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Original Article

Expression of XB130 in human ductal breast cancer

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Abstract: Objectives: XB130 is involved in gene regulation, cell proliferation, cell survival, cell migration, and tumorigenesis. In the present study, we first evaluated the expression of the XB130 and its prognostic significance in breast cancer. Then we evaluated whether XB130 could be a target for therapy in breast cancer. Materials and methods: Immunohistochemistry was used to assess the level of XB130 protein in surgically resected, formalin-fixed, paraffin-embedded breast cancer specimens. Associations between XB130 and the postoperative prognosis of patients with breast cancer were evaluated. We evaluated the effect of XB130 inhibited by RNA interference on proliferation, invasion and apoptosis in vitro in a metastatic subclone of MCF-7 breast cancer cell line (LM-MCF-7). The effect of XB130 silencing alone or in combination with gemcitabine on LM-MCF-7 cells apoptosis was also investigated. Results: XB130 protein was present in the cytoplasm of malignant cells, and not in the normal breast tissues. There was correlation between the presence of XB130 in tumour cells and lymph node status, tumor classification and clinical stage. XB130 expression level was significantly associated with recurrence-free and overall survival. Furthermore, multivariate Cox regression analyses revealed that positive XB130 was an independent risk factor for overall survival and recurrence free survival. XB130 silencing alone inhibits tumor growth and induces apoptosis in the LM-MCF-7 cells. Depletion of the XB130 in combination with gemcitabine resulted in marked apoptotic and necrotic cell death in LM-MCF-7 cells. Conclusions: XB130 could be useful as a prognostic marker of recurrence-free and overall survival in invasive breast cancer, as well as for the response to chemotherapy.

Keywords: Breast cancer, XB130, invasion, apoptosis, gemcitabine

Introduction

Breast cancer is by far the leading cause of cancer death in women throughout the world and its incidence continues to rise [1, 2]. The main reasons consist of high propensity to metastasize at an early stage and the acquired resistance to a wide range of anticancer agents [3]. Once the cancer has spread beyond the breast and loco-regional lymph nodes, it is seemed to be incurable [4]. In such cases, chemotherapy or radiotherapy considered to be the main treatment, but accompanied by various adverse effects. This fact emphasizes the importance of selecting sensitive diagnostic and prognostic markers in the early stage and more efficient targeted treatment for this disease.

XB130 is a newly identified adaptor protein that is strongly expressed in the spleen and thyroid of humans, while it shows weak expression in the kidney, brain, lung, and pancreas [5]. XB130 has been detected in human esophageal squamous cell carcinoma (ESCC) [6], follicular and papillary thyroid carcinoma, as well as in human lung carcinoma cell lines [7]. In ESCC cells, expression of XB130 may affect cell cycle progression and impact prognosis of patients with ESCC [6]. In thyroid and lung cancer cells, XB130 has been implicated as a substrate and regulator of tyrosine kinase-mediated signaling and in controlling cell proliferation and apoptosis [7]. In the gastric cancer, reduced XB130 protein expression is a prognostic biomarker for shorter survival and a higher recurrence rate in patients with GC, as well as for the response to chemotherapy [8]. However, in patients with HCC, protein expression of XB130 is not associated with the postoperative prognosis of patients with HCC [9].

Takeshita et al has found XB130 could promote growth of cancer cells by regulating expression of tumor suppressive miRNAs and their targeted genes [10]. Shiozaki et al has recently

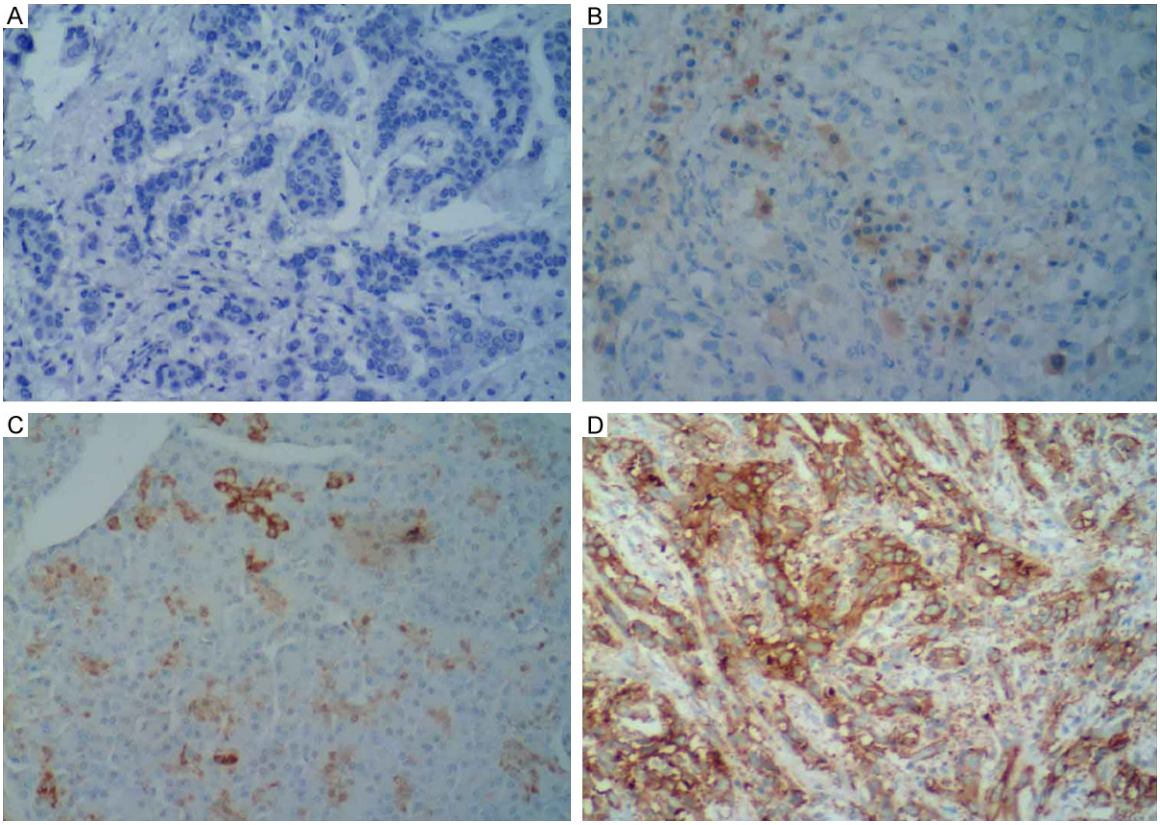

Figure 1. Up-regulation of XB130 protein is illustrated in tissues from patients with breast cancer. Representative immunohistochemical staining of XB130 is shown (A-D). (A) XB130 staining was negative. (B) Weak positive staining of XB130. (C) Moderate staining of XB130. (D) Strong XB130 staining.

reported XB130 can regulate cell proliferation and survival through modulating selected down-stream signals of PI3K/Akt pathway [11]. XB130 is also a novel Rac- and cytoskeleton-regulated and cytoskeleton-regulating adaptor protein that exhibits high affinity to lamellipodial (branched) F-actin and impacts motility and invasiveness of tumor cells [12]. It is suggested that XB130 is involved in gene regulation, cell proliferation, cell survival, cell migration, and tumorigenesis [13].

In this study, we first discuss the relationship between XB130 expression and clinicopathological characteristics in breast cancer. Then we discussed whether XB130 could be a target for breast cancer therapy.

Materials and methods

Patients and specimens

All breast tissues, including 24 paired adjacent normal tissues and 140 primary breast cancer tissues were collected from 140 breast cancer patients. Surgery was performed from January 2002 to December 2007 at the affiliated hospital of Weifang medical college. No patients received chemotherapy or radiotherapy prior to the operation. This study was approved by the Institutional Review Board of the Qilu hospital, Shandong University and or able consent was obtained from all participants. All patients received conventional postoperative treatments, depending on the extents of the disease. Patients without axillary lymph node involvement were treated with operation alone, while patients with axillary lymph node involvement received six courses of adjuvant chemotherapy with cyclophosphamide/methotrexate/fluorouracil regiment. Patients with positive nodes or tumor size ≥5 cm received postoperative radiation. The patients with ER+/PR+ tumor were treated for 2-5 years with tamoxifen. The patient characteristics including age (median: 48.9 years), menopausal status, clinical stage (TNM classification defined by the International Union against Cancer, UICC, 2003) were assessed by the surgical pathologists.

Table 1. Correlation of high XB130 expression with clinicopathological parameters

Characteristics	XB130 expression			*P*-value
	No. of patients	Positive (%)	Negative (%)	
Age (years)				NS
≤50	57	31 (54.4)	26 (45.6)	
>50	83	56 (67.5)	27 (32.5)	
Tumor size (cm)				NS
≤2	36	19 (52.8)	17 (47.2)	
>2	104	68 (65.4)	36 (34.6)	
Tumor classification				0.024
T1-T2	119	71 (59.7)	48 (40.3)	
T3-T4	21	16 (76.2)	5 (23.8)	
Clinical stage				0.0084
I-II	96	48 (50)	48 (50)	
III-IV	44	39 (88.6)	5 (11.4)	
Lymph node status				0.0013
Positive	68	56 (82.3)	12 (17.7)	
Negative	72	31 (43)	41 (57)	
ER status				NS
Positive	96	56 (58.3)	40 (41.7)	
Negative	44	31 (70.4)	13 (29.6)	
PR status				NS
Positive	95	58 (61)	37 (39)	
Negative	45	29 (64.4)	16 (35.6)	
Her-2 status				NS
Positive	33	21 (63.6)	12 (36.4)	
Negative	107	66 (61.7)	41 (38.3)	
Menopausal				NS
Pre-menopausal	73	43 (58.9)	30 (41.1)	
Post-menopausal	67	44 (65.7)	23 (34.3)	

NS: statistical significance.

Cell line and culture

The breast cancer cell line LM-MCF-7 (a metastatic subclone of MCF-7 breast cancer cell line) were maintained in RPMI 1640 (Gibco, Carlsbad, CA) medium containing 10% fetal bovine serum (Gibco) [14]. In our pre-experiment, high levels of XB-130 was detected in the XB130 cells.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were cut into 4-micron-thick sections, stained with haematoxylin and eosin (H&E). Histological classification was made by two pathologists based on World Health Organization criteria and breast invasive ductal carcinomas were selected exclusively for analysis. Sections were deparaffinized in xylene and soaked in a series of graded alcohols for rehydration. Epitope retrieval was achieved by pretreatment with sodium citrate buffer, pH6.0, in a microwave for XB130. Slides were incubated at 4°C overnight with anti-XB130 (dilution 1:100). In the negative controls, primary antibodies were omitted and replaced by PBS.3% hydrogen peroxide was used for 30 minutes to inactivate endogenous peroxidase activity. Thereafter, sections were treated with peroxidase-conjugated goat anti-rabbit antibodies. Counterstaining was carried out using haematoxylin. Results were evaluated independently by two investigators with no prior knowledge of the patient data. Sections were visualized under a bright-field microscope (Olympus), and staining intensity and subcellular localization were evaluated twice in a blinded manner based on a pre-agreed staining scoring standard from specialized pathologists.

The staining intensity was scored as 0 (negative, -), 1 (weak, +), 2 (medium, ++), or 3 (strong, +++). For statistical analysis, the results were presented as a positive (strong positive staining 3+ and moderately positive staining 2+) or a negative (weak positive 1+ and negative staining 0) for tumor cells.

siRNA transfection

siRNA was designed according to XB130 sequence as described previously [15]. LM-MCF-7 cells were transfected with 20 nM pooled XB130 siRNAs using the oligofectamine reagent (Invitrogen, Shanghai, China) according to a novel approach described previously [16]. Forty-eight hours after transfection, the cells were harvested for western blot analysis of the knockdown level of the exogenous proteins by siRNA. For targeting and detection of the endogenous gene level, cells were transfected with 20 nM siRNA, with a GFP siRNA as control.

In vitro cell invasion assay

In vitro Matrigel invasion assays were done using 6.5-mm Costar transwell chambers

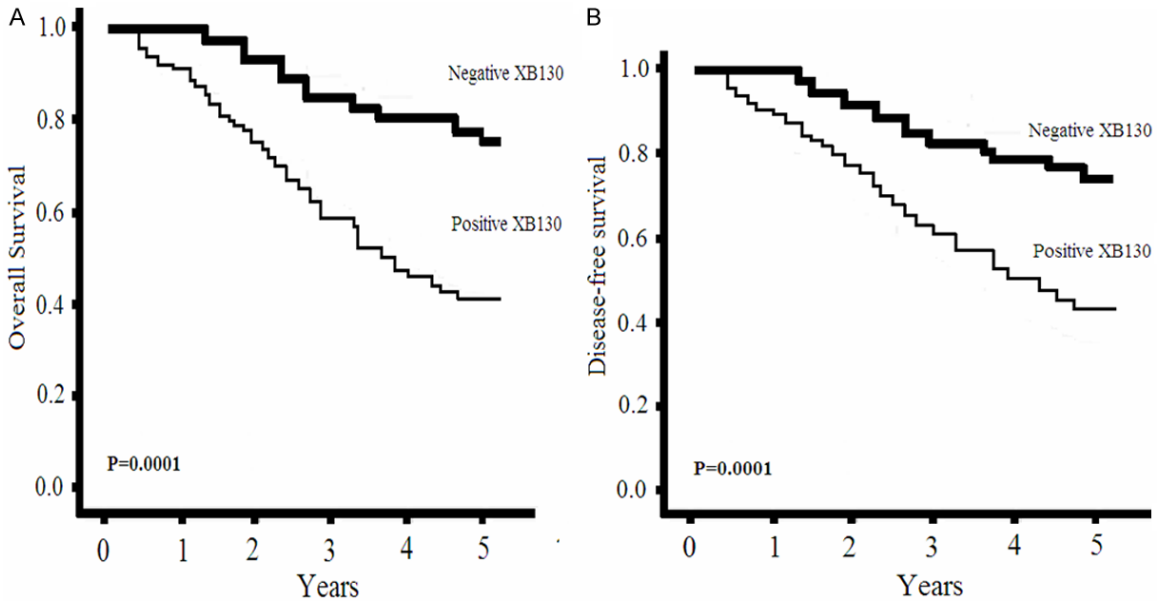

Figure 2. Kaplan-Meier overall and disease-free survival curves were calculated according to XB130 expression levels. A. Differences in cumulative overall survival are observed between patients with Positive and Negative XB130 expression. B. Differences in cumulative recurrence-free survival are observed between patients with Positive and Negative XB130 expression. *P* values were obtained using the log-rank test.

(8-um pore size). The Transwell filters were coated with appropriate Matrigel. After the Matrigel solidified at 37°C, 1×10^5 cells (LM-MCF-7/siRNA-XB130, LM-MCF-7/siRNA-GFP and LM-MCF-7) were seeded onto the Matrigel. After 24-hour incubation, the filter was gently removed from the chamber and the noninvasive cells on the upper surface were removed by wiping with a cotton swab. The cells that invaded the Matrigel and attached to the lower surface of the filter were fixed with methanol and stained with Giemsa solution. The number of cells attached to the lower surface of the polycarbonate filter was counted at 200 magnification under a light microscope. Each type of cell was assayed in triplicate.

Cell proliferation assay

The cells were seeded onto 96-well plates at 4,000 per well in culture medium (100 uL). After culturing for various durations, cell numbers were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously [17]. Briefly, at each time point, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (10 μL; 5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The reaction was stopped by adding 100 μL of 0.04 N HCl in isopropanol to each well, with vigorous mixing to solubilize colored crystals produced by the reaction. The absorbance at 570 nm to absorbance at 630 nm as reference wave was measured by a multi well scanning spectrophotometer. Each data point is the average of six determinations and each experiment was repeated thrice.

Apoptosis assays

For flow cytometry analysis, cells were collected at the indicated times PI, washed once with PBS, and suspended in 0.5 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The suspension was filtered through a nylon mesh and then adjusted to a final concentration of 0.1% (w/v) RNase and 50 ug/ml propidium iodide. Apoptotic cells were quantified by FACScan cytometer. The DNA fragmentation assay was carried out as described previously [18].

Western blotting

Western blotting was performed using rabbit anti-XB130 antibody (PradoWalnut, CA, USA) and β-actin (Santa Cruz, CA). Immunoreactive bands were visualized by the enhanced chemiluminescence method (Amersham) with a western blotting detection system (Kodak Digital

Table 2. Univariate logistic regression analysis of XB130 expression

	Recurrence-free survival			Overall survival		
Variable	No. of patients	Median (95% CI)	*P*	No. of patients	Median (95% CI[a])	*P*
XB130 expression			0.0001			0.0001
Positive	87	42.6 (37.7-49.3)		87	43.1 (36.8-50.4)	
Negative	53	58.4 (51.3-62.2)		53	58.9 (51.6-61.8)	
Tumor size (cm)			0.026			0.004
≤2	36	51.8 (45.7-60.2)		36	53.3 (44.6-61.4)	
>2	104	56.5 (44.8-62.9)		104	56.1 (44.3-63.6)	
Lymph node status			0.0001			0.0001
Negative	68	51.2 (47.0-52.8)		68	52.5 (47.8-53.5)	
Positive	72	34.4 (30.6-47.7)		72	34.9 (31.2-48.2)	

Table 3. Multivariate Cox proportional hazards analysis for recurrence-free survival and overall survival according to XB130 expression

	Recurrence-free survival			Overall survival		
Variable	No. of patients	RR (95% CI)	*P*	No.of patients	RR (95% CI[a])	*P*
XB130 expression			0.016			0.029
Positive	87	1.000		87	1.000	
Negative	53	0.53 (0.314-0.97)		53	0.61 (0.34-1.562)	
Lymph node status			0.003			0.003
Negative	68	1.000		68	1.000	
Positive	72	3.14 (1.76-5.137)		72	3.21 (1.83-4.915)	

Science, Rochester, NY, USA) and were quantified by Image software Quantity One v4.6.2.

Statistical assay

Data analysis was performed with SPSS 11.0 (SPSS Inc., Woking, UK) with $P<0.05$ for significance. Data are expressed as mean ± S.D. from at least three experiments and analyzed by Student's t test. Relation between XB130 and clinicopathologic features were analyzed by Fisher exact test (two variables) or Chi-square tests (three or more variables), and all tests were two-tailed. Univariable survival analysis (disease-specific survival, disease-free survival and metastasis-free survival) were made with the log rank test and all results were displayed in Kaplan-Meier. A Cox Proportional Hazards Model was performed to observe the independent prognostic value of immunoexpression of XB130.

Results

Increased expression levels of XB130 in breast cancer

To investigate XB130 for use as a potential biomarker and therapeutic target for breast cancer, XB130 expression levels in breast cancer and normal tissues were detected by immunohistochemistry. As shown in **Figure 1**, XB130 staining was mainly moderate or strongly positive in invasive ductal carcinoma tissues but not in normal breast tissues. XB130 was expressed in cytoplasm, suggesting that XB130 may be involved in breast tumorigenesis.

Association of XB130 expression with clinicopathological features of breast cancer

The correlations between the expression of XB130 and the clinicopathological features of breast cancer are summarized in **Table 1**. Overall, 87 of the 140 cases (62.1%) showed positive expression of XB130 in the tumor tissues (strong positive staining 3+ and moderately positive staining 2+), whereas 53 (37.9%) of the cases showed negative expression (weak positive 1+ and negative staining 0). Generally, XB130 staining density was significantly higher in invasive ductal carcinoma tissues than in ductal carcinoma in situ tissues (**Table 1**). A positive correlation between the expression of XB130 and positive lymph node was observed ($P=0.0013$). Furthermore, there were also significant associations for the tumor classifica-

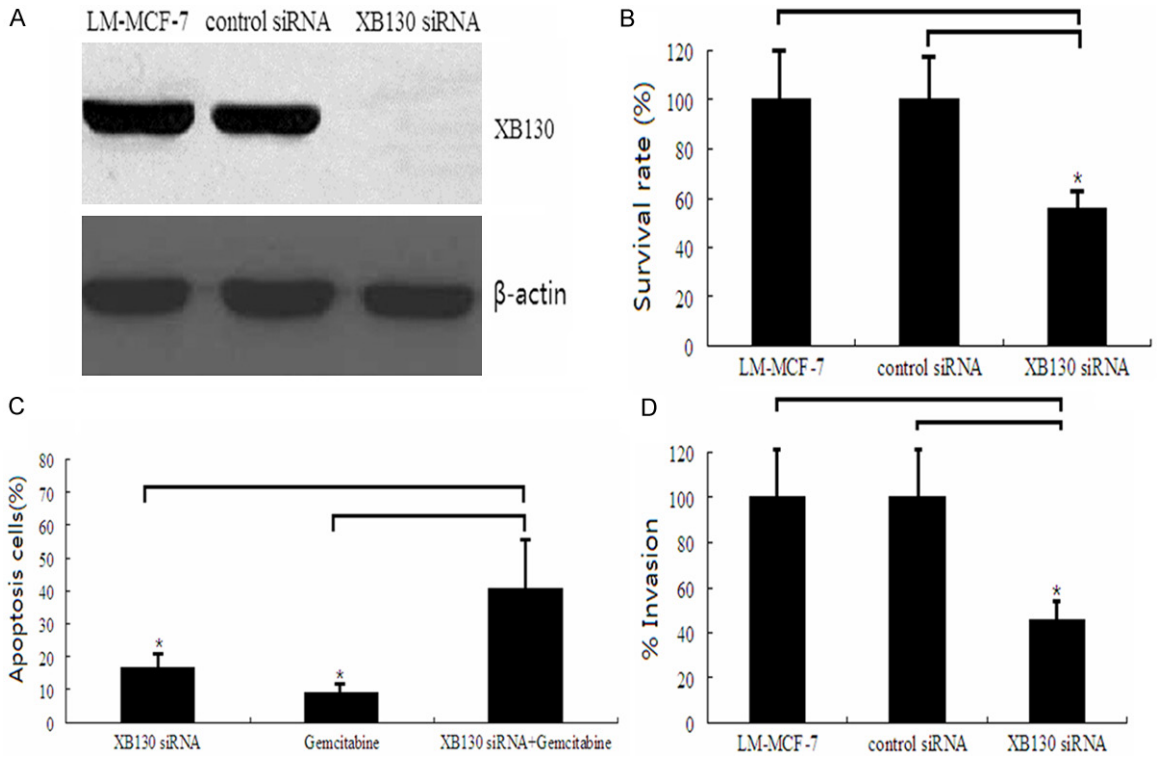

Figure 3. Effect of XB130 silencing on LM-MCF-7 growth, apoptosis, invasion and gemcitabine-induced cell death. A. LM-MCF-7 cells were transfected into XB130 siRNA or control siRNA for 48 hours. Western blot was used to detected XB130 expression after siRNA transfected. B. The effect of XB130 silencing by XB130 siRNA transfection on proliferation in the LM-MCF-7 cells. C. The effect of XB130 silencing by XB130 siRNA transfection or/and combined with gemcitabine treatment on apoptosis in the LM-MCF-7 cells. D. The effect of XB130 silencing by XB130 siRNA on invasion in the LM-MCF-7 cells.

tion (P=0.024) and clinical stage (P=0.0084) in patients who had positive XB130 expression compared with patients who had negative XB130 expression. There was no significant association between XB130 expression and the other clinical features (**Table 1**).

High XB130 expression is associated with poor prognosis

Kaplan-Meier survival curves showed that patients who had positive XB130 expression were more likely to have a shorter overall survival (P=0.0001, **Figure 2A**) and recurrence-free survival (P=0.0001, **Figure 2B**) compared with patients who had negative XB130 expression, suggesting that XB130 overexpression may be associated with a poor clinical prognosis. Patients who had positive XB130 had a poor recurrence-free survival (P=0.0001) compared with patients who had negative XB130 expression (univariate analysis) (**Table 2**). Overall survival examined by Cox univariate analysis also indicated that positive XB130 was significantly associated with shorter survival (P=0.0001). Univariate Cox regression analyses showed that tumor size, lymph node status, and XB130 expression level were significantly associated with recurrence-free and overall survival, whereas other clinical characteristics lost their predictive significance. Furthermore, multivariate Cox regression analyses revealed that positive XB130 was an independent risk factor for overall survival and recurrence free survival (**Table 3**). Patients who had positive XB130 were prone to have an early recurrence compared with patients who had negative XB130 expression (**Table 2**).

Knockdown of XB130 inhibits growth of LM-MCF-7 cells in vitro

To investigate the function of XB130 sliencing in breast cancer cell survival, the XB130 siRNA was transiently transfected into LM-MCF-7 cells. Significantly reduced XB130 protein level was found in the LM-MCF-7/XB130 siRNA cells (**Figure 3A**). Because XB130 has been found to

be linked to the ptomotion of cell proliferation and XB130 sliencing has been found to be linked to the inhibition of cell proliferation [7, 8]. We analyzed the cell proliferation rate in XB130-silenced LM-MCF-7 cells. XB130-silenced LM-MCF-7 cells showed a significantly decreased cell proliferation rate (**Figure 3B**).

Knockdown of XB130 induces apoptosis of LM-MCF-7 cells in vitro

To examine whether XB130 sliencing influences cell death, cell death was detected in XB130-silenced LM-MCF-7 cells by flow cytometry. XB130-silenced LM-MCF-7 cells showed significantly increased cell apoptosis rate compared with LM-MCF-7-mock transfectant (**Figure 3C**). Taken together, these results indicated that inactivation of XB130 may induce apoptosis.

Knockdown of XB130 inhibits invasion of LM-MCF-7 cells in vitro

We tested whether XB130 sliencing affected the invasion capabilities of LM-MCF-7 cells by using an in vitro invasion assay. Cells were seeded in the upper part of a Matrigel-coated invasion chamber in a reduced (5%) FCS concentration. After 24 h, cells that migrated in the lower chamber containing a higher (10%) FCS concentration were stained and counted. In XB130-silenced LM-MCF-7 cells, invasion was significantly reduced (**Figure 3D**). The results show that XB130 modulates invasion of LM-MCF-7 cells in vitro.

Knockdown of XB130 enhances gemcitabine-induced cell death

To investigate the role of XB130 on the gemcitabine-mediated cellular response, we attempted to down-regulate its expression by employing siRNA targeting the XB130. Protein expression analysis indicated that XB130 was completely inhibited after 2 days from the initial siRNA transfection (**Figure 3A**). Flow cytometry analysis was conducted to measure cell death in response to gemcitabine treatment in XB130 knockdown cells (**Figure 3C**). Treatment with 50 nM gemcitabine for 72 h led to 9% cell death. Gemcitabine treatment in XB130-reduced cells resulted in 38% cell death. Preliminary experiments conducted with control siRNA neither modified the percentage of control LM-MCF-7 cells in sub-G1 nor the expression of the XB130 (data not shown). They indicate that LM-MCF-7 cells are sensitized to gemcitabine treatment following cellular depletion of the XB130 by RNA interference.

Discussion

Both clinicopathological markers and molecular classification have clearly indicated that breast cancer is a heterogeneous disease with distinct clinical outcomes [19]. To this end, hormone receptors (ER and PR) and Her-2/neu have been used with certain degrees of success as the biomarkers. While the existing predictive markers have vastly improved our ability to manage breast cancer patients and improved outcomes by matching therapeutic strategies with the types of breast cancer and existence of risk factors, it has also become evident that additional markers are needed to improve patient outcomes.

XB130 has been recently cloned as a molecular homologue of AFAP-110 [5]. Although the regulation and potential functions of this protein have just begun to be elucidated, it has already emerged as a unique and functionally multifaceted adaptor molecule. Recently, expression of XB130 has found in a variety of cell lines derived from thyroid, lung, esophageal, pancreatic, and colon cancers. Shi et al [8] has reported that XB130 low expression patients might be sensitive to cisplatin and irinotecan. The clinical prospective of this study includes: XB130 may act as GC prognostic biomarker for its low expression implicating for unfavorable outcomes, and assessment of XB130 expression may help guide clinical medication in GC. In the esophageal squamous cell carcinoma (ESCC), expression of XB130 in ESCC cells may affect cell cycle progression and impact prognosis of patients with ESCC [6]. In the current study, we examined a cohort of 140 Chinese breast cancer specimens and report the evidence that XB130 correlated with more aggressive breast cancer. Our data suggest that XB130 may serve as a marker for poor prognoses. XB130 expression level was significantly associated with recurrence-free and overall survival. Furthermore, multivariate Cox regression analyses revealed that positive XB130 was an independent risk factor for overall survival and recurrence free survival. No correlation was found between XB130 and PR, ER

and Her-2 levels. To our knowledge, this is the first IHC study to investigate the potential utility of XB130 as a biomarker of breast cancer among Chinese patients.

In WRO thyroid cancer cells, knockdown of XB130 using small interfering RNA inhibited G1-S phase progression, induced spontaneous apoptosis, and enhanced intrinsic and extrinsic apoptotic stimulus-induced cell death. Growth of tumors in nude mice formed from XB130 shRNA stably transfected WRO cells were significantly reduced, with decreased cell proliferation and increased apoptosis [7]. In gastric cancer, targeting XB130 by shRNA sensitized the GC cells to 5-FU, cisplatin and irinotecan. The cell viability in sh-XB130 and wild-type groups was both suppressed by all three chemotherapeutic agents in a dose dependent way [8]. It is suggested that inhibition of XB130 alone or in combination with other approaches could be as a promising therapeutic strategy.

In the present study, we found that the knockdown of XB130 expression inhibited the invasion capability of LM-MCF-7 cell lines in vitro. XB130 significantly affected cell proliferation and apoptosis of the LM-MCF-7 cell lines in agreement with the data on the proliferation of XB130-silenced WRO thyroid cancer cells [7].

Resistance to gemcitabine-based chemotherapy is one of the cause of treatment failure in human breast cancer. XB130 status is recently found to be associated with chemotherapeutic agents sensitivity, suggesting that additional factors may be involved. Our studies here showed LM-MCF-7 cell is resistant to gemcitabine-mediated cellular response. However, downregulation XB130 by siRNA significantly attenuated the ability of gemcitabine-induced apoptosis, and this was dependent of XB130 expression. These results support the hypothesis that XB130 is a determinant of gemcitabine sensitivity, and suggest that XB130 overexpression contributes to chemoresistance.

In conclusion, this IHC study of IP with 140 specimens of Chinese breast cancer provided the first evidence that XB130 is highly expressed in the tumor cells. XB130 could be useful as a prognostic marker of lymph node metastasis, tumor classification, clinical stage, decreased overall survival and increased recurrence-free, as well as for the response to chemotherapy. XB130 is an important mediator in breast cancer development and may be a novel therapeutic target for breast cancer.

Disclosure of conflict of interest

None.

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