AXL upregulates c-Myc expression through AKT and ERK signaling pathways in breast cancers

XIAOBAI SUN[1], HONG CHEN[2], SHULING YOU[1], ZHIKANG TIAN[3], ZHAOYU WANG[3], FULIN LIU[3], WENYI HU[3], HAO ZHANG[3], GUOAN ZHANG[4], HONGLI ZHAO[5] and QINGWEI GUO[6]

[1]Department of Pathology, Jinan Adicon Clinical laboratory, Jinan, Shandong 250000; [2]Clinical Laboratory, The Third People's Hospital of Jinan, Jinan, Shandong 250132; [3]College of Basic Medicine, Jining Medical University, Jining, Shandong 272067; [4]Institute of Forensic Medicine and Laboratory Medicine, Jining Medical University, Forensic Science Center of Jining Medical University, Jining, Shandong 272067; [5]Department of Digestive System, Shandong Institute of Parasitic Diseases, Shandong First Medical University and Shandong Academy of Medical Sciences, Jining, Shandong 272067; [6]Department of Hematology, Jinan Children's Hospital, Jinan, Shandong 250132, P.R. China

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Abstract. Breast cancer (BC) is common worldwide. c-Myc and AXL are both overexpressed in BC, promoting its progression. The present study aimed to investigate the role of AXL in c-Myc expression in BC. Overexpression of AXL increased c-Myc expression while knockdown of AXL decreased c-Myc expression as determined by western blot analysis. Pharmaceutical inhibition of AXL also suppressed c-Myc expression. AKT and ERK inhibitor LY294002 and U0126 suppressed c-Myc expression, respectively. AXL overexpression which activates AKT and ERK signaling, upregulates c-Myc expression, while kinase-dead AXL which cannot activate AKT and ERK signaling, does not upregulate c-Myc expression, emphasizing the important role of these two signaling pathways in c-Myc upregulation. Finally, expression data of BC tissues from The Cancer Proteome Atlas displayed an association between AXL and c-Myc. Taken together, the present study revealed that AXL upregulates c-Myc expression through AKT and ERK signaling pathways in BC.

Introduction

AXL is a member of the TAM (Tyro3, AXL, Mer) family of receptor tyrosine kinases (RTKs), and is activated by its ligand Gas6, which leads to activation of several downstream signaling pathways, depending on the cell types, mainly phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK). AXL has also been implicated in the pathophysiology of numerous cancers including breast, gastric, prostate, ovarian and lung (1-4). It enhances cancer cell survival, angiogenesis, metastasis and drug-resistance (2,4,5).

c-MYC is a basic helix-loop-helix leucine zipper transcription factor that dimerizes with MAX to bind DNA and regulate gene expression (6). c-Myc plays an essential role in cell cycle progression (7-9), cellular differentiation (10), metabolism (11), cancer progression (12) and drug resistance (13).

More than 1 million new breast cancer (BC) cases are diagnosed each year worldwide and account for most cancer-related deaths in women (14). Among BCs, some basal-like BCs are estrogen receptor negative, progesterone receptor negative and HER2 negative, causing triple-negative BC (TNBC), which accounts for 10-20% of all BC cases. These tumors are aggressive with poor prognosis due to ineffective target therapies.

In BC, 30-50% of tumors overexpress c-MYC (15), and it is considered that c-Myc is an enhancer of tumorigenesis in BC. Notably, c-MYC is elevated in TNBC compared with other cancer subtypes (ER^+ (estrogen receptor alpha positive), $HER2^+$ (human epidermal growth factor receptor 2 positive) (16).

AXL has been reported to be overexpressed in BC, and is a negative prognostic indicator for survival in patients with BC (17). AXL is also an inducer of Epithelial-to-mesenchymal transition-induced downstream effector, that is required for BC metastasis and progression (1,18). AXL also mediates BC cells resistance to epidermal growth factor receptor-targeted therapies (19). Thus, AXL represents a promising target in BC therapies (20).

Based on the observation that both c-Myc and AXL overexpressed in TNBC, it was hypothesized that as a receptor tyrosine receptor, AXL maybe regulate c-Myc expression in BC. It was revealed that AXL could upregulate c-Myc expression in an AKT- and ERK-dependent manner.

Correspondence to: Dr Hongli Zhao, Department of Digestive System, Shandong Institute of Parasitic Diseases, Shandong First Medical University and Shandong Academy of Medical Sciences, Jining, Shandong 272067, P.R. China
E-mail: xushulinfeng@163.com

Dr Qingwei Guo, Department of Hematology, Jinan Children's Hospital, Jinan, Shandong 250132, P.R. China
E-mail: gqw2007@163.com

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Materials and methods

Cell lines and reagents. 293T cells, HeLa cells, MDA-MB-231 cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. 293T cells, HeLa cells, MDA-MB-231 cells were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS) (Zhejiang Tianhang Biotechnology Co., Ltd.). MCF7 cells were purchased from Procell Life Science & Technology and cultured in MEM medium (cat. no. PM150410; Procell Life Science & Technology) supplemented with 0.01 mg/ml insulin (cat. no. PB180432; Procell Life Science & Technology) and 10% FBS (cat. no. 164210-500; Procell Life Science & Technology). All the cells were maintained in a humidified atmosphere at 37˚C with 5% CO_2. Cell line authentication was achieved by genetic profiling using the polymorphic short tandem repeat (STR) method at the Forensic Science Center, Jining Medical University (Shandong, China). R428 was purchased from MedChemExpress and used as previously described (21). LY294002 and U0126 were purchased from Beyotime Institute of Biotechnology.

Western blotting. Total cellular proteins were extracted from cells with RIPA buffer containing protease inhibitors and phosphatase inhibitors (all from Beyotime Institute of Biotechnology), and the quantity was determined by BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Then 10 μg proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Beyotime Institute of Biotechnology). The membrane was blocked for 1 h at room temperature in 5% non-fat milk solution and incubated overnight at 4˚C with the following primary antibodies: AXL (1:1,000; cat. no. 8661), phosphorylated (p)-AKT (1:1,000; cat. no. 4060), AKT (cat. no. 4691; all from Cell Signaling Technology, Inc.), ERK1 + ERK2 (1:10,000; cat. no. ab184699), p-ERK1 + ERK2 (1:10,000; cat. no. ab76299; both from Abcam) and c-Myc antibody (1:1,000; cat. no. ab32072; Abcam). Following incubation with primary antibodies, membranes were incubated with peroxidase-conjugated goat anti-rabbit (cat. no. ZB-2301) or goat anti-mouse (cat. no. ZB-2305; both from OriGene Technologies, Inc.) secondary antibodies (1:5,000) at room temperature for 1 h. Immunoblot bands were visualized using ECL reagent (Beyotime Institute of Biotechnology). GAPDH (1:1,000; cat. no. TA-08; OriGene Technologies, Inc.) was included as an endogenous protein loading control.). All the experiments were repeated three times. ImageJ software (National Institutes of Health, version 1.53m) was used for the densitometric analysis.

Nuclear and cytoplasmic protein extraction. Nuclear and cytoplasmic protein were extracted using nuclear and cytoplasmic protein extraction kit (cat. no. P0027) from Beyotime Institute of Biotechnology according to the manufacturer's instructions. These experiments were repeated three times.

Plasmids, short hairpin (sh)RNA and transfection. AXL plasmid (cat. no. 105932) or AXL kinase-dead plasmid (cat. no. 105934; both from Addgene, Inc.) were gifts from Rosa Marina Melillo (22). Transfection of plasmids (AXL or empty vector) was performed using Lipo8000 (cat. no. C053; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, Lipo8000 and vector were incubated in DMEM without FBS for 5 min and added the cell medium for 4 h. ~44-48 h later, cells were harvested for analysis. Lentivirus for shRNA targeting AXL (shAXL-3) (5'-GGGTGACAATGTGGGAGATTG-3') and Control viruses (Cont; 5'-TTCTCCGACGTGTCACGT-3') were purchased from Shanghai GenePharma Co., Ltd., and the titer for both of them was 10^9 TU/ml. For viral transfection, viruses (5 μl/ml) and polybrene (2 μg/ml) were added to the cell medium (MOI=~5 for HeLa and MDA-MB-231 cells), after 3 days cells were treated with puromycin (2 μg/ml) for ~1 week to select transfected cells. Then, cells were cultured at puromycin (0.5 μg/ml) for maintenance.

Analysis of protein expression of AXL and c-Myc from The Cancer Proteome Atlas (TCPA). TCPA is a resource for cancer functional proteomics data over a large number of tumor and cell line samples using reverse-phase protein arrays as a part of The Cancer Genome Atlas Project (23). To analyze the association between AXL and c-Myc in BCs, their protein expression data was downloaded from TCPA (n=901) (https://www.tcpaportal.org/tcpa/download.html). The samples whose AXL expression marked with NA (n=156) were excluded. The expression association between AXL and c-Myc was analyzed by SPSS (version 13.0; SPSS, Inc.) using Pearson's correlation analysis (n=745). To further analyze the samples without c-Myc amplification, data of c-Myc amplification of the samples were extracted from cBioportal (https://www.cbioportal.org/). After excluding these samples with c-Myc amplification, the expression association between AXL and c-Myc was analyzed by SPSS using Pearson's correlation analysis (n=648).

Statistical analysis. Data are presented as mean ± standard deviation (SD). Statistical differences between experimental and control groups were analyzed using an unpaired two-tailed Student's t-test (2 groups) or a one-way ANOVA (>2 groups) followed by a multiple post hoc comparisons test (Dunnett's test) using GraphPad Prism 8 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

AXL increases c-Myc expression. To determine the association of AXL and c-Myc, AXL was overexpressed in MCF7 and 293T cells which did not express AXL, and it was revealed that exogenous AXL increased c-Myc expression (Fig. 1A and B). Furthermore, c-Myc expression was revealed to be dependent on AXL amount transfected; increasing AXL transfection led to elevated c-Myc expression (Fig. 1C and D). c-Myc is located in the nucleus, therefore the expression of cytoplasmic and nuclear c-Myc was explored. It was identified that nuclear c-Myc was upregulated in response to AXL overexpression (Fig. 1E). The present results also showed a part of AXL was also located in the nucleus, which was consistent with previous results (24).

To further determine the association between AXL and c-Myc, AXL expression was knocked down in two AXL-high expression cell lines, HeLa and MDA-MB-231.

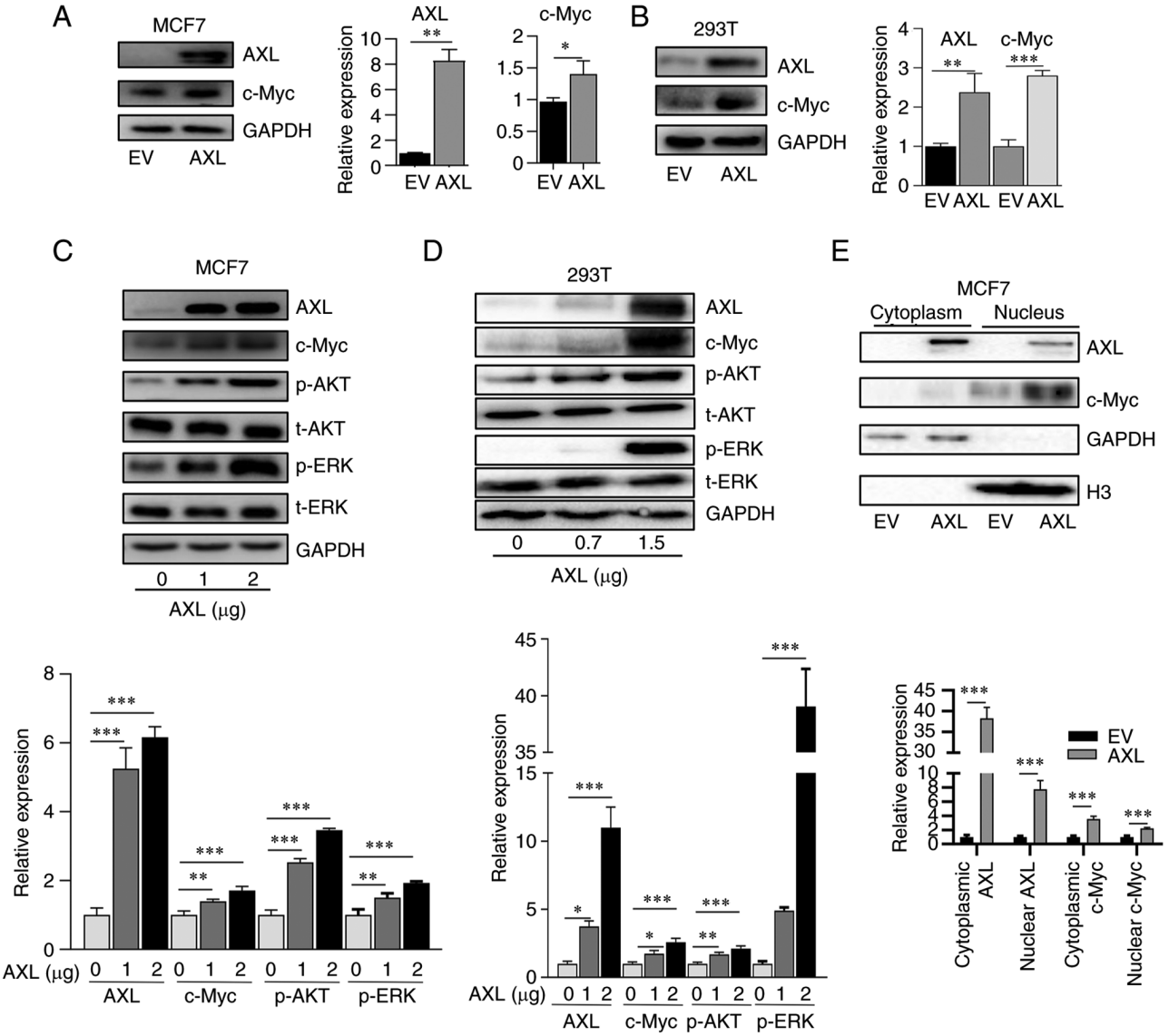

Figure 1. AXL overexpression leads to upregulation of c-Myc. (A and B) MCF7 and 293T cells were transfected with 2 μg EV or AXL plasmid. After 48 h, cells were harvested and immunoblotted. (C and D) MCF7 cells and 293T cells were transfected with increasing amount of AXL plasmid. After 48 h, cells were harvested and immunoblotted. (E) MCF7 cells were transfected with 2 μg EV or AXL plasmid. After 48 h, cytoplasmic and nuclear part of cells were harvested and immunoblotted. EV, empty vector; p-, phosphorylated. *P<0.05, **P<0.01 and ***P<0.001.

AXL knockdown using shRNA decreased c-Myc expression in both cell lines (Fig. 2A and B). Re-overexpression of AXL in shRNA-knockdown HeLa and MDA-MB-231 recovered c-Myc expression (Fig. 2C and D). R428, a selective AXL inhibitor (25), suppressed c-Myc expression in a dose-dependent manner (Fig. 2E and F). Thus, it was concluded that AXL could increase c-Myc expression.

AXL increases c-Myc expression through AKT and ERK pathway. AKT and ERK are the main downstream signaling pathways of AXL (4,26). It was observed that overexpression of AXL is accompanied by activation of AKT and ERK pathways, which are manifested by p-AKT and p-ERK, respectively (Fig. 1C and D). Thus, to understand the mechanisms underlying AXL-upregulated c-Myc, MDA-MB-231 cells were first treated with AKT and ERK inhibitors LY294002 and U0126, respectively, and it was revealed that LY294002 and U0126 suppressed the activation of AKT and ERK, respectively (Fig. 3A and B). Moreover, both inhibitors suppressed c-Myc expression in a time-dependent manner, suggesting that both pathways are important in maintaining c-Myc expression (Fig. 3A and B). Subsequently, it was investigated whether both signaling pathways are involved in AXL-upregulated c-Myc. Overexpression of AXL led to c-Myc upregulation; however, overexpression of kinase-dead AXL (K567R) did not increase c-Myc expression as markedly as AXL. AXL activated AKT and ERK signaling while AXL-KD activated AKT weakly and did not activate ERK signaling (Fig. 3C and D). These results suggested both AKT and ERK signaling are important for c-Myc upregulation by AXL.

AXL is associated with c-Myc expression in patients with BC. To further study the association between AXL and c-Myc, protein expression data from patients with BC of TCPA (23) was examined. It was revealed that there is a trend association between AXL and c-Myc (P=0.059) (Fig. 4). In BCs, c-Myc protein expression was closely correlated with c-Myc

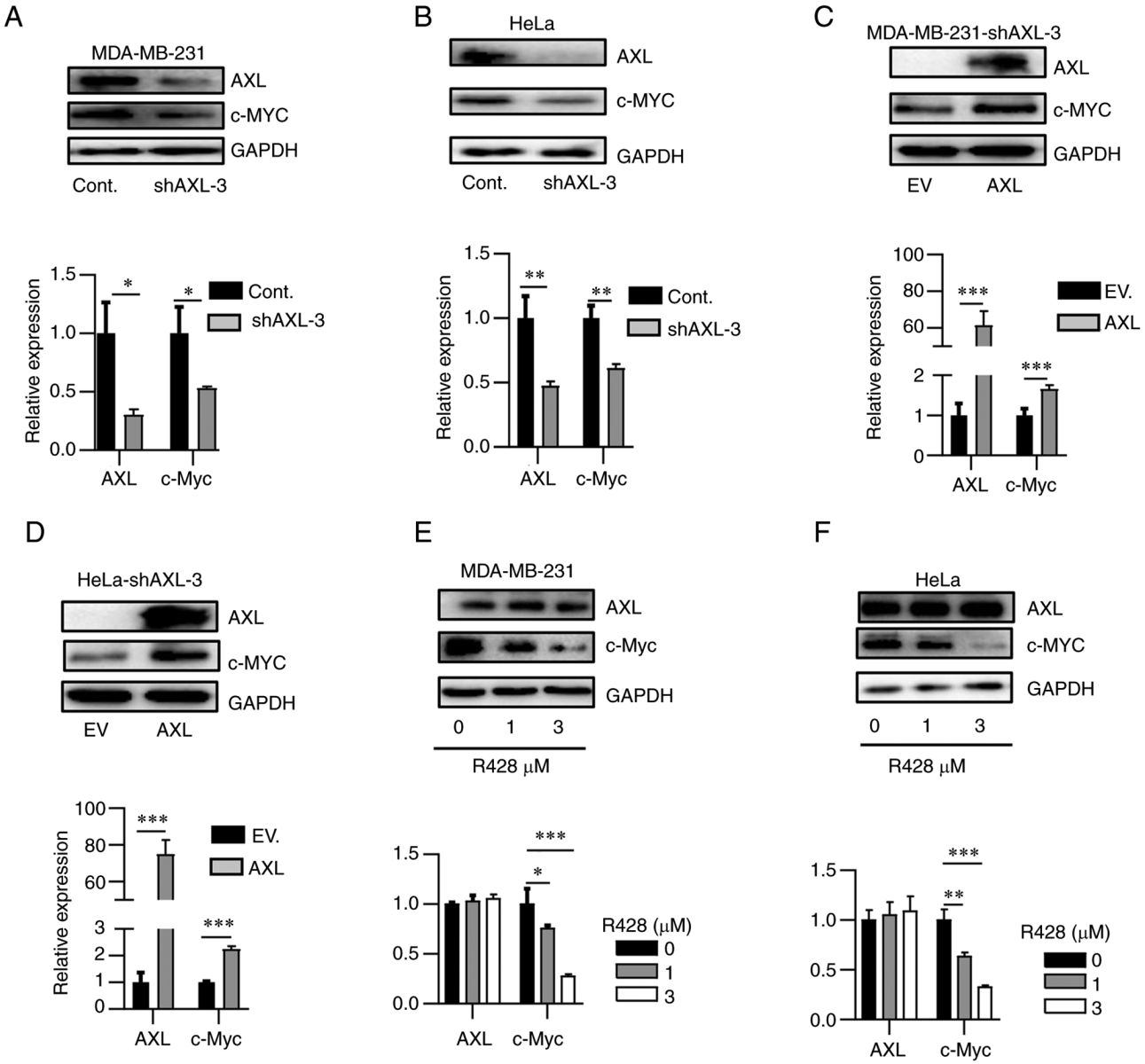

Figure 2. AXL knockdown leads to downregulation of c-Myc. (A and B) MDA-MB-231 and HeLA cells were transfected with control shRNA (Cont) or AXL-targeting shRNA (shAXL-3). After 48 h, cells were harvested and immunoblotted. (C and D) MDA-MB-231 and HeLA cells stably expressing shAXL-3 were transfected with AXL plasmid. After 48 h, cells were harvested and immunoblotted. (E and F) Western blotting for MDA-MB-231 and HeLA cells treated with or without R428 for 24 h. sh-, short hairpin; EV, empty vector. *P<0.05, **P<0.01 and ***P<0.001.

amplification (27), suggesting a subset of c-Myc overexpression is caused by c-Myc amplification, and it was hypothesized that c-Myc in this part of tumors was more dependent on amplification but not signaling pathways. Indeed, when this subset of samples was excluded, AXL and c-Myc were associated with each other significantly (P=0.020) (Fig. 4).

Discussion

c-Myc is now known to be one of the most frequently dysregulated oncogenes. It is overexpressed by different mechanisms in 60-70% of human solid and hematopoietic tumors (7,11,12). It was found in a pan-cancer copy-number analysis to be the third most amplified gene in human cancers (11). Besides copy-number amplification, numerous dysregulated signaling pathways also contribute to the overexpression of c-Myc, such as Wnt-APC pathway found in human colon carcinoma (28) and NOTCH signaling pathway found in T cell leukemia (29). c-MYC contributes to the genesis of numerous human cancers. It has been revealed to be linked to numerous key biological processes of cancer cells including proliferation, stem cell properties, metabolism, metastasis, cancer immune and genome instability (11,12,30).

In the present study, a new mechanism of c-Myc overexpression in BC cells was demonstrated, in which AXL upregulates c-Myc expression through the AKT and ERK signaling pathways. The results are in accordance with those of Hong *et al* (13), which displayed a close association between AXL and c-Myc in esophageal adenocarcinoma.

In the present study, to the best of our knowledge, exogeneous AXL was first overexpressed in 293T and MCF7 cells, both of which do not express AXL. As a result, c-Myc expression was

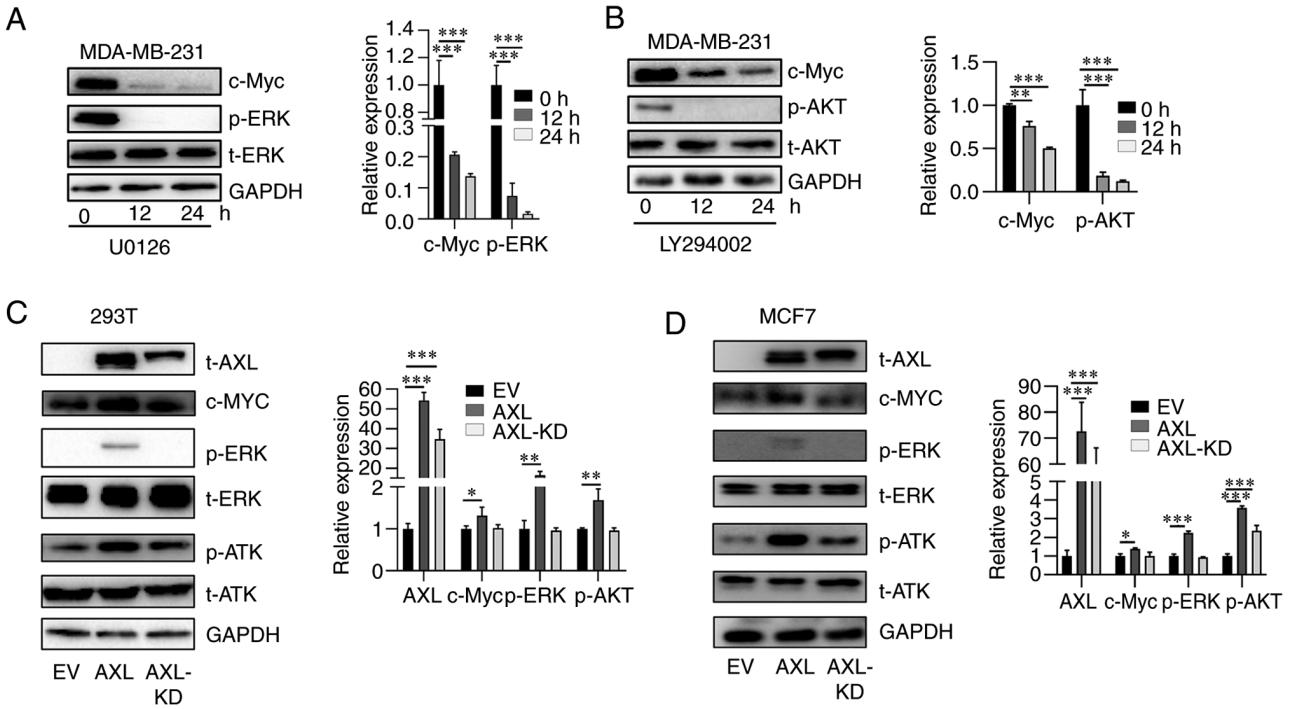

Figure 3. AXL activates AKT and ERK signaling to increase c-Myc expression. (A and B) Western blotting for MDA-MB-231 cells treated with U126 (20 μM) or LY294002 (30 μM) (B) for the indicated time. 293T cells were transfected with 2 μg pcDNA4.1, AXL and AXL-KD plasmids for 48 h, and indicated proteins were immunoblotted. (C and D) 293T or MCF7 cells were transfected with 2 μg pcDNA4.1 EV, AXL plasmid or AXL kinase dead (AXL-KD) plasmid. After 48 h, cells were harvested and immunoblotted. EV, empty vector; p-, phosphorylated. *P<0.05, **P<0.01 and ***P<0.001.

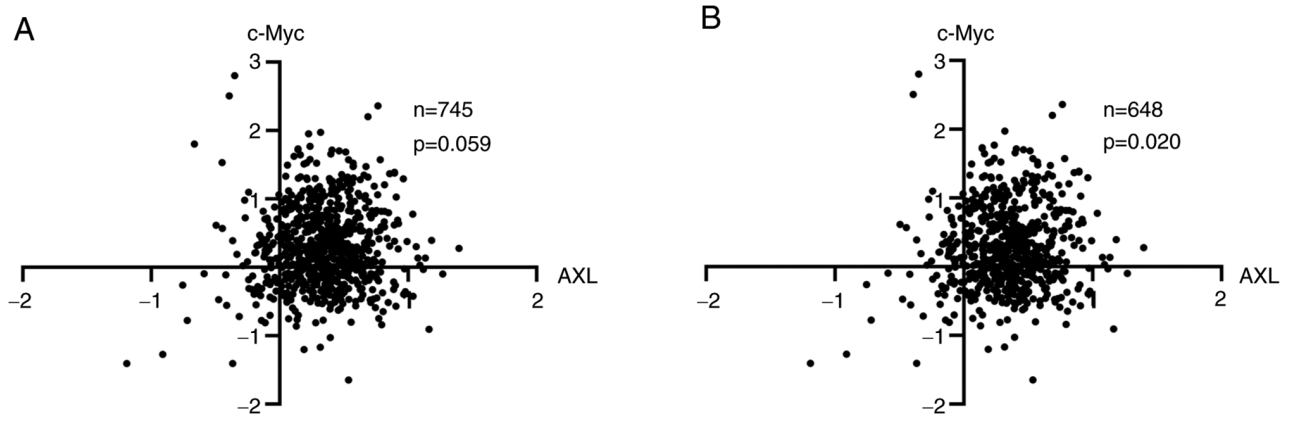

Figure 4. AXL expression is associated with c-Myc in breast cancers in TCPA. (A) Protein expression data of AXL and c-Myc were extracted from TCPA. The correlation between them is calculated by SPSS using Pearson's correlation analysis. (B) After samples with c-Myc amplification were excluded, the correlation between AXL and c-Myc was calculated by SPSS using Pearson's correlation analysis. TCPA, The Cancer Proteome Atlas.

upregulated in a dose-dependent manner. Conversely, knockdown of AXL expression in MDA-MB-231 and HeLa cells decreased c-Myc expression. Furthermore, R428, a selective AXL inhibitor, also suppressed c-Myc expression. Collectively, these results supported a role of AXL in c-Myc upregulation in BC cells. Furthermore, the association between AXL and c-Myc was supported by data from tumor tissues of patients with BC. To understand the mechanism underlying c-Myc upregulation by AXL, the AKT and ERK pathways were examined. It was revealed that both of the two pathways are important for c-Myc upregulation by AXL. Nevertheless, the detailed mechanisms must be further studied. These experiments were not performed, which is a limitation to the present study.

In conclusion, in the present study, it was demonstrated that AXL upregulates c-MYC expression through activation of the AKT and ERK signaling in BC cells.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GZ, HoZ and QG designed the study. XS, HC, SY, ZT, ZW, FL, WH and HaZ performed the experiments and analyzed the data. GZ, QG and HoZ prepared the manuscript. All authors read and approved the final manuscript. HoZ and QG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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