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Expression of glucose-regulated protein 78 as prognostic biomarkers for triple-negative breast cancer

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Running title: GRP78 as prognostic biomarkers for TNBC

Keyworlds: GRP78, proliferation, Prognosis, Triple-negative breast cancer, metastasis
Abstract

Introduction: glucose-regulated protein78 (GRP78) is a stress-induced endoplasmic reticulum chaperone protein. It is closely related to the occurrence, development, proliferation, differentiation and drug resistance of breast cancer. However, the association and clinicopathological features between GRP78 and triple negative breast cancer (TNBC) remain to be studied.

Material and Methods: Clinical and pathological characteristics and overall survival were analyzed retrospectively in 179 surgically resected TNBC patients. GRP78 was detected by immunohistochemistry (IHC) using breast cancer tissue microarrays (TMAs), and the association between GRP78 levels and clinicopathological factors and prognosis was analyzed. Furthermore, GRP78 expression in human TNBC and NTNBC cell lines was detected by Western blot and qRT-PCR. After Si-GRP78 knocked-down GRP78 in MDA-MB-231 and BT549 cell lines, cell proliferation was detected using Cell Counting Kit-8 (CCK-8) and cell colony formation was detected by crystal violet staining, respectively.

Results: GRP78 was expressed in triple negative breast cancer (TNBC). GRP78 expression was significantly associated with invasive, distant metastasis and proliferation of TNBC ($P < 0.05$). In addition, patients with positive GRP78 expression had shorter overall survival (OS) and disease-free survival (DFS). And the high expression of GRP78 was significantly associated with disease-free survival (DFS) in patients with TNBC ($P < 0.001$).

Conclusions: These findings improve our understanding of the expression pattern of GRP78 in TNBC and clarify the role of GRP78 as promising prognostic biomarkers for triple-negative breast cancer.
**Introduction**

Breast cancer is the most prevalent cancer in women and the second leading cause of cancer death worldwide (Cai et al., 2018). Breast cancer can be initially classified into three different types based on the presence or absence of estrogen receptors (ERs), progesterone receptors (PRs), and the human epidermal growth factor receptor 2 (Her2/neu) (Carlson et al., 2012; Garcia-Estevez and Moreno-Bueno, 2019). Hormone receptor-positive breast cancers that express ER and/or PR constitute approximately 60% of all breast cancers (Buzdar, 2009). The Her2/neu receptor is overexpressed in approximately 20% of all breast cancer cases, while triple-negative breast cancer (TNBC) constitute approximately 20% of breast cancer cases and are negative for the expression of ER, PR, and Her2/ Neu (Slamon et al., 2001; Anders and Carey, 2009). It is widely accepted that TNBC is one of the most aggressive subtypes, often associated with poor patient outcome due to the development of metastases in secondary organs, such as the lungs, brain, and bone. And there is still lack of effective diagnostic and prognostic markers for TNBC (Slamon et al., 2001; Buzdar, 2009). Traditionally, due to the lack of ER, PR, and Her2/Neu expression, the ineffectiveness of current breast cancer targeted therapies as well as due to the challenges in identifying key molecular drivers of TNBC progression, chemotherapy has been the foundation of treatment for patients with this disease over the last decades. Despite its sensitivity to chemotherapy, TNBC is associated with a higher risk of distant recurrence, high rates of metastases, higher probability of relapse and worse overall survival (OS) compared to other subtypes (Ovaricek et al., 2001; Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2005). Metastatic spread of tumor cells is a highly complicated, yet poorly understood process, and is consisted of multiple steps, including the acquisition of invasive properties through genetic and epigenetic alterations, the angiogenesis, the tumor–stroma interactions, intravasation through the basement membrane, survival in the circulation, and extravasation of some cancer cells to distal tissues (Nguyen and Massagué, 2007). The molecular complexity of the metastatic process in combination with the lack of effective targeted therapies for TNBC metastasis have fostered significant research efforts during the
past few years to identify molecular “drivers” of this lethal cascade. TNBC and other cancers are characterized by altered glucose metabolism, and the tumor microenvironment is marked by impaired blood flow and hypoxia, all of which can cause endoplasmic reticulum (ER) stress. glucose-regulated protein78 (GRP78) is a multifunctional protein with activities far beyond its well-known role in the unfolded protein response (UPR) which is activated after ER stress in the cells (Neophytou et al., 2005). GRP78 is involved in several aspects of cancer development including tumor survival and proliferation, chemoresistance, angiogenesis, and metastasis (Lee, 2014). Many tumor cells overexpress GRP78 on the outer plasma membrane. In addition, in different types of cancer, such as those of prostate, breast, and melanoma origins, abnormally high GRP78 expression is correlated with tumor resistance, greater risk for cancer recurrence, and an overall decrease in patient survival (Pfaffenbach and Lee, 2011). In a transgene-induced endogenous mammary tumor model, GRP78 haploinsufficiency resulted in delayed tumor latency, decreased tumor proliferation, and increased apoptosis (Wang, Ye, et al., 2010). Strikingly, in mice harboring bi-allelic conditional knockouts of both GRP78 and PTEN in the prostate epithelium, prostate tumorigenesis was potently arrested, providing the first evidence that GRP78 is required for tumorigenesis driven by loss of PTEN and activation of the PI3K/AKT oncogenic pathway (Fu et al., 2008). Indeed, the ligation of cell-surface GRP78 by antibody slowed growth rate and blocked PI3K/AKT signaling (Misra and Pizzo, 2008). Recently, some studies have found that the expression of GRP78 is positively correlated with the invasiveness of breast cancer cell lines. After down-regulating the GRP78 gene table, the invasion and metastasis ability of breast cancer cells will be significantly reduced (Dong, Stapleton et al., 2011). These findings suggest that GRP78 may be a prognostic factor and therapeutic target for breast cancer patients.

In this study, we explored the expression pattern of GRP78 in TNBC. Our results indicate that GRP78 is associated with invasiveness, proliferation and metastasis of triple-negative breast cancer. Our study provides evidence that GRP78 could be prognostic factors and potential therapeutic targets in TNBC.
METHODS

Cell lines and culture

Human MDA-MB-231, BT549, MDA-MB157, SUM159PT TNBC cells and MCF-7, T47D, SKBR3, NTNBC cells, were purchased from the American Type Culture Collection (Manassas, VA, USA), and grown in DMEM or RPMI. Human non-tumor mammary epithelial cell lines MCF-10A was cultured according to the suppliers’ instructions. TNBCs and MCF-7 were cultured in high glucose–DMEM medium, T47D, and SKBR3 were maintained in RPMI 1640 medium, both purchased from (DMEM, Invitrogen, CA, USA). All the cells were incubated in 5% CO2 atmosphere and 95% relative humidity at 37 °C. All of these cell lines were authenticated by the standard short tandem repeat DNA typing.

Human tissue specimens and patient information

This study was approved by the Ethics Committees of Sun Yat-Sen University Cancer Center and Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. From October 2005 to September 2011, 179 pathologically diagnosed triple-negative breast cancer tissues were obtained at the Cancer Center of Sun Yat-sen University. Tissue samples were formalin-fixed, routinely processed, and embedded in paraffin by standard methodology after obtained during surgery and were stored in the Department of Specimen and Resource in Sun Yat-Sen University Cancer Center. They provided complete well-documented clinical histories and follow-up data, and had surgery, meanwhile most of the patients also had preoperative chemotherapy and radiotherapy. Patient characteristics are detailed in Table 1, and clinical outcomes were collected until death, censorship, or loss to follow-up. The date of death and the date of relapse were used to calculate estimate overall survival (OS) and disease-free survival (DFS). Disease-free survival (DFS) was defined as the period between the time of surgery and that of recurrent or
metastatic disease. Breast tumor tissue cores were collected from each patient, and
used to construct a TMA. Tissue microarrays (TMAs) were constructed as follow: in
brief, histological slides were retrieved and reviewed, and Chips of 1mm diameter
were punched from cancer tissues. Clinical parameters and overall survival data were
obtained from patient’s medical records.

Immunohistochemistry (IHC) analysis and scoring system

Briefly, the tissue microarrays (TMAs) Baked at 65°C for two and a half hours, Then,
The sections were then removed from paraffin in xylene (100%) solution, rehydrated
in a descending ethanol series (100%, 95%, 85%, and 75% ethanol) and water, and
subjected to heat-mediated antigen retrieval with citrate buffer for 5 min at 100°Cin a
pressure boiler. (Dilution1:100). Next Endogenous peroxidase activity was blocked
through incubating the slides for 10 minutes with Peroxidase blocker, followed by
treatment with Non-<animal immune serum for 1 hour to reduce nonspecific binding.
The sections were then incubated with primary antibodies GRP78 (AF5366, Affinity,
USA [ dilution:1:200]) overnight at 4°C. After 36 hours, Rewarming, washed by
phosphate buffer saline (PBS). the sections were incubated 1 hour with rabbit
antibody (E030120-02, EARTHOX, USA, [Dilution: 1:500]), After DAB (Dako,
Glostrup, Denmark). staining 6 min, in order to Color development. The sections were
counterstained with hematoxylin, and the slides were then dehydrated with graded
ethanol( 85%,95%,100%)for 3 min and mounted, Finally, the sections were dried at
65°Cfor 5 minutes.

Immunohistochemical analysis and scoring

The GRP78 expression was evaluated on the basis of the ratio of positive cells per
specimen and staining intensity. According to the percentage of positive cells and the
intensity of staining, the staining of GRP78 and triple negative breast cancer was
divided into three groups: negative, positive and strong positive. Two independent
investigators scored these sections (double-blind). The staining intensity and
percentage of GRP78 positive cells was assessed and scored independently by two experienced pathologists who were blinded to patient outcomes and clinicopathological information. The intensities of GRP78 staining were scored between 0 and 3 according to the standards of 0 = negative, 1 = weak, 2 = moderate and 3 = strong. The percentages of GRP78 positive cells in 3 representative high-power fields of individual samples were analyzed. Scores ranging from 0 to 12 were obtained by multiplying the percentage of positive cells by the staining intensity (0–3). The percentage of positive cells were scored as follows: 0= 0% stained, 1=1-25% stained, 2 = 26-50% stained, and 3 = 51-75% stained, 4 = 75% -100% stained. The marker scores for intensity and percentage of positive cells were multiplied. and samples were divided into three grades: 0-1=absent (−), 2-5=low expression (+), and 6-12=high expression (++). For survival analysis, GRP78 expression levels were dichotomized as low expression and high expression with the cutoff set at the 50th percentile.

Western blotting
Protein was extracted using RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 0.5% sodium deoxychlorate, 0.1% SDS and 1.0% Igepal CA-630 (NP-40)) with a proteinase inhibitor (500 mM phenylmethylsulfonyl fluoride), spun down at 14,000 × g for 30 min at 4 °C. were measured with the Protein BCA Assay Kit (Bio-Rad, USA). Samples were boiled for 10 minutes, the protein samples were separated by 10% sodium dodecylsulfate–PAGE and subsequently blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membrane was blocked in 5% blocking buffer (5% nonfat milk and 0.1% Tween-20 in PBS) for 2 h at room temperature, and then incubated with primary antibodies at 4°C overnight. Western blots were performed using antibodies for anti-GRP78(AF5366) from Affinity (USA) and anti-β-actin (AF7018) from Affinity (USA), and then membranes were further incubated 1 hour in a secondary antibody (1:5000 dilution).
**Quantitative RT-PCR analysis (qRT-PCR)**

Total RNA of cell lines was extracted using TRIZol reagent (Invitrogen, USA) according to the manufacturer's instructions. qRT-PCR was conducted with SYBR Premix Ex Taq™ (Takara, Japan) and an All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia) using Bio-Rad IQ™5 Multicolor Real-Time PCR Detection System (USA). The threshold cycle (CT) value for GRP78 were normalized against the CT value for internal control GAPDH. The relative foldchange in expression with respect to a control sample was calculated by the 2^−ΔΔCT method. The following human primers (Generay Bioteach CO. Ltd, shanghai, China) were used in this study.

**GRP78**

F: 5’-TTTGTTTGCCCACCTCCA-3’

R: 5’-CATCAAGTTCTTGCCGTTCA-3’

**GAPDH**

F: 5’ GACATCCGCAAAGACCTG 3’

R: 5’ GGAAGGTGGACAGCGAG 3’

**Cell counting kit-8 (CCK-8) assay**

Cell Proliferation Assay: The effect of GRP78 on MDA-MB-231, BT549 cells proliferation was examined by CCK-8 assay. In brief, MDA-MB-231 and BT549 cells were seeded in 96-well plates at a density of 10^3 cells/well. After 24 h incubating, 10uL of CCK-8 solution was added to the plated cells which were incubated at 37°C for 2 h. OD values at 450 nm were measured using a microplate reader (BioTek) to reflect cell proliferation.
Colony formation assay

The colony formation assays also revealed the proliferation promote role of GRP78 in both MDA-MB-231 and BT549 cells. MDA-MB-231 and BT549 were seeded at a density of $10^3$ in 6-well plates, colonies were grown for 3-4 days, fixed with 4% paraformaldehyde for 15 min, stained with crystal violet, Subsequently, photographed and counted.

Knockdown of GRP78

The silence efficiency of these four different siRNA sequences was analysed 48 h post-transfection via quantitative RT-PCR and compared with negative control siRNAs that did not target any known gene. The siRNA sequence with the strongest silence efficiency on GRP78 was used for the follow-up study. And levels of GRP78 gene silencing were assessed 24h, 48h and 72h post transfection by qPCR respectively. 48h was selected to be the time-point for observation in later experiment. Cell were transfected using RNAiMAX (TranSheepBio-Tech Co., Ltd. Shanghai, China) according to the manufacturer’s protocol in a final concentration of 50 nM. After transfection of cells for 48 hours, the transfection efficiency was analyzed by Western blot and qPCR.

Statistical analysis

Statistical analysis was performed using SPSS version 25.0 (IBM Corporation, Armonk, NY, USA). Using Pearson's $\chi^2$ test to compare the association between the clinicopathological characteristics, and performing Fisher's exact test as needed. Two-tailed Pearson correlation was used to examine the correlation of GRP78 and TNBC expression. Comparisons of clinicopathological variables between different GRP78 and TNBC expression groups were performed using Chi-Square tests.
Two-sided P-values of < 0.05 were considered to be statistically significant. Survival analysis was performed using the Kaplan-Meier method with the log-rank test and multivariate Cox proportional hazard model. Univariate and multivariate analyses were used to assess the association between TNBC and GRP78, age, menstruation, T stage, pathological grade, Ki67, P53. Then, factors significantly associated with GRP78 in univariate analysis were then entered in a multivariate analysis to identify variables associated with independent prognostic values for DFS and OS. Risk ratios and 95% confidence intervals were recorded for each marker. Results were considered statistically significant when $P < 0.05$ was obtained.

Result

**GRP78 was expressed in breast cancer cells**

To explore the expression pattern of GRP78 in breast cancer, and human non-tumor mammary. However, we used Western blot and qRT-PCR to demonstrate the expression of GRP78 in cell lines. Expression of GRP78 were detected in four TNBC cell lines, three NTNBC cell lines and human non-tumor mammary epithelial cell lines MCF-10A. The result showed that GRP78 was expressed in breast cancer cell lines and human non-tumor mammary. [Figures 1, a and b].

**Knockdown of GRP78 inhibits proliferation of triple-negative breast cancer**

To explore the function of GRP78 in TNBC, we explore the role of GRP78 in proliferation in TNBC cell lines, (MDA-MB-231 and BT549). We knocked down GRP78 expression, the inhibition was successful with si-GRP78 on MDA-MB231, and BT549, (Fig. 3a-d). Then the proliferation ability of these two cell lines was detected by CCK8 assay. A CCK-8 assay showed that GRP78 knockdown significantly inhibited cell proliferation (Fig. 3e, f). $P<0.05$. GRP78 knockdown also
reduced the colony formation ability of the cells (Fig. 3g, h). $P<0.05$.

**The expression of GRP78 in human Triple-negative breast cancer and its correlation with patient characteristics**

We used 179 TNBC specimens to further investigate the expression of GRP78 in TNBC and its relationship to the pathological features of clinical specimens. Patient feature details are summarized in Table 1. Detection of GRP78 expression in triple negative breast cancer by IHC and representative results are shown in Table 2. Table 2 shows the expression of GRP78 in triple negative breast cancer (TNBC). We then determined whether GRP78 expression was associated with clinicopathological tumor features, including age, Menopause, Histological grade, lymph node (LN) status, Ki67 expression, and T stage. The results showed that GRP78 expression was associated with T stage, distant metastasis and Lymph node status (values were $P = 0.0018$, 0.006 and 0.005, respectively) [Table 3]. In order to further study the differences between them, they were compared multiple times. The results showed that GRP78 was associated with T stage ($P = 0.007$) [Table 3]. In the expression of GRP78-positive patients, they are more likely to have greater distant metastasis, suggesting that GRP78 may play a key role in the development and progression of breast cancer. In fact, the expression of GRP78 was not associated with, age, Menopause, Histological grade, lymph node (LN) status, Ki67 expression. The results demonstrate that GRP78 is associated with poor clinical outcomes in triple-negative breast cancer. Thus, to analyze the significance of GRP78 in the clinical prognosis of triple-negative breast cancer patients is very important.
The expression of GRP78 is correlated with poor clinical outcome of TNBC.

In univariate survival analysis, Kaplan-Meier survival curve was used and log-rank test was performed to calculate P values in determining clinicopathological variables whether they had significant impacts on patient survival. According to Kaplan Meier survival analysis was performed using overall survival (OS) [Figures 2b] and disease-free survival (DFS) [Figures 2c]. The results showed that patients with positive GRP78 expression had lower OS and DFS than those with negative GRP78 expression (both OS and DFS were $P = 0.000$) [Figures 2b and 2c]. At the same time, GRP78 is positively correlated with Triple-negative breast cancer expression. In a word, these results indicated that the expression of GRP78 is significantly associated with shorter survival in patients with triple-negative breast cancer.

Discussion

Metastasis accounts for most cancer-associated death (Zhang et al., 2019). Breast cancer is associated with high mortality due to tumor metastasis, and Distant metastases account for 90% of breast cancer-related deaths (Ye et al., 2019). Triple-negative breast cancers (TNBCs), as a subgroup of breast cancer, accounts for 10–20% of all cases of breast carcinoma, due to exhibits the most aggressive metastatic behavior and limited treatment options (Wang, Kar et al., 2018; Gupta, Sareyeldin et al., 2019). In TNBCs, metastatic rates are high to visceral organs, in addition, cerebral metastasis is more common (Gupta, Sareyeldin et al., 2019). Patients with TNBCs have higher rates of distant metastasis, more aggressive and a poorer prognosis compared with patients with hormone receptor positive and/or human epidermal growth factor receptor2 positive disease (Lee and Djamgoz, 2018, Chiharu Moriya et al., 2018). Because of currently cytotoxic chemotherapy is the only approved treatment option for TNBC (Sharma, 2016; Cinkaya et al., 2016; Gupta, Sareyeldin et al., 2019). thus, TNBC is more difficult to treat and more likely
to disseminate and recur, and its survival rate tends to be lower than other forms of breast cancer (Sharma, 2016; Jia et al., 2017; Shimelis et al., 2018; Al-Mahmood et al., 2018). Therefore, it is urgent to find useful biomarkers that can predict the metastatic potential of TNBC and serve as prognostic indicators or targets for treatment (Lv et al., 2019; Tang et al., 2019).

At the present study, no study has been dealt with the expression of GRP78 and its correlation with clinicopathological features in TNBC patients. Interestingly, in our study, we used 179 TNBC specimens to investigate the expression of GRP78 in TNBC and its relationship to the pathological features of clinical specimens. We found that GRP78 expression was associated with T stage, distant metastasis, and Lymph node status (values were $P = 0.0018$, $0.006$ and $0.005$, respectively). We also found that patients with positive GRP78 expression was associated with OS and DFS in triple-negative breast cancer, and patients with triple-negative breast cancer who were positive for GRP78 had shorter OS and a higher rate of distant DFS metastasis. It was indicated that TNBCs have a higher metastatic rate, are more aggressive, and have a poorer prognosis.

However, several studies have revealed that HSPA5/GRP78/BiP is involved in cell survival or tumor progression (Chang et al., 2016; Kim et al., 2018), including proliferation, tumor invasion, metastasis, drug resistance, and angiogenesis (Lee, 2014; Casas, 2017; Wang, 2018; Zhang et al., 2018; Dauer, Sharma et al., 2019). For these reasons, HSPA5 is an emerging therapeutic target in cancer development (Casas, 2017). Glucose-regulated protein 78 (GRP78), (also known as BiP and HSPA5), is a protein chaperone, GRP78 is located mainly in the ER, acts as a master in ER-stress. It is belonging to the heat shock protein (HSP) family (Cook et al., 2016; Shen, Ha et al., 2017; Hebert-Schuster et al., 2018). GRP78 expression is maintained at low basal levels in adult tissues, it is strongly induced in tumors (Kim et al., 2018; Aran et al., 2018). Moreover, many experiments have shown that GRP78 is overexpressed in many tumors and is closely related to the risk of tumor invasion, metastasis, and recurrence (Ran et al., 2017).
carcinoma (Mashima et al., 2014), prostate cancer (Dauer, Sharma et al., 2018), pancreatic cancer (Kuang et al., 2016), Colorectal cancer (Takahashi et al., 2011), as well as breast cancer (Bailly and Waring, 2019). Clearly, GRP78 has been suggested as a cancer-targeting marker because it is found on the cell surface of tumors, but not in normal organs (Kim et al., 2018). Also, many studies have shown that GRP78 has significantly reduced cell proliferation, invasion, metastasis and drug resistance after knocking down in many tumor cells. Cell proliferation is one of the important features in cell life (Shen, Chen et al., 2019; Li et al., 2005), it report that GRP78 is expressed elevated in malignant glioma specimens and human malignant glioma cell lines, correlating with their rate of proliferation (Pyrko et al., 2007). Thus, GRP78/BiP, is critical for tumor proliferation, survival (Dong, Ni et al., 2008). and research reports: GRP78 is a marker for poor prognosis in breast cancer patients and has an important role in cancer progression, including the promotion of drug resistance and metastasis (Chang et al., 2016). As well as, GRP78 also expresses the same invasion and metastasis as triple-negative breast cancer in triple-negative breast cancer.

In this study, we first identified that GRP78 knockdown attenuated the proliferation the TNBC cell lines MDA-MB 231 and BT549, which was associated with the invasiveness of GRP78. we knocked down GRP78 expression, then the proliferation ability of these two cell lines was detected by CCK8 assay. A CCK-8 assay showed that GRP78 knockdown significantly inhibited cell proliferation. at the same time, the proliferation promoting effect of TNBC was also determined by colony formation, GRP78 knockdown also reduced the colony formation ability of the cells.

**Conclusion**

In this study, our results indicate that GRP78 is involved in the aggressiveness and proliferation of TNBC. Our study provides insight into the role and molecular
mechanisms of GRP78 in TNBC and may reveal that GRP78 is a prognostic factor and potential therapeutic target for TNBC.

**Ethics statement**

All procedures performed in this study involving human participants were approved by Ethics Committees of Sun Yat-Sen University Cancer Center and the First Affiliated Hospital of University of South China and were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent about the researchable use of the clinical data was obtained from each participant patient. All patient data were anonymous and de-identified prior to analysis.

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**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**


confers chemoresistance to pancreatic cancer cells. Mol Oncol. 12, 1498-1512.
Figure 1. The express of GRP78 in breast cancer cells. a. Four TNBC cell lines and three NTNBC cells were subjected to Western blotting, expression levels of GRP78 were assessed by Western blot (above) β-actin was used as a loading control.

b. Expression levels of GRP78 were determined by qRT-PCR. Quantitative real-time PCR results of the relative expression level of GRP78 in TNBC and NTNBC. GAPDH was used as an internal control. All of the data are shown as the means ± s.e.m. *P < 0.05, **P < 0.01.

Figure 2. The expression of GRP78 in Triple-negative breast cancer. a. The expression of GRP78 were detected by IHC using breast cancer TMAs of 179 patients. For GRP78, cytoplasmic staining intensity was marked as negative (score 0), weak (score 1), moderate (score 2), or strong (score 3). scale bars; 50 µm. b. Kaplan-meier curve of the shorter overall survival (OS) for 179 studied patients. c. Kaplan-meier curve of the shorter disease-free survival (DFS) for 179 studied patients.

Figure 3. The role of GRP78 in the proliferation of MDA-MB-231 and BT549 cell lines. MDA-MB-231 and BT549 cell lines were transfected with small interfering si-RNA directed against GRP78 or control. The efficiency of siRNA was determined by Western blot and qRT-PCR analysis (Fig. 3a-d). After transfecting the cells with siRNA against GRP78 for 48 hours, cells proliferation was detected using cck-8(Fig. 3e-f) and colony formation were detected using crystal violet staining (Fig. 3g-h). (n = 3 independent experiments). *P<0.05.
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TNBC: Triple-negative breast cancer
Table 2. Correlation of GRP78 expression with clinicopathologic characteristics of TNBC patients

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<tr>
<td>G3</td>
<td>66</td>
<td>29</td>
<td>37</td>
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<td><strong>T classification</strong></td>
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</tr>
<tr>
<td>T1-T2</td>
<td>52</td>
<td>21</td>
<td>31</td>
<td>0.018</td>
</tr>
<tr>
<td>T3-T4</td>
<td>127</td>
<td>51</td>
<td>76</td>
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<tr>
<td><strong>Lymph node status</strong></td>
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</tr>
<tr>
<td>Negative</td>
<td>92</td>
<td>33</td>
<td>59</td>
<td>0.006</td>
</tr>
<tr>
<td>Positive</td>
<td>87</td>
<td>39</td>
<td>48</td>
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<tr>
<td><strong>Distant metastasis</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>149</td>
<td>50</td>
<td>99</td>
<td>0.001</td>
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<tr>
<td>Positive</td>
<td>30</td>
<td>22</td>
<td>8</td>
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<tr>
<td><strong>Ki67 status</strong></td>
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<td></td>
</tr>
<tr>
<td>&gt;14%</td>
<td>47</td>
<td>15</td>
<td>32</td>
<td>0.786</td>
</tr>
<tr>
<td>≤14%</td>
<td>132</td>
<td>57</td>
<td>75</td>
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Table 3. Univariate and multivariate Cox regression analysis of GRP78 and survival in TNBC patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariate analysis</th>
<th></th>
<th></th>
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<th>Multivariate analysis</th>
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<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>P</td>
<td>HR</td>
<td>95% CI</td>
<td>P</td>
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<tr>
<td>Age (&gt;50 vs. ≤50 years)</td>
<td>0.561</td>
<td>0.278-1.133</td>
<td>0.107</td>
<td>NA</td>
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<tr>
<td>Menopause (No vs. Yes)</td>
<td>0.632</td>
<td>0.314-1.276</td>
<td>0.201</td>
<td>NA</td>
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<tr>
<td>Histological grade (G3 vs. G1-2)</td>
<td>1.547</td>
<td>0.809-2.958</td>
<td>0.188</td>
<td>NA</td>
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<tr>
<td>T stage (T3-4 vs. T1-2)</td>
<td>3.496</td>
<td>1.240-9.860</td>
<td><strong>0.018</strong></td>
<td>1.966</td>
<td>1.200-3.221</td>
<td><strong>0.007</strong></td>
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<tr>
<td>Lymph node status (Positive vs. Negative)</td>
<td>2.640</td>
<td>1.329-5.245</td>
<td><strong>0.006</strong></td>
<td>1.327</td>
<td>0.900-1.958</td>
<td>0.153</td>
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<tr>
<td>Ki67 expression (High vs. Low)</td>
<td>1.137</td>
<td>0.451-2.865</td>
<td>0.786</td>
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<tr>
<td>GRP78 expression (High vs. Low)</td>
<td>3.299</td>
<td>1.439-7.564</td>
<td><strong>0.005</strong></td>
<td>2.765</td>
<td>1.164-6.564</td>
<td><strong>0.001</strong></td>
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</tbody>
</table>

NA: not analyze; * P <0.05, statistically significant.