contrary to previous reports (26). This suggests that Axl expression is not associated with the clinical outcome of luminal and non-luminal type breast cancer. However, a correlation between Axl expression and breast cancer prognosis may indeed exist. The follow-up time in the present study was short, as the 5-year survival rate of patients with breast cancer is relatively high [currently 90.6% for those diagnosed in 2006 (30)]. A larger sample size with a longer follow-up time is required to make more concrete conclusions. Non-luminal (triple-negative and Basal) breast cancer is clinically more aggressive, with higher rates of recurrence and worse prognostic outcomes, compared with other types of breast cancer (31-33). The current study demonstrated that Axl expression was higher in luminal type breast cancer and positively correlated with histological grade. Luminal breast cancer often presents a better prognostic outcome, however the cancer histological grade positively correlates with tumor malignancy. Therefore, further study is required to determine whether there is a significant association between expression of Axl and the prognosis of patients with luminal type breast cancer.

In conclusion, the present study demonstrated that Axl is highly expressed in breast cancer tissues and is associated with poorly differential tumors, indicating that it serves an important role in the carcinogenesis and development of breast cancer. High Axl expression was significantly correlated with ER and PR status, indicating that Axl may contribute to the upregulation of ER and PR. However, its exact mechanism of action warrants further investigation. No obvious connection was demonstrated between levels of Axl expression and the prognosis of patients with breast cancer. Whether Axl expression is correlated with the prognosis of luminal type breast cancer patients requires further investigation. In the future, if a significant correlation is proven; Axl may be a promising biomarker for breast cancer malignancy and prognosis.

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