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Original Article

Expression of G protein-coupled estrogen receptor 30 (GPR30) and extracellular signal-regulated kinase 1/2 (p-ERK1/2) in the endometrioid endometrial cancer and their correlation

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Abstract: Purpose: This study aimed to explore the expressions of G protein-coupled estrogen receptor 30 (GPR30) and extracellular signal-regulated kinase 1/2 (p-ERK1/2) in the endometrioid endometrial cancer (EEC) and evaluate their correlation. Methods: Expressions of GPR30 and p-ERK1/2 were measured in 22 cases of EEC, 20 cases of atypical hyperplasia endometrium (EAH) and 20 cases of normal endometrium. Results: The positive rate of GPR30 expression in EEC was significantly higher than in normal endometrium and EAH ($P<0.05$), and significant difference was also observed between normal endometrium and EAH ($P<0.05$). Significant difference in GPR30 expression was also found between EECs with different depths of myometrial invasion: the deeper the myometrial invasion, the higher the positive rate was ($P<0.05$). GPR30 expression had no relationship with other clinicopathological characteristics ($P>0.05$). The positive rate of p-ERK1/2 expression in EAH was significantly higher than in normal endometrium, but it in EEC was comparable to that in normal endometrium and EAH ($P>0.05$). p-ERK1/2 expression was not related to clinicopathological characteristics ($P>0.05$). The positive rate of GPR30 and p-ERK1/2 co-expression was 5.0% in normal endometrium, and there was no relationship between GPR30 expression and p-ERK1/2 expression ($P>0.05$). The positive rate of GPR30 and p-ERK1/2 co-expression was 35.0% and 45.6% in EAH and EEC, respectively, and positive relationship was observed between GPR30 expression and p-ERK1/2 expression in both groups ($P<0.05$). Conclusion: GPR30 may exert its effects via regulating p-ERK1/2 signaling pathway in EEC.

Keywords: Endometrioid endometrial cancer, G protein-coupled estrogen receptor, extracellular signal regulated kinase1/2, immunohistochemistry

Introduction

Endometrial cancer (EC) is a common malignancy in women [1]. About 72% of EC patients are diagnosed at stage I-II, but 28% of EC patients are diagnosed when regional or distal metastasis has been present (about 20% at stage III and about 8% at stage IV) [2]. Of different types of EC, endometrioid endometrial cancer (EEC) is a major one and accounts for 80-90%. Long term estrogen stimulation in the absence of antagonism of progesterone is a major cause of EEC. Recent studies identify a 7-transmembrane G protein-coupled estrogen receptor (GPR30) in human breast cancer cells. It can rapidly bind to trace estrogen to activate intracellular second messengers and mitogen-activated protein kinases (MAPK), the rate of which is dozens of times rapider than the transduction through classic ERα [3]. Thus, this belongs to the rapid non-genomic transduction [4]. Estrogen may rapidly bind to GRP30 to activate transmembrane signal transduction and exert its non-nuclear effect. Extracellular signal-regulated kinase (ERK) is a subfamily of MAPK. Excess activation of ERK is closely related to the tumorogenesis [5]. ERK1 and ERK2 are two important members of ERK family and

involved in several intracellular processes including proliferation, differentiation and metabolism. ERK1/2 can be activated by several mitogens including growth factors, to promote the transcription and expression of oncogenes and genes related to the regulation of cell cycle, leading to the proliferation of cancer cells and the inhibition of their apoptosis [6]. Phosphorylated ERK (p-ERK) is the activated form of ERK. After entering the cells, p-ERK1/2 may initiate the expression of some oncogenes such as c-jun, c-fos and c-myc, which then promotes cells to progress from G1 phase to S phase, resulting in malignant proliferation. Acconcia et al [7] used estrogen and tamoxifen (TAM) to induce the cytoskeleton remodeling and migration of EC cells and found E2 and 4-OHT could rapidly activate ERK1/2, C-Srk and FAK signaling pathway to alter the cytoskeleton of EC cells and promote their migration and invasion. The binding of estrogen to GPR30 may activate ERK via Src, Raf, Ras and Mek, leading to the cell proliferation and prolongation of cell cycle. Filardo et al [8] found that, in GPR30 positive MCF-7 breast cancer cells, estrogen could activate ERK, but estrogen failed to activate ERK in MDA-MB-231 breast cancer cells negative for GRP30. After transfection with GRP30 in MDA-MB-231 breast cancer cells, estrogen was able to activate ERK. These findings suggest that GPR30 plays an important role in the ERK/MAPK signaling pathway.

In the present study, immunohistochemistry was performed to detect the GPR30 and activated p-ERK1/2 in normal endometrium, atypical hyperplasia endometrium (EAH) and endometrioid endometrial cancer (EEC), analyze the correlation between GPR30 and p-ERK1/2 and explore the role of GPR30 in the pathogenesis of EEC and the non-nuclear regulation of estrogen, which may provide theoretical evidence for the clinical therapy of EEC.

Materials and methods

Clinical characteristics

Patients (n=22) who received surgery due to ECC in the Dongfang Hospital of Tongji University between January 2009 and February 2012 were included. The median age was 55.0 years (range: 46-68 years). ECC was staged according to the FIGO staging system. G1 EEC was found in 5 patients, G2 EEC in 12, and G3 EEC in 5. Patients (n=20) with EAH were included. The median age was 47.5 years (range: 40-56 years). In control group, normal endometrium was collected from 20 patients who received surgery due to benign uterine diseases. The median age was 48.5 years (range: 40-61 years). Chemotherapy, radiotherapy and hormone therapy were not performed in these patients before study. The age was comparable among three groups.

Detection of GPR30 and p-ERK1/2 in the endometrium

SP method was used for immunohistochemistry for GPR30 and p-ERK1/2 according to manufacturer's instructions. Antigen retrieval was conducted with a microwave oven. There were positive control group (known positive) and negative control group (primary antibody replaced with PBS). Rabbit anti-human GPR30 polyclonal antibody (ab98075; Abcam, USA; 1:50), rabbit anti-human p-ERK1/2 polyclonal antibody (BS4621; Bioworld, USA; 1:50), HRP conjugated goat anti-rabbit secondary antibody (111-035-003; Jackson, USA), immunohistochemistry kit (SP9001) and DAB detection kit (Beijing Zhongshan GoldenBridge Biotech Co., Ltd) were used in the present study.

Determination of protein expression

Under a light microscope, p-ERK1/2 was mainly found in the cytoplasm and less found in the nucleus. p-ERK1/2 positive cells had yellow-brown or brown granules. Protein expression was determined in a blind manner. Six fields were randomly selected from each section at a magnification of ×400, and the proportion of positive cells was determined. The protein expression was determined according to previously reported [9] on the basis of staining intensity and proportion of positive cells: (1) staining intensity: 0, no positive cells; 1, weakly positive (+); 2, moderately positive (++); 3, strong positive (+++); (2) proportion of positive cells: At least 100 cells were counted in 6 fields rich in cancer cells: 1, <1%; 2, 1-10%; 3, 11-33%; 4, 34-66%; 5, 67-100%. The sum of above scores was used as a final score. The final score of 5-8

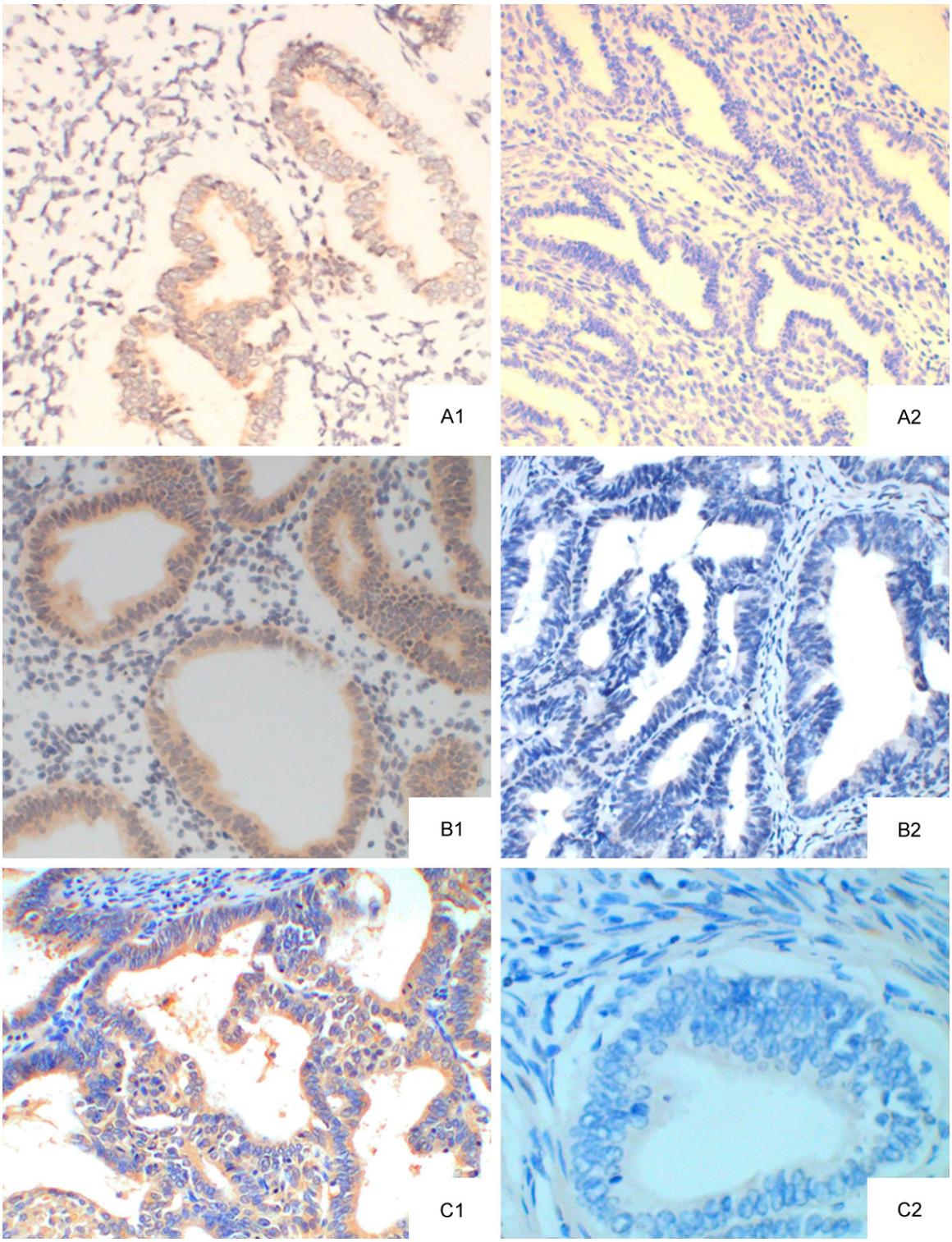

Figure 1. GPR30 protein expression (SP method; ×20). A: normal endometrium; A1: GPR30 positive; A2: GPR30 negative; B: EAH; B1: GPR30 positive; B2: GPR30 negative; C: EEC; C1: GPR30 positive; C2: GPR30 negative

suggests positive. Scoring was conducted by 2 pathologists blind to this study, and consensus was obtained after consultation if there was discrepancy.

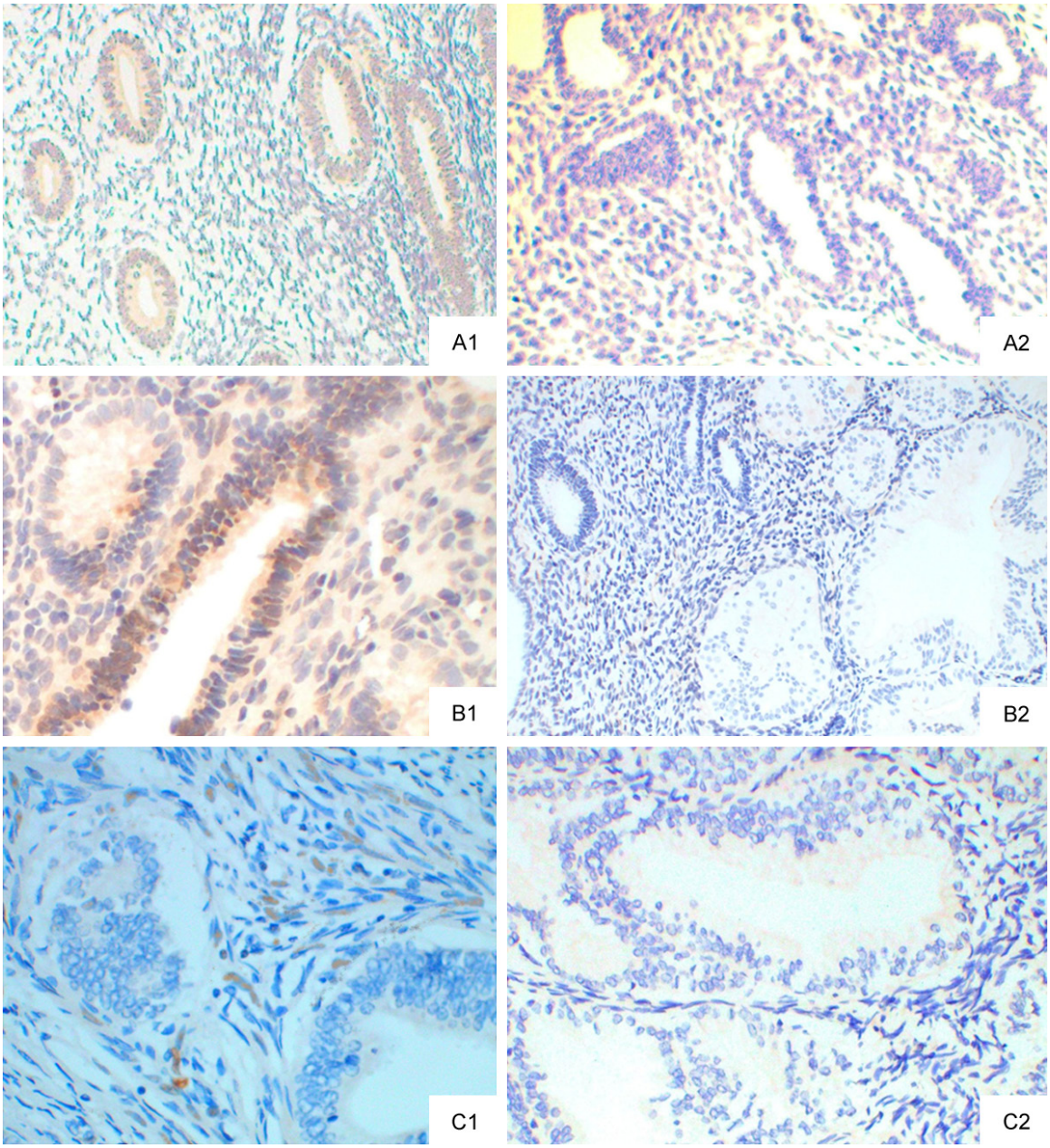

Figure 2. p-ERK1/2 protein expression (SP method; ×20). A: normal endometrium; A1: p-ERK1/2 positive; A2: p-ERK1/2 negative; B: EAH; B1: p-ERK1/2 positive; B2: p-ERK1/2 negative; C: EEC; C1: p-ERK1/2 positive; C2: p-ERK1/2 negative.

Statistical analysis

Statistical analysis was conducted with SPSS version 17.0. Qualitative data are expressed as rate and compared with chi square test. Correlation analysis was performed with Spearman rank correlation analysis with r=0 as a criterion for correlation. A value of $P<0.05$ was considered statistically significant.

Results

GPR30 and p-ERK1/2 expression in the endometrium

GPR30 expression mainly localizes in the cytoplasm. Most G protein-coupled receptors are transmembrane receptors, but a variety of studies propose that GPR30 mainly localizes in

Table 1. GPR30 and p-ERK1/2 expression in the endometrium of different groups (n %)

Group	n	GPR30			p-ERK1/2		
		Positive (n %)	Negative (n %)	P	Positive (n %)	Negative (n %)	P
Normal	20	3 (15)	17 (85)	0.04[a]	6 (30)	14 (70)	0.03[a]
EAH	20	8 (40)	12 (60)	0.036[b]	13 (65)	7 (35)	0.207
EEC	22	16 (72.7)	6 (27.3)	0.001[c]	10 (45.5)	12 (54.5)	0.306

Note: a. P<0.05 vs. EAH group; b. P<0.05 vs. EEC group; c. P<0.05 vs. normal control group.

the endoplasmic reticulum or Golgi bodies [10]. In EEC, GPR30 expression was mainly found in the cytoplasm and cell membrane of EEC cells, cells strong positive for GPR30 were brown, and a lot of positive cells were found in EEC. In EAH, GPR 30 expression was mainly found in the cytoplasm and membrane (predominantly in cytoplasm), and moderately positive was found. In normal endometrium, weak GPR30 expression was found, cells were mainly light yellow, and GPR30 expression was predominantly found in the cytoplasm (**Figure 1**). The positive rate of GPR30 expression was 15.0% (3/20), 40% (8/20) and 72.7% (16/22) in normal endometrium, EAH and EEC, respectively. The positive rate of GPR30 expression in EEC was significantly higher than in other groups (P<0.05). The positive rate of GPR30 expression in EAH group also increased markedly as compared to control group (P<0.05).

In normal endometrium, weak p-ERK1/2 expression was noted, cells were light yellow, and p-ERK1/2 expression was mainly found in the cytoplasm. In EAH, p-ERK1/2 expression increased significantly, cells were yellow-brown or brown, and p-ERK1/2 expression was found in the cytoplasm and nucleus. In EEC, p-ERK1/2 expression was mainly found in the nucleus and cytoplasm (predominantly in nucleus), and positive cells were yellow-brown and showed spotty distribution. In normal endometrium, p-ERK1/2 expression was not observed in the cytoplasm and nucleus (**Figure 2**). The positive rate of p-ERK1/2 expression was 30% (6/20), 65% (13/20) and 45.5% (10/22) in normal endometrium, EAH and EEC, respectively. In normal endometrium, low p-ERK1/2 expression was found although there was still p-ERK1/2 expression. In EAH group, p-ERK1/2 expression increased significantly as compared to normal endometrium, (P<0.05). In EEC group, the p-ERK1/2 expression was moderate and comparable to that in other groups (P>0.05) (**Table 1**).

Correlation of GPR30 and p-ERK1/2 expression with clinicopathological characteristics of EEC patients

Results showed the GPR30 expression was significantly different in EEC patients with different depths of myometrial invasion: the deeper the myometrial invasion, the higher the positive rate of GPR30 expression was (Spearman correlation analysis; P=0.003). However, GPR30 expression had no relationship with other clinicopathological characteristics. (P>0.05). In addition, p-ERK1/2 had no correlation with any clinicopathological characteristic (**Table 2**).

Correlation between GPR30 expression and p-ERK1/2 expression

In normal endometrium, the proportion of GPR30 and p-ERK1/2 co-expression was 5.0% (1/20), and there was no correlation between GPR30 expression and p-ERK1/2 expression (r_s=0.031, P>0.05). In EAH, the proportion of GPR30 and p-ERK1/2 co-expression was 35.0% (7/20), and positive relationship was found between GPR30 expression and p-ERK1/2 expression (r_s=0.599, P<0.05). In EEC group, the proportion of GPR30 and p-ERK1/2 co-expression was 45.6% (10/22), and positive relationship was noted between GPR30 expression and p-ERK1/2 expression (r_s=0.559, P<0.05) (**Table 3**).

Discussion

GPR30 was independently identified and cloned in 1990s by different groups. GPR30 gene is mapped to 7p22 and its full length mRNA is 2604 bp. The full length mRNA includes a long open reading frame of 1128 bp which encodes GPR30 protein of 375 amino acids. Studies have revealed that GPR30 has a high homology with G Protein-Coupled Receptors (GPCRs) [11, 12]. GPR30 is a functional membrane receptor of estrogen [13, 14]. GPR30 mediates the non-genomic effects of estrogen which are involved in a variety of physiological and pathological processes in cells.

Table 2. Correlation of GPR30/p-ERK1/2 expression with clinicopathological characteristics of EEC patients

Clinicopathological characteristics	GPR30			p-ERK1/2		
	Positive (n %)	Negative (n %)	P	Positive (n %)	Negative (n %)	P
Age (yr)			0.432			0.122
≤55	10 (71.4)	4 (28.6)		5 (62.5)	3 (37.5)	
>55	6 (75)	2 (25)		5 (35.7)	9 (64.3)	
Menstruation			0.220			0.122
Premenopausal	5 (62.5)	3 (37.5)		9 (75)	3 (25)	
Menopausal	11 (78.6)	3 (21.4)		5 (50)	5 (50)	
FIGO stage			0.068			0.149
I	6 (60)	4 (40)		4 (40)	6 (60)	
II	5 (71.4)	2 (28.6)		2 (28.6)	5 (71.4)	
III	5 (100)	0 (0)		4 (80)	1 (20)	
Histological grade			0.500			0.500
G1	4 (80)	1 (20)		2 (40)	3 (60)	
G2	8 (66.7)	4 (33.3)		6 (50)	6 (50)	
G3	4 (80)	1 (20)		2 (40)	3 (60)	
Myometrial invasion			0.003*			0.115
≤1/2	6 (50)	6 (50)		4 (33.3)	8 (66.7)	
>1/2	10 (100)	0 (0)		6 (60)	4 (40)	
Lymph node metastasis			0.348			0.241
Yes	4 (80)	1 (20)		3 (60)	2 (40)	
No	12 (70.6)	5 (29.4)		7 (41.2)	10 (58.8)	

Note: *P<0.05 (Spearman correlation analysis).

Table 3. Correlation between GPR30 expression and p-ERK1/2 expression in the endometrium of different groups

p-ERK1/2 (n)	GPR30 (n)					
	Normal endometrium		EAH*		EEC*	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	1	5	8	5	10	0
Negative	2	12	0	7	6	6
r_s	0.031		0.599		0.559	
P	0.898		0.005		0.007	

Note: *P<0.05 (Spearman correlation analysis).

There is evidence showing that GPR30 plays important roles in the follicular development, adrenal gland development and protection against liver injury as well as regulation of cell proliferation. Of important, GPR30 is also involved in the occurrence and progression of cancers. E2 and selective agonist of GPR30 (G1) may activate GPR30 to promote the proliferation of ovarian cancer cells and thyroid cancer cells. The binding of E2 to GPR30 may activate GPR30 and the intracellular downstream cascade, which leads to Ca^{2+} influx, production of cAMP and phosphatidyl inositol, activation of ERK1/2 and subsequent initiation of gene transcription and its regulation, exerting the rapid non-genomic effect of estrogen [15, 16]. Thus, we speculated that GPR30 may activate MAPK signaling pathway to play important roles in the occurrence and development of EEC. Investigators have found the GPR30 expression and the biological activities of GPR30 in EC cell lines (HEC50, HEC-1A, Ishikawa, KLE, RL95-2 and H38) [17, 18]. Lin et al [19] postulated that GPR30 could mediate the estrogen induced rapid activation of PI3K or MAPK in EC cells and had crosstalk with nuclear hormone receptor SF-1 or LRH-1 signaling pathway. GPR30 mediated the E2 or tamoxifen induced activation of EGFR-MAPK/Erk1/2 signaling pathway in HEC-1A cells and Ishikawa cells, in which the c-fos expression was up-regulated, leading to the proliferation of

EC cells. The crosstalk between GPR30 and SF-1 leads to the focal aggregation of estrogen and together with classic ER signaling pathway regulates the TAM induced proliferation of EC cells and even the tumorogenesis. Smith et al [20] detected GPR30 expression in 46 patients with pathologically proven EC, and their results showed GPR30 expression was very low in benign endometrial lesions and EC with good differentiation, but GPR30 expression increased markedly in EC with poor differentiation or deep invasion (such as uterine papillary serous adenocarcinoma, clear cell carcinoma and carcinosarcoma).

Our results showed GPR30 was mainly expressed in the cytoplasm and membrane of glandular epithelium. In normal endometrium and EAH, the GPR30 expression was moderate to low. However, most EEC tissues had a high GPR30 expression, and the proportion of GPR30 positive patients among EEC patients was significantly higher than in patients with normal endometrium and EAH patients ($P<0.05$). In addition, GPR30 expression in EAH group also increased markedly as compared to patients with benign endometrial lesions ($P<0.05$). These findings were consistent with previously reported. Our results showed the GPR30 expression increased gradually from normal endometrium to EAH and then to EEC, suggesting that GPR30 plays an important role in the estrogen induced tumorogenesis of endometrium. In patients with high GPR30 expression, trace estrogen binds to GPR30 to exert its non-genomic effects, leading to the sustained hyperplasia of endometrium or even tumorogenesis. Correlation analysis showed GPR30 expression was positively related to the depth of myometrial invasion ($P<0.05$): the deeper the myometrial invasion, the higher the GPR30 expression was. This suggests that GPR30 expression is closely related to the invasion of EEC. Further analysis revealed that GPR30 expression had no relationship with pathological stage and lymph node metastasis ($P>0.05$). These findings were different from those reported by Smith et al, but consistent with findings in some other studies. This indicates that GPR30 is not directly involved in the biobehaviors of EEC. On the basis of above findings, we speculate that GPR30 plays crucial roles in the occurrence and development of EEC, and GPR30 is involved in the estrogen induced activation of several signaling pathways, which is one of mechanisms underlying the proliferation of cancer cells and the progression of cancers [21]. However, the complex mechanisms are required to be elucidated in future studies.

Few studies have been conducted to investigate whether ERK1/2 signaling pathway is involved in the tumorogenesis of endometrium and whether abnormal activation of ERK1/2 signaling pathway is related to the pathogenesis of EC. Some investigators proposed that estrogen could rapidly activate MAPK/ERK1/2 signaling pathway via the non-genomic mechanism, leading to the increase in p-ERK expression, elevated proliferation of cells, inhibition of cell apoptosis, which are involved in the occurrence and development of EC. Treeck et al proposed that estrogen could stimulate the proliferation of EC cells via the ERK1/2 signaling pathway [22]. Desouki et al [23] detected ERK1/2, C-Jun and P38 expressions in 33 patients with EC and 38 patients with benign endometrial lesions. Their results showed the progression from normal endometrium into EC had not relationship with MAPK signaling pathway activation. Mizumoto et al [24] detected p-ERK expression in 63 patients with EC, and results showed 63.5% of patients had p-ERK expression of different extents. Vantaggiato et al [25] found that cells with ERK2 knock out showed a poor proliferation, suggesting that ERK mediates the cell proliferation. The activated ERK enters the nucleus and then activate transcription factors such as NF-κB, leading to the increase in Cyclin D1 expression and the subsequent proliferation of cells [26, 27]. Carcamo et al [28] revealed that reduction in ERK2 expression could decrease Cyclin D1 expression, arrest cells in G0/G1 phase and reduce cell proliferation.

Our results showed the positive rate of p-ERK1/2 expression was 30.0%, 65.0% and 45.5% in normal endometrium, EAH and EEC, respectively. Normal endometrium had a low p-ERK1/2 expression, indicating that ERK signaling pathway is in a physiological status. The p-ERK1/2 expression increased significantly in EAH as compared to normal endometrium ($P<0.05$). This indicates there is abnormal ERK1/2 expression during the progression from normal endometrium to EAH, which leads to the uncontrollable cell proliferation and the final atypical hyperplasia. In the present study, p-ERK1/2 expression was moderate to low in EEC, which was different from the high p-ERK1/2 expression in breast cancer and colon cancer [29]. The proportion of p-ERK1/2

positive patients in EEC patients (45.5%) was slightly lower than in EAH patients (65%) (P>0.05). Moreover, the p-ERK1/2 expression was comparable between EAH and normal endometrium (P>0.05). Desouki et al postulated that the increased activated ERK in EEC might be related to the anti-estrogen effects of TAM in the endometrium, but had no relationship with the progression of EEC. There is evidence showing that estrogen may rapidly activate MAPK/ERK signaling pathway via the non-genomic mechanism, to increase p-ERK1/2 expression, stimulate cell proliferation, and inhibit cell apoptosis, which are involved in the occurrence and development of EEC. We speculate that there were two different regulatory mechanisms of ERK1/2 in the EAH and EEC: in EAH, aberrant ERK1/2 activation may promote the atypical hyperplasia. When the ERK1/2 expression reaches a threshold, the negative feedback is initiated, causing the irreversible malignant transformation as demonstrated by reduced p-ERK1/2 expression in EEC in the present study. ERK1/2 signaling pathway is one of pathways involved in the regulation of EEC cells, but there is no non-hormone dependent aberrant activation as observed in breast cancer. In the present study, correlation analysis showed p-ERK1/2 expression had no relationship with age, menstruation state, FIGO stage, histological differentiation, depth of myometrial invasion and lymph node metastasis, which was similar to findings of Mizumoto et al. Thus, we speculate that ERK1/2 signaling pathway is not directly involved in the progression of EEC.

In vitro experiments showed GPR30 mediated the activation of MAPK/Erk1/2 signaling pathway in ER positive or negative endometrial cell lines [30]. In the present study, the GPR30 expression was positively related to p-ERK1/2 expression in EAH and EEC (r=0.599 and 0.559, respectively; P<0.05). This correlation might play an important role in the progression of EAH and might belong to an early molecular event in tumorogenesis. We speculate that ERK1/2 may exert synergistic effect with GPR30 in the occurrence and development of EEC, and p-ERK1/2 may be a key point in the GPR30 related estrogen induced activation of signaling pathway. However, the specific mechanism underlying the interaction between GPR30 and ERK1/2 are required to be further investigated. To study the correlation between GPR30 and p-ERK1/2 may provide new targets for the therapy of EEC.

Disclosure of conflict of interest

None.

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