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Original Article

Expression of glucose regulated protein 78 in human triple negative breast cancer and its clinical implications

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Abstract: Recently, it was reported that glucose regulated protein 78 (GRP78) may predict survival and responsiveness to chemotherapy or endocrine therapy in breast cancer. However, the expression of GRP78 and another glucose regulated protein-GRP94, and their association with clinical and prognostic implications in triple negative breast cancer (TNBC) are still not clear. In this study, we performed immunohistochemistry and statistical analyses on cancer tissues and paired adjacent normal-like tissues from 71 patients with TNBC. We found that (1) the expression level of GRP78 and GRP94 in TNBC cancer tissues was significantly higher than paired adjacent normal-like tissues ($P<0.01$; $P<0.05$); (2) GRP78 positive staining presents not only in cytosol of TNBC cancer epithelial cells but also in myoepithelial cells of normal breast tissues; (3) GRP78 high-expression was of positive correlation with expression of HIF-1α ($P<0.05$) but not with VEGF in TNBC; (4) patients with high GRP78 expression displayed more lymph node metastasis ($P<0.05$) and poor duration of survival ($P<0.05$). These findings are thought to substantiate the possibility of using GRP78 as a marker or a target treatment candidate for TNBC.

Keywords: GRP78 triple negative breast cancer HIF-1α metastasis

Introduction

Triple negative breast cancer (TNBC) is a subtype of human breast cancer (HBC), characterized by lacking the expression of estrogen receptor (ER), progesterone receptor (PR) and the absence of human epidermal growth factor receptor (HER2) overexpression or gene amplication [1]. It includes about 10-20% of all HBC [2, 3]. Moreover, TNBC is more aggressive with early local and distant recurrence, visceral metastases comparing with other HBC. Unfortunately, since TNBC is heterogeneous form of HBC, until now it lacks specific markers. This feature also limits the success of targeted therapy, so patients always have a poor outcome, especially those with advanced TNBC.

To date, researchers have tried to identify specific molecular features to subtype TNBC. Traditional category classifies TNBC as basal-like, normal-like and claudin-low subtype using markers such as CK5, epidermal growth factor receptor (EGFR), vimentin, P-cadherin and so on [4-8]. Pathologists tried to subtype TNBC by histological subclassifications like atypical medullary carcinoma and central acellular zone [9]. Recently, using gene-expression analyses, six distinguishable TNBC subtypes have been identified by Lehmann in 2011: 2 basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL) and a luminal androgen receptor (LAR) subtype [10]. As they reported in that research, BL1 and BL2 subtypes were responsive to cisplatin. M and MSL subtypes might be sensitive to AR antagonist [10]. Additionally, McNamara reported that the presence of androgen synthesizing pathways in addition to AR expression in TNBC predicted a better clinical outcome by suppressing cell proliferation [11]. Up to now, molecular markers like BRCA1/2, EGFR, VEGF,

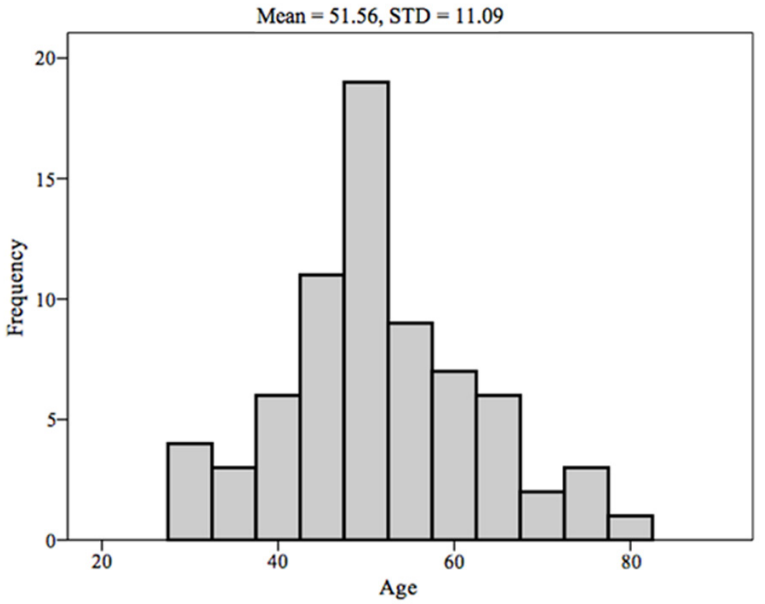

Figure 1. Histogram of ages of patients.

mTOR and Src have been used to investigate target treatment [12-16]. However, what we know about TNBC is far from enough, more specific molecules or active biologic pathways will be required as the targets of new biological drugs.

The glucose regulated protein 78 (GRP78, also known as BiP and HSPA5) and GRP94 (also known as GP96 and HSP90B1) are stress-inducible molecular chaperones. They are located in the endoplasmic reticulum. Functionally, GRP78 is a crucial regulator of endoplasmic reticulum function due to its roles in protein folding and assembly, retrograde transport across the endoplasmic reticulum membrane of aberrant proteins [17, 18]. In cell stress response, GRP78 binds to accumulated misfolded proteins thereby, having to release the three endoplasmic reticulum stress transducers-protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). These released proteins lead cells to activate the UPR pathway [19]. For tumorigenesis, GRP78 controls processing and maturation of various cell surface receptor proteins and secretory proteins that are critical for response to proliferative signal in cancer cells. On the other hand, the expression of GRP94 is increased under chronic exposure to reactive oxygen species (ROS) in breast cancer cells. Moreover, GRP94 was reported to show higher expression in recurrent human breast cancers than in paired primary neoplasias [20]. In mouse model of breast cancer, heterozygous knockout of *Grp78* prolongs latency period and impedes tumor growth by reducing tumor cell proliferation, angiogenesis and by inducing apoptosis [21]. In addition, overexpression of GRP78 and GRP94 is associated with higher pathological grade and aggressive behavior in human cancers including breast, colon, liver and prostate [22-27]. However, the expression of GRP78 and GRP94, as well as their relation with clinical and pathological factors, has not been explicitly defined in TNBC.

In the present study, we investigated that the expression of GRP78, GRP94, VEGF, HIF-1α and the relation with clinicopathological features by examining 71 cases mastectomy specimens obtained from patients with TNBC in our hospital. We found that the level of GRP78 and GRP94 expression in TNBC cancer tissues was statistically higher than paired adjacent normal-like tissues. GRP78 positive staining presents not only in cytosol of TNBC cancer epithelial cells but also in myoepithelial cells of normal breast tissues. We also observed that GRP78 expression was positively correlated with HIF-1α expression in the TNBC tumor tissues. Finally, GRP78 expression was associated with more lymph node metastasis and poor prognoses in TNBC in our research.

Materials and methods

Patients and specimens

Paraffin-embedded tumor samples of 71 patients with primary resected triple negative breast cancer were obtained from First Affiliated Hospital of Dalian Medical University between September 2002 and April 2013. The follow-up data were collected up to July 2015. None of the patients had received radiotherapy or chemotherapy before surgical resection and all of the patients were treated with routine chemotherapy after the operation. All of these were carried out by curative operation and

Table 1. Correlations between GRP78 expression and clincopathological variables

Variable	*n*	GRP78^{-}	GRP78^{1+}	GRP78^{2+}	GRP78^{3+}	*P*-value
Age						
<35 years	5	3	1	0	1	0.6007
>35 years	66	34	18	10	4	
Tumor size						
T1	32	19	9	2	2	0.4722
T2	36	16	10	7	3	
T3	3	2	0	1	0	
Lymphovascular invasion status						
Negative	45	26	15	4	1	0.0267
Positive	26	11	4	6	4	

Table 2. Relation between immunohistochemical markers on normal tissues and on tumor tissues

Variable	-	1+	2+	3+	*P*-value
GRP78					
Normal	70	1	0	0	2.5E-11
Tumor	37	19	10	5	
GRP94					
Normal	27	31	12	1	0.0279
Tumor	3	36	20	12	
VEGF					
Normal	58	12	1	0	2.2E-16
Tumor	7	46	17	1	
HIF-1α					
Normal	68	3	0	0	3E-14
Tumor	26	41	3	1	

examined by two pathologists with a specialization in breast pathology. This study was performed with the approval of Dalian Medical University Ethics Committee.

Tumor tissues of all cases were fixed in 4% paraformaldehyde solution (pH 7.0) for period not exceeding 24 h and were paraffin embedded. 4 μm-thick sections were cut, mounted on 3-aminopropyl triethoxysilane coating-microscope slides and dried 37°C for 30 minutes for immunohistochemistry. The histological type and grade of tumors were determined by hematoxylin-eosin staining.

Antibodies

Polyclonal rabbit anti-human GRP78 antibody was from Cell Signaling. Polyclonal rabbit anti-human GRP94 antibody and monoclonal mouse anti-HIF1α antibody were from Abcam. Polyclonal rabbit anti-VEGF antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse anti-human ER antibody, monoclonal mouse anti-human PR antibody and monoclonal mouse anti-human HER2/neu antibody were from Dako.

Statistical analysis

All data were analyzed with SPSS statistics software (Version 13.0, Chicago, IL, USA). Relationships between the markers of interest and clinicopathological parameters were evaluated using Pearson's χ^2 text, Fisher's exact test, and Pearson correlation coefficients. Pearson correlation coefficient values were considered no correlation (0.0-0.2); a low degree of correlation (0.2-0.4); a moderate degree of correlation (0.4-0.6); a marked degree of correlation (0.6-0.8); or a high correlation (0.8-1.0). Survival analysis was conducted by using Kaplan-Meier method and compared using log-rank test. All *P*-values were two-sided, and the *P*-value was considered statistically significant less than 0.05.

Results

Patient characteristics

The ages of patients were from 30 to 80, with mean 51.56 and standard deviation 11.09 (histogram is illustrated by **Figure 1**). There were 37 cases with GRP78-negative (52.1%) and 34 cases with GRP78-positive (47.9%) (**Table 1**). The mean ages were statistically different between patients with positive and negative expression for GRP78 (49.9 and 53.6, respectively; P = 0.048). It suggests that GRP-positive patients seem to have younger onset age than GRP-negative patients in our study.

Immunohistochemistry

We performed immunohistochemistry in 71 cases TNBC specimens and their corresponding adjacent normal tissues. We found that

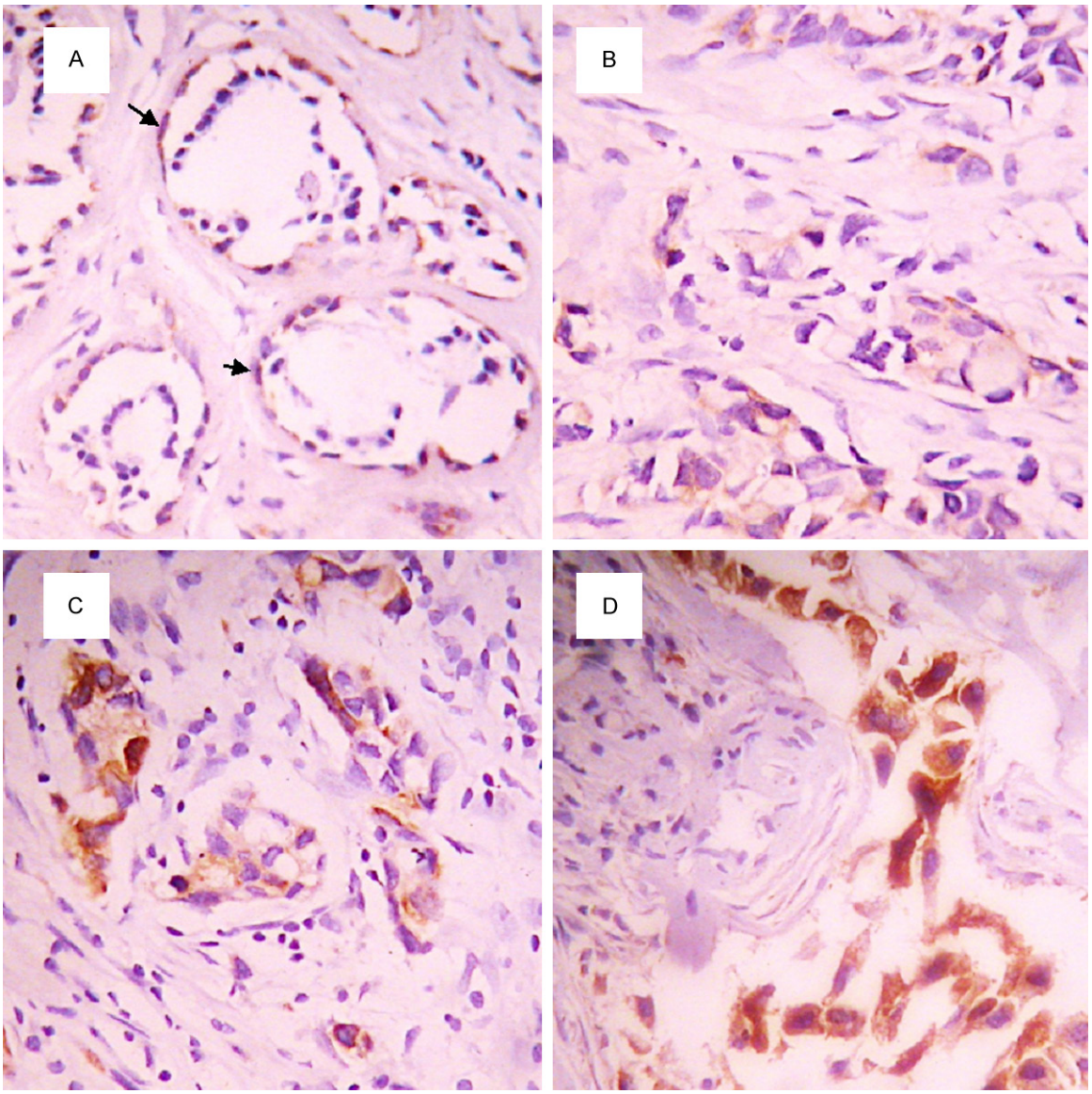

Figure 2. Representative immunohistochemical staining for GRP78 in TNBC (400× magnification). A. GRP78 expression was observed in myoepithelial cells (cytosol) of adjacent normal tissues; B. Weakly positive; C. Moderate positive; D. Strongly positive cytoplasmic staining for GRP78 in cancer cells. Arrow means the myoepithelial cells of normal-like breast tissues stained by GRP78.

GRP78 was overexpressed in 47.89% of tumor samples (34 of 71) comparing with normal tissues (1/71) (47.89% vs 1.41%; P<0.01) (**Table 2**). The GRP78 protein appeared to be expressed in cytoplasmic components of tumor cells (**Figure 2B**, **2C**). Meanwhile, the GRP78 positive staining was also observed in cytoplasm of myoepithelial cells of the adjacent normal breast tissues. Whereas epithelial cells showed no staining (**Figure 2A**). In contrast, GRP94 displayed similar cytoplasmic staining pattern. However, it did not present in myoepithelial cells of normal breast tissues (**Figure 3A-D**; **Table 2**). In addition, the cancer cells of TNBC were also stained more strongly by HIF-1α or VEGF comparing with adjacent normal-like epithelial cells (**Figure 4A-D**; **Table 2**).

Correlations between GRP78 expression and clinicopathological features

We performed statistic analysis with GRP78 expression and clinical features including onset age, tumor size and lymphovascular invasion status. The positive lymphovascular invasion

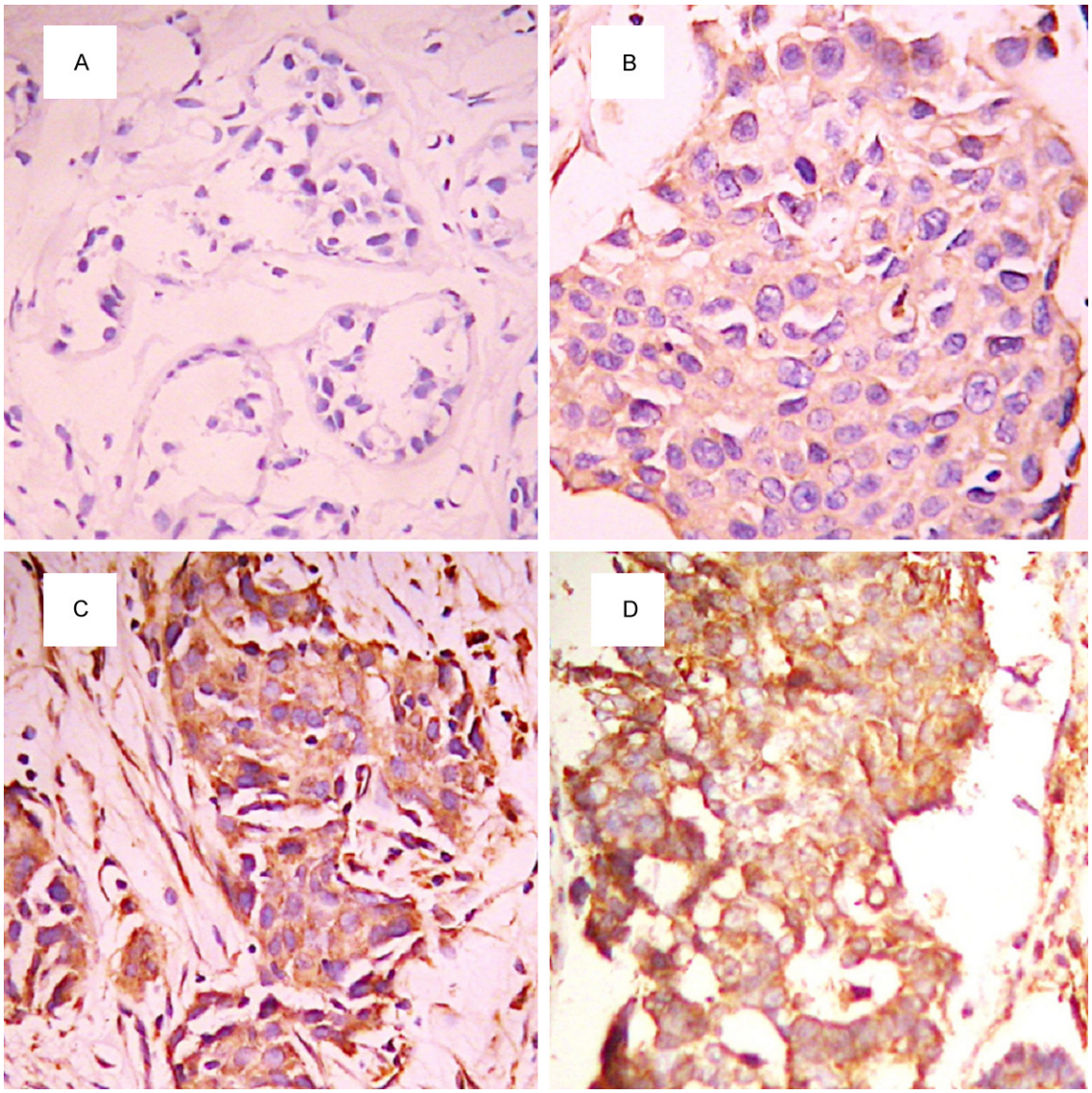

Figure 3. Representative immunohistochemical staining for GRP94 in TNBC (400× magnification). A. Negative for GRP94 in normal breast tissue; B. Weakly positive; C. Moderate positive; D. Strongly positive cytoplasmic staining for GRP94 in cancer tissues of TNBC.

status had statistically high-expression rate for GRP78 than negative group (P = 0.0267, **Table 1**).

Relations between GRP78 expression and VEGF or HIF-1α

We also estimated the relations between GRPs expression and VEGF or HIF-1α. We found a significantly positive correlation between GRP78 and HIF-1α (correlation coefficient = 0.26, P = 0.028). However, correlations between GRP78 and VEGF, HIF-1α and VEGF (**Table 3**) or GRP94 and other three markers (data not shown) were not statistically significantly different.

Survival outcome

Survival analysis was conducted by dividing the patients into two subgroups according to negative and positive for GRP78. Subgroup analysis demonstrated that GRP78 expression was associated with over-all survival function in TNBC (P = 0.042, log rank test, **Figure 5**). It suggested that GRP78 expression was associated with poor prognoses in TNBC in our research.

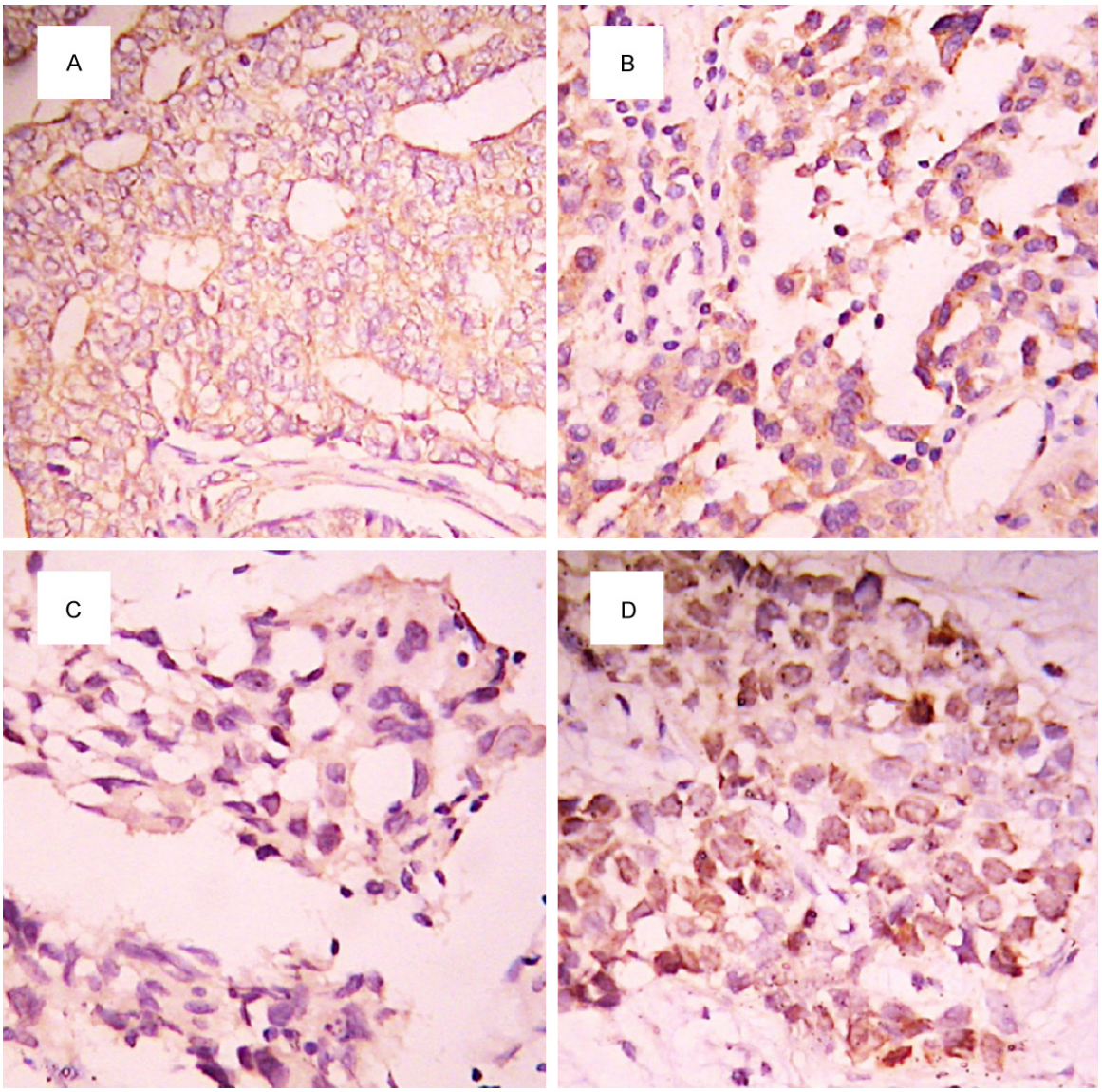

Figure 4. Representative immunohistochemical staining for VEGF or HIF-1α in TNBC (400× magnification). Weakly positive (A) and Strongly positive (B) cytoplasmic staining for VEGF; Weakly positive (C) and strongly positive (D) nuclear staining for HIF-1α.

Table 3. Relation among GRP78, VEGF and HIF-1α on tumor

Variables	Correlation coefficients	*P*-value
GRP78 vs VEGF	0.128	0.288
GRP78 vs HIF-1α	0.26	0.028
VEGF vs HIF-1α	0.141	0.241

Discussion

Recently, many researchers focus on identifying new molecules as potential clinical markers or therapeutic targets for TNBC, try to uncover the association between clinical implication and new targets. Unfortunately, as the heterogeneous character of TNBC, there is no explicit classification to guide treatment like other types of HBC yet.

GRP78 and GRP94 are two kinds of glucose-regulated proteins. They control processing and maturation of a widely various proteins that are crucial for the ability of cancer cell to respond to extrinsic proliferative signals. This mechanism can help cancer cells to survive against hypoxic acidic or glucose starvation conditions and immunological response of the host [29, 30]. Previous study reported by

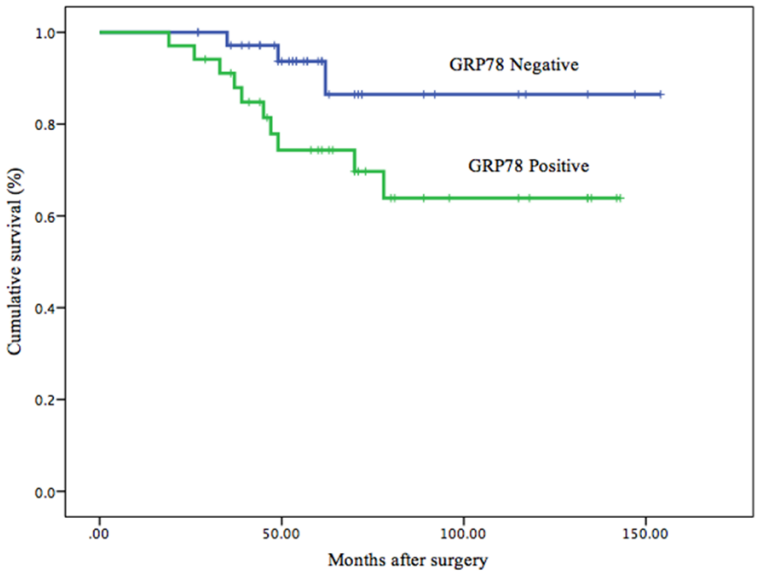

Figure 5. Overall survival for patients according to GRP78 expression (P = 0.042, log rank test).

Melendez showed surface positive GRP94 expression in breast cancer cell lines but not nonmalignant cell lines, whereas GRP78 in both [31]. In contrast, Fernandez have observed that overexpression of GRP78 was inclined to appear in most of the more aggressive ER-tumors on surgical breast tissues [22]. The present study demonstrated that both GRP78 and GRP94 were overexpressed in TNBC tumor tissues comparing with normal tissues. In addition, it appeared to be expressed only in cytosol of TNBC tumor cells but not cell surface. These results suggest these two kinds of GRPs may play critical roles in TNBC. Further statistic analysis showed GRP78 expression was associated with serious lymphovascular invasion status and poor prognoses in TNBC. Hence, our findings provide insights into using GRP78 as a new specific marker and a potential molecular target of TNBC. However, the underlying mechanisms of GRP78 involved in the tumorigenesis of TNBC are required to know in the follow work.

Meanwhile, the GRP78 positive staining was also observed in cytoplasm of myoepithelial cells of the adjacent normal breast tissues. Whereas normal glandular-epithelial cells showed no staining. Up to now, several myoepithelial markers have been investigated like p63, maspin, P-cadherin, actin, S-100 protein, HMW-CK and CD109 [32, 33]. A follow-up study should be necessary to elucidate the specificity and sensitivity of GRP78 comparing with other myoepithelial markers. In addition, because morphological features that the cytoplasm of myoepithelial cells was packed with abundant endoplasmic reticulum [34], we doubt if GRP78 localizes in endoplasmic reticulum of normal breast myoepithelial cells.

Similarly, colon cancer tissues showed high expression levels of GRP78 comparing with adjacent normal tissue, Kuo et al. verified GRP78 may induce human colon cancer tumor growth through HIF-1α/VEGF/VEGFR2 pathway in vitro [35]. Data from our study showed that a positive correlation between levels of GRP78 and HIF-1α in TNBC cancer tissues. GRP78 expression was significantly related to the expression of HIF-1α in TNBC. It demonstrated that GRP78 might play an important function in TNBC development and metastasis through a defense mechanism for the survival against hypoxic acidic condition. More studies should be performed to clear this mechanism in future.

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Disclosure of conflict of interest

None.

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