GRP78 positively regulates estrogen-stimulated cell growth mediated by ER-α36 in gastric cancer cells

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Abstract. Estrogen receptor (ER)-α36, a novel isoform of ER, primarily mediates non-classical estrogen signaling. It has been reported that ER-α36-mediated growth stimulating signals are involved in the malignancy of gastric tumor cells. However, the mechanism underlying the regulation of ER-α36 function in development of gastric cancer remains to be elucidated. The present study investigated the role of 78 kDa glucose-regulated protein (GRP78) in the regulation of ER-α36 expression and signaling during the growth of gastric tumor cells. It was demonstrated that GRP78 expression was detectable in gastric cancer tumor tissues, and was positively-correlated with tumor stage, lymphatic metastasis and ER-α36 expression ($P<0.05$). An increased growth rate, and increased expression of ER-α36 and the cell cycle regulator cyclin D1 was detected in cells with GRP78 overexpression (SGC-High78 cells). SGC-High78 cells are more sensitive to estrogen compared with SGC-Control cells. Therefore, the results of the present study demonstrated that GRP78 positively regulated ER-α36 expression and signaling with cell growth in gastric cancer, which is involved in gastric carcinogenesis.

Introduction

Gastric tumor is the type of tumor with the third highest mortality rate worldwide and is the second most frequently-diagnosed type of cancer in China (1,2). Traditionally, gastric tumor was considered to be unrelated to estrogen signaling. Epidemiological studies reported a male predominance in gastric cancer (male/female ratio of 2-3:1), and it has been proposed that there may be a protective function of estrogen in gastric tumorigenesis (3,4). However, studies into the expression patterns of estrogen receptor (ER)-α in samples from gastric tumor patients were inconsistent (5,6). ER-α expression was frequently low and variable (0-62.5%) in gastric cancer specimens (5). ER-β was considered to be an inhibitory factor in the invasiveness of gastric tumor. Therefore, ER-β-positivity has been proposed as a prognostic marker (6). A previous study demonstrated that a novel isoform, ER-α36, was expressed in specimens from patients with gastric cancer (7). Upregulated expression of ER-α36 was positively associated with large size, increased nuclear fission, increased proliferation marker protein Ki-67 expression and decreased E-cadherin expression (8). However, the potential role of ER-α36 in gastric carcinogenesis remains to be determined.

ER-α36 is predominantly expressed in the cytoplasm and at the cell membrane, unlike ER-α which is primarily in the cell nucleus (9-11). In breast cancer, ER-α36-mediated signaling positively regulates ER-positive stem/progenitor cells (12), and it serves an important role in the malignant growth of ER-negative breast tumor cells via the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway (13). In gastric tumor cells, ER-α36 conducts biphasic estrogen signaling (14). A decreased concentration of estrogen (0.1 nM) has been demonstrated to promote cell growth, while a high concentration (1 μM) inhibited cell growth (8,15). However, the mechanism underlying estrogen signaling in cell growth of the gastric tumor is still unclear.

The 78 kDa glucose-regulated protein (GRP78) is a stress-inducible chaperone, and maybe induced under tumor microenvironmental stress conditions (16). GRP78 has been implicated in cancer cell growth, invasion, metastasis and angiogenesis (15,17,18). In gastric carcinoma, GRP78 overexpression is positively-correlated with larger tumor size, increased invasion and advanced stage (19). Targeting GRP78 in gastric cancer leads to a more effective therapeutic outcome (20). GRP78 expression suppressed apoptosis induced by serine/threonine-protein kinase BIK in estrogen-deprived breast cancer cells (21). GRP78 expression is induced by treatment with estrogen in endometrial cancer cells (22). A previous study demonstrated that elevated endoplasmin (GRP94) expression, another protein in the heat shock protein family, was correlated with tumor malignancy and upregulated

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expression of ER-α36 in gastric tumor cells (23,24). In breast cancer, GRP94 was reported to positively-regulate ER-α36 expression, and enhance cell proliferation and invasion (25). However, the potential role and mechanism through which GRP78 may regulate ER-α36 signaling remains unclear.

In the present study, GRP78 and ER-α36 expression patterns in samples from patients with gastric tumor, in addition to the correlation between their expression levels and clinicopathological features, were analyzed. GRP78, ER-α36 and cyclin D1 expression in established gastric tumor cells with overexpressed GRP78, and the cell growth of these cells following treatment with estrogen, were additionally investigated.

Materials and methods

Reagents. 17β-estradiol (E2) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). E2 was dissolved in absolute alcohol (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) at a concentration of 10 mM, and then stored at -20˚C for cell treatment. The rabbit polyclonal antibody recognizing GRP78 (cat. no. ab21685) was from Abcam (Cambridge, UK). The mouse monoclonal Cyclin D1 antibody was purchased from ProteinTech Group, Inc. (Chicago, IL, USA; cat. no. 60186-1-Ig). The rabbit polyclonal ER-α36 antibody was provided by D. Zhaoyi Wang, Shenogen Pharma Group (Beijing, China). The antibody was generated using the custom service provided by the Pacific Immunology (Ramona, CA, USA) using the last 20 amino acids of ER-α36 encoded by exon 9 which are unique to ER-α36 as an immunogen. The produced antibody was purified using an affinity column consisting of immunogen peptides (9-11). The monoclonal β-actin antibody (cat. no. sc-47778) and the horseradish peroxidase-conjugated antibodies (cat. nos. sc-2004 and sc-2005) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). A lentiviral expression vector (Lenti-HSPA5) and a lentiviral vector expressing GFP alone (LV-control) were constructed and produced by Shanghai GeneChem Co., Ltd. (Shanghai, China). The SuperPicture 3rd Gen Immunohistochemistry kit was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Enhanced Bicinchoninic Acid (BCA) Protein Assay kit and radioimmunoprecipitation assay (RIPA) buffer were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture and treatments. SGC7901 cells were obtained from Tongji Medical College (Wuhan, China). A stable cell line with overexpressed GRP78 (SGC-High78 cells) and a control cell line were generated by Shanghai GeneChem Co., Ltd. SGC7901 and SGC-High78 cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal calf serum (FCS; Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China) in a 5% CO_2 atmosphere at 37˚C. For E2 treatment, the cells were maintained in phenol red-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 5% charcoal-stripped FCS (Biological Industries, Beit-Heamek, Israel) for 6 h at 37˚C, and then in 2% charcoal-stripped FCS for 24 h at 37˚C prior to experiments; the same volume of alcohol was used as the control.

Cell proliferation assay. Cells ($3x10^3$/well) were seeded and then treated with 0.1 nM E2 for 5, 7 and 9 days were assessed using the Scepter™ 2.0 automated cell counter (Merck KGaA). All experiments were repeated three times with three 6-well plates for each point.

Table I. Association between GRP78 expression, clinicopathological features of gastric carcinoma, and ER-α36 expression.

	GRP78 expression		
Factor	Positive	Negative	P-value
Age, years			
≤60	61	20	2.83
>60	34	21	
Sex			
Male	78	22	11.91
Female	17	19	
Tumor size, cm			
≤5	50	15	2.96
>5	45	26	
Histological differentiation			
High differentiation	65	29	0.07
Low differentiation	30	12	
T stage			
T2-3	67	29	<0.01
T4	28	12	
N stage			
N0	20	8	0.04
N1-3	75	33	
ER-α36			
Positive	77	33	0.01
Negative	18	8	

GRP78, 78 kDa glucose-regulated protein; T, tumor; N, node; ER-α36, estrogen receptor-α36.

Gastric tumor samples. Tissue from 136 patients with gastric cancer between January 2006 and December 2010 were obtained from the Jiangda Pathology Institute (Wuhan, China) with Institutional Review Board approval and written informed consent. The samples were obtained from 100 men and 36 women aged between 34 and 82 years (mean age, 56.84 years), and all samples were fixed in 10% formalin at room temperature for 1 day prior to paraffin-embedding. No patient had received any anticancer therapy prior to surgery. Tumor size, differentiation and staging were assessed according to the classification system of the World Health Organization (2013).

Tissue microarray. Representative areas of the tumors were identified by hematoxylin and eosin (H&E)-staining of the sections obtained from patients. Briefly, a 0.6-mm in diameter tissue core block (1 per donor) was punched out of each sample and transferred to a recipient block (novel paraffin block containing a maximum of 130 patient core samples), using a

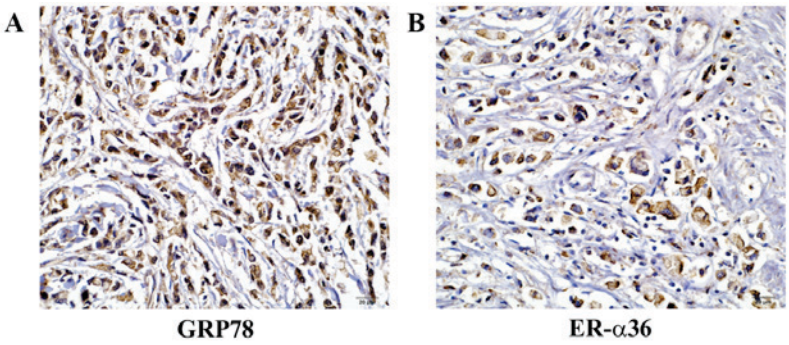

Figure 1. Immunohistochemical staining of gastric cancer tissues. Representative findings of (A) GRP78 and (B) ER-α36 (scale bar, 20 μm). GRP78, 78 kDa glucose-regulated protein; ER-α36, estrogen receptor-α36.

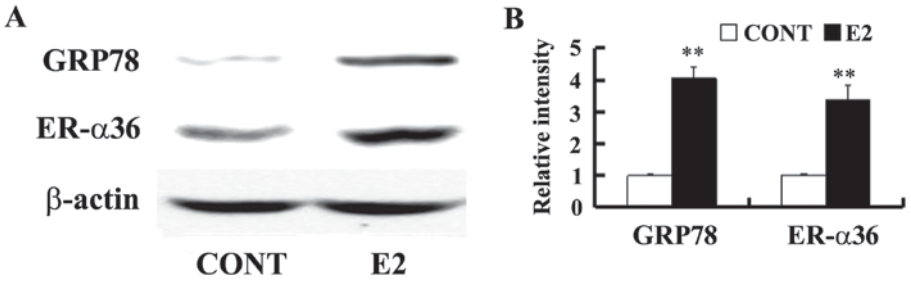

Figure 2. GRP78 and ER-α36 expression following treatment with E2. The SGC7901 cells were treated with E2 at a concentration of 0.1 nM for 24 h, and the same volume of alcohol was used as the control. The expression levels of GRP78 and ER-α36 were measured by (A) western blotting and (B) quantitative analysis. Data are expressed as the mean ± standard error of the mean from three independent experiments. **P<0.01 vs. control. GRP78, 78 kDa glucose-regulated protein; ER-α36, estrogen receptor-α36; CONT, control; E2, 17β-estradiol.

tissue microarrayer MTA-1 (Beecher Instruments, Inc., Sun Prairie, WI, USA). Consecutive 4-μm-thick sections were cut from the recipient block and transferred to polylysine-coated glass slides. H&E staining (Mayer's hematoxylin for 2 min and 1% eosin for 30 sec at room temperature) was performed on the tissue microarray to check the quality of the sections prior to experiments.

Western blot analysis. Western blotting was performed as previously described (26,27). Cells were harvested, washed and lysed in RIPA buffer. Following determination of the protein concentration using the BCA kit, the samples were separated using SDS-PAGE on a 10% gel and then blotted to polyvinylidene fluoride filters (EMD Millipore, Billerica, MA, USA). The filters were blocked in buffer containing 5% nonfat milk for 1 h, and detected with appropriate primary antibodies at 4˚C overnight. The dilutions of the antibodies were as follows: GRP78, 1:1,000; ER-α36, 1:1,000; cyclin D1, 1:1,000; and β-actin, 1:5,000. The blots were subsequently probed with secondary antibodies for 1 h at 37˚C, visualized using enhanced chemiluminescence, and quantitatively analyzed using Totallab version TL120 analysis software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

Immunohistochemistry assay. Immunohistochemical analysis was performed as previously described (7). The slides were dewaxed in xylene and gradually rehydrated. Antigen retrieval was performed in EDTA buffer (pH 8.0) and by boiling in a water bath for 20 min. The samples were rinsed, incubated with antibodies against GRP78 (1:400) or ER-α36 (1:400) overnight

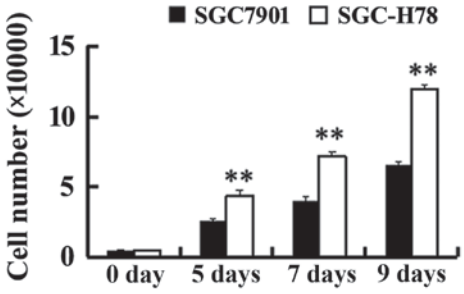

Figure 3. Growth of the gastric cancer SGC7901 cell variants. The growth of SGC-H78 cells overexpressing recombinant 78 kDa glucose-regulated protein and SGC-Control cells were detected on different days. Data are expressed as the mean ± standard error of the mean from three independent experiments. **P<0.01 vs. SGC7901. SGC-H78, SGC-High78.

at 4˚C, and with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin; 1:100; cat. no. A16096; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min, prior to counterstaining with hematoxylin at room temperature for 5 min. The slides were independently evaluated using a light microscope (Olympus BX51; x10 ocular magnification) by two pathologists in a blinded manner.

Statistical analysis. The association between GRP78 expression, clinical pathological features and ER-α36 expression was examined using the Pearson χ^2 test. SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of

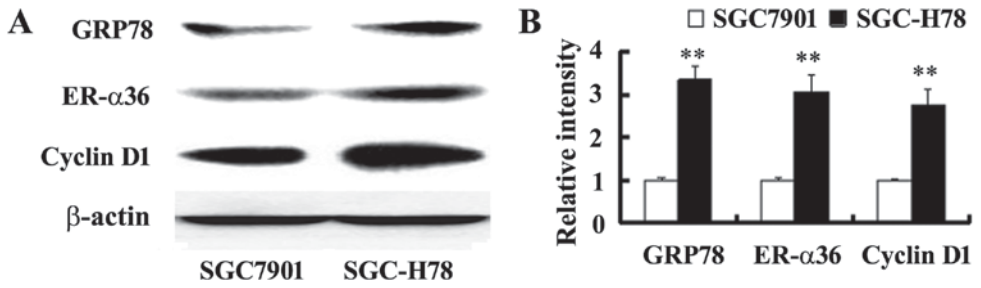

Figure 4. Western blot analysis of GRP78, ER-α36 and cyclin D1 expression in SGC7901 cell variants. The expression levels of GRP78, ER-α36 and cyclin D1 were measured in SGC-H78 and SGC-Control cells by (A) western blotting and (B) quantitative analysis. Data are expressed as the mean ± standard error of the mean from three independent experiments. **P<0.01 vs. SGC7901. ER-α36, estrogen receptor-α36; GRP78, 78 kDa glucose-regulated protein; SGC-H78, SGC-High78.

variance, followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Association between GRP78, ER-α36 expression and clinicopathological properties of gastric tumor samples. GPR78 expression was assessed in 136 specimens by immunohistochemical analysis. GRP78 and ER-α36 were detected in the cytoplasm of gastric cancer cells (Fig. 1). GRP78 expression (2^+ or 3^+) was observed in 95 of the cases of gastric carcinoma (95/136; 69.85%). ER-α36 expression (2^+ or 3^+) was observed in 110 out of the 136 cases (80.88%) (Table I).

Analysis of the association between GRP78 expression and the clinical pathological characteristics of gastric cancer specimens was performed. High GRP78 was positively-associated with tumor stage (P<0.01) and an increased incidence of lymphatic metastasis (P<0.05), although no association was observed with age, gender, histological differentiation and tumor size (P>0.05). Compared with female patients, GRP78 positivity was detected in more male patients (male-to-female ratio, 2.78:1; Table I).

A positive association between GPR78 and ER-α36 expression (P<0.05; Table I) was observed, suggesting that GPR78 and ER-α36 may be involved in gastric tumorigenesis.

Estrogen induces GRP78 and ER-α36 expression. Estrogen-deprived SGC7901 cells were cultured in the presence of E2 at a concentration of 0.1 nM for 24 h to determine whether estrogen is able to regulate GRP78 expression. GRP78 expression was assessed by western blotting. It was demonstrated that a low concentration of E2 upregulated GPR78 and ER-α36 expression in SGC7901 cells (Fig. 2).

Increased ER-α36 and cyclin D1 expression, and enhanced growth in GRP78 expressing cells. In order to study the role and potential mechanism of GRP78 in the growth of gastric tumor cells, SGC-High78 cells that overexpressed recombinant GRP78 and SGC-Control cells were examined for cell growth. It was observed that SGC-High 78 cells exhibited a higher growth rate compared with SGC7901-Control cells (Fig. 3). A significant increase in ER-α36 and cyclin D1 expression was noted in the cells with overexpressed GRP78, compared with SGC-Control cells (Fig. 4), indicating that upregulated ER-α36 expression in GPR78-expressing cells may be important for the increased cell growth of GPR78-expressing cells.

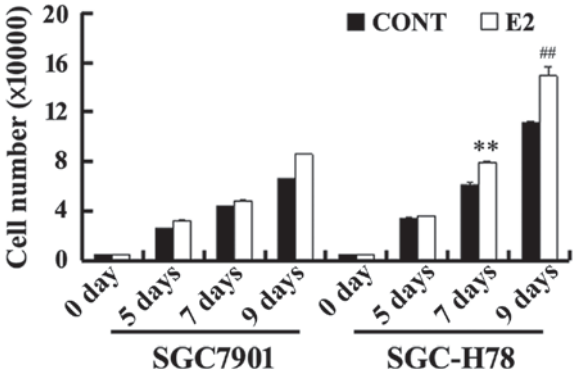

Figure 5. Growth of SGC7901 and SGC-H78 cells following treatment with E2. SGC-H78 and SGC-Control cells were treated with E2 for different durations and were counted. Data are expressed as the mean ± standard error of the mean from three independent experiments. **P<0.01 vs. SGC7901 on day 7; ##P<0.01 vs. SGC7901 on day 9. SGC-H78, SGC-High78; E2, E2, 17β-estradiol.

GRP78 induces ER-α36 and cyclin D1 expression via estrogen in gastric tumor cells. In order to confirm the function of GRP78 in the responsiveness of gastric tumor cells to estrogen SGC-High78 cells overexpressing recombinant GRP78 and SGC7901-Control cells were treated with E2 at a concentration of 0.1 nM for different time periods, and cell growth was examined. As presented in Fig. 5, SGC-High78 cells exhibited an increased growth rate with treatment with estrogen compared with SGC7901-Control cells. Western blot analysis illustrated that E2 upregulated the levels of GRP78, ER-α36 and cyclin D1 expression, and these increases were more marked in SGC-High78 cells compared with those in SGC-Control cells (Figs. 5 and 6). The results of the present study suggested that overexpressed GRP78 promoted the growth of gastric tumor cells via upregulation of ER-α36 signaling.

Discussion

ER-α36 expression has been reported in gastric, breast, lung and endometrial cancer, and its function is associated with the carcinogenesis and progression of these tumors (7,11,28,29).

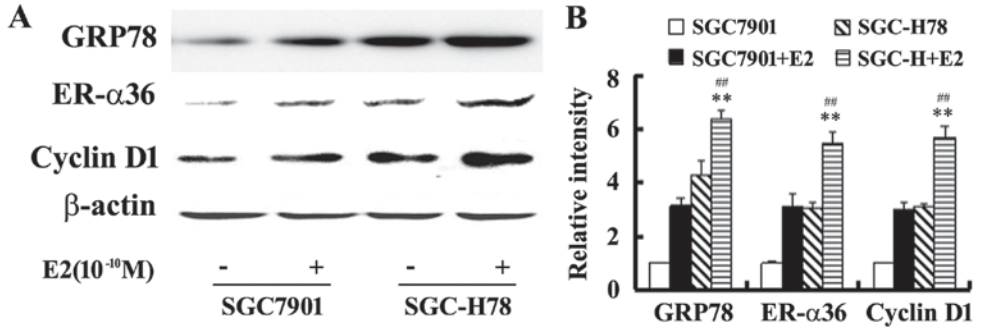

Figure 6. GRP78, ER-α36 and cyclin D1 expression in SGC-H78 and SGC-Control cells following treatment with E2. SGC-H78 and SGC-Control cells were treated with E2 at a concentration of 0.1 nM for 24 h. The expression levels of GRP78, ER-α36 and cyclin D1 were measured by (A) western blotting and (B) quantitative analysis. Data are expressed as the mean ± standard error of the mean from three independent experiments. **P<0.01 vs. SGC-H78; $^{\#\#}$P<0.01 vs. SGC7901+E2. SGC-H78, SGC-High78; E2, E2, 17β-estradiol; ER-α36, estrogen receptor-α36; GRP78, 78 kDa glucose-regulated protein.

In gastric tumor, increased ER-α36 expression was associated with more advanced lymphatic metastasis (7). ER-α36 enhanced the growth of gastric tumor cells by augmenting proto-oncogene tyrosine-protein kinase Src (Src) signaling and upregulating cyclin D1 expression (14). In the present study, GRP78 and ER-α36 were expressed in gastric tumor specimens. Estrogen promoted gastric cancer cell growth and upregulated GRP78 and ER-α36 in SGC7901 cells. The result of the present study suggested an involvement of GRP78 in the estrogen-enhanced growth of gastric tumor cells via the ER-α36 signaling pathway.

ER-α36 is primarily expressed in the cytoplasm and at the plasma membrane. ER-α36 mediates the membrane-initiated rapid estrogen pathway and inhibits genomic estrogen signaling mediated by ER-α66 and ER-β, and it functions as an important factor in the increased cell growth and tumorigenesis of breast cancer stimulated by estrogen (9,30). Estrogen has been demonstrated to stimulate the growth of gastric cancer cells (14,15). It has been reported that cells with high levels of ER-α36 require lower concentrations of estrogen (in the pM range) to enhance cell growth, compared with cells expressing low levels of the receptor (13). In the present study, a low concentration of estrogen (equivalent to the level observed in postmenopausal women) was demonstrated to promote gastric tumor cell growth and to increase GRP78 and ER-α36 expression, which provided a potential explanation for the observed male predominance in gastric tumor and a possible mechanism underlying postmenopausal ER-α36-mediated rapid estrogen signaling in gastric tumorigenesis.

It was additionally demonstrated in the present study that GRP78 expression was positively associated with tumor stage, increased lymphatic metastasis and ER-α36 expression in gastric carcinoma specimens. In addition, a higher growth rate, and increased levels of ER-α36 and cyclin D1, were detected in cells with GRP78 overexpression. Cells with overexpressed GRP78 were more sensitive to treatment with estrogen and the growth rate of these cells was higher, with increased ER-α36 and cyclin D1 expressions compared with SGC-Control cells. The present findings suggested that ER-α36 may be positively regulated by GRP78, and may be involved in the cell growth of gastric tumors. A recent report indicated that GRP94, a scaffold protein, stabilized cell membrane ER-α36 and upregulated its levels in breast cancer (25). Targeting GRP94 with a specific small interfering RNA or a specific monoclonal inhibited ER-α36-driven cell growth *in vitro* and *in vivo* (25). A previous study reported that the GRP94 expression level was upregulated by ER-α36 in gastric cancer cells (23,24). In established gastric cancer cells with knockdown of ER-α36 expression, GRP94 was markedly reduced (23). ER-α36 was reported to be involved in the testosterone-stimulated activation of the MAPK/ERK and phosphatidylinositol 3-kinase/RAC-α serine/threonine protein kinasesignaling pathways in endometrial cancer Hec1A cells (29). E2 induced MAPK/ERK activation via a mechanism involving ER-α36 and the epidermal growth factor receptor/Src/SHC transforming protein 1complex (31). Therefore, it is possible that there exists a positive regulatory loop between GRPs and ER-α36 expression, although the mechanism underlying their association with tumorigenesis requires further investigation.

In conclusion, GRP78 expression was positively correlated with advanced tumor stage, increased lymphatic metastasis and increased ER-α36 expression in specimens from patients with gastric tumors. ER-α36-mediated signaling positively regulated by GRP78 enhanced cell growth in gastric tumors. The results of the present study thereby provided evidence that GRP78 may function as an important regulator in the estrogen-enhanced growth of gastric tumor through ER-α36 signaling.

Acknowledgements

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