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Original Article

Expression of G protein-coupled estrogen receptor in gastric cancer tissues

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Abstract: Objectives: Estrogen possesses important functions in gastric carcinogenesis. However, the expression of G protein-coupled estrogen receptor (GPR30) in gastric cancer (GC) has never been investigated. This study aimed to compare the expression of GPR30 and estrogen nuclear receptors in GC tissues, matched tumor-adjacent tissues and normal gastric mucosa. Methods: Gastric mucosal biopsies were obtained by endoscopy from patients with GC and functional dyspepsia. Expression of GPR30, estrogen receptor α (ERα) and estrogen receptor β (ERβ) was measured by immunohistochemistry (IHC) and real-time polymerase chain reaction. Results: GPR30 was detected in GC and noncancerous tissues. IHC showed a significantly higher GPR30 expression in GC tissues (29 of 38, 76.3%) compared with matched adjacent tissues (11 of 38, 28.9%) and normal gastric tissues (5 of 21, 23.8%). Overexpression of GPR30 was observed in intestinal type GC rather than in diffuse type (88.5% versus 54.5%, $P = 0.047$). The mRNA level of GPR30 was also higher in GC tissue than in adjacent ($P = 0.001$) and normal tissues ($P = 0.047$). No significant differences in the expression of ERα and ERβ were found among the three groups. Conclusions: This study is the first to examine the expression of GPR30 in normal gastric mucosa and cancer tissue and demonstrate that GPR30 expression was increased in patients with intestinal type GC. Our findings may provide information on the significance of gender difference in GC patients.

Keywords: GPR30, estrogen, estrogen receptor, gastric cancer

Introduction

The incidence and mortality rates of gastric cancer (GC) have sharply declined, but GC remains the fourth most common cancer and second leading cause of cancer death worldwide [1, 2]. In China, the incidence of GC ranks third among all malignant tumors, and the mortality rate of GC remains high [3]. Given these reasons, new prevention strategies are necessary. The effect of gender difference on the risk of developing GC has been investigated in several studies, and the incidence of GC is significantly lower in females than in males [4, 5]. A recent meta-analysis showed that longer exposure to estrogen of either ovarian or exogenous origin may decrease the risk of GC [6]. Estrogen and other sex hormones are speculated to be involved in GC carcinogenesis.

17-β estradiol is the most common endogenous estrogen that mediates multiple effects throughout the human body and regulates a wide variety physiological processes in both women and men [7]. These classic actions of estrogen are achieved by binding two nuclear estrogen receptors, namely, estrogen receptor α (ERα) and estrogen receptor β (ERβ), which commonly function as ligand-activated transcription factors to mediate genomic effects [8]. However, estrogen also mediates rapid transmembrane signaling pathways [9], which are independent of ERα and ERβ.

G protein-coupled estrogen receptor (GPR30) is a novel seven-transmembrane receptor that binds estrogen with high affinity and activates rapid intracellular response. GPR30 is widely expressed in the reproductive system, heart, nerve, lung, liver, kidney and bone marrow. GPR30 is also expressed in a number of cancer tissues [10, 11]. Previous studies demonstrated that high expression of GPR30 in malignant breast and ovarian tumors was associated with disease progression and poor survival [12, 13].

Table 1. Primer sequences for real-time PCR

Gene	Primer	Primer sequence (5'-3')	PCR product (bp)	Sequence accession no.
GPR30	Forward	CAACCTCGCCGCCTTCTC	172	NM_001505.2
	Reverse	CTGCTCGGTGCTGTCTGG		
ERα	Forward	CCTGATGATTGGTCTCGTCTG	185	NM_000125.3
	Reverse	GGCACACAAACTCCTCTCC		
ERβ	Forward	ATACCTTCCTCCTATGTAGAC	197	NM_001437.2
	Reverse	TGTGATAACTGGCGATGG		
β-actin	Forward	ACTTAGTTGCGTTACACCCTT	156	NM_001101
	Reverse	GTCACCTTCACCGTTCCA		

GPR30 has never been assessed in tissues of human stomach and GC. Therefore, this study evaluated the expression of GPR30 and classic estrogen receptors in normal gastric mucosa, GC tissues and matched tumor-adjacent tissues.

Materials and methods

Subjects and samples

This study was approved by the Research Ethics Committee of The Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, China. Written informed consent was obtained from all subjects. Specimens of tumor and matched adjacent tissues were obtained from patients with GC. Adjacent tissues were collected at least 5 cm away from the tumor location. Normal tissue specimens were obtained from the gastric antrum of patients with functional dyspepsia (FD). Patients were clinically diagnosed at the Department of Gastroenterology by two experienced physicians from March 2012 to November 2013. The histological diagnosis and tumor classification were given at the Department of Pathology by experienced pathologists according to the Lauren criteria. Tumor specimens were used only when the pathological diagnosis of gastric adenocarcinoma was confirmed. All specimens were collected by upper gastric endoscopy, immediately frozen in liquid nitrogen and stored at -70°C until use. Rapid urease test or breath test was used to detect *Helicobacter pylori* infection, which was defined as a positive result from any of the two methods.

Immunohistochemistry (IHC)

Tissues were fixed in 10% formalin, embedded in paraffin and cut into 4 μm thickness. Immunostaining of GPR30, ERα and ERβ were carried out following the procedure previously described [14]. In brief, tissue sections were dewaxed and hydrated using xylenes, graded ethanols, water and PBS. Antigen retrieval was performed by heating the sections in citrate buffer for 8 min. To block nonspecific binding sites, we incubated the sections with 10% goat serum for 30 min in a humidified chamber. The sections were then incubated overnight with antibodies against GPR30 (rabbit polyclonal antibody; Abcam, Hong Kong; 1:200), ERα (Santa Cruz Biotechnology, USA; 1:100) and ERβ (Santa Cruz Biotechnology, USA; 1:100). The sections were washed three times with PBS for 5 min, blocked for endogenous peroxidase activity with 3% hydrogen peroxide, incubated with goat anti-rabbit antibodies (ZSGB Biotechnology, Beijing, China), and washed again with PBS. The sections were then dyed with diaminobenzidine and counterstained with hematoxylin. The negative controls were incubated with PBS instead of primary antibody. The immunostained sections were viewed under an Olympus DP20 microscope (Olympus, Japan), and the number of positive cells was quantified at 200 × magnification. Positive status for GPR30, ERα and ERβ was defined as having cytoplasmic or nuclear staining in at least 10% of cells.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the frozen tissue using TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. The extracted total RNA was then stored at 4°C for future analysis. The amount of RNA was estimated by UV spectrophotometry (NanoDrop Technologies, USA) at 260 nm. A sample of total RNA (2 μg) was used to synthesize cDNA using a PrimeScript RT Master Mix (TaKaRa, Japan).

Quantitative real-time PCR was performed with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using SYBR® Premix Ex Taq™ (TaKaRa, Japan) in accordance with the manufacturer's protocol. **Table 1**

Table 2. Immunostaining results of GPR30, ERα and ERβ

Subjects	Total number	GPR30			ERα			ERβ		
		+	-	*P*	+	-	*P*	+	-	*P*
Cancer Tissue	38	29	9		10	28	0.820**	15	23	0.054**
Adjacent Tissue	38	11	27	< 0.05*	11	27		25	13	
Normal Tissue	21	5	16	< 0.05*	7	14		13	8	

*Compared with GC tissue by Mann-Whitney test; **Compared among three groups by Kruskal-Wallis test.

shows the primers used in the present study. In brief, 25 μl of PCR reactions was carried out comprising 12.5 μl of SYBR green, 1 μl of PCR forward, 1 μl of PCR reverse primers, 2 μl of cDNA and 8.5 μl of dd H_2O. The amplification protocol was 3 min at 95 C, followed by denaturation at 94°C for 30 s, annealing at 55 C or 56°C for 30 s and extension at 72°C for 30 s for a total of 45 amplification cycles. The amplification specificity was confirmed by melting curve analysis. β-actin was used as an endogenous internal standard control. All experiments were repeated twice, and relative mRNA quantities of GPR30, ERα and ERβ were normalized to β-actin expression.

Statistical analysis

All data were expressed as mean ± SD and analyzed using SPSS 16.0 software (SPSS Inc., Chicago). The comparison of IHC staining results and differences within groups were performed using Kruskal-Wallis and Mann-Whitney U tests as appropriate. One-way ANOVA and Dunnett t test were conducted to compare the mRNA expression level among the three groups and assess the differences within groups, respectively. *P* value less than 0.05 was considered statistically significant.

Results

Clinicopathological data of subjects

This study comprised 38 patients (male 27, female 11) with GC and 21 patients (male 15, female 6) with FD (**Table 2**). GC patients aged 36 years to 76 years (median 55). Of the 38 GC patients, 24 were positive for *H. pylori* infection. GC patients were subdivided according to the Lauren classification: intestinal type (26), diffuse type (11) and mixed type (1). Nine FD patients aged 32 years to 65 years (median 47) were positive for *H. pylori* infection.

Immunohistochemical results of GPR30

Table 2 shows that both cancer and noncancerous gastric mucosa showed immunoreactive staining for GPR30. In gastric biopsies, GPR30 was localized in the cytoplasm of cells found in both the epithelium and lamina propria (**Figure 1**). Positive staining was detected in 29 of 38 (76.3%) GC tissues, 11 of 38 (28.9%) matched adjacent tissues and 5 of 21 (23.8%) normal gastric tissues. Compared with noncancerous tissues, GC tissues showed a significantly higher expression of GPR30 than matched adjacent tissues ($P < 0.05$) and normal gastric tissues ($P < 0.05$). No differences were found between tumor adjacent tissues and normal tissues ($P = 0.673$).

Association of GPR30 expression with clinicopathological parameters

We examined the association of GPR30 staining with the clinicopathological parameters in GC tissues (**Table 3**). Statistical analysis showed that the percentage of positive GPR30 staining in GC tissues was 78.2% (18 of 23) in older patients and 73.3% (11 of 15) in younger patients (P = 0.730). In GC patients, the percentage of positive GPR30 staining in GC tissues was 72.7% (8 of 11) in females and 77.8% (21 of 27) in males ($P = 0.743$). The percentage of positive GPR30 staining in GC tissues was 79.1% (19 of 24) in *H. pylori* positive patients and 71.4% (10 of 14) in *H. pylori* negative patients; the difference was not statistically significant ($P = 0.593$). However, the percentage of positive GPR30 staining was significantly higher ($P = 0.047$) in intestinal type GC (88.5%, 23 of 26) than in diffuse type GC (54.5%, 6 of 11).

Immunohistochemical results of ERα and ERβ

In immunohistochemical staining (**Figure 2**), ERα was positive in 10 of 38 (26.3%) tumor tissues, 12 of 38 (31.5%) matched adjacent tissues and 7 of 21 (33.3%) normal gastric tissues. The percentage of positive staining for ERβ was 39.5% (15 of 38) in tumor tissues, 65.8% (25 of 38) in adjacent tissues and 61.9% (13 of 21) in normal tissues. ERα expression

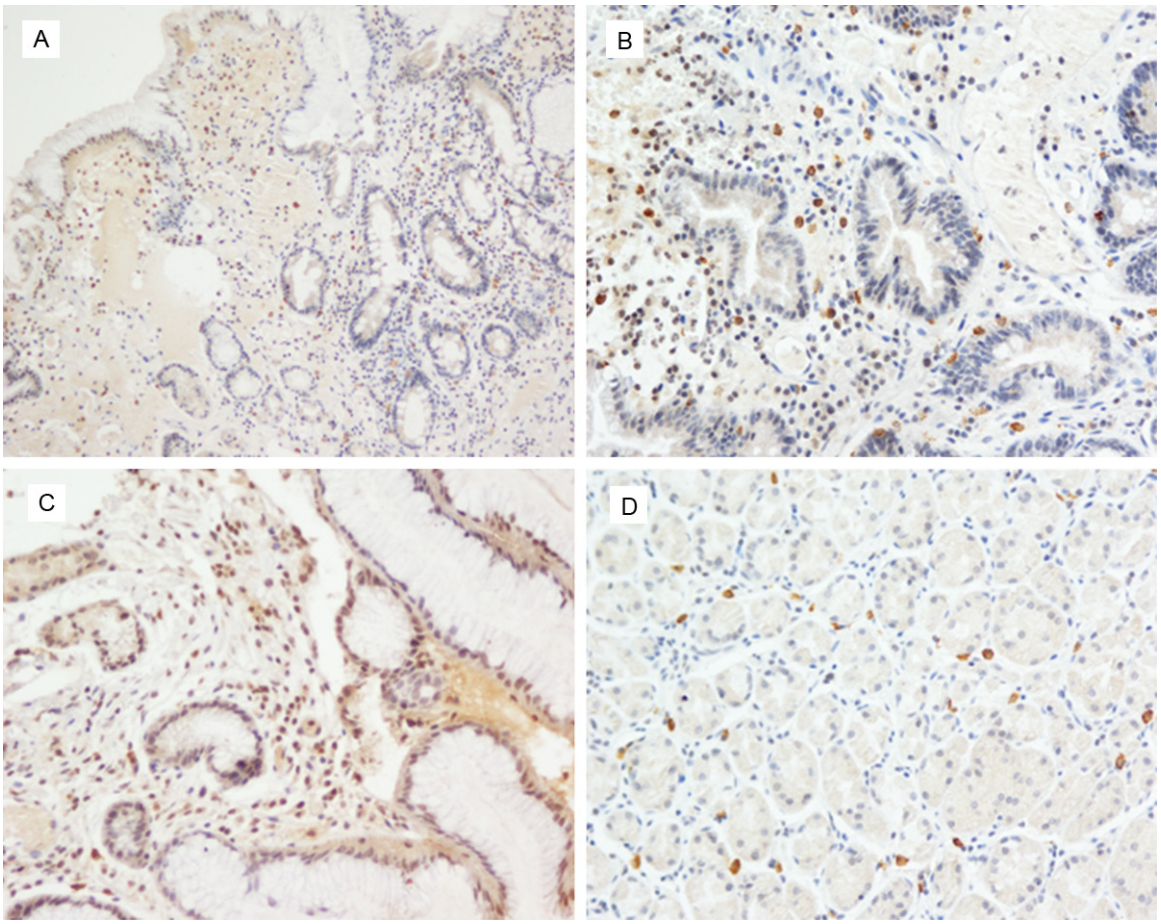

Figure 1. Representative immunostaining of GPR30. Positive staining of GPR30 in GC tissues was visualized under 200 × (A) and 400 × (B) magnification. Positive staining of GPR30 was shown in adjacent (C) and normal gastric (D) tissues under 400 × magnification.

Table 3. Association of GPR30 expression with clinicopathological factors in GC patients

Factors	Number	GPR30 in GC patients		
		Positive	Negative	*P*
Age (years)				
< 50	15	11	4	0.730
≥ 50	23	18	5	
Gender				
Male	27	21	6	0.743
Female	11	8	3	
H. pylori				
Positive	24	19	5	0.593
Negative	14	10	4	
Subtypes				
Intestinal type	26	23	3	0.047*
Diffuse type	11	6	5	

*Difference of GPR30 expression in intestinal type GC compared with diffuse type GC.

showed no significant difference among the three groups (P = 0.820). ERβ expression was lower in cancer tissues than in noncancerous tissues, but the difference was not statistically significant (*P* = 0.054) (**Table 2**). Interestingly, GPR30 was positively stained in 13 biopsies (72.2%) of 18 cancer tissues with negative staining for both ERα and ERβ.

Relative mRNA expression of GPR30, ERα and ERβ

We detected the mRNAs of GPR30, ERα and ERβ in all GC tissues, matched adjacent tissues and normal gastric tissues by real-time PCR. **Figure 3** shows that the relative mRNA level of GPR30 in GC tissues was 0.722 (95% CI 0.621-0.844). Compared with GC tissues, the relative mRNA levels of GPR30 in noncancerous tissues were significantly lower in tumor adjacent

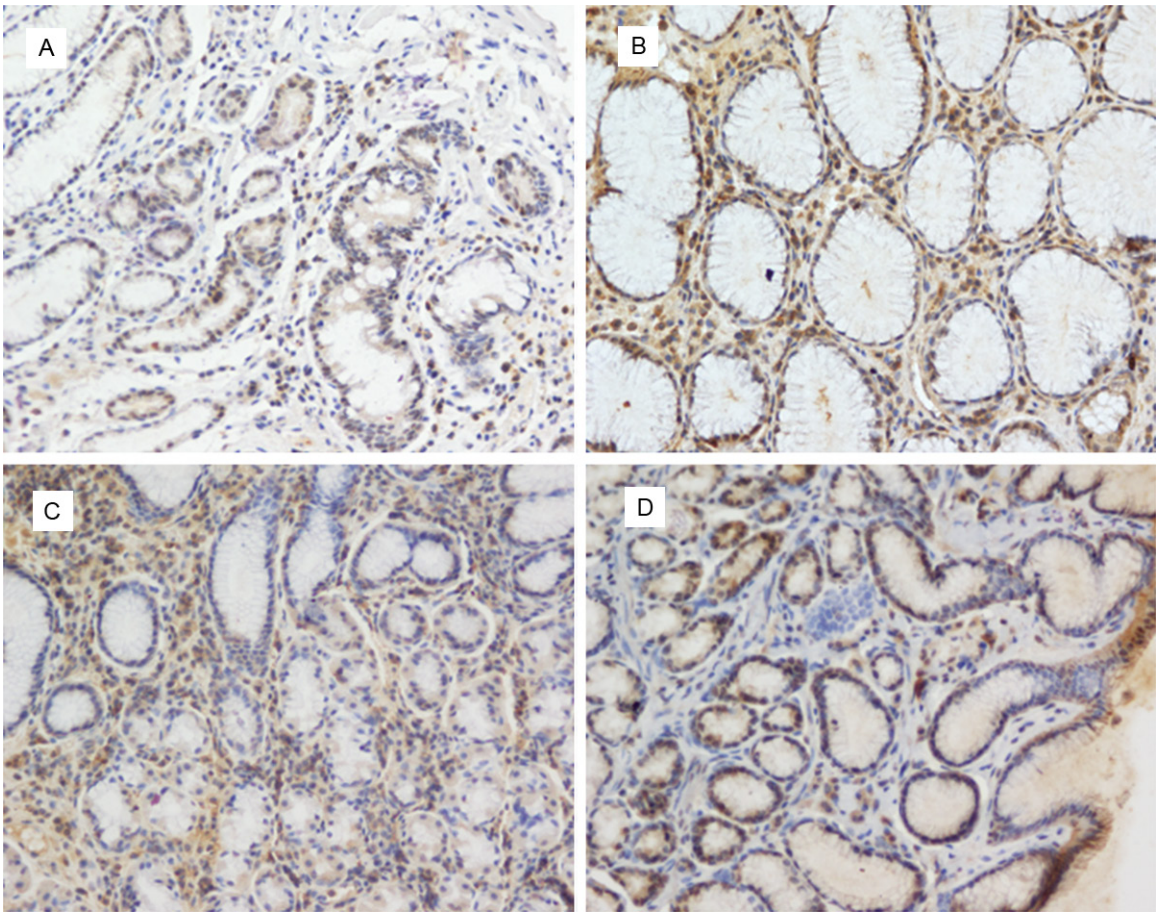

Figure 2. Representative immunostaining of ERα and ERβ. ERα (A) and ERβ (C) were detected in GC tissues. ERα (B) and ERβ (D) were detected in normal gastric tissues under 400 × magnification.

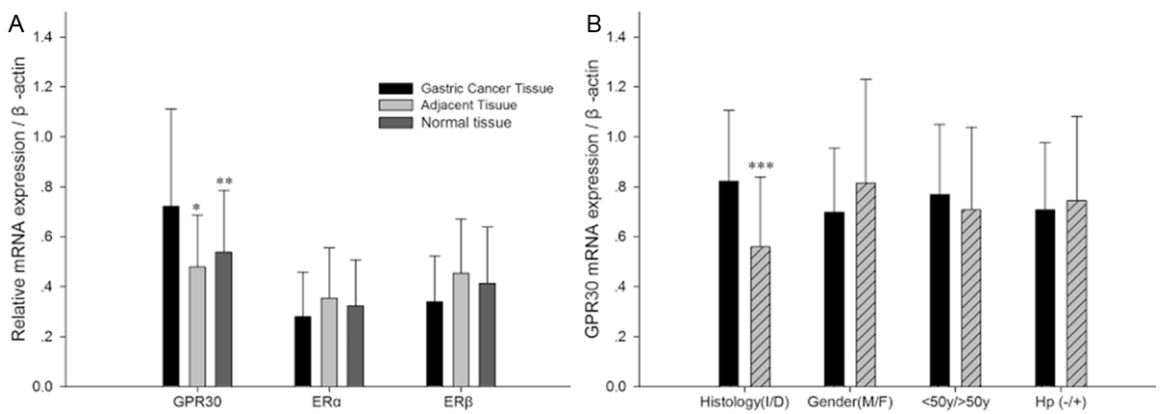

Figure 3. Relative mRNA expression of GPR30, ERα and ERβ. A: The mRNA level of GPR30 in GC tissue was significantly higher than that of matched adjacent tissue (*P = 0.001) and normal GC (**P = 0.047). No differences in mRNA levels were found for ERα and ERβ among the three groups. B: Relative mRNA expression of GPR30 in GC patients. GPR30 expression was increased in GC patients with intestinal type (I) than in diffuse type (D). No differences were found among gender (M/F = male/female), age (< 50 y/> 50 y = younger than 50 years old/older than 50 years old) and *H. pylori* infection (−/+ = negative/positive).

tissues (0.497; 95% CI 0.428-0.565, P = 0.001) and normal gastric tissues (0.539; 95% CI 0.427-0.651, P = 0.047). We also found that the mRNA levels of GPR30 were significantly

higher in intestinal type GC than in diffuse type GC ($P = 0.014$). The mRNA expression of GPR30 was not associated with age, gender and *H. pylori* infection. Compared with noncancerous tissues, no differences in mRNA levels were observed in cancer tissues that were positive for ERα ($P = 0.247$) or ERβ ($P = 0.055$).

Discussion

The male predominance of GC remains an unexplained phenomenon. The onset of intestinal type GC in females is about 10 years to 15 years delayed compared with males [5]. Sex hormones, particularly estrogen, is suggested to be a protective factor against GC, and estrogen receptors located in the stomach may be involved in carcinogenesis [4]. This study demonstrated that GPR30 was abundantly expressed in human gastric mucosa and overexpressed in GC tissues compared with adjacent tissues and normal gastric tissues.

GPR30, a G protein-coupled membrane receptor binding with estrogen, was first described in 1990s [15, 16] and detected in the reproductive system, lung, heart and nervous tissues [17]. In several studies, GPR30 expression is changed in the malignant tumors of the reproductive system [12, 18]. Although the function of GPR30 in cancer growth is unclear, GPR30 expression correlates with clinical characteristics, metastasis and poor outcome [19]. A recent study found that GPR30 is overexpressed in lung tumors and responsible for some of the proliferation signals induced by estrogen [20]. GPR30 expression in human stomach and its relationship with gastric carcinogenesis have never been investigated. IHC and real-time PCR showed that GPR30 was expressed in tumor tissues of GC and the normal gastric mucosa. GPR30 was also overexpressed in GC tissues compared with noncancerous tissues. The function of GPR30 in the gastric mucosa is unclear, but our results suggested that the effects of estrogen on gastric carcinogenesis may be correlated with changes of GPR30 expression in the stomach.

We analyzed the relationship between the expression of GPR30 and clinical characteristics of the patients. GPR30 expression was not significantly associated with gender, age or status of *H. pylori* infection in GC tissue. However, a significantly higher GPR30 expression was observed in the tissues of intestinal type GC than that of diffuse type GC. Therefore, GPR30 could be a biomarker for intestinal type GC. We supposed that the GPR30-mediated effects of estrogen may be correlated with low risk of women to develop intestinal type GC. Further investigation of GPR30 as a potential target for estrogen and GC prevention is necessary.

IHC and real-time PCR detected the expression of the traditional estrogen nuclear receptors (ERα and ERβ) in the three groups of GC tissue, adjacent tissue and normal mucosa, but the expression was not significant. Several studies have examined the expression of traditional estrogen receptors in GC tissue, but the expression pattern of ERα and ERβ in GC remains unclear [21, 22] because neither ERα nor ERβ was proved to be the target of estrogen acting on GC cells [23]. Recent studies [24, 25] showed that high endogenous estrogen exposure reduces the risk of the intestinal type gastric adenocarcinoma in women. However, the present study showed no differences in ERα and ERβ expression between cancer and noncancerous tissues. No sufficient evidence was found to confirm that traditional estrogen receptors were mediators of the protective effects from estrogen exposure. GPR30 was detected in tissues that were negatively stained with both ERα and ERβ using IHC, suggesting that GPR30 may function independently as a target of estrogen in GC. Our findings may support a new direction for understanding the function of estrogen in gastric carcinogenesis.

In summary, the expression of the estrogen receptor GPR30 was higher in patients with intestinal type than that with diffuse type GC. Therefore, GPR30 may be a potential biomarker for GC patients with intestinal type GC. However, further investigations are needed to verify our findings.

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Disclosure of conflict of interest

None.

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