

Expression of ER α 36 in gastric cancer samples and their matched normal tissues

JIANJUN WANG^{1*}, JIAJIA LI^{2*}, RENGUI FANG¹, SHUDUO XIE³, LINBO WANG³ and CHAOYANG XU^{2,3}

¹Department of Surgical Oncology, The Chunan County First Hospital, Zhejiang 312030; ²Department of Breast and Thyroid Surgery, Shaoxing People's Hospital, The First Affiliated Hospital of Shaoxing University, Zhejiang 312000; ³Department of Surgical Oncology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Zhejiang 310000, P.R. China

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Abstract. Estrogen receptor α 36 (ER α 36) is believed to mediate membrane-initiated effects of estrogen signaling, and promote cell growth and resistance to tamoxifen treatment. However, few studies are available regarding ER α 36 expression in gastric cancer. In the present study, we evaluated the expression of ER α 36, as well as estrogen receptor α 66 (ER α 66), in gastric cancer and its correlation with clinicopathological parameters. Real-time polymerase chain reaction (PCR) was applied to detect the expression of ER α 66 and ER α 36 mRNA in 45 pairs of samples of gastric cancer tissues and matched normal tissues. The $\Delta\Delta$ CT method was used to evaluate the relative quantity of target mRNA expression. Among the 45 pairs of samples of gastric cancer tissues and matched normal tissues adjacent to the tumor, the ER α 36 mRNA levels in normal tissues were significantly higher than those observed in gastric cancer tissues ($p=0.040$). Additionally, the expression of ER α 66 mRNA levels between gastric cancer tissues and matched normal tissues had no statistically significant difference. We confirmed that ER α 36 mRNA was expressed in the four gastric cancer cell lines, and ER α 66 mRNA was expressed in two of the four gastric cancer cell lines. According to the tissue and cell findings, it was suggested that the expression level of ER α 36 is greater than that of ER α 66 in gastric cancer. In conclusion, the expression of ER α 66 and ER α 36 in gastric cancer tissues and cells was confirmed in this study. A decreased expression of ER α 36 mRNA in gastric cancer tissues may be one of the factors affecting tumorigenesis in gastric cancer patients.

Introduction

Findings of recent studies have shown that there is a possible correlation of estrogen with the biological activity of gastric cancer cells (1), and that the expression of estrogen receptor α 66 (ER α 66) may correlate with poorer prognosis among patients with gastric cancer (2).

ER α 36, a novel variant of the full-length 66 kDa ER α 66, has one of the most crucial roles in cell growth and differentiation in various types of cancer (3). This variant differs from ER α 66 by lacking the transcriptional activation domains (AF-1 and AF-2), but retains the partial dimerization and ligand-binding domains and DNA-binding domain.

ER α 36 enhances oncogenesis, and promotes cell growth and survival during endocrine therapy in breast cancer (4). The expression of ER α 36 was subsequently detected in breast (5), colorectal (6) and endometrial cancer (7). Furthermore, unlike ER α 66, which is often detected in the cell nucleus, ER α 36 is located in the cytoplasm and plasma membrane. As a result, ER α 36 mediates the membrane-initiated effects of estrogen signaling cascades and stimulates cell growth (3,8). These features make ER α 36 an attractive target for antibody-based therapy.

The expression of ER α 66 has been detected in gastric cancer cell lines as well as in normal and cancer tissues. However, the physiological role of ER α 66's possible involvement in the etiology of gastric cancer remains to be clarified. Recently, it was reported that the effect of tamoxifen treatment in ER α 66-positive breast tumors could be prevented by ER α 36. A similar event may occur in other types of cancer, including gastric cancer. Therefore, understanding the existence and expression status of ER α 36 may have significant implications in the prognosis and treatment of gastric cancer.

Although ER α 36 has been extensively studied in other types of cancer, no investigation has been conducted in gastric cancer. We hypothesize that ER α 66 and its splicing variant ER α 36 may play a role in the oncogenesis of gastric cancer. The present study was undertaken to examine the expression of ER α 36 and ER α 66 in gastric cancer tissues by using a validated specific and sensitive real-time quantitative PCR assay. In this study, we examined tissue from 45 cases of gastric cancer to observe the potential difference of ER α 66 and

Correspondence to: Dr Chaoyang Xu, Department of Breast and Thyroid Surgery, Shaoxing People's Hospital, The First Affiliated Hospital of Shaoxing University, Zhejiang 312000, P.R. China
E-mail: xuchaoyang2001@126.com

*Contributed equally

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ER α 36 expression in gastric cancer tissues and their matched normal tissues, and to assess the correlation between ER α 66 and ER α 36 expression and clinicopathological characteristics in gastric cancer patients.

Materials and methods

Case selection. Specimens were obtained from 45 patients who underwent curative resection of gastric cancer at the Department of Surgical Oncology of the Sir Run Run Shaw Hospital, Zhejiang University College of Medicine, China, between July 2007 and November 2009. Informed consent was obtained from all patients, and the study was conducted according to the guidelines of the Hospital Ethics Committee. The patients comprised 26 males and 19 females, aged 35-81 years (mean 60.0). The correlation between the expression of ER α 36 and ER α 66 and clinicopathological parameters including age, gender, differentiation state, location and pTNM pathological classification according to the International Union against Cancer (UICC) (9) were evaluated. The clinicopathological characteristics of the 45 cases are shown in Table I.

Cell culture. Four gastric cancer cell lines, AGS, MKN-45, NCL-N87 and SGC-7901, were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G and 100 mg/ml streptomycin.

RNA extraction and cDNA synthesis. Total RNA was extracted from freshly frozen gastric tissues using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA was reverse-transcribed into single-strand complementary DNA (cDNA) using Moloney-murine leukemia (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). Briefly, the RNA was denatured by heating for 5 min at 70°C, cooled on ice, and then used for reverse transcription (2 μ g of total RNA, 25 U of RNase inhibitor, 0.5 mM each of dNTPs, 1.5 μ M reverse primer and 200 U of M-MLV reverse transcriptase in a total volume of 25 μ l). For reverse transcription, tubes were incubated at 42°C for 60 min, followed by rapid cooling.

Real-time quantitative PCR. Real-time RT-PCR analyses were performed with the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Reaction mixture (25 μ l) containing 2 μ l of cDNA template, 1 μ l each of sense and anti-sense primers and 1X SYBR-Green Universal PCR Mix was amplified as follows: denaturation at 95°C for 10 min and 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 40 sec. Real-time quantitative PCR was performed in triplicate for each sample and a mean value of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to calculate mRNA levels. Quantitative analysis was performed using the comparative CT method (10,11). The ER α 66 and ER α 36 mRNA copy numbers in normal and tumor tissues were normalized to mRNA copy numbers of the housekeeping gene, GAPDH to give a value of Δ CT. This final value was to determine changes in the expression of ER α 66 and ER α 36 in each sample. The primer sequences for ER α 66 were: forward 5'-AAGAAAGAACACATCAGCAGTAAAGCT-3'; and reverse 5'-GGGCTATGGCTTGTTAAACAT-3'. The

Table I. Clinicopathological characteristics of 45 patients with gastric cancer.

Clinicopathological characteristics	Case (n)
Age	
≤60	25
>60	20
Gender	
Male	26
Female	19
Histological type	
Differentiated	22
Undifferentiated	23
Location	
Upper or whole	15
Middle or lower	30
Tumor size (cm)	
≤5.5	24
>5.5	21
Outside of serosal	
Yes	5
No	40
Node stage	
N0-1	21
N2-3	24

primer sequences for ER α 36 were: forward, 5'-CCAAGAATGTTCAACCACAACCT-3'; and reverse 5'-GCACGGTTCATT AACATCTTTCTG-3'. The primers for GAPDH were obtained as previously described (12). Fluorescent data were converted i) into RQ measurements, which represent relative expression, ii) automatically by the SDS system software and iii) exported to Microsoft Excel. Thermal dissociation plots were examined for biphasic melting curves, indicative of whether primer dimers or other non-specific products may be contributing to the amplification signal.

Statistical analysis. Statistical analysis was conducted using the statistical program SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Pre-treatment characteristics were analyzed using the two-tailed χ^2 test. The two-tailed t-test was used to evaluate the correlation between ER α 36 expression and the clinicopathological parameters.

Results

Real-time quantitative PCR of the expression of ER α 36 and ER α 66 in gastric cancer cells. To evaluate mRNA expression of ER α 66 and ER α 36 in cancer cells, we detected four gastric cancer cell lines. As shown in Fig. 1, ER α 66 mRNA was detected in two cell lines, AGS and NCI-N87. By contrast, ER α 36 mRNA was detected in the four cell lines. Consistent with the clinical data, the expression of ER α 36 mRNA was more predominant than the ER α 66 mRNA expression.

Table II. Relative quantity of ER α 36 mRNA and ER α 66 mRNA in gastric cancer tissues and matched normal tissues.

	Tumor tissue	Normal tissues	P-value
Relative ER α 36 expression	1.73 \pm 5.85	10.54 \pm 2.70	0.040
Relative ER α 66 expression	(7.87 \pm 15.66) $\times 10^{-3}$	(4.30 \pm 6.98) $\times 10^{-3}$	0.135

Table III. Correlation between the expression of ER α 36 mRNA and the number of metastasis lymph nodes, tumor size.

	ER α 36 expression level ≤ 0.237	ER α 36 expression level > 0.237	P-value
Number of metastasis lymph nodes	11.4 \pm 11.3	7.3 \pm 7.1	0.150
Tumor size	6.4 \pm 2.4	5.2 \pm 2.6	0.100

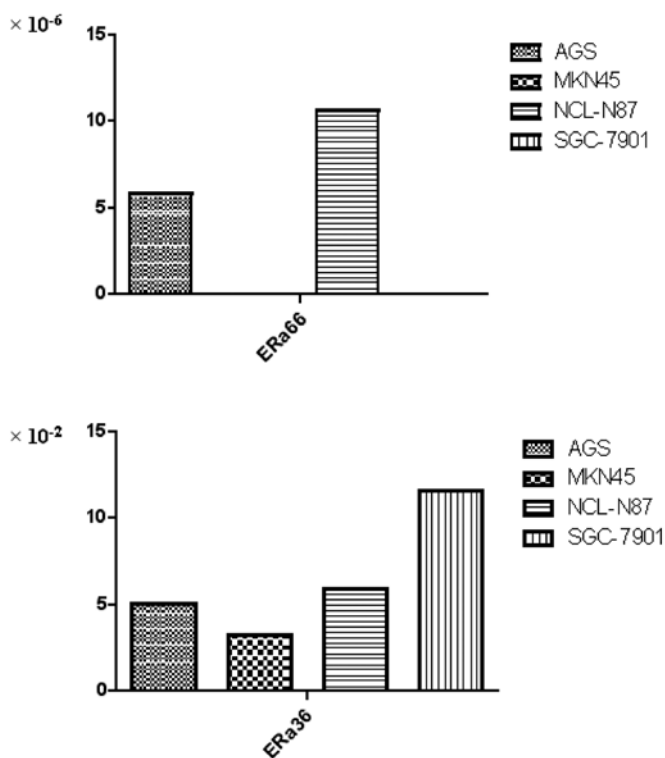


Figure 1. ER α 66 and ER α 36 mRNA expression level in gastric cancer cells (AGS, MKN45, NCL-N87, SGC-7901).

Expression of ER α 36 and ER α 66 mRNA in gastric cancer tissues by real-time PCR. Among the 45 pairs of samples of gastric cancer tissues and matched normal tissues adjacent to the tumor, the level of ER α 66 of the former was similar to that of the latter, and no significant associations were found between ER α 66 mRNA expression in gastric cancer tissues and normal tissues ($p=0.135$).

As shown in Table II, of the 45 samples of gastric cancer tissues and matched normal tissues adjacent to the tumor, expression of ER α 36 was detected in the total samples. In normal tissues, the ER α 36 mRNA levels ranged from 0.029 to 157.696 with a median of 2.016. In gastric cancer tissues, the ER α 36 mRNA levels ranged from 0.004 to 39.233 with a median of 0.237. The ER α 36 mRNA levels in normal tissues

were significantly higher than those observed in gastric cancer tissues ($p=0.040$). Moreover, we found that the expression of ER α 36 mRNA was higher than that of ER α 66 mRNA in gastric cancer tissues and their matched normal tissues.

Correlation between ER α 36 and clinicopathological parameters. According to the median expression level of ER α 36, the 45 cases of gastric cancer were divided into two groups, the high ER α 36 expression group (ER α 36 expression level > 0.237) and the low ER α 36 expression group (ER α 36 expression level ≤ 0.237). The mean number of metastasis lymph nodes in the high ER α 36 group was lower than that in the low ER α 36 expression group (11.4 vs. 7.3), but the differences among them were not statistically significant ($p=0.150$) (Table III). Moreover, tumor size varied between the high ER α 36 expression group versus the low ER α 36 expression group (6.4cm vs. 5.2 cm), but the difference was also not statistically significant ($p=0.099$) (Table III).

Discussion

In the present study, we found the relative quantity of ER α 36 mRNA and ER α 66 mRNA in 45 samples of gastric cancer tissues as determined by real-time PCR. ER α 36 mRNA was expressed more predominantly than ER α 66 mRNA in gastric cancer and normal tissues adjacent to the tumor.

Recent studies have shown conflicting results of ER α expression in gastric cancer (13,14). Moreover, when using the immunohistochemical method, the expression of ER α gastric cancer tissues showed marked variability (0-62.5%) among a number of studies (15-17). These data suggested that a more reliable and sensitive method was required to evaluate the ER α expression in gastric cancer tissues, particularly those with low expression levels. In the current study, real-time quantitative PCR was used to compare the expression of ER α 66 and its splice variant ER α 36 mRNA in 45 cases of gastric cancer and their matched normal tissues, which allows the detection of ER α expression in stomach tissues at a low level. In our study, the expression of another ER α 66 splice variant, ER α 46 mRNA, was also detected; however, it was found in neither the gastric cancer cells nor the gastric cancer tissues.

Estrogen not only modulates cell proliferation in classic estrogen-sensitive tissues, but also in other tissues such as

the lungs (18), colon (19) and stomach (15,16). An epidemiological study showed that tamoxifen, an anti-estrogen agent, may increase the incidence of gastric cancer, which suggested that estrogen may be involved in the pathogenesis of gastric cancer (20). However, few studies have reported the expression of ER α 66 and its variant forms in gastric cancer.

In the present study, we determined not only ER α 66, but also, for the first time, its splicing variant ER α 36 mRNA in gastric cancer samples and their matched normal tissues by real-time quantitative PCR assay. Furthermore, we correlated these findings with the clinicopathological parameters of the gastric cancer samples.

The expression levels of ER α 66, between gastric cancer tissues and normal tissues did not exhibit a significant difference, and the expression level was extremely low. ER α 36 had a differential expression level between normal and cancer tissues, suggesting that ER α 36 plays a more significant role in stomach tumorigenesis, and the decrease in this variant was significantly correlated with increased tumor size. This result suggests that ER α 36 is involved in gastric cancer proliferation.

Recently, it was reported that aromatase expression in gastric cancer cells, and cancer cells in the presence of testosterone, produced estradiol in a short incubation period, suggesting estrogen is also localized in human gastric cancer tissues (21). However, a randomized, controlled study of adjuvant tamoxifen therapy in gastric cancer found that estrogen receptor α expression is an independent prognostic factor. By contrast, tamoxifen had no effect on overall survival in gastric cancer patients; furthermore, treatment with tamoxifen significantly decreased the survival time of patients with estrogen receptor α -positive tumors (22).

It is notable that breast cancer patients with ER α 66 expression-positive tumors that also express high levels of ER α 36 are less likely to benefit from tamoxifen treatment (4). In our study, ER α 36 mRNA was expressed more predominantly than ER α 66 mRNA in gastric cancer tissues, which may be one of the factors impacting on the function of tamoxifen treatment in gastric cancer patients.

The human ER α 36 is known to mediate membrane-initiated estrogen and antiestrogen signaling, such as the mitogen-activated protein kinase (MAPK) signaling pathway, which may provide an explanation for the antiestrogen resistance observed in breast cancer patients. Similar results may present in gastric cancer patients. Furthermore, elucidation of the roles of the estrogen receptor and its variant in gastric cancer may contribute to diagnosis and treatment.

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