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Original Article

ER-α36 mediates gastric cancer cell invasion

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Abstract: Estrogen evidently exerts a protective role against gastric cancer. Accordingly, we evaluated the relationship between the expression of the estrogen receptor ER-α36 and the clinicopathologic features in gastric cancer. ER-α36 expression levels differed among the tumor center, invasion front, and vascular metastases. The effects of E2β (17β-Estradiol, E2β) on invasion ability in SGC7901, High36 (with ER-α36 upregulation), and Low36 (with ER-α36 downregulation) cells were evaluated using Transwell assays. Furthermore, the c-Src signaling pathway was inhibited using PP2 and the effects on E2β-induced increases in E-cadherin, MMP2, and MMP9 were evaluated using western blotting. ER-α36, c-Src, MMP2, and E-cadherin levels were also evaluated in tumor xenografts. We found that 0.1 nM E2β promoted gastric cancer cell invasion by reducing E-cadherin expression and increasing MMP2 and MMP9 levels. The upregulation of ER-α36 promoted gastric cancer cell invasion and the downregulation of ER-α36 reduced the invasive ability of cells. The levels of ER-α36, c-Src, and MMP2 were the highest in tumor xenografts using High36 cells, intermediate in tumor xenografts using SGC7901 cells, and lowest in tumor xenografts using Low36 cells. The opposite results were obtained for E-cadherin expression. ER-α36 enhanced gastric cancer cell invasion by the activation of membrane-initiated c-Src signaling pathways. In particular, treatment with E2β and ER-α36 influenced gastric cancer cell invasion. Furthermore, c-Src was involved in the ER-α36-mediated estrogen signaling pathway and cell invasion.

Keywords: Gastric cancer, ER-α36, invasion, c-src, invasion

Introduction

The incidence of gastric cancer is high, with approximately 989,600 new cases, accounting for approximately 8% of all new cancer cases, and 738,000 deaths annually [1]. The incidence is higher in men than in women with a male-to-female ratio of between 2:1 and 3:1 [1-4]. Environmental risk factors for gastric cancer, such as smoking, dietary factors, and Helicobacter pylori infection, cannot explain the sex-based difference in incidence [5-7]. Further research has shown that the risk of gastric cancer is higher in men than in women before menopause, but after menopause, the incidence is similar between women and men [8]. The risk of developing gastric cancer is lower in individuals treated with estrogen replacement therapy than in those who do not receive such treatment [9-12]. These findings suggest that estrogen has a protective effect against gastric cancer.

The effects of estrogen are mediated by estrogen receptors, including ER-α and ER-β [13]. ER-α includes three main isoforms: ER-α66, ER-α46, and ER-α36 [14]. ER-α36 is expressed in human gastric adenocarcinoma tissues and gastric cancer cell lines, such as AGS, BGC823, MKN45, and SGC7901, and ER-α36 expression is significantly correlated with tumor invasion and lymph node metastasis in gastric cancer [15]. We have found that ER-α36 increases gastric cancer cell proliferation by the activation of membrane-initiated c-Src signaling pathways and direct interactions with c-Src [16]. Glucose-regulated protein 94 is a downstream effector of ER-α36-mediated estrogen signaling and may be involved in ER-α36 function during gastric carcinogenesis [17]. These previous results support an important role of ER-α36 in gastric cancer.

In this study, we investigated the mechanisms by which ER-α36 functions in the gastric cancer

cell line SGC7901 and in human gastric cancer tissues, and demonstrated the role of the c-Src pathway in the invasion of gastric cancer cells stimulated by ER-α36-mediated mitogenic estrogen signaling.

Materials and methods

Reagents

17β-estradiol (E2β) and PP2 (a c-Src inhibitor) were obtained from Sigma (St. Louis, MO, USA). A rabbit polyclonal anti-ER-α36 antibody was kindly provided by Prof. Zhaoyi Wang at Guilin Medical College. The anti-c-Src antibody (sc-19), anti-p-c-Src antibody (sc-81521), anti-p-c-Src antibody (sc-16846-R), anti-E-cadherin antibody (sc-52328), anti-MMP2 antibody (sc-13594), anti-MMP9 antibody (sc-21733), and anti-β-actin antibody (sc-47778) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The appropriate secondary antibodies [goat anti-mouse IgG-HRP (sc-2005) and goat anti-rabbit IgG-HRP (sc-2004)] were obtained from Santa Cruz Biotechnology. RIPA buffer and the Enhanced BCA Protein Assay Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). PVDF membranes were obtained from Millipore (Billerica, MA, USA). Lipofectamine2000 was obtained from Invitrogen (Carlsbad, CA, USA).

Cell lines

The human gastric cancer cell line SGC7901 was obtained from the Chinese Academy of Medical Sciences Cell Center of Basic Medicine (Beijing, P. R. China). Recombinant SGC7901 cell lines (with low ER-α36 expression and high ER-α36 expression) were generated in our laboratory, as described previously [15].

Cell culture

All cells were maintained in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Before treatment with E2β, the medium was replaced with phenol-red-free RPMI 1640 medium containing 2% FBS for 2 to 3 days and serum-free medium for 6 h.

Transwell assay

To examine invasion in the presence or absence of estrogen, cells maintained for 3 days in phenol red-free RPMI 1640 medium plus 2% FBS were treated with E2β (0.1 nM) and/or PP2 (10 μM) or ethanol as a vehicle control. Cell migration through Matrigel-coated filters was measured using Transwell chambers (Corning Incorporated, Corning, NY, USA) with 8-μm-pore polycarbonate filters coated with the Matrigel matrix. SGC7901 cells were trypsinized and seeded onto the upper chambers in medium containing 2% FBS (1×10^5 cells/well in 100 μl) and treated with estrogen and/or PP2. The lower chambers were filled with medium containing 10% FBS (600 μl). Cells were allowed to migrate for 12 h at 37°C. Then, the upper side of the filter was carefully washed with cold PBS and non-migrated cells at the top of the filter were removed using a cotton swab. Subsequently, non-migrating cells on the upper surface of the membrane were removed by gently scrubbing with a cotton swab, and the invading cells on the lower surface were fixed with 100% methanol and stained with Giemsa (Sigma) for 15 min. Stained cells were counted in five fields imaged at ×400 magnification. The average number of cells per field was used to determine the total number of migrated cells. All experiments were repeated three times.

Tumor samples and tissue microarray

Paraffin-embedded samples of gastric cancer tissues obtained from 138 patients between 2010 and 2019 (Department of Pathology, Affiliated Hospital of Guilin Medical College) were used after obtaining the approval of the Institutional Review Board of affiliated Hospital of Guilin Medical College. Tumor tissues used for immunohistochemistry (IHC) were fixed in 10% neutral formalin, embedded in paraffin, processed, and stained with hematoxylin and eosin (H&E). The IHC samples (n = 138) were obtained from 96 men and 42 women aged 30-59 years (mean, 56.5 years). None of the patients had received any anticancer treatment prior to surgery. Histologic differentiation, T stage, N stage, and M stage were evaluated according to the clinical-pathologic classification of the World Health Organization (2012). Targeted tissue areas of 138 tumors were marked on H&E-stained sections. One tissue core of 1.0 mm in diameter and 3 to 4 mm in depth was removed from each block using a manual microarray device (Beecher Instruments, Silver Spring, MD, USA) and 138 total tissue cores were inserted into the recipient

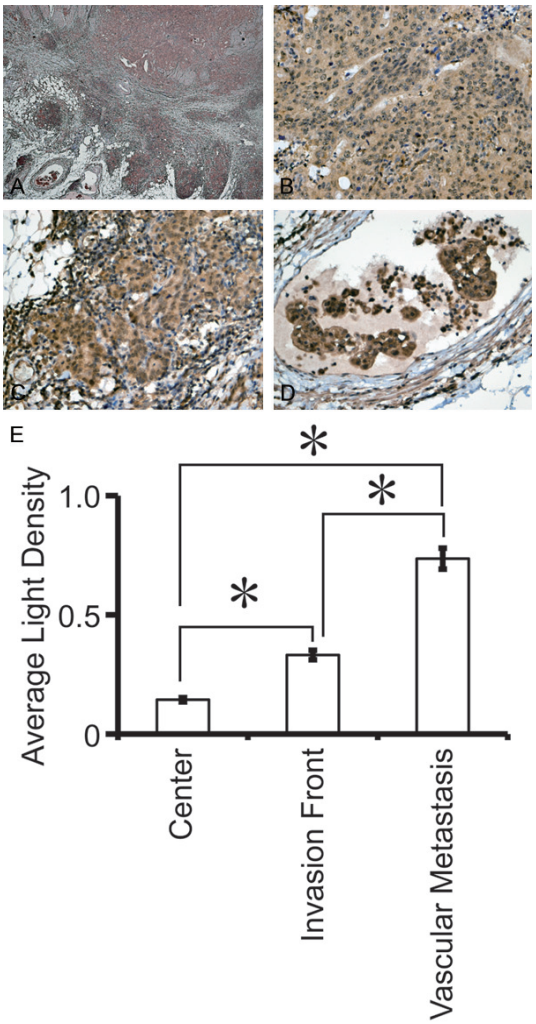

Figure 1. ER-α36 expression in gastric cancer tissues. (A) ER-α36 expression differed between the center of the tumor, invasion front, and vascular metastasis (40×). (B) ER-α36 expression was the lowest in the center of the tumor (400×), (C) intermediate in the invasion front (400×), and (D) highest in the vascular metastasis (400×). (E) The mean density of positive staining, as measured using Image-Pro Plus 6.0. Data are presented as means ± SD, n = 20. *P < 0.05.

paraffin-block. The tissue microarray was sectioned at a thickness of 4 μm. Then, 40 cases were randomly selected from the ER-α36-positive tissues to evaluate differences in ER-α36 expression among the invasion front, center, and vascular metastasis.

Immunohistochemistry and quantitative analysis of protein expression

Briefly, 5-μm paraffin-embedded tissue sections were dewaxed in xylene and rehydrated in a concentration gradient of ethanol (100%, 95%, 90%, 80%, and 70% in PBS, 5 min each). Antigen retrieval was performed by incubating the slides with 100 mM sodium citrate solution (pH 6.0) for 20 min. Tumor tissues were stained with an anti-ER-α36 antibody, followed by avidin-biotin-immunoperoxidase visualization. Cell nuclei were stained with hematoxylin. Positively stained cells were observed using an Olympus microscope (Tokyo, Japan) at ×400 magnification. Immunostained slides were evaluated by two pathologists independently in a blinded manner. In most cases, the evaluations of the two pathologists were identical. Any discrepancies were resolved by re-examination and consensus. The intensity of staining for ten random fields at 400× magnification was measured and documented using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA). The mean density values for the digital image (×400) were designated as the representative ER-α36 staining intensity. First, the intensity was corrected by the standard optical density. Second, single colors were manually selected, the background gray level was set to 150 in all slices, and 150-255 (maximum) signals were counted. Third, the positive area and density (mean) were determined. Finally, the average optical density of tissue areas from selected fields was subjected to statistical analysis using SPSS 12.0. The data are summarized in **Figure 1**.

Western blot assay

For western blot analysis, cells were washed with cold PBS and lysed with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.25 mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF) supplemented with protease inhibitors (Sigma). The protein concentrations were determined using an Enhanced BCA Protein Assay Kit. Cell lysates were mixed with loading buffer, separated on a 12% SDS-PAGE, and transferred to a PVDF membrane. The membranes were probed with various primary antibodies, appropriate secondary antibodies, and visualized with enhanced chemiluminescence detection reagents (DNR Bio-Imaging Systems, Jerusalem, Israel). The densities of protein bands were assessed using TotalLab (Nonlinear Dynamics Technical, Durham, NC, USA).

Nude mouse xenograft assay

Male nude mice (BALB/c-nu/nu nude mice, 20-25 g) were purchased from the Hubei

Table 1. Relationship between ER-α36 expression and clinicopathologic features of gastric cancer

Factor	ER-α36 expression		*P*-value
	positive	negative	
Sex			
Male	80	16	0.014
Female	27	15	
Age			
≤ 50 years	34	17	0.019
> 50 years	73	14	
T Stage			
T1	8	12	0.125
T2	16	10	
T3	30	12	
T4	29	21	
N Stage			
N0	26	18	P < 0.01
N1-3	81	13	
M Stage			
M0	38	19	0.01
M1	69	12	

Experimental Animal Center, China. All experimental procedures were approved by the Animal Care and Use Committee at the affiliated Hospital of Guilin Medical College, Guangxi province, China. All experimental procedures were performed in compliance with National Institutes of Health guidelines on the ethical use of animals. The following cell lines were used: SGC7901, an ER-α36 upregulated SGC-7901 cell line (High36), and an ER-α36 knockdown SGC7901 cell line (Low36). Approximately 5×10^5 cells resuspended in PBS were subcutaneously implanted into the dorsal skin of nude mice. Tumor volume (V) was measured using calipers every 4 days and was calculated as V = length × width (cm^2). After 24 days, all animals were euthanized. The tumors were removed and weighed. All tumor tissues were retained for western blotting and IHC.

Statistical analysis

Statistical analysis was performed using SPSS 12.0. Results are presented as means ± SD of three replicates. Comparisons were performed using the Student's t-test and analysis of variance (ANOVA). Differences were considered significant when P < 0.05. All experiments were repeated at least three times to ensure the reproducibility of the results.

Results

Relationship between ER-α36 expression and clinicopathologic features in gastric adenocarcinoma

The expression patterns of ER-α36 were examined in 138 gastric carcinoma samples using IHC. ER-α36 expression was predominantly detected in the cytomembrane and cytoplasm of gastric carcinoma cells (**Figure 1**). Positive ER-α36 expression was detected in 107 of the 138 cases (77.53%) (**Table 1**). Correlations between ER-α36 expression and clinicopathologic features were also investigated. ER-α36 expression was correlated with an older age (median age, 56.5 years old; range 30-59 years, P < 0.05), sex (male:female ratio 2.29:1; P < 0.05), histological type (ER-α36 expression was higher in the intestinal type; P < 0.05), tumor invasion (P < 0.05), and lymph node metastasis (P < 0.05) but not with distant metastasis (P > 0.05; **Table 1**). We chose 20 cases in which the center of the tumor, invasion front, and vascular metastasis were visible in a single slice to analyze differences in ER-α36 expression among sites. We found that ER-α36 expression was highest in the area of vascular metastasis (OA = 0.7358 ± 0.03679), moderate in the invasion front (OA = 0.3316 ± 0.01658), and lowest at the center of the tumor (OA = 0.1437 ± 0.0072) (n = 20) (**Figure 1**).

C-Src is involved in ER-α36-mediated mitogenic estrogen signaling in gastric cancer cells

To determine the mechanisms by which ER-α36 mediates the estrogen-stimulated migration of gastric cancer cells, three gastric cancer cell lines (SGC7901, High36, and Low36) were treated with the c-Src inhibitor PP2 (10 μM). After stimulation with 0.1 nM E2β, migration was higher in High36 cells than in SGC7901 and Low36 cells. These results suggest that ER-α36 is involved in the migration of gastric cancer cells. Additionally, PP2 inhibited migration stimulated by 0.1 nM E2β in all cell lines, indicating that c-Src is involved in the ER-α36 signaling pathway (**Figure 2**).

ER-α36, MMP2, MMP9, and E-cadherin were expressed in tumor xenografts

To determine tumor invasion ability, all cell lines (1 million cells per nude mouse) were independently transplanted subcutaneously into the

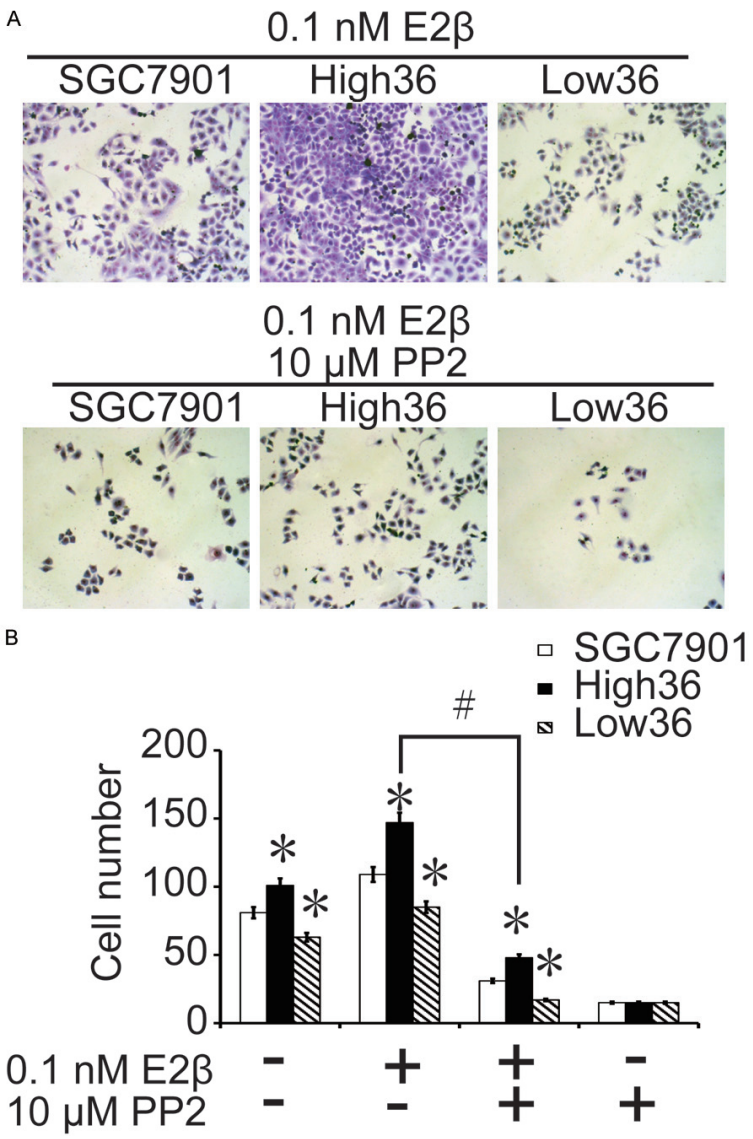

Figure 2. PP2 inhibited the increase in invasion induced by E2β in response to ER-α36 treatment in SGC7901 cells. A. E2β-induced SGC7901, High36, and Low36 cell invasion was evaluated using a Transwell assay. PP2 inhibited E2β-induced changes in SGC7901, High36, and Low36 cell invasion, as determined by the Transwell assay. B. Effects of E2β and/or PP2 on SGC7901, High36, and Low36 cell invasion ability (*P < 0.05, compared with SGC7901; #P < 0.05, compared with each other).

skin of the dorsal body of three nude mice. All experimental procedures were approved by the Animal Care and Use Committee at the Affiliated Hospital, Guilin Medical University. All experimental procedures were performed in compliance with the National Institutes of Health guidelines on the ethical use of animals. The growth of the transplanted tumors was monitored every 4 days and tumors were detected from day 8. After 24 days, nude mice were euthanized and tumors were excised. Weights were highest for the tumors using High36 cells (1.487 ± 0.075 g), intermediate for SGC7901 (0.874 ± 0.045 g), and lowest for Low-36 (0.545 ± 0.027 g) (all $P < 0.05$). The expression levels of ER-α36, c-Src, MMP2, and E-cadherin in xenografts were examined using IHC (**Figure 3**). Levels of ER-α36, c-Src, and MMP2 were highest for High-36 cells, intermediate for SGC7901 cells, and lowest for Low36 cells. The opposite results were obtained for E-cadherin. These results further indicated that ER-α36-mediated signaling plays an important role in the migration of gastric cancer cells, presumably by c-Src, MMP2, and E-cadherin.

C-Src is involved in ER-α36-mediated mitogenic estrogen signaling in gastric cancer cells

A western blot analysis of phospho-specific c-Src showed that E2β increased phosphorylation at Tyr416 of c-Src and decreased phosphorylation at Tyr527 of c-Src (**Figure 4**). PP2 decreased c-Src phosphorylation at Tyr416 induced by E2β and increased phosphorylation at Tyr527 (**Figure 4**). The activation of c-Src was related to the expression levels of ER-α36, suggesting that the c-Src signaling pathway is involved in estrogen signaling mediated by ER-α36 in gastric cancer cell invasion.

C-Src is involved in ER-α36-mediated MMP2, MMP9 and E-cadherin expression in gastric cancer cells

We next examined whether c-Src contributes to the induction of MMP2, MMP9, and E-cadherin expression in gastric cancer cells in response

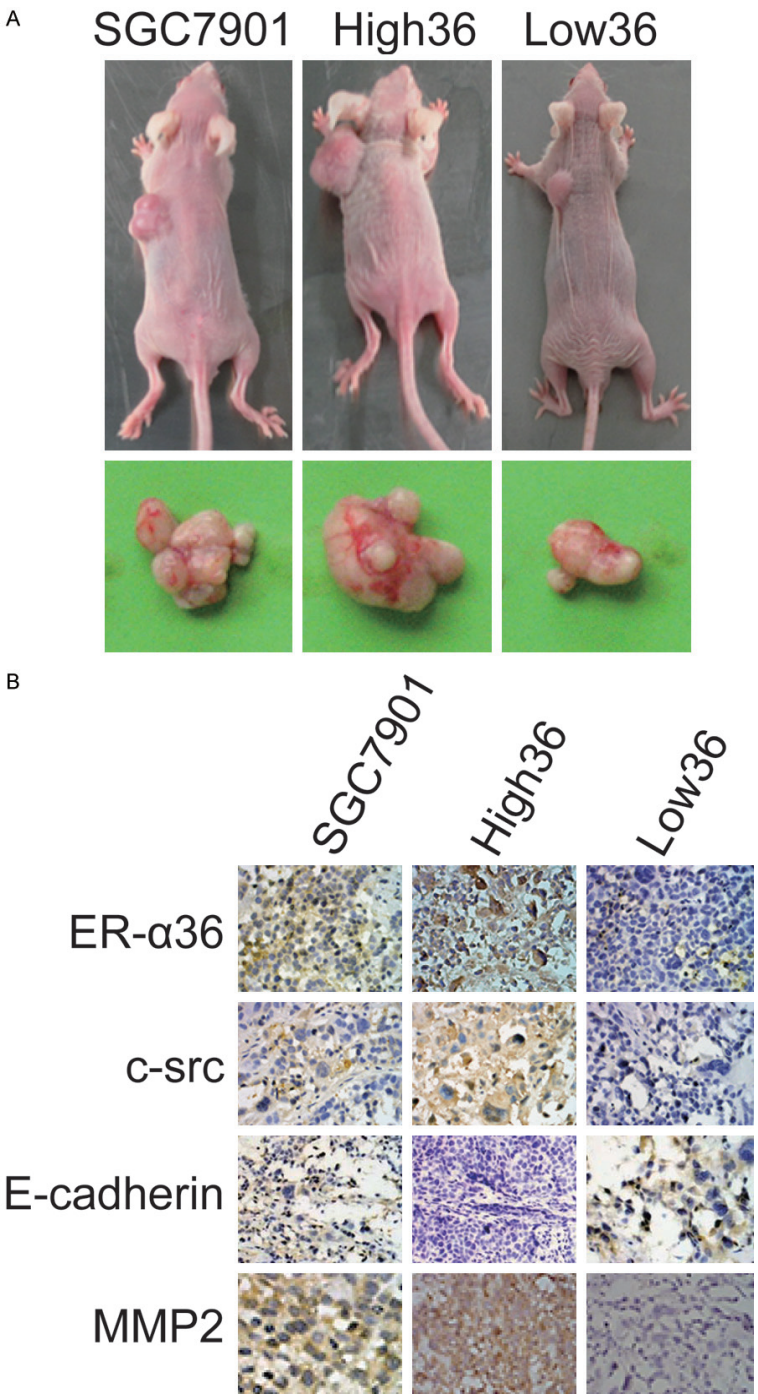

Figure 3. ER-α36, c-Src, E-cadherin, MMP2, and MMP9 expression in xenografts. A. Xenografts in nude mice. B. Protein expression of ER-α36, c-Src, E-cadherin, MMP2, and MMP9 in xenografts in nude mice.

to estrogen. SGC7901 cells were treated with 0.1 nM E2β and PP2 for 48 h and a western blot analysis was performed to examine c-Src, MMP2, MMP9, and E-cadherin expression. The increases in MMP2 and MMP9 expression and decrease in E-cadherin expression induced by 0.1 nM E2β were inhibited by PP2 and with no effects on c-Src expression. These results suggested that c-Src is involved in the induction of MMP2, MMP9, and E-cadherin expression by 0.1 nM E2β in gastric cancer cells (**Figure 5**).

Discussion

Epidemiologic investigations have shown that gastric cancer is the second leading cause of cancer-related death [1, 18]. It has typically been considered an estrogen-independent tumor [19, 20]. However, a growing number of epidemiological studies have shown that the ratio of men to women with gastric cancer ranges from 2:1 to 3:1 [1, 4, 18, 21, 22]. Gastric cancer is therefore a mysterious phenomenon that predominantly affects men [5]. However, this sex difference disappears when comparing men with postmenopausal women; estrogen levels and other known risk factors cannot explain this observation [5]. The incidence of gastric cancer is delayed in women before the age of menarche and later menopause and the incidence increases in the postmenopausal period [5]. From menarche to menopause, increased fertility in women decreases the incidence of gastric cancer. For example, the incidence of gastric cancer is high in nuns [5]. These findings indicate that estrogen may be an important determinant of the incidence of gastric cancer.

Estrogen acts by interacting with estrogen receptors. Our results showed that ER-α36 mediated the estrogen-induced invasion of gastric cancer cells, consistent with our previous results showing that ER-α36 expression is significantly correlated with tumor invasion and

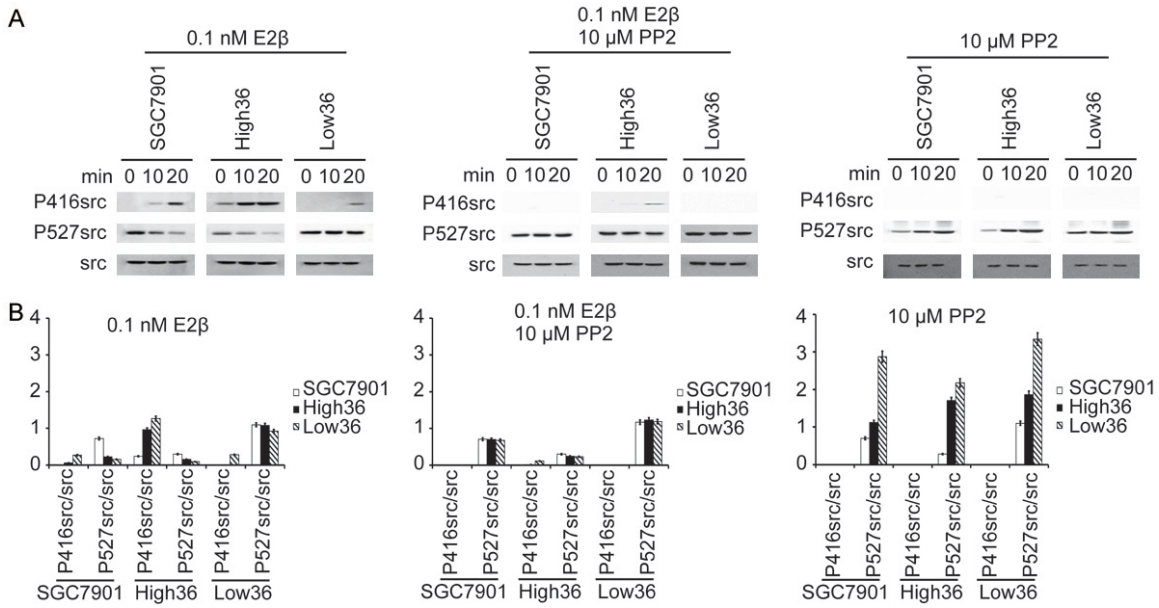

Figure 4. PP2 inhibited the activation of c-Src induced by E2β. A. E2β (0.1 nM) and PP2 (10 μM) stimulation of SGC7901, High36, and Low36 at 0, 10, and 20 min. Cell lysates were analyzed with an anti-p416-c-Src antibody and anti-p527-c-Src antibody. The anti-c-Src antibody was used to ensure equal loading. B. Western blot analysis of p-416-c-Src and p-527-c-Src in SGC7901, High36, and Low36.

lymph node metastasis in gastric cancer [15]. We further found that c-Src was involved in ER-α36-mediated mitogenic estrogen signaling in gastric cancer cell invasion, in accordance with our previous results for gastric cancer cell growth [16]. We also observed that ER-α36 expression was associated with older age, male sex, intestinal type, tumor invasion, and lymph node metastasis but not with distant metastasis (**Table 1**). The lack of a correlation with distant metastasis may be explained by the small sample size of human gastric cancer tissues involving distant metastasis (only 17 cases).

MMP2 is a collagenase that promotes tumor growth and invasion by digesting the extracellular matrix around the tumor tissue [23]. A previous study has shown that MMP-9 can degrade type IV collagen, which is a major constituent of the basement membranes of blood vessels, and promotes lymph node metastasis, especially in gastric cancer [24]. In this study, we found that MMP2 and MMP9 expression were induced by 0.1 nM E2β in gastric cancer cells and was the highest in High36 cells, intermediate in SGC7901 cells, and lowest in Low36 cells.

E-cadherin is the primary cell adhesion molecule in the epithelium, and a loss of expression increases lymph node metastasis in gastric cancer [25]. We found that E-cadherin expression was the highest in Low36 cells and the lowest in High36 cells, and similar results were found in tumor xenografts.

C-Src is a non-receptor protein tyrosine kinase that transduces signals involved in a variety of cellular processes, such as cell adhesion, invasion, growth, and differentiation [16]. We found that c-Src was involved in changes in MMP2, MMP9, and E-cadherin expression induced by 0.1 nM E2β in gastric cancer cells. Moreover, the expression levels of ER-α36, c-Src, MMP2, and MMP9 were the highest in High36 cells, intermediate in SGC7901 cells, and lowest in Low36 cells. Furthermore, we found that ER-α36 interacted with c-Src when SGC7901 cells were stimulated by E2β.

Overall, our results demonstrate that ER-α36 is involved in gastric cancer cell invasion through c-Src, MMP2, MMP9, and E-cadherin.

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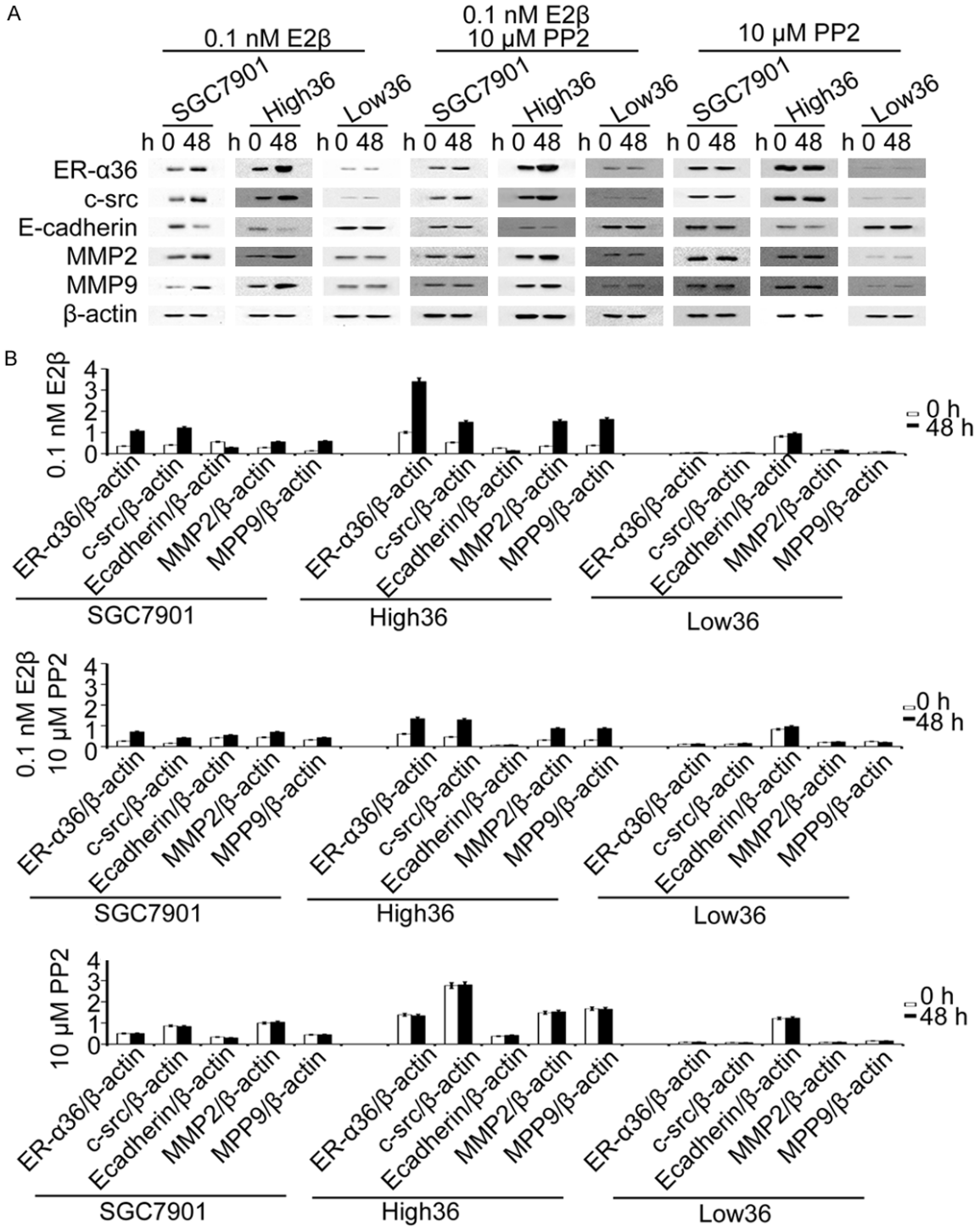

Figure 5. PP2 inhibited the expression of ER-α36, c-Src, E-cadherin, MMP2, and MMP9 induced by E2β in SGC7901, High36, and Low36 cells. A. E2β (0.1 nM) and/or PP2 (10 μM) treatment of SGC7901, High36, and Low36 at 0 h and 48 h. Cell lysates were analyzed with an anti-ER-α36 antibody, anti-c-Src antibody, anti-E-cadherin antibody, anti-MMP2 antibody, and anti-MMP9 antibody. The anti-β-actin antibody was used to ensure equal loading. B. Western blot analysis of ER-α36, c-Src, E-cadherin, MMP2, and MMP9 in SGC7901, High36, and Low36 cells.

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Disclosure of conflict of interest

None.

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