Expression of estrogen receptor and estrogen receptor messenger RNA in gastric carcinoma tissues

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INTRODUCTION

Gastric carcinoma is the most common cause of cancer mortality in China\[1-6\] and is responsible for approximately 160,000 deaths annually. During the last 10 years, there has been no improvement in survival after the diagnosis of gastric cancer with an overall 5-year survival rate of 20%. Surgery remains the primary treatment of choice. However, the disease is often advanced at first presentation, and only 30-40% of patients undergoing surgery will have a curative resection. The failure of surgery on the disease has led to the use of chemotherapy and radiotherapy as adjuvant or palliative means, but their value is limited because of toxicity and lack of efficacy\[7-12\]. Since Jensen discovered the existence of estrogen receptor (ER) in the cytoplasm of human mammary cancer cells in 1960, many researchers have also discovered the presence of ER in some gastric cancer cells, suggesting that these cells can be controlled and regulated by sex hormones. From this we can infer that some cases of gastric cancer are hormone-dependent tumors, and this has stimulated the use of the anti-estrogen compound in its treatment. In this study, the expression of ER, ERmRNA in gastric cancer tissues was examined by immunohistochemistry and in situ hybridization, respectively, and the association of their expression and clinical significance at molecular pathological level was also investigated.

MATERIALS AND METHODS

Specimens

Thirty specimens of gastric cancer tissue were collected from The General Surgical Department and The Tumor Surgical Department of the First hospital of Xi’an Jiaotong University. All the cases were pathologically proved to be gastric carcinoma. Of the patients, 15 were females and 15 males. Their age ranged from 42 to 70 and the average age was 58.4. Pathologically 2 cases were papillary adenocarcinoma, 12 tubular adenocarcinoma, 13 poorly differentiated adenocarcinoma, and 3 signet ring cell carcinoma. According to Borrmann classification, 6 cases were type I, 8 type II, 8 type III and another 8 type IV.

ERmRNA in situ Hybridization

The slides were treated with 3-amino propyltri-ethoxy saline (APES) and with polyllysine. The slides were deparaffinized, hydrated and treated with 30 mL/L H2O2, at room temperature for 10 minutes to eliminate the endogenous peroxidase. The slides were incubated with freshly diluted protease K (1:1 000 with 0.01 mol/L Tris buffer saline (TBS)) at 37 °C for 5 to 15 minutes. After being washed with distilled water three times, the slides were treated with 2 g/L glycine for 5 minutes, washed with PBS for 5 minutes, fixed with 40 g/L polymethanol for 30 minutes, and washed again with PBS for 5 minutes, dehydrated with gradient alcohol, and then washed with DEPC, treated with digoxin-labeled probe in 90-100 °C water for 5 to 10 minutes, and then taken out and immediately put in shimmered ice for 5 minutes. After the slides became dry in the air, 10 µL in situ hybridization solution containing digoxin-labeled probe was added onto each slide.
and the hybridization was conducted in a humidified box for 20 hours. The slides were then washed twice with 2×SSC at 20-30 °C for 5 minutes and with 1×SSC at 37 °C for 10 minutes, incubated with mouse anti-digoxin at 20-37 °C for 30 minutes and washed with 0.5 mol/L PBS three times, each for 2 minutes. The slides were then incubated with anti-mouse biotin IgG at 20-37 °C for 20 minutes, washed with 0.5 mol/L PBS three times, each for 2 minutes and again incubated with SABC at 20-37 °C for 20 minutes, washed with 0.5 mol/L PBS four times, each for 5 minutes. The color reaction was developed with the addition of DAB, and the slides were counter-stained with hematoxylin and sealed with xylene.

Negative control: No estrogen receptor probe in the hybridization solution. The slices showed color directly without any solution added. Hybridization solution was replaced by reserve hybridization solution containing no probe.

Positive control: The specimens from 3 women with mammary cancer and 3 with ovarian cancer, all under 45 years old, were collected and treated in the same way as in the gastric cancer specimens.

**ER Immunohistochemistry**

Consecutive 5 μm thick sections were stained with HE and by immunohistochemistry separately. The deparaffinized sections were washed with PBS three times, soaked in 30 mL/L hydrogen dioxide solution for 10 minutes to eliminate the endogenous peroxidase, washed with PBS three times, digested with 10 g/L trypsin for 15 minutes (37 °C), washed with PBS three times, heated to 95 °C in pH 6.0 citric acid buffer solution for 10 minutes before cooled down to room temperature, and then washed three times with PBS, and then blocked with serum (45 °C). The sections were then incubated with the first antibody (1:50) over night, washed three times with PBS, incubated with biotin-labeled secondary antibody and then washed with PBS. The sections were finally incubated with streptavidin biotin peroxidase complex, the color reaction was developed with the addition of DAB, and the slides were counter-stained with hematoxylin and sealed transparently.

Positive cells from *in situ* hybridization appeared yellow and the positive stain was mainly located in the nuclei and cytoplasm around the nuclei. Immunohistochemically positive cells appeared brown yellow and the positive stain was mainly located in the nuclei and cytoplasm. The average positive rate in every case was calculated in 5 high-power fields. When 10 % or more of the cancer cells were stained positive in a labeled slice, it was defined as ER or ERmRNA positive.

**Statistical analysis**

All data were analyzed with SPSS 8.0 statistical software (including the accurate four square table probability method and similar χ² test) and *P*<0.05 was considered to have statistical significance.

**RESULTS**

Immunohistochemically stained positive cells looked brown yellow in cytoplasm. The distribution of ER positive cells and the intensity of positive reaction were uneven (Figure 1). The smooth muscle cells and the lymphocytes in the interstice and the mucosa membrane beside the cancer tissue appeared negative. The positively expressed ERmRNA were mainly located in cytoplasm and nuclei of cancer cells, next to the interstice (Figure 2). The number of positive cells was different in different fields. It was greater in some fields, with 34 positive cells in a high power field, but in other fields, the positive cells were scarce or absent. There were weakly hybridized positive signals in interstitial smooth muscle cells and lymphocytes. The tissue beside the cancer appeared negative.

ER positive gastric cancer tissues both in men and women were more common in Borrmann type IV, histologically it was more common in poorly differentiated adenocarcinoma and signet ring cell carcinoma (*P*<0.05). ERmRNA positive cells were found in Borrmann type I, II, III and IV (*P*<0.05). ERmRNA expression was also found in tubular adenocarcinoma, poorly differentiated adenocarcinoma and signet ring cells (*P*<0.05, Table 1).

**Figure 1** ER positive expression in gastric carcinoma tissue SABC>400.

**Figure 2** ER mRNA positive expression in gastric carcinoma tissue *in situ* hybridization ×100.

ER expression had a high positive rate in females above 55 years old and in males under 55 years old (*P*<0.05). And ERmRNA expression had a high positive rate in both males and females above 55 years old (*P*>0.05, Table 1). Diffusely growing gastric cancer had a high ER positive rate (*P*<0.05). Both the diffusely and non-diffusely growing gastric cancers had a high positive expression rate of ERmRNA (*P*<0.05, Table 1). The increase of ER positive rate is associated with the increase of the involved lymph nodes including the upper and lower parts of pylorus, the greater and lesser curvatures of the stomach and the lymph nodes on both sides of cardia (*P*<0.05). There seemed to be a tendency that the increase of ERmRNA positive rate is associated with the increase of the number of the involved lymph nodes (*P*>0.05, Table 1).

To compare the immunohistochemistry results with *in situ* hybridization results, ER positive rate was 40.0 % (M/F: 33.3 % vs 46.7 %), and ERmRNA positive rate was 80.0 % (M/F: 73.3 % vs 86.7 %, *P*<0.05, Table 1).
DISCUSSION

In 1960 Lensen reported for the first time that after injecting a physiological dose of $^3$H-E$_2$ into the hypoderm of a young mouse, the amount of $^3$H-E$_2$ found in the tissues of uterus, vagina and other parts was far greater than that found in blood plasma. This proved for the first time that ER protein was present in the tissues of uterus and vagina. When estrogen enters target cells, it first combines with its receptor in cytoplasm, then forms a compound of receptor protein-estradiol which then enters cell nucleus, binds to the chromatin and affects the transcription of DNA. To sexual and non-sexual target organs, estrogen may be a hormone promoting splitting. There are proved documents that gastric cancer cells have sex hormone receptors and may be controlled and regulated by sex hormones, suggesting that gastric cancer in some cases is hormone-dependent tumor, but the molecular mechanisms underlying carcinogenesis are still largely unknown.[13-17]. A lot of documents have proved that molecular biology plays an important role in the development and metastasis of some cancers, such as endometrial adenocarcinoma,[18-20] lung cancer[21], breast cancer[22,23], apocrine carcinoma[24], leukemia[25] and prostate cancer[26,27]. But there are very few studies on the ERmRNA expression in gastric cancer tissues.

With ER examination on the specimens of ten primary gastric cancer patients (6 men and 4 women), Tokunaga discovered that 2 cases were ER(+) and the patients were women with histological undifferentiated cancer. Yanzuoshaner used the PAP method to analyze 140 specimens of primary gastric cancers, such as endometrial adenocarcinoma, and the results showed that the expression of estrogen receptor-alpha and ER-beta mRNAs in human gastric cancers, and the results showed that the expression of estrogen receptor-alpha and ER-beta mRNAs were higher in female than in male. Thus we can conclude that female gastric cancer patients are more easily affected by estrogen than male gastric cancer patients. Our results are similar to the results reported by other scholars.

Many foreign researchers think that compared with other methods in examining ER protein, the molecular hybridizations in examining ERmRNA has a higher sensitivity[28-31]. By using Northern hybridization method Hankin found that the ERmRNA positive rate of breast cancer was 87.0 %. But by using the $^3$H-estradiol method to examine the ER protein the positive rate was only 46.0 %. The two methods showed a marked difference. In our study, the immunohistochemical examination showed that the high expression of ER protein was most common in poorly differentiated adenocarcinoma and signet ring cell carcinoma. In situ hybridization showed more evidence to support[13]. Takano et al reported the expression of estrogen receptor-alpha and ER-beta mRNAs in human gastric cancers, and the results showed that the expression of estrogen receptor-alpha and ER-beta mRNAs were changed in 20 cases (49 %) and unchanged in 21 cases (51 %). The incidences of lymph node metastasis and liver metastasis were significantly higher in changed cases than in unchanged cases[14]. One fourth gastric cancers were ER positive compared with breast cancers, and gastric cancer nuclear receptors were also smaller than that of breast cancers in number[22,23]. Of 95 cases of male patients with gastric cancers, 12 were ER(+) (12.6 %), of 45 cases of female patients 10 were ER(+) (22.2 %), 11.5 % of men under 50 years old were ER(+) and 14.5 % of men above 50 years old were ER (+). There was no marked difference between them, most of the ER(+) cases were Borrmann II, and most cases of the histological type were undifferentiated[15-16].

Our results of the present study showed that ER positive rate was 40.0 % and ER-mRNA positive rate was 80.0 % in gastric cancers. ER(+) was related to lymph node metastasis and gastric cancer growth patterns. Furthermore, we also discovered that the positive rates of gastric cancer ER, ERmRNA were higher in female than in male. Thus we can conclude that female gastric cancer patients are more easily affected by estrogen than male gastric cancer patients. Our results are similar to the results reported by other scholars.

### Table 1  Relationship between ER, ERmRNA expression and pathology in gastric cancer

| Pathology                  | Male         |          | Female        |          | Total        |          |
|----------------------------|--------------|----------|---------------|----------|--------------|----------|
|                            | n ER(+) %    | ERmRNA(+) % | n ER(+) %    | ERmRNA(+) % | n ER(+) %    | ERmRNA(+) % |
| Borrmann I                 | 3            | 1        | 3.33         | 3        | 3            | 1        |
|                            | 4            | 2        | 25.0         | 3        | 4            | 2        |
| H-E                        | 4            | 2        | 25.0         | 3        | 4            | 2        |
| Diffused                   | 6            | 3        | 50.0         | 4        | 6            | 3        |
| Poorly differentiated      | 7            | 4        | 57.1         | 6        | 7            | 4        |
| Signet ring cell           | 1            | 1        | 100.0        | 1        | 1            | 1        |
| Lymph node 0               | 3            | 0        | 0            | 0        | 3            | 0        |
| Nondiffused                | 9            | 2        | 22.2         | 6        | 9            | 2        |
| Involvement ≤ 3            | 4            | 1        | 25.0         | 3        | 4            | 1        |
| Age ≤ 55                   | 6            | 3        | 50.0         | 4        | 6            | 3        |
| >6                         | 2            | 2        | 100.0        | 2        | 2            | 2        |
| Total                      | 15           | 5        | 33.3         | 11       | 7            | 15       |

### Notes

- Borrmann I: 3 cases, 2 ER(+) (66.7 %), 6 ERmRNA(+) (100.0 %)
- Borrmann II: 4 cases, 8 ER(+) (83.3 %), 4 ERmRNA(+) (100.0 %)
- Borrmann III: 3 cases, 3 ER(+) (100.0 %), 3 ERmRNA(+) (100.0 %)
- Borrmann IV: 8 cases, 8 ER(+) (100.0 %), 8 ERmRNA(+) (100.0 %)
- Overall: 15 cases, 12 ER(+) (80.0 %), 15 ERmRNA(+) (100.0 %)
that ERmRNA had a high positive expression rate, which was also found in tubular adenocarcinoma and poorly differentiated adenocarcinoma of histological hype. What is more noteworthy is that in 13 cases of ER(−) gastric cancer tissues in situ hybridization examination showed that ERmRNA was (+). The reason may be that in situ hybridization probe could hybridize with mRNA which directs the synthesis of protein of irregular quality lacking function. The protein may lack the epitope that can be identified by the ER monoclonal antibody, and there may be defects present in ER protein synthesis after transcription. ERmRNA positive signal was also present in interstitial smooth muscle cells and lymphocytes, which suggests that estrogen can regulate not only epithelial cells but also interstitial cells.

We speculate that ERmRNA expression has greater value than ER protein expression in clinical application because of the high sensitivity of in situ hybridization and the strong ERmRNA expression in gastric cancer, which can be used to judge the prognosis of tumor and predict the effectiveness of endocrine therapy for gastric cancer.

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