Abstract. To examine the expression patterns of ER-α36 and Cyclin D1 in human gastric cancer tissues and to investigate the effects of ER-α36-mediated estrogen signaling on the growth of gastric cancer cells, 117 samples of formalin-fixed and paraffin-embedded gastric cancer tumor tissues and 40 fresh gastric cancer tumor tissues were analyzed with immunohistochemistry assay and western blot analysis. ER-α36 expression was well correlated with gender (male:female ratio 2.88:1, \( P=0.01 \)), invasion to serosa \( (P=0.01) \) as well as Cyclin D1 expression \( (P<0.01) \). The effects of different concentrations of estrogen on the growth of different gastric cancer cells and normal gastric cells as well as gastric cancer SGC7901 cells with different levels of ER-α36 expression were examined. SGC7901 cells with high levels of ER-α36 expression exhibited estrogen hypersensitivity, high growth rate and high levels of Cyclin D1 expression while SGC7901 cells with knocked-down levels of ER-α36 expression were insensitive to estrogen stimulation, grew slowly and expressed less Cyclin D1. Our results indicate that ER-α36 mediates biphasic estrogen signaling in the growth of gastric cancer cells.

Introduction

Gastric cancer is one of the most common cancers worldwide, accounting for ~8% new cancers diagnosed each year \((1,2)\). The epidemiology studies indicate that the male predominance of gastric cancer is a global phenomenon \((male:female ratio 2:1 \text{ or } 3:1) \) \((3)\). The predominance is also related to a delay of 10-15 years in the appearance and onset of gastric cancer in females compared with males \((3,4)\). From adolescence to menopause, women with multiple fertility have relatively less frequent incidence of gastric cancer, while the incidence of gastric cancer was high in nuns \((5)\). Oral contraceptives and estrogen replacement therapy reduced the incidence of gastric cancer in women \((1,6)\). The incidence of gastric cancer in the prostate cancer patients treated with estrogen was lower than non-treated patients \((1,6)\). The incidence of gastric cancer increased in women receiving oophorectomy and decreased in women receiving estrogen replacement therapy \((1,6)\). Recent epidemiological survey results also indicated an association between the estrogen level and histological type of gastric; intestinal type gastric cancer occurred more often in middle-aged males \((1,6,7)\). Animal experimental results also showed that in rats treated with the chemical N-methyl-N’-nitro-N-nitrosoguanidine \((MNNG)\), the incidence of gastric cancer in male rats was higher than in female \((8)\). However, estrogen treatment reduced the incidence of MNNG-induced gastric cancer in male rats while oophorectomy increased the incidence of MNNG induced gastric cancer in female rats. In addition, estrogen treatment reversed precancer lesions of gastric mucosa induced by MNNG \((8)\). Taken together, estrogen signaling is involved in development of human gastric cancer, which is regulated by endogenous estrogen level.

Estrogen signaling is mediated by estrogen receptors, ER-α and ER-β. ER-α has three isoforms, namely, ER-α66 (the original estrogen receptor), ER-α46 and ER-α36 \((9,10)\). ER-α36 lacks the transcription activation domain AF-1 and AF-2 but retains the DNA binding domain, receptor dimerization domain and part of the ligand-binding domains \((9)\). Previously, we reported that ER-α36 is expressed in normal gastric and gastric cancer tissues, which was correlated with TNM stage and metastasis of gastric cancer cells \((3,10,11)\). However, the function and underlying mechanism of ER-α36-mediated estrogen signaling in the growth of gastric cancer have not been established. Here, we examined the expression patterns of ER-α36 and Cyclin D1 in human gastric cancer samples and studied effects of different concentrations of estrogen on growth of various gastric cancer cells.

Keywords: gastric cancer, estrogen, ER-α36, Cyclin D1
Materials and methods

Cell culture. The human gastric cancer cell lines AGS, BGC823 and SGC7901 and the normal gastric cell line GES-1 were obtained from Chinese Academy of Medical Sciences Cell Center of Basic Medicine (Beijing, China). Gastric cancer SGC7901 cells with different levels of ER-α36 expression, SGC7901/High36 and SGC7901/Low36 cell lines with high or low levels of ER-α36 expression, respectively, were generated and characterized as described before (10). SGC7901, SGC7901/High36 and SGC7901/Low36 cells were maintained in DMEM medium (Invitrogen, USA) containing 10% fetal bovine serum (FBS, Invitrogen) at 37°C in a 5% CO2 atmosphere. Before treated with indicated concentrations of E2 or ethanol vehicle as a control, cells were maintained for 3 days in phenol red-free DMEM plus 2.5% dextran-charcoal stripped fetal calf serum. Following treatment for 7 days, the cells were trypsinized and counted with the ScepterTM 2.0 handheld automated cell counter (Merck KGaA, Darmstadt, Germany). Assays were performed in three dishes for each time-point and all experiments were repeated three times.

Tumor sample and tissue microarray. Frozen tumor samples obtained from 40 gastric cancer patients between 2009 and 2012 (Jiangda Pathology Institute), and paraffin-embedded samples of gastric cancer obtained from 117 patients between 2006 and 2012 (Jiangda Pathology Institute) were used for this study with the approval of the Institutional Review Board of Jianghan University. Tumor tissues used for immunohistochemistry (IHC) were fixed in 10% neutral formalin, embedded in paraffin, processed and stained with hematoxylin and eosin (H&E). Tissues for western blot analysis were snap-frozen in liquid nitrogen and were kept at -150°C. The 117 samples for IHC included 83 men and 34 women aged 30-59 years (mean age, 57.3 years) and the samples for western blot analysis were from 28 men and 12 women aged 30-59 years (mean age, 56.2 years). None of the patients had received any anticancer treatment prior to surgery. Tumor size, histological differentiation, T stage and N stage were evaluated according to the clinicopathological classification of the World Health Organization (2010). Targeted tissue areas of 117 tumors were marked on H&E-stained sections. One tissue core, 1.0 mm in diameter and 3-4 mm in depth were removed from each block using a manual microarray device (Beecher Instruments, Silver Spring, MD, USA) with a total of 117 tissue cores inserted into the recipient paraffin-block. The tissue microarray was sectioned at 4-micron thickness.

Western blot analysis. For western blot analysis, cells were washed with cold PBS and lysed with the 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 and phosphatase inhibitors from Sigma. Tumor tissues were dissected and homogenized in the lysis buffer. The protein concentrations were determined with an Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Cell lysates were mixed with loading buffer (Beyotime Institute of Biotechnology), separated on 12% SDS-PAGE gels and transferred to PVDF membranes (0.22 µm, Millipore, Billerica, MA, USA). The membranes were probed with various primary antibodies, appropriate secondary antibodies, and visualized with enhanced chemiluminescence (ECL, Beyotime Institute of Biotechnology) detection reagents (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). The densities of protein bands were assessed with Totallab analysis software (Nonlinear Dynamics Technical, NC, USA).

The anti-ER-α36 antibody was kindly provided by Professor Zhaoyi Wang at Creighton University. Anti-Cyclin D1 antibody (SC-718) and anti-β-actin antibody (SC-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

RNA extraction and reverse transcription PCR. Total RNA was extracted from the frozen tissues using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. Total RNA was then reverse transcribed into cDNA by reverse transcription-PCR kit (Shengong, Shanghai, China). Following reverse transcription, PCR reaction was carried out with 32 cycles using the following conditions: 94°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. Cyclin D1 primers and β-actin primers were designed using Primer 5.0 (Primer Biosoft International, Palo Alto, CA, USA) and were used simultaneously in the same reaction. The following primers were used: Cyclin D1 forward primer 5'-ATGGAAC ACCAGCTCCTGTG-3'; Cyclin D1 reverse primer 5'-ACCTC CAGCATCCAGGTGGC-3'; β-actin forward primer 5'-ATGA TGATATCGCCCGGC-3'; β-actin reverse primer 5'-GTAC

Table I. Relationships of ER-α36 expression with clinicopathological features and Cyclin D1 expression in gastric carcinomas.

<table>
<thead>
<tr>
<th>Factors</th>
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<th>P-value</th>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 years</td>
<td>7</td>
<td>0.04</td>
</tr>
<tr>
<td>&gt;50 years</td>
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<td></td>
</tr>
<tr>
<td>Gender</td>
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<td>0.01</td>
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<tr>
<td>Female</td>
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<tr>
<td>Tumor size</td>
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<td>0.20</td>
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<tr>
<td>&gt;5 cm</td>
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<td>Invasion to serosa</td>
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<tr>
<td>Negative</td>
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<td>Negative</td>
<td>79</td>
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Factors Negative Positive P-value
ATGGCTGGGGTGTTGA-3’. PCR products (200 bp for Cyclin D1 and 395 bp for β-actin) were separated on a 1.5% agarose gel and stained with Gelred (SBS Genetech Co., Beijing, China). The densities of DNA bands were determined with analysis software (Biostep Photoimpact, Beijing, China).

Statistical analysis. The statistical analysis was performed by SPSS 12.0 software. Results are shown as the mean ± SD in three replicate samples and compared to 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.

Results

Relationship between ER-α36 and Cyclin D1 expression and clinic pathological features in gastric adenocarcinomas. The expression patterns of ER-α36 and Cyclin D1 were examined in 117 samples of gastric carcinoma with IHC. ER-α36 expression was detected predominantly in the cytomembrane and cytoplasm of gastric carcinoma cells while Cyclin D1 was detected predominantly in the nuclei of gastric carcinoma cells. Positive expression of Cyclin D1 was observed in 81 of the 117 samples (69.2%), and ER-α36 expression was detected in 101 of the 117 cases (86.23%) (Table I and Fig. 1). A strong correlation was found between Cyclin D1 and ER-α36 expression in IHC (\( P<0.01 \), Table I), suggesting that Cyclin D1 may be one of the downstream effectors of ER-α36-mediated estrogen signaling.

The expression patterns of ER-α36 and Cyclin D1 were also examined in frozen gastric cancer tissues. ER-α36 and Cyclin D1 expression were examined in 40 cases of frozen gastric cancer tissues with western blot analysis and IHC (Fig. 2). Positive expression of ER-α36 was detected in 32 of the 40 samples (80%) in western blot analysis and 31 of the 40 samples (77.5%) in immunohistochemistry. We also observed positive expression of Cyclin D1 in 29 of the 40 samples (72.5%) in western blot analysis and 27 of the 40 samples (77.5%) in immunohistochemistry. A strong correlation was also found between Cyclin D1 and ER-α36 expression in western blot analysis (\( P<0.05 \)).

The correlation between ER-α36 expression and other clinicopathological features was also investigated. ER-α36 expression was correlated with the older patients (median age, 57.3 years old; range from 30 to 59 years old, \( P=0.04 \)), gender (male:female ratio 2.88:1; \( P=0.01 \)), invasion to serosa (\( P=0.01 \)), but not with tumor size, histological differentiation and lymph node metastasis (\( P>0.05 \); Table I).

Estrogen stimulated growth of gastric cancer cells. First, we examined ER-α36 expression in different human gastric cancer cell lines and the normal gastric cell line GES-1 using western blot analysis. As shown in Fig. 3A, ER-α36 expression was the lowest in GES-1 cells and the highest in AGS cells with the second in BGC823 cells and the third in SGC7901 cells. To determine the effects of estrogen on growth of gastric cancer cells, gastric cancer cells lines AGS, BGC823, SGC7901 and the normal gastric cell line GES-1 were treated with different concentrations of 17β-estradiol (E2β) for 7 days and the cell number was determined. All gastric cancer cells treated with E2β exhibited an increased growth rate compared with cells treated with vehicle while normal gastric GES-1 cells only slightly responded to E2β at \( 10^{-8} \) M of E2β. The dose-response curves of these cells to E2β displayed a non-monotonic or
biphasic pattern; increasing concentrations of E2β that initially stimulated cell growth but inhibited cell growth at higher concentrations (Fig. 3C). E2β at 10^{-16} M started to weakly stimulate the growth of AGS cells that express high levels of endogenous ER-α36 while BGC823 and SGC7901 cells required 10^{-14} and 10^{-12} M, respectively. The result suggested that ER-α36 expression is involved in the sensitivity of gastric cancer cells to estrogen.

**Correlation between estrogen expression and Cyclin D1 expression in gastric cancer.** SGC7901 cells with different levels of ER-α36 expression. We also used different concentrations of estrogen to treat SGC7901, SGC7901/High36 and SGC7901/Low36 cells for 6 and 12 h to examine Cyclin D1 mRNA expression, and for 24 and 48 h to detect Cyclin D1 protein expression. As shown in Figs. 5 and 6, the 10^{-12} M of E2β-induced Cyclin D1 expression in SGC7901 cells, which peaked at 10^{-10} M and decreased at 10^{-8} M. In SGC7901/High36 cells, however, 10^{-14} M of E2β induced Cyclin D1 expression, which peaked at 10^{-12} M and decreased at 10^{-10} M.
Figure 5. Cyclin D1 mRNA expression of SGC7901 cells, High36 cells and Low36 cells. Cyclin D1 mRNA expression was assessed by reverse transcription-PCR analysis (A and B) and quantitative analysis (C and D) in SGC7901 cells, High36 cells and Low36 cells. The levels of Cyclin D1 mRNA expression were normalized against that of β-actin. The SGC7901, High36 and Low36 cells with various ER-α36 expression were stimulated by vehicle, 10^{-16}, 10^{-14}, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M of E2β in 6 and 12 h. The densities of DNA bands were examined with the analysis software and the relative values were determined. The data are expressed as mean ± SD from three independent experiments. *P<0.05.

Figure 6. Cyclin D1 protein expression of SGC7901 cells, High36 cells and Low36 cells. Cyclin D1 protein expression was assessed by western blot analysis (A and B) and quantitative analysis (C and D) in SGC7901 cells, High36 cells and Low36 cells. The levels of Cyclin D1 protein expression were normalized against that of β-actin. The SGC7901, High36 and Low36 cells with various ER-α36 expression were stimulated by vehicle, 10^{-16}, 10^{-14}, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M of E2β in 24 and 48 h. The data are expressed as mean ± SD from three independent experiments. *P<0.05.
(Figs. 5 and 6). The SGC7901/Low36 cells required higher concentrations of E2β (10⁻¹⁰ M) to induce Cyclin D1 expression, which was decreased at 10⁻¹⁰ M (Figs. 5 and 6). Our results indicated that induction of Cyclin D1 expression by estrogen in gastric cancer cells also exhibited a biphasic pattern, which is influenced by expression levels of ER-α36.

Discussion

Despite advances in various diagnostic tools and therapy, the 5-year relative survival rate of gastric cancer is still low (1.12-1.14). Epidemiology studies have shown the male predominance of gastric cancer (3). The predominance is also shown by a delay of 10-15 years in the appearance and onset of gastric cancer in females compared with males (3,4). Accumulating evidence suggested that estrogen played a protective role in the incidence of gastric cancer. However, the underlying mechanisms of male dominance in gastric cancer have not been established.

Our study showed that ER-α36 expression was correlated well with male patients (P=0.01), invasion to serosa (P=0.01) and Cyclin D1 expression (P<0.01) in gastric cancer tissues. Our results thus suggested that ER-α36 may be involved in observed male predominance in human gastric cancer. We also found gastric cancer cells were stimulated by estrogen, which exhibited a biphasic growth curve, and cells with high levels of ER-α36 expression are more sensitive to estrogen and required less estrogen to stimulate cell growth compared to cells express lower levels of ER-α36.

Cyclin D1 is an important regulatory factor for cell cycle progression that is required to mediate the G1 to S transition and cell cycle progression (15-17). Cyclin D1 overexpression has been documented in several carcinomas, including gastric cancer (18-21). It has been reported that a gender difference in MNNG-induced rat gastric carcinogenesis is involved in the gender difference of Cyclin D1/cdk4 expression (22). Here, we found that Cyclin D1 expression is induced by ER-α36-mediated estrogen signaling in gastric cancer cells and is also well correlated with ER-α36 expression in human gastric tumor tissues. We found estrogen induction of Cyclin D1 expression also exhibited a biphasic pattern; induces Cyclin D1 expression at low concentrations and failed to do so at high concentrations, consistent with growth curves of gastric cancer cells in response to estrogen. Our results thus indicated that Cyclin D1 may be one of the downstream effectors of ER-α36 mediated mitogen estrogen signaling in gastric cancer cells.

The findings that gastric cells with high levels of ER-α36 require pM even fM range estrogen to stimulate cell growth and estrogen at µM range to inhibit cell growth while cells with low levels of ER-α36 respond to estrogen at µM range and fail to respond to estrogen at nM range may provide a molecular explanation to the male dominance in the incidence of human gastric cancer and a mechanism for the protection role of estrogen in gastric cancer.

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References