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Estrogen receptor-α36-mediated rapid estrogen signaling regulates 78 kDa glucose-regulated protein expression in gastric carcinoma cells

ZHENGQI FU[1,2*], XUMING WANG[1*], ZHAOYI WANG[3] and LIJIANG LIU[1,2]

[1]Department of Pathology and Pathophysiology, School of Medicine; [2]Jiangda Pathology Institute, Jianghan University, Wuhan, Hubei 430056; [3]Shenogen Pharma Group, Beijing 102206, P.R. China

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Abstract. To determine whether estrogen receptor-α36 (ER-α36) -mediated rapid estrogen signaling is associated with 78 kDa glucose-regulated protein (GRP78) expression in gastric cancer, 86 samples of gastric tumor tissue with corresponding normal and tumor-adjacent tissues were used to examine expression patterns of GRP78 and ER-α36. Immunohistochemistry demonstrated that 55/86 (63.95%) patients with gastric carcinoma, and western blot analysis revealed that GRP78 was upregulated in 15/20 (75%) of tumor specimens. GRP78 expression was positively associated with ER-α36 expression, the male sex and lymph node metastasis ($P<0.05$). Estrogen treatment increased GRP78 and ER-α36 expression, as well as GSK-3β phosphorylation in established gastric cancer SGC-7901 cells. The steady-state level of GRP78 protein expression and the level of phosphorylated GSK-3β at Ser9 were decreased in SGC-7901 cells with ER-α36 knockdown. Forced expression of ER-α36 in SGC-7901 cells, however, led to an increase in GRP78 expression and GSK-3β phosphorylation. It may therefore be concluded that ER-α36-mediated rapid estrogen signaling positively regulates GRP78 expression, presumably via the GSK-3β pathway, which may be associated with gastric carcinogenesis.

Introduction

Gastric cancer is one of the most common types of cancer in China and worldwide (1-3). Epidemiological evidence indicates that the incidence rate of gastric cancer is higher in males than that in females (4). The onset of gastric cancer in males occurs 10-15 years earlier compared with females; however, following menopause, the incidence rate of gastric cancer increases and becomes equally prevalent between men and women (4). It has been reported that estrogen of ovarian or exogenous origin, may suppress gastric cancer (5). The results of a Swedish cohort study of male patients with prostate cancer reveled that estrogen exposure reduced the risk of gastric cancer (6). It has therefore been postulated that estrogen may serve a protective role in development of gastric cancer.

There are two estrogen receptors (ERs); ER-α and ER-β. Several studies have investigated the role of estrogen signaling in gastric cancer, as well as its potential mechanisms and clinical relevance (7). However, reports on the function of ERs in the development of gastric cancer have been inconsistent (8).

ER-α36, a truncated 36-kDa form of ER-α, has been reported to be upregulated in gastric cancer and, furthermore, ER-α36 expression is associated with lymph node metastasis in gastric cancer (9-13). Compared with ER-α66, ER-α36 is primarily expressed in the cytoplasm and at the plasma membrane, mediating rapid, non-genomic estrogen signaling (11-13). A previous study revealed that gastric cancer cells with an increased expression of ER-α36 are more sensitive to estrogen compared with cells expressing decreased levels of ER-α36 (14). ER-α36 mediates rapid estrogen signaling to promote cell growth via the phosphoinositide 3-kinase/RAC serine/threonine-protein kinase and the mitogen-activated protein kinase/extracellular signaling-related kinase signaling pathways (15). However, the exact molecular mechanisms by which ER-α36 functions in the carcinogenesis of gastric cancer remain unclear.

The 78 kDa glucose-regulated protein (GRP78) is an endoplasmic reticulum chaperone that functions as a regulator of intracellular calcium, protein folding and the unfolded-protein response (16,17). Additionally, GRP78 is a stress-response protein that is upregulated disturbances to cellular homeostasis, including reduced levels of oxygen or glucose, and is translocated from the endoplasmic reticulum to other cellular locations, including the nucleus or the plasma membrane, where it functions as a membrane receptor to transduce proliferation, invasion, apoptosis, inflammation and immunity signals (16,17). GRP78 overexpression has been

Correspondence to: Dr Lijiang Liu, Jiangda Pathology Institute, Jianghan University, 8 Sanjiaohu Road, Wuhan, Hubei 430056, P.R. China
E-mail: liulijiang@163.com

*Contributed equally

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documented in a number of cancer cell lines and tissues and is associated with aggressive growth and invasive properties (18-20). Upregulation of GRP78 expression is associated with increased lymph node metastasis and poor prognosis in patients with gastric cancer (21). Knockdown of GRP78 expression in established gastric cancer cells attenuates invasion *in vitro* and tumor metastasis *in vivo* (22). These results indicate that GRP78 serves an important role in the progression of gastric carcinoma and has the potential to be used as an effective marker for aggressive disease and poor prognosis in patients with gastric carcinoma. It has been reported that estrogens also regulate GRP78 expression in endometrial cancer cells (23). GRP78 overexpression was detected in samples from aggressive ER-negative breast cancer, but not in those from benign human breast lesions (24). Elevated GRP94 expression was also reported to be associated with cancer progression and ER-α36 expression in gastric and breast cancer (25,26). Crosstalk between GRP94, ER-α36 and HER2 forms positive feedback loops in breast cancer, which may affect tumor growth, metastasis and drug resistance (26). Inhibition of GRP94 expression with siRNA or monoclonal antibody (mAb) blocked the GRP94-ER-α36 interaction and inhibited breast cancer growth and invasion *in vitro* and *in vivo* (26). HER2 signaling activated ER-α36 transcription, which mediates non-genomic estrogen and anti-estrogen (tamoxifen) signaling and stimulated cell proliferation (9-13,27). Although the induction of GRP protein expression by estrogen signaling has been documented, the functions and underlying mechanisms of the induction of GRP78 expression by estrogen in gastric cancer have not been elucidated (28).

The present study examined the expression of GRP78 and ER-α36 in tumor samples from gastric cancer patients and their association with sex and lymph node metastasis. GRP78 expression and glycogen synthase kinase 3β (GSK-3β) phosphorylation were also examined in gastric cancer cells with different levels of ER-α36 expression.

Materials and methods

Reagents. 17β-estradiol (E2) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rabbit anti-ER-α36 antibody was provided by the Shenogen Pharma Group Ltd. (Beijing, China). The antibody was generated using the custom service provided by the Pacific Immunology (Ramona, CA, USA) using the final 20 amino acids of ER-α36 encoded by exon 9. The produced antibody was purified using an affinity column consisting of immunogen peptides (11-13). The rabbit anti-GRP78 antibody (cat. no. ab21685) was obtained from Abcam (Cambridge, UK). Rabbit anti-phospho-GSK-3β at Ser9 (Ser9-GSK-3β; cat. no. 9323) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-β-actin antibody (cat. no. sc-47778), goat anti-mouse horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2005) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2004) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Bicinchoninic acid protein detection kit, the chemiluminescent substrate kit and polyvinylidene difluoride membranes were obtained from Pierce (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A SuperPicture 3rd Generation Immunohistochemical (IHC) Detection kit was obtained from Invitrogen (Thermo Fisher Scientific, Inc.). Radioimmunoprecipitation assay (RIPA) buffer and enhanced chemiluminescence reagents were obtained from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture, treatment, and lysate preparation. The human gastric adenocarcinoma cell line SGC-7901 was obtained from the Department of Immunology of Tongji Medical College (Wuhan, China). Gastric cancer cells expressing low levels of ER-α36 (SGC-7901-Low36 cells) were established using small hairpin RNAs (shRNAs). The ER-α36-specific shRNA expression vector was constructed by cloning the DNA oligonucleotides (5'-TTAACCGTACCACTCTGCTGATTGATATCCGTCAG CAGAGTGGTACGGTTA-3') targeting the 3'-untranslated region of ER-α36 cDNA into the pRNAT-U6.1/neo expression vector purchased from GenScript Biotech Corporation (Piscataway, NJ, USA). A gastric cancer cell line overexpressing ER-α36 (SGC-7901-High36 cells) was established via transfection with an ER-α36 expression vector, as previously described (11,13,25,29). SGC-7901, SGC-7901-Low36 cells and SGC-7901-High36 were all maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2. Prior to E2 treatment, cells were cultured at 37˚C in phenol-red-free medium (Gibco; Thermo Fisher Scientific, Inc.) with 5% charcoal-stripped FBS (Biological Industries, Kibbutz Beit-Haemek, Israel) for 6 h, and then in 2% charcoal-stripped FBS for 24 h, then cells were treated with 10^{-10} M E2 for 24 h.

Gastric tumor samples. Frozen tumor tissues of 20 patients with gastric cancer collected between January 2009 and December 2010 and the paraffin-embedded tissues of 86 patients with gastric cancer collected between January 2006 and December 2010 were obtained from the Jiangda Pathology Institute (Wuhan, China). Written informed consent was obtained from all patients and the study protocols were approved by The Ethics Committee of the School of Medicine, Jianghan University (Wuhan, China). The histological type was adenocarcinoma in all cases. Tumor tissues used for IHC staining were fixed in 10% neutral formalin at room temperature for 24 h, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The tissues for western blotting were snap-frozen in liquid nitrogen and stored at -150˚C. Frozen samples were obtained from 13 men and 7 women aged 34-78 years (mean, 60.1 years), and the formalin-fixed paraffin-embedded samples for IHC were obtained from 56 men and 30 women aged 34-82 years (mean, 57.13 years). No patient received any anticancer treatment prior to surgery. Tumor size, histological differentiation, size and location of the primary tumor (T stage) and lymph node involvement (N stage) were all evaluated according to the clinicopathological classification outlined by the World Health Organization (2013) (25).

Tissue microarray. Representative areas of solid tumors were identified in H&E-stained sections. A 0.6-mm diameter tissue core was punched out from each block and transferred

Table I. Associations between GRP78 expression, clinicopathological features of gastric carcinomas and ER-α36 expression.

Factors	GRP78 expression		P-value
	Positive	Negative	
Age, years			>0.05
≤60	34	14	
>60	21	17	
Sex			0.01
Male	36	20	
Female	19	11	
Tumor size, cm			>0.05
≤5	25	7	
>5	30	24	
Histological differentiation			>0.05
High	45	24	
Low	10	7	
T stage			>0.05
T2-3	46	21	
T4	9	10	
N stage			0.01
N0	11	6	
N1-3	44	25	
ER-36			0.01
Positive	49	27	
Negative	6	4	

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

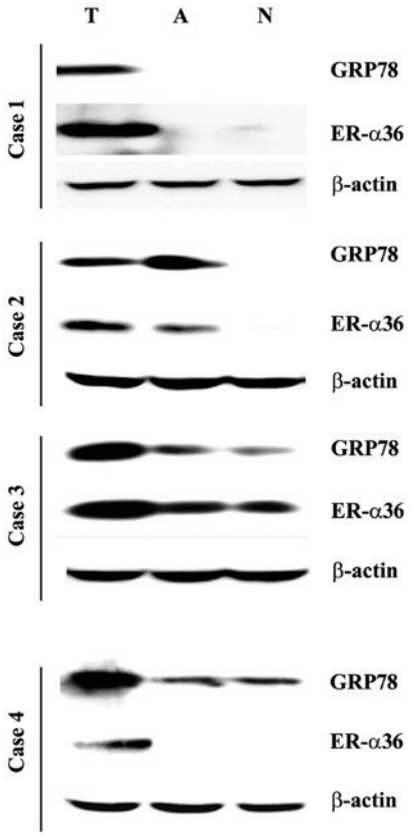

Figure 1. GRP78 and ER-α36 expression in specimens of human gastric adenocarcinoma. The levels of GRP78 and ER-α36 expression in tumor tissues, adjacent nonmalignant tissues, and corresponding normal tissues were assessed using western blotting. GRP78, 78 kDa glucose-regulated protein; ER-α36, estrogen receptor-α36; T, tumor tissue; A, non-malignant tissue; N, normal tissue.

to a recipient block with 86 samples using an MTA-1 tissue microarray (Beecher Instruments, Inc., Sun Prairie, WI, USA). Sections 4 μm thick were consecutively sliced from the recipient block and transferred to polylysine-coated glass slides. H&E staining with 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, such as integrity of tumor tissues, thickness and dye uniformity, of the sections prior to experiments.

Western blot analysis. Western blot analysis was conducted as previously described (25). Briefly, cells were washed with cold PBS and lysed with RIPA buffer. The tumor tissues were dissected and homogenized using RIPA buffer. The protein concentration was determined using the bicinchoninic acid kit. Proteins (20 μg GRP78, 20 μg ER-α36 and 30 μg Ser9-GSK-3β) were separated using SDS-PAGE (10% gel) and transferred to polyvinylidene fluoride membranes. Membranes were incubated with the aforementioned primary antibodies at 4˚C overnight. Blots were subsequently washed, incubated with the appropriate secondary antibodies for 1 h at 37˚C and visualized using enhanced chemiluminescence. The Total Lab analysis software (version TL120; Total Lab Ltd., Newcastle upon Tyne, UK) was used to perform densitometry analysis of protein bands.

Immunohistochemistry assays. The tissue slides were de-paraffinized with xylene for 15 min, and gradually rehydrated in an ethanol series (100, 100, 95, 95 and then 80% alcohol; 3 min each). Endogenous peroxidase activity was blocked by incubating with 3% hydrogen peroxide/methanol buffer for 10 min at room temperature. Antigen retrieval was performed by immersing slides in an ethylenediamine tetraacetic acid buffer (pH 8.0) followed by boiling in a water bath at 100˚C for 25 min. Slides were rinsed with PBS and then incubated with a rabbit anti-GRP78 antibody (dilution, 1:400) or with a rabbit anti-ER-α36 antibody (dilution, 1:400) overnight at 4˚C. Slides were washed and incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (dilution, 1:100; cat. no. A16096; Invitrogen; Thermo Fisher Scientific, Inc.), for 1 h at 37˚C. Diaminobenzidine was used as a chromogen, and the slides were counterstained with hematoxylin at room temperature for 5 min.

GRP78 and ER-α36 staining was scored as follows: 0, no staining or staining observed in <10% of tumor cells; 1+, faint/barely perceptible staining detected in ≥10% of tumor cells; 2+/3+, moderate or strong staining, respectively, observed in ≥10% of tumor cells. A score of 0 or 1+ was

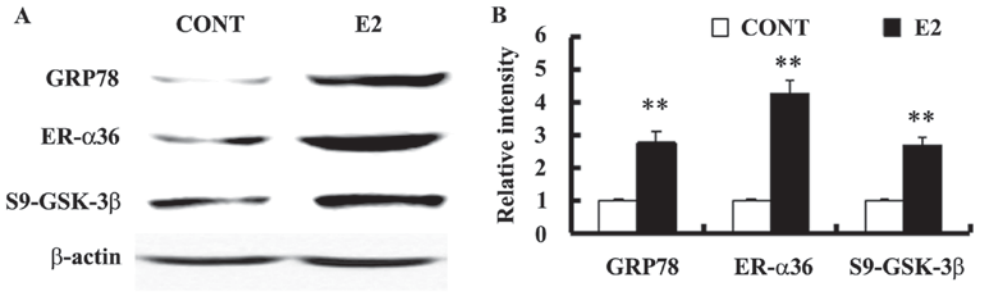

Figure 2. E2 treatment increases GRP78 and ER-α36 expression and the level of phosphorylated GSK-3β at Ser9 in human gastric adenocarcinoma SGC-7901 cells. Cells were treated with 10^{-10} M E2 for 24 h and same concentration of alcohol was used for the control group. GRP78 and ER-α36 expression, as well as levels of GSK-3β phosphorylation at Ser9 were measured by (A) western blotting and (B) quantitative analysis of these blots. n=3, **P<0.01 vs. control. E2, 17β-estradiol; GRP78, 78 kDa glucose-regulated protein; ER-α36, estrogen receptor-α36; GSK-3β, glycogen synthase kinase 3β.

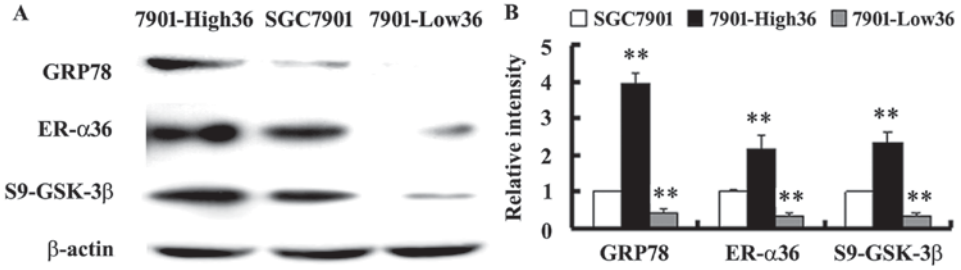

Figure 3. ER-α36 regulates GRP78 expression and GSK-3β phosphorylation. GRP78 expression and GSK-3β activity were assessed by (A) western blotting and (B) quantitative analysis in SGC-7901 cells with ER-α36-knockdown, cells with forced expression of ER-α36, and control cells transfected with an empty vector. n=3, **P<0.01 vs. SGC-7901. GRP78, 78 kDa glucose-regulated protein; ER-α36, estrogen receptor-α36; GSK-3β, glycogen synthase kinase 3β; 7901-Low36, ER-α36-knockdown SGC-7901 cells; 7901-High36, ER-α36-overexpressing SGC-7901 cells.

considered negative, and a score of 2+ or 3+ was considered positive. The immunostained slides were evaluated independently by two pathologists in a double-blinded manner. In the majority of cases, the evaluations of the two pathologists were identical; discrepancies were resolved by re-examination and consensus (25).

Statistical analysis. Data are presented as the mean ± standard deviation. The association between GRP78 expression, clinicopathological characteristics, and ER-α36 expression was determined using Pearson's χ^2 test. Statistical analysis was performed using one-way analysis of variance, followed by least significant difference tests. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA).

Results

Association between GRP78 and ER-α36 expression and clinicopathological characteristics of gastric adenocarcinomas. The expression of GRP78 and ER-α36 was assessed in gastric tumor tissues, adjacent non-tumor tissues and corresponding normal tissues using western blot analysis. Compared with the paired normal tissues, higher levels of GRP78 and ER-α36 were observed in 15/20 (75%) and 13/20 (65%) tumor specimens, respectively, whereas 11/20 (55%) cases co-expressed the two proteins (Fig. 1).

The expression pattern of GRP78 was also examined in 86 specimens using IHC. High levels of GRP78 expression (2+ or 3+) were observed in 55 cases of gastric carcinoma patients (55/86; 63.95%). The IHC assay also revealed that ER-α36 was expressed in 76 specimens (76/86; 88.37%; Table I).

Analysis of the associations between GRP78 expression and clinicopathological properties of gastric adenocarcinoma was then performed. GRP78 expression was significantly associated with male and lymph node metastasis (P<0.05), but not with other features including age, tumor size, tumor stage and histological differentiation (P>0.05). GRP78 was expressed at greater levels in males compared with females, at a ratio of 1.87:1 (P<0.05; Table I). GRP78 expression was positively associated with increased lymph node metastasis (P<0.05, Table I). A significant association was also detected between GRP78 and ER-α36 expressions (P<0.05; Table I).

Involvement of GRP78 and GSK-3β in ER-α36-mediated rapid estrogen signaling. Estrogen-deprived SGC-7901 cells were treated with 10^{-10} M E2 for 24 h to assess whether estrogen regulates GRP78 expression. Levels of GRP78 expression and GSK-3β phosphorylation at Ser9 were also assessed using western blotting. E2 treatment increased the expression of GRP78 and ER-α36, as well as the level of GSK-3β phosphorylation at Ser9 in SGC-7901 cells (Fig. 2).

GRP78 expression and GSK-3β phosphorylation in gastric cancer cells expressing different levels of ER-α36. To confirm the involvement of ER-α36 in regulation of GRP78 expression, GRP78 expression and GSK-3β phosphorylation was assessed in SGC-7901-Low36 cells and in SGC-7901-High36 cells. Fig. 3 demonstrates the decrease of the levels of GRP78 expression and the phosphorylation of GSK-3β at Ser9 in the SGC-7901-Low36

cells compared with the control cells transfected with an empty vector. In cells overexpressing ER-α36, GRP78 expression and Ser9-phosphorylated GSK-3β were significantly increased (Fig. 3). The results of the present study therefore indicate that GRP78 expression is positively regulated by ER-α36-mediated signaling, presumably via the GSK-3β pathway.

Discussion

Epidemiological evidence indicates a consistent predominance of gastric cancer in men worldwide (4). It has been postulated that estrogen may serve a protective role in gastric tumorigenesis (4-6). Estrogen receptors are nuclear receptors that mediate estrogen signaling in different tissues (11-15). Dysregulation of ER-mediated estrogen signaling is reported to be associated with development of gastric cancer (9,10,14). However, ER-α66 expression in gastric cancer is inconsistent, with variable patterns (0-62.5%) (30,31). ER-β expression, however, is reported to be associated with lower tumor stage, negative perineural invasion and lower risk of recurrence (7). A previous study indicated that estrogen receptors are present at low levels in gastric tumors and normal tissues (32). Thus, their roles in gastric carcinogenesis may be limited.

ER-α36, a novel variant of ER-α, was demonstrated to be highly expressed in specimens of human gastric cancer and its expression was positively associated with invasion to serosa, lymph node metastasis and cyclin D1 expression (14). In ER-α36-knockdown SGC-7901 cells, downregulated ER-α36 expression was associated with smaller tumor size, decreased nuclear fission and reduced GRP94 expression (25). In the present study, a high level of ER-α36 expression was detected in specimens from patients with gastric carcinoma, which was positively associated with GRP78 expression.

GRP78 is a stress-inducible molecular chaperone that is upregulated in cancer cells with increasing hypoxia, nutrient starvation and acidosis (16,17). GRP78 is overexpressed in different human cancer types, including gastric cancer, and is associated with the growth, invasion and metastasis of tumors (16-20). Inhibition of GRP78 activities reduces tumor formation of gastric cancer cells in xenograft models and suppresses proliferation, invasion and drug resistance in gastric cancer cells (33). In the present study, high levels of GRP78 expression were observed in specimens from gastric carcinoma. GRP78 expression was positively associated with the male sex, metastasis to the lymph node and ER-α36 expression. Estrogen treatment increased GRP78 and ER-α36 expression. The steady state level of GRP78 protein was decreased in SGC-7901-Low36 cells; however, in SGC-7901-High36 cells, GRP78 expression was upregulated. These results indicate that ER-α36-mediated rapid estrogen signaling is associated with the regulation of GRP78 expression in gastric cancer cells, which is consistent with a previous report that estrogens regulate GRP78 expression (23). It has been reported that the C-terminal domain of GRP94 interacts directly with ER-α36 at the cell membrane of breast cancer cells and that this interaction stabilizes ER-α36, thereby increasing ER-α36 signaling and cell growth (26). It was reported that targeting GRP94 with small interfering RNA or a specific monoclonal antibody blocked the GRP94-ER-α36 interaction, inhibiting breast cancer growth *in vitro* and *in vivo* (26). Taken together, these results suggest that GRPs are associated with ER-α36 and its signaling, which serves a role in gastric carcinogenesis.

GSK-3β is a serine/threonine kinase, and its activity is regulated by the dynamic balance between activating Tyr216-phosphorylation and inactivating Ser9-phosphorylation. GSK-3β is associated with the regulation of a number of cellular processes, including cell growth, stem cell renewal and apoptosis (34,35). Dysregulation of the GSK-3β signaling pathway is associated with the onset and progression of various tumors (34,35). It has previously been reported that activation of GSK-3β in tumors may be pro- or anti-apoptotic, depending on the cell type or subcellular localization (34). In certain cases, suppression of GSK-3β phosphorylation via growth factor-stimulated signaling or downregulation of GSK-3β expression is associated with cancer progression (35). In addition, GSK-3 was reported to negatively regulate the activity of proto-oncogenic proteins and cell cycle regulators via the Hedgehog and Wnt/β-catenin signaling pathways (36). ERs have also been identified as GSK-3 substrates: Silencing of GSK-3α or GSK-3β resulted in ER-α downregulation and a resultant reduction in its activity in ER-positive breast tumor cells (37). GSK-3β is also activated during endoplasmic reticular stress (38). The ability of GRP78 to bind to GSK-3 and tau are increased with elevated GRP78 expression *in vivo* and *in vitro* (39). In hepatocellular adenoma, novel links between endoplasmic reticular stress, decreased cyclin D expression and elevated ER-α36 and GSK-3β expressions were detected (40). The present study demonstrates that GRP78 and ER-α36 expression and GSK-3β phosphorylation at Ser9 are increased in gastric cancer SGC-7901 cells following estrogen treatment. A significant decrease in GRP78 expression and GSK-3β phosphorylation at Ser9 was observed in ER-α36-knockdown cells compared with the SGC-7901-control cells. In cells overexpressing ER-α36, levels of GRP78 and GSK-3β phosphorylation at Ser9 were significantly increased. These results suggest that ER-α36-mediated estrogen signaling is associated with the regulation of GRP78 expression, presumably via the GSK-3β pathway.

In conclusion, increased GRP78 expression is associated with the male sex, lymph node metastasis and ER-α36 expression in gastric cancer. GRP78 is regulated by ER-α36-mediated rapid estrogen signaling via the GSK-3β pathway, which may also be associated with gastric carcinogenesis.

Acknowledgements

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