AXL is a marker for epithelial-mesenchymal transition in esophageal squamous cell carcinoma

GUOAN ZHANG[1*], XIA KONG[2*], MENG WANG[3], HONGLI ZHAO[4], SHA HAN[5], RONGHANG HU[6], JIAN HUANG[7] and WEN CUI[1]

[1]Cancer Pathology Research Institute, Jining Medical University, Jining, Shandong 272067; Departments of [2]Pharmacy and [3]Oncology, Shandong Jining No. 1 People's Hospital, Jining, Shandong 272011; [4]Department of Gastroenterology, Shandong Control Center for Digestive Diseases, Jining, Shandong 272033; [5]Life Science Experimental Center, Jining Medical University, Jining, Shandong 272067; [6]Department of Thoracic Surgery; [7]Central Laboratory, Affiliated Hospital of Jining Medical University, Jining, Shandong 272029, P.R. China

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Abstract. Esophageal squamous cell carcinoma (ESCC) is a common cancer in China and certain other parts of the world with a dismal prognosis for affected patients. AXL is a member of the TYRO3-AXL-MER family of receptor tyrosine kinases, and has been revealed to be an important mediator of epithelial-mesenchymal transition (EMT) in several types of cancer. However, to the best of our knowledge, its function in EMT in ESCC cells has not yet been examined. The present study employed two independent ESCC mRNA profile datasets and revealed that AXL is associated with several EMT markers. Gene Set Enrichment Analysis indicated that EMT occurs more in ESCC with high AXL expression. Analysis on another dataset demonstrated further that increased expression of AXL in ESCC is associated with increased migratory ability. Collectively, the results of the present study provide evidence that AXL is a marker for EMT in ESCC.

Introduction

AXL is a member of the TYRO3-AXL-MER family of receptor tyrosine kinases, which includes two other members: TYRO3 and MER. AXL is activated by its ligand, growth arrest-specific 6, which in turn triggers several downstream signaling pathways depending on the cell type, primarily phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase/extracellular-signal-regulated kinase, nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) signaling pathways. It exerts a wide array of functions including cell survival, proliferation, migration and adhesion (1). AXL has also been implicated in the pathophysiology of a number of types of cancer, including breast, gastric, prostate, ovarian and lung (2-4). AXL expression promotes cancer cell survival, angiogenesis, metastasis and drug resistance (3,5).

AXL is significantly associated with epithelial-mesenchymal transition (EMT) in several tumors. RNA sequencing data of 643 cancer cell lines demonstrated that AXL expression is markedly associated with a mesenchymal phenotype (6). AXL expression is increased in mesenchymal cells compared with epithelial cells in non-small cell lung cancer (NSCLC) cell lines (7). Furthermore, AXL downregulation in A549 and H460 mesenchymal cells leads to enhanced expression of epithelial cadherin (E-cadherin) and decreased expression of vimentin and neural cadherin (N-cadherin), which are features of mesenchymal-epithelial transition (7,8), suggesting that AXL is involved in maintaining EMT. AXL has also been identified to regulate EMT in other types of cancer, including breast (9,10), prostate (11), ovarian (12) and pancreatic (13,14).

China is among the countries at highest risk of esophageal squamous cell carcinoma (ESCC) (15). ESCC is the fourth most common cancer in China, accounting for ~13% of all cancer cases in 2015 (16). ESCC is frequently associated with a high risk of recurrence and high mortality rate and the 5-year survival rate is <20% (17).

The function of AXL in ESCC has been demonstrated only recently (18-20). AXL gene (19) and protein (19,20) expression were upregulated in ESCC cells compared with normal adjacent cells, and were identified to be associated with tumor progression, increased risk of mortality and distant metastasis (20). AXL was also identified to be consistently overexpressed in ESCC cell lines (19). Knockdown of AXL expression inhibited cell proliferation, survival, migration and invasion *in vitro* and *in vivo*, and those effects were mediated by the AKT/NF-κB and AKT/glycogen synthase kinase-3β signaling pathways (19). Overexpression of AXL

Correspondence to: Dr Wen Cui, Cancer Pathology Research Institute, Jining Medical University, 16 Beihu Road, Jining, Shandong 272067, P.R. China
E-mail: cuiwenmdd@163.com

*Contributed equally

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also mediated resistance to the PI3Kα inhibitor BYL719 by activating signaling pathways in ESCC cells (18).

However, the function of AXL in EMT in ESCC cells is not well-documented. To the best of our knowledge, the present study provides the first evidence that AXL is a marker for the mesenchymal phenotype in ESCC using online mRNA profile data.

Materials and methods

Gene expression data. GSE47404 (21), GSE23400 (22) and GSE21293 (23) gene expression data were obtained from the Gene Expression Omnibus database (GEO; www.ncbi.nlm.nih.gov/geo). Further information on the sample size and the microarray platforms used for the creation of these datasets is presented in Table I. Processed expression data and information of corresponding platforms were downloaded and used further. Expression values (mRNA $\log_2$ intensity) for each gene were examined manually and used for further study.

Gene Set Enrichment Analysis (GSEA). GSEA is a computational software that determines whether predefined set of genes exhibit statistically significant concordant differences between two biological states (http://software.broadinstitute.org/gsea/index.jsp) (24). AXL expression was determined on the basis of GSE47404 and GSE23400 datasets. If two probe sets correspond to AXL, AXL expression is calculated as the average of the value of the two probe sets. Following determination of the mean expression of AXL, the samples were designated AXL-high ESCC (samples with an above-average AXL value) or AXL-low ESCC (samples with a below-average AXL value). AXL-low samples served as the control samples. This collection of gene sets summarized and represented specific well-defined biological states or processes, and was generated by a computational methodology based on identifying gene set overlaps and retaining genes that display coordinate expression across several datasets (25). All the parameters were set to their default values. A false discovery rate (FDR) value <0.25 was considered to indicate significant enrichment. The procedure followed for the GSEA is presented in Fig. 1.

Association of AXL expression and cancer cell invasive ability in GSE21293. GSE21293 displayed the mRNA profiles of 34 genetically engineered human esophageal cells with different invasive abilities (23). To determine whether AXL is associated with the invasive ability of ESCC cells, AXL expression in the samples was examined and the mean expression value was calculated, and samples were divided into two groups according to their AXL expression value as aforementioned: AXL-high and AXL-low. AXL-low samples acted as the control samples. The samples were also divided into two groups according to their invasive ability: Invasive and non-invasive. Fisher's exact test was used to examine their association. Furthermore, AXL expression in the invasive and non-invasive groups was also compared using Mann-Whitney U test.

Statistical analysis. Data were analyzed using SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA). Spearman's correlation was used to analyze the association between AXL mRNA and mRNA of mesenchymal markers. Fisher's Exact Test was applied to determine the correlation between AXL expression and invasion ability of genetically engineered esophageal cells. Mann-Whitney U test was used to assess the expression difference of AXL mRNA in invasive and non-invasive esophageal cells. $P<0.05$ was considered to indicate a statistically significant difference.

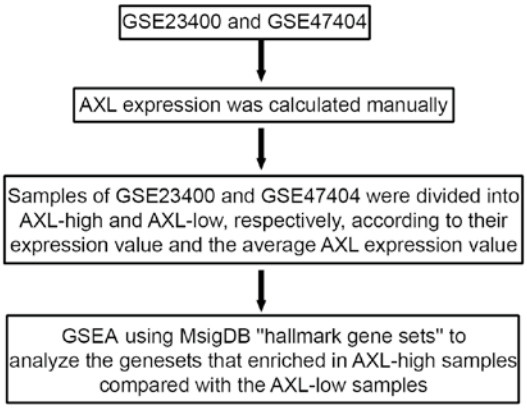

Figure 1. Schematic diagram of the procedure for the GSEA. GSEA, Gene Set Enrichment Analysis; MsigDB, The Molecular Signatures Database.

Results

AXL mRNA expression is associated with certain mesenchymal markers. To investigate the function of AXL in EMT, the association of AXL with several EMT markers, including E-cadherin, vimentin, fibronectin, snail family transcriptional repressor 1 (SNAI1), snail family transcriptional repressor 2 (SNAI2), twist family basic helix-loop-helix transcription factor 1 (TWIST1), zinc finger E-box-binding homeobox 1 (ZEB1) and N-cadherin, was examined (Fig. 2). It was revealed that AXL mRNA expression in GSE47404 and GSE23400 datasets is associated with several EMT markers, including vimentin ($P=1.00\times10^{-6}$ for GSE47404 and 1.00×10^{-6} for GSE23400; Fig. 2B and J), fibronectin ($P=2.22\times10^{-4}$ for GSE47404 and 1.00×10^{-6} for GSE23400; Fig. 2D and L), TWIST1 ($P=0.01$ for GSE47404 and 1.90×10^{-4} for GSE23400; Fig. 2G and O) and ZEB1 ($P=0.04$ for GSE47404 and 1.00×10^{-6} for GSE23400; Fig. 2H and P). However, no association between AXL expression and E-cadherin (Fig. 2A and I) or SNAI2 (Fig. 2F and N) was identified in either dataset. A significant association was identified between AXL and SNAI1 in GSE47404 ($P=2.03\times10^{-3}$; Fig. 2E), but not in GSE23400 ($P=0.67$; Fig. 2M). Additionally, the association between AXL and N-cadherin was significant in GSE23400 ($P=6.59\times10^{-3}$; Fig. 2K), but not in GSE47404 ($P=0.74$; Fig. 2C). These preliminary results suggest that AXL may be involved in EMT in ESCC.

EMT gene set is enriched in the ESCC group with high AXL expression. To further determine the function of AXL in EMT in ESCC, GSEA was performed to examine whether the AXL-high and the AXL-low expression groups display a different EMT state in ESCC. The GSEA of the present

Table I. Gene expression data.

Dataset accession no.	Description	Platform
GSE47404	mRNA profiles of 71 primary tumor tissues from patients with ESCC from Japan	Whole Human Genome 4X44K Agilent G4112F microarray
GSE23400	mRNA profiles of 51 pairs of primary tumor tissues from patients with ESCC and adjacent normal tissues from China (only mRNA expression profiles from patients with ESCC were used in this study)	Human Genome U133 Set microarray
GSE21293	mRNA profiles of invasive and non-invasive genetically engineered human esophageal cells with hTERT and p53 mutations and ERFG overexpression	Human Genome U133Plus 2.0 microarray

ESCC, esophageal squamous cell carcinoma; hTERT, human telomerase reverse transcriptase; EGFR, epidermal growth factor receptor.

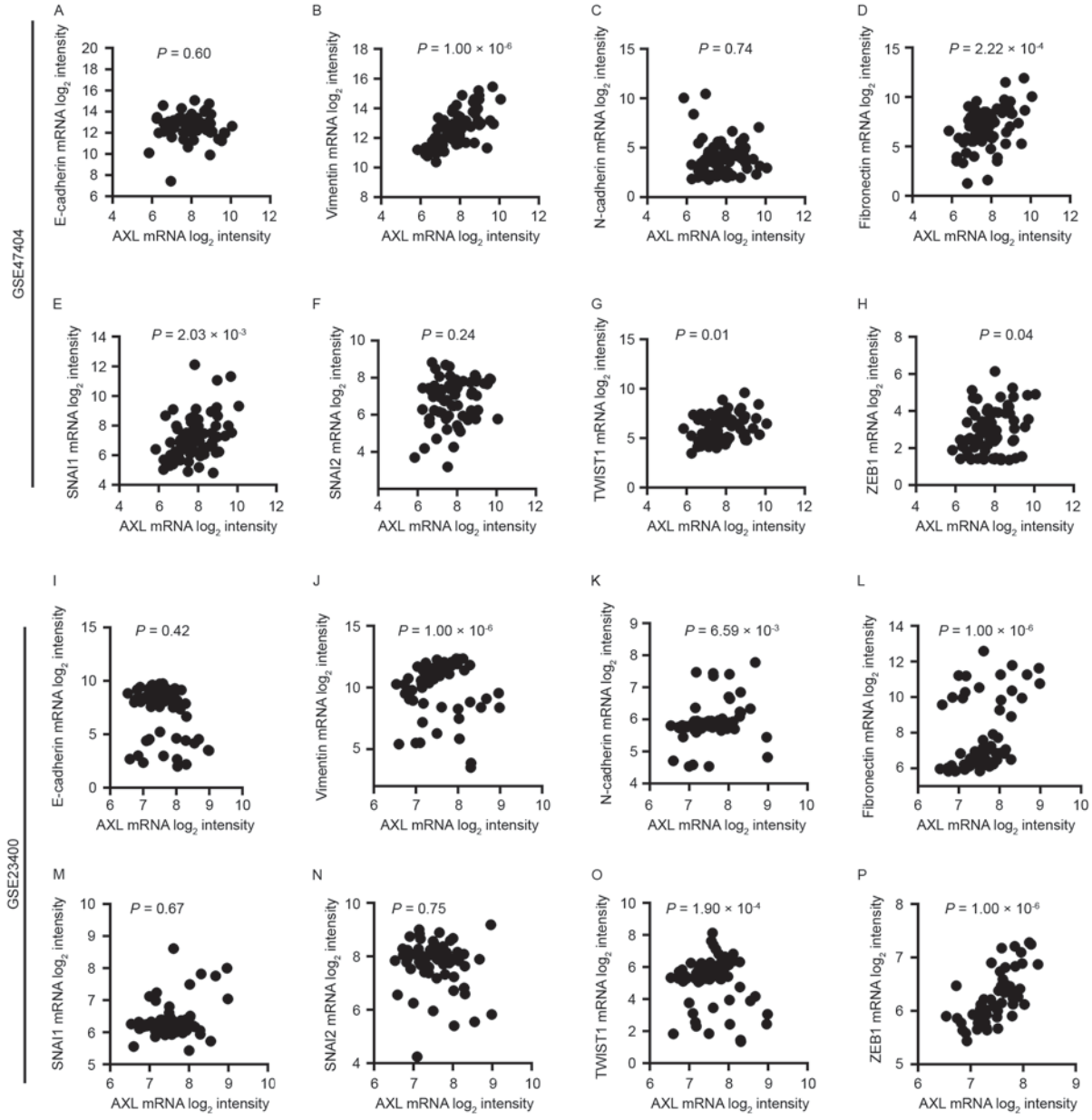

Figure 2. Association between the expression of AXL and (A) E-cadherin, (B) vimentin, (C) N-cadherin, (D) fibronectin, (E) SNAI1, (F) SNAI2, (G) TWIST1 and (H) ZEB1 in the GSE47404 dataset. Association between the expression of AXL and (I) E-cadherin, (J) vimentin, (K) N-cadherin, (L) fibronectin, (M) SNAI1, (N) SNAI2, (O) TWIST1 and (P) ZEB1 in the GSE23400 dataset. Results were assessed by Spearman's correlation. E-cadherin, epithelial cadherin; N-cadherin, neural cadherin; SNAI, snail family transcriptional repressor; TWIST1, twist family basic helix-loop-helix transcription factor 1; ZEB1, zinc finger E-box-binding homeobox 1.

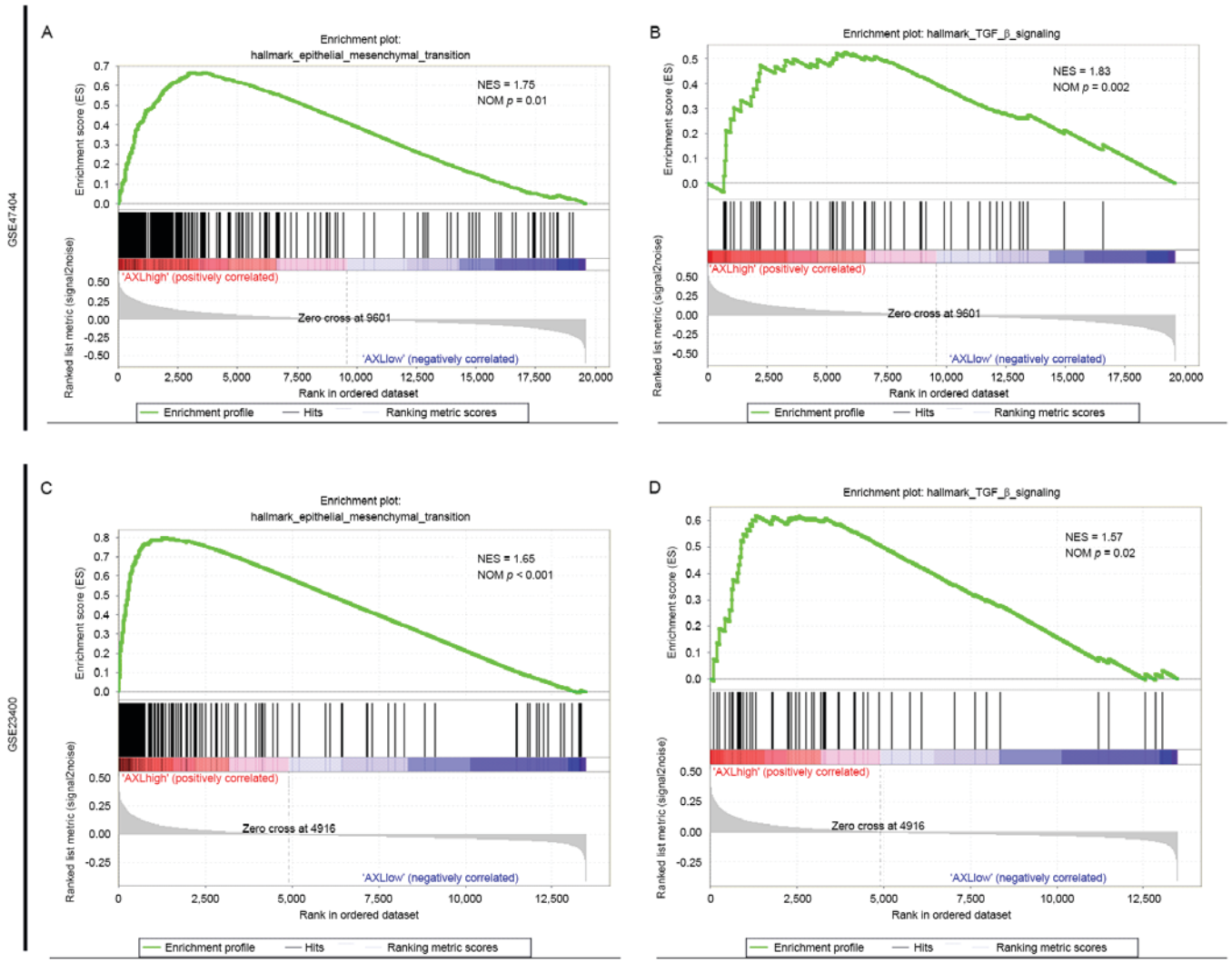

Figure 3. Gene Set Enrichment Analysis demonstrated that the (A) epithelial-mesenchymal transition gene set and the (B) TGFβ gene set were enriched in the ESCC group with high AXL expression in the GSE47404 dataset. Gene Set Enrichment Analysis demonstrated that the (C) epithelial-mesenchymal transition gene set and the (D) TGFβ gene set were enriched in the ESCC group with high AXL expression in the GSE23400 dataset. The barcode plot indicates the position of the genes in each gene set. The horizontal bar in graded color from red to blue indicates positive and negative correlation with AXL expression. The vertical axis in the lower plot indicates Ranked List Metric. NES, normalized enrichment score; NOM, nominal; TGFβ, transforming growth factor-β.

study utilized 'Hallmark gene sets [h.all.v5.2.symbols.gmt (Hallmarks)]' as the gene sets to be analyzed.

The results revealed that in GSE47404, the ESCC group with high AXL expression levels displayed a more marked mesenchymal state compared with the ESCC group with low AXL expression levels (Fig. 3A, and Table II). Furthermore, transforming growth factor-β signaling, which has a predominant function in EMT in cancer (26,27), is also enriched in the AXL-high expression group (Fig. 3B and Table II). Analyze on GSE23400 also showed similar pattern (Fig. 3C and D and Table II). STAT3 signaling, which is reported to serve a function in EMT in ESCC (28), is also highly enriched in the AXL-high expression group in the two datasets (Table II).

AXL expression is associated with increased invasive ability of ESCC cells. A prominent feature of EMT is to confer enhanced invasive ability on cancer cells. Therefore, the present study assessed the association between AXL expression and the invasive ability of ESCC on the basis of the GSE21293 dataset (23). This dataset includes normal esophageal cells, which are genetically engineered with human telomerase reverse transcriptase, epidermal growth factor receptor over-expression and p53 mutations, in order to immortalize them and display differential invasion. According to their ability to invade, these cells were divided into invasive and non-invasive groups. As presented in Table III, the AXL-high expression group displayed increased ability of invasion ($P=2.61x10^{-4}$). AXL expression in non-invasive and invasive groups was also compared and it was revealed that the invasive group expressed significantly increased AXL levels ($P=1.95x10^{-4}$; Fig. 4).

Discussion

EMT is a fundamental process in body development, and in the differentiation of multiple tissues and organs (29). EMT endows cancer cells with invasive properties and with stem-cell like characteristics, prevents drug-induced apoptosis and senescence, and contributes to immunosuppression (29,30). The EMT state defined by the E-cadherin/vimentin ratio has

Table II. Gene Set Enrichment Analysis on GSE47404 and GSE23400 with hallmark gene sets.

Dataset accession no.	Gene set name	NES	NOM P-value	FDR Q-value
GSE47404	HALLMARK_ALLOGRAFT_REJECTION	1.87	<0.001	0.15
GSE47404	HALLMARK_NOTCH_SIGNALING	1.85	<0.001	9.59×10^{-2}
GSE47404	HALLMARK_COMPLEMENT	1.84	<0.001	6.73×10^{-2}
GSE47404	HALLMARK_COAGULATION	1.84	<0.001	5.04×10^{-2}
GSE47404	HALLMARK_TGF_BETA_SIGNALING	1.83	1.95×10^{-3}	4.38×10^{-2}
GSE47404	HALLMARK_IL2_STAT5_SIGNALING	1.82	<0.001	3.95×10^{-2}
GSE47404	HALLMARK_ANGIOGENESIS	1.79	7.39×10^{-3}	4.98×10^{-2}
GSE47404	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	1.75	1.13×10^{-2}	4.67×10^{-2}
GSE47404	HALLMARK_IL6_JAK_STAT3_SIGNALING	1.73	5.93×10^{-3}	4.73×10^{-2}
GSE47404	HALLMARK_INTERFERON_GAMMA_RESPONSE	1.73	1.79×10^{-2}	4.58×10^{-2}
GSE47404	HALLMARK_INFLAMMATORY_RESPONSE	1.71	1.38×10^{-2}	4.83×10^{-2}
GSE47404	HALLMARK_UV_RESPONSE_DN	1.64	1.24×10^{-2}	7.29×10^{-2}
GSE47404	HALLMARK_KRAS_SIGNALING_UP	1.63	5.78×10^{-3}	7.14×10^{-2}
GSE47404	HALLMARK_APOPTOSIS	1.62	3.12×10^{-2}	6.76×10^{-2}
GSE47404	HALLMARK_INTERFERON_ALPHA_RESPONSE	1.62	3.43×10^{-2}	6.54×10^{-2}
GSE47404	HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.53	8.74×10^{-2}	0.10
GSE47404	HALLMARK_HYPOXIA	1.38	0.11	0.20
GSE23400	HALLMARK_COAGULATION	1.71	5.86×10^{-3}	7.27×10^{-2}
GSE23400	HALLMARK_KRAS_SIGNALING_UP	1.71	<0.001	3.64×10^{-2}
GSE23400	HALLMARK_UV_RESPONSE_DN	1.66	1.91×10^{-3}	4.74×10^{-2}
GSE23400	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	1.65	<0.001	4.51×10^{-2}
GSE23400	HALLMARK_APICAL_JUNCTION	1.58	4.10×10^{-3}	8.88×10^{-2}
GSE23400	HALLMARK_TGF_BETA_SIGNALING	1.57	1.74×10^{-2}	7.69×10^{-2}
GSE23400	HALLMARK_ANGIOGENESIS	1.55	2.10×10^{-2}	8.26×10^{-2}
GSE23400	HALLMARK_MYOGENESIS	1.54	3.97×10^{-2}	7.94×10^{-2}
GSE23400	HALLMARK_ALLOGRAFT_REJECTION	1.54	4.45×10^{-2}	7.36×10^{-2}
GSE23400	HALLMARK_COMPLEMENT	1.49	3.75×10^{-2}	0.10
GSE23400	HALLMARK_IL6_JAK_STAT3_SIGNALING	1.47	6.22×10^{-2}	0.11
GSE23400	HALLMARK_IL2_STAT5_SIGNALING	1.45	<0.001	0.11
GSE23400	HALLMARK_APOPTOSIS	1.45	9.77×10^{-3}	0.11
GSE23400	HALLMARK_INFLAMMATORY_RESPONSE	1.41	7.68×10^{-2}	0.13
GSE2340	HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.36	0.01	0.17
GSE23400	HALLMARK_INTERFERON_GAMMA_RESPONSE	1.31	0.18	0.23
GSE23400	HALLMARK_PROTEIN_SECRETION	1.31	0.12	0.21

NOM P-values indicate differences between AXL-high ESCC group and AXL-low ESCC group for the corresponding gene set. FDR Q-values indicate the estimated probability that the normalized enrichment score represents a false positive finding. FDR, false discovery rate; IL, interleukin; Jak, Janus kinase; NES, normalized enrichment score; NOM, nominal; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TNF, tumor necrosis factor; UV, ultraviolet.

been demonstrated to be associated with a poor 5-year survival rate of patients with ESCC (31).

AXL has been demonstrated to be an important factor in certain tumors including NSCLC, breast cancer, prostate cancer, myeloid leukemia and ovarian cancer (2,3,12,32). AXL enhances tumor proliferation, promotes EMT and induces drug resistance (2,3,32). Clinical trials for melanoma, NSCLC and acute myeloid leukemia are ongoing to determine the

Table III. Association between AXL expression and invasive ability of genetically engineered esophageal cells.

	Invasive ability			
AXL expression	Non-invasive	Invasive	Total	P-value
AXL-low	19	2	21	
AXL-high	4	10	14	
Total	23	12		2.61×10^{-4}

Results were assessed using Fisher's exact test.

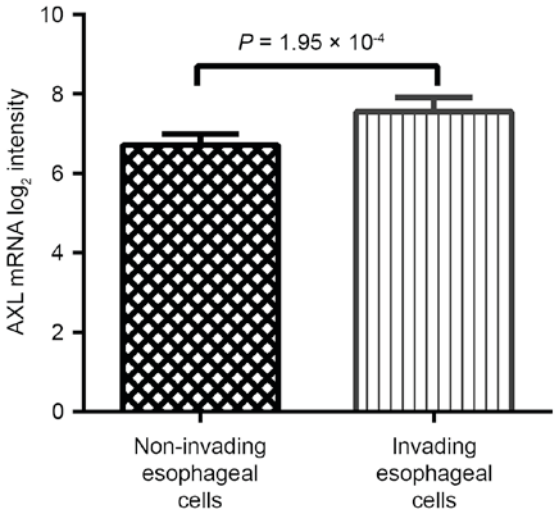

Figure 4. Expression of AXL in non-invasive and invasive esophageal cells. Results were assessed by Mann-Whitney U test.

safety and efficiency of AXL inhibitors (https://clinicaltrials.gov/ct2/results?cond=&term=BGB324&cntry1=&state1=&Search=Search).

The function of AXL in ESCC has only recently been identified (18-20). However, its function in EMT in ESCC is even less well-elucidated. The present study provides preliminary evidence from three independent mRNA array studies indicating that AXL is a marker for the mesenchymal state of ESCC. According to the results of the present study, it was revealed that AXL expression is associated with certain EMT markers, including vimentin, fibronectin, TWIST1 and ZEB1. However, AXL was not associated with other EMT markers, including E-cadherin. These results are consistent with those in a study by Wilson *et al* (6) which demonstrated that AXL mRNA is associated with vimentin mRNA expression in a group of 643 human cancer cell lines. In contrast, Sato *et al* (33) indicated that AXL expression is associated with E-cadherin, but not vimentin, in lung adenocarcinomas. Additionally, the ESCC group with high AXL expression was enriched with the EMT gene set as revealed by GSEA. Another data cohort revealed that AXL is markedly associated with the invasive ability of genetically engineered esophageal cells. Collectively, the results of the present study suggest that AXL is an EMT marker for ESCC. However, further studies, including *in vitro* and *in vivo* cancer models, are required to confirm those results. As mRNA and protein expression are not necessarily associated (34), experiments analyzing AXL protein levels in ESCC are required.

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