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Original Article

High expression of GPER1, EGFR and CXCR1 is associated with lymph node metastasis in papillary thyroid carcinoma

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Abstract: Clinical and epidemiological studies have shown that estrogen may be involved in the development and progression of papillary thyroid carcinoma (PTC). G protein-coupled estrogen receptor 1 (GPER1) is a novel seven-transmembrane estrogen receptor that functions alongside traditional nuclear estrogen receptors (ERs) to regulate the cellular responses to estrogen. The purpose of this study was to examine GPER1, EGFR and CXCR1 expression in PTC and to assess the association of their expression with clinicopathological indicators. GPER1, EGFR and CXCR1 protein expression in 129 PTCs, 61 nodular hyperplasia and 118 normal thyroid tissue specimens were analyzed using immunohistochemistry. The protein expression levels of these three molecules were up-regulated in PTCs. High protein expression of GPER1, EGFR and CXCR1 was significantly correlated with lymph node metastasis (LNM) ($P \leq 0.001$). Furthermore, GPER1, EGFR and CXCR1 protein expression were correlated with one another. Concomitant high expression of these molecules had stronger correlation with LNM than did each alone ($P = 0.002$ for GPER1/EGFR, $P = 0.013$ for GPER1/CXCR1, $P = 0.018$ for EGFR/CXCR1 and $P < 0.001$ for GPER1/EGFR/CXCR1). Additionally, GPER1, EGFR and CXCR1 mRNA expression was assessed in 30 PTCs, 10 nodular hyperplasia and 10 normal thyroid tissue specimens using real-time RT-PCR. GPER1, EGFR and CXCR1 mRNA expression levels were up-regulated in PTCs, and high mRNA expression of GPER1, EGFR and CXCR1 was significantly correlated with LNM ($P < 0.001$ for all these three molecules). These results demonstrated that the evaluation of GPER1, EGFR and CXCR1 expression in PTC may be useful in predicting the risk of LNM.

Keywords: GPER1, EGFR, CXCR1, papillary thyroid carcinoma

Introduction

Clinical and epidemiological studies have shown that papillary thyroid carcinoma (PTC) accounts for 80% of thyroid malignancy, and is three times more common in women than in men, with the greatest gender difference observed during reproductive age and the decreased incidence after menopause [1]. The elevated risk was reported in women who used estrogen for gynecological problems [2]. These data strongly suggest that estrogen may be involved in the development and progression of PTC, as largely demonstrated in breast, endometrial and ovarian carcinoma [3].

G protein-coupled estrogen receptor 1 (GPER1), formerly known as GPR30, is a novel seven-transmembrane receptor belonging to the G protein-coupled receptor family, binds estrogen with high affinity and functions alongside the traditional nuclear estrogen receptors (ERα and ERβ) to regulate cellular and physiological responsiveness to estrogen [4]. Activation of GPER1 leads to multiple intracellular responses related to growth, differentiation, and proliferation [4, 5]. In addition to estrogen, GPER1 also binds tamoxifen and fulvestrant, two ER inhibitors widely used in the clinic, which creates an agonistic response through cross-talk with growth factor receptors and other signaling molecules and is involved in resistance to classical endocrine therapy in estrogen-related cancers [6, 7]. GPER1 is widely expressed in numerous tissues throughout the body and is often highly

Table 1. Primers used for real-time RT-PCR

Gene	Primers	Product size
GPER1	Forward: 5'-AGTCGGATGTGAGGTTCAG-3'	240 bp
	Reverse: 5'-TCTGTGTGAGGAGTGCAAG-3'	
EGFR	Forward: 5'-AGCTTCTTGCAGCGATACAGCTCAGAC-3'	106 bp
	Reverse: 5'-TGGGAACGGACTGGTTTATGTATTCAGG-3'	
CXCR1	Forward: 5'-GCAGCTCCTACTGTTGGACA-3'	214 bp
	Reverse: 5'-GGGCATAGGCGATGATCACA-3'	
GAPDH	Forward: 5'-GGAGTCCACTGGCGTCTTCA-3'	191 bp
	Reverse: 5'-GGGGTGCTAAGCAGTTGGTG-3'	

expressed in cancer cell lines, particularly those from aggressive tumors [4], and has been shown to be an important prognostic factor in breast, endometrial, ovarian cancers [8-10]. High expression of GPER1 in these estrogen-related tumors has been associated with metastases and poor survival. However, so far, no study dealt with GPER1 expression and its correlation with clinicopathological features of PTC.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase important in transducing extracellular signals from the cell surface to the cell interior, mediating crucial processes such as cell proliferation, differentiation, migration and apoptosis [11]. Dysregulated expression of EGFR can lead to aberration of homeostatic cellular processes, resulting in malignant transformation of cells [12]. Studies have shown that EGFR is transactivated through stimulation of GPER1 to generate a survival response, facilitating proliferation, invasion and migration in several types of cancer [4, 7, 13]. Overexpression of EGFR is frequently found in epithelial cancers such as breast cancer [14], esophageal squamous cell carcinomas [15] and papillary thyroid cancer [16], and high expression of EGFR occurs at an advanced stage of malignancy characterized by metastatic competence and poor prognosis.

CXCR1 is one of two high-affinity receptors for the CXC chemokine interleukin-8 (IL-8), a major mediator of immune and inflammatory responses implicated in many disorders, including tumor growth [17]. CXCR1 is mainly expressed in neutrophils and is originally characterized by its ability to induce chemotaxis of leukocytes. However, CXCR1 has been shown to act on multiple cell types, as our recent study showed that CXCR1 is involved in invasion and migration of ER-negative breast cancer cells through cross-talk with GPER1 via EGFR-ERK/PI3K pathway [18]. Moreover, it was found that overexpression of CXCR1 is correlated with drug-resistance, invasion, and metastasis in several solid tumors [19].

Given their associations with biological aggressiveness in several human tumors, the purpose of this study was to examine GPER1, EGFR and CXCR1 expression in PTC, to assess association of GPER1, EGFR and CXCR1 expression with several clinicopathological indicators, and to evaluate potential usefulness of these three molecules in prediction for invasion, metastasis and progression of PTC.

Materials and methods

Case selection and tissue sample preparation

Tumor specimens for immunohistochemistry were obtained from 129 PTC patients who underwent thyroidectomy in the Department of Surgery, the First Affiliated Hospital, Chongqing Medical University, between Jan 2010 and Jan 2013. There were 32 men and 97 women with a median age of 45 years. According to histopathologic diagnosis, there were 80 classic PTC, 21 follicular variant of PTC, 15 tall cell variant of PTC and 13 oncocytic variant of PTC. The size of primary tumor ranged from 0.3 to 6.0 cm (2.28 ± 1.38). According to AJCC classification [20], there were 69 patients with stage I and stage II, 60 with stage III and stage IV. Sixty-eight patients were confirmed to have lymph node metastasis (LNM). Besides, benign thyroid disease specimens were obtained from 61 patients with nodular hyperplasia. 118 normal thyroid tissues were taken from the contralateral lobe of PTC specimens, which exhibit apparently normal morphology as a control. The study protocol was approved by the Research Ethics Committee of Chongqing Medical University and informed consent was obtained from all patients.

Tumor specimens for real-time RT-PCR were obtained from 30 PTC patients between Jan. 2012 and Jan. 2013, including 15 PTCs without LNM and 15 PTCs with LNM. The benign thyroid

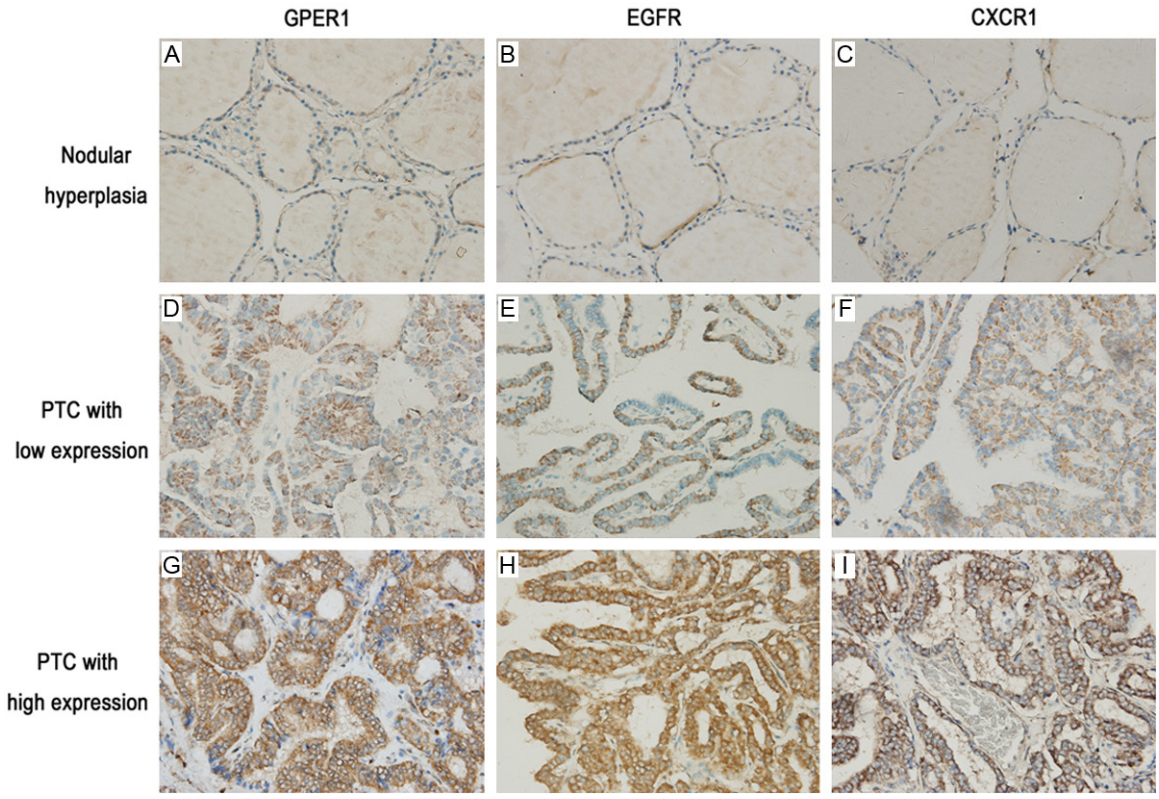

Figure 1. Immunohistochemical staining for GPER1, EGFR and CXCR1. Columns correspond to immunostaining for GPER1, EGFR and CXCR1, respectively. The first row exhibits weak staining of nodular hyperplasia tissues with the indicated antibody (A-C); the succeeding rows show moderate staining (D-F) and strong staining (G-I) of GPER1, EGFR and CXCR1 in PTCs. All the pictures are in high-power fields (×400).

disease specimens were obtained from 10 patients with nodular hyperplasia. For controls, 10 normal thyroid tissue specimens were used. All specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C up to subsequent real-time RT-PCR.

Tissue microarray

Formalin-fixed, paraffin-embedded blocks were routinely prepared from surgical specimens of PTC, nodular hyperplasia and normal thyroid tissue. Representative areas containing tumor, nodular hyperplasia or normal thyroid tissue were identified by a pathologist. Duplicate tissue cores with a diameter of 0.6 mm were taken from each specimen (Beecher Instruments, Silver Springs, USA) and arrayed on a recipient paraffin block, using standard procedures [21]. Serial 5-μm-thick sections were cut with a Leica microtome (Leica Microsystems, Wetzlar, Germany) and mounted onto polylysine-coated slides.

Immunohistochemical staining

Sections from TMA blocks were dewaxed and hydrated. Antigen retrieval was achieved by microwaving in 0.01 M citrate buffer (pH 6.0) for 10 min. After microwave treatment, the slides were treated with 3% hydrogen peroxide for 30 min to block the endogenous peroxidase and followed by blocking with 10% normal goat serum (50062Z, Invitrogen, USA) in PBS at room temperature for 1 h. The slides were then incubated overnight at 4°C in the primary rabbit polyclonal anti-GPER1 antibody (1:100 dilution, ab39742; Abcam, USA), anti-EGFR antibody (1:100 dilution, BS1533; Bioworld Technology, USA), or anti-CXCR1 antibody (1:100 dilution, bs-1009R; Bioss, China). For negative isotype controls, the sections were incubated in rabbit immunoglobulin G (1:1000, NI01-100UG; Merck Millipore, Germany). After defrosting at 37°C for 30 min, the slides were washed with PBS and incubated with a secondary biotinylated goat-anti-rabbit antibody (ZB-2010; Zho-

Table 2. Immunohistochemical analysis of GPER1, EGFR and CXCR1 expression in 129 PTCs, 61 nodular hyperplasia and 118 normal thyroid tissue specimens according to the scoring system

Score	GPER1			EGFR			CXCR1		
	Normal thyroid tissue *n* (%)	Nodular Hyperplasia *n* (%)	PTC *n* (%)	Normal thyroid tissue *n* (%)	Nodular Hyperplasia *n* (%)	PTC *n* (%)	Normal thyroid tissue *n* (%)	Nodular Hyperplasia *n* (%)	PTC *n* (%)
0									
Negative	65 (55.1)	32 (52.5)	3 (2.3)	67 (56.8)	36 (59.0)	5 (3.9)	70 (59.3)	38 (62.3)	6 (4.7)
+									
1	48 (40.7)	20 (32.8)	8 (6.2)	48 (40.7)	18 (29.5)	10 (7.8)	46 (39.0)	19 (31.1)	11 (8.5)
2	5 (4.2)	7 (11.5)	12 (9.3)	3 (2.5)	6 (9.8)	14 (10.9)	2 (1.7)	4 (6.6)	16 (12.4)
3	0 (0)	2 (3.3)	15 (11.6)	0 (0)	1 (1.6)	17 (13.2)	0 (0)	0 (0)	19 (14.7)
4	0 (0)	0 (0)	19 (14.7)	0 (0)	0 (0)	18 (14.0)	0 (0)	0 (0)	16 (12.4)
++									
6	0 (0)	0 (0)	18 (14.0)	0 (0)	0 (0)	16 (12.4)	0 (0)	0 (0)	20 (15.5)
8	0 (0)	0 (0)	21 (16.3)	0 (0)	0 (0)	20 (15.5)	0 (0)	0 (0)	16 (12.4)
+++									
9	0 (0)	0 (0)	22 (17.1)	0 (0)	0 (0)	19 (14.7)	0 (0)	0 (0)	17 (13.2)
12	0 (0)	0 (0)	11 (8.5)	0 (0)	0 (0)	10 (7.8)	0 (0)	0 (0)	8 (6.2)

The immunohistochemical scores in PTCs, nodular hyperplasia and normal thyroid tissue specimens were determined as the multiplication of proportion score and intensity score.

Table 3. Correlation of GPER1, EGFR and CXCR1 protein expression with clinicopathological parameters in 129 PTCs

Characteristics	Case (n)	GPER1			EGFR			CXCR1		
		Low	High	*P* value	Low	High	*P* value	Low	High	*P* value
Tissue type										
Normal thyroid tissue	118	118	0		118	0		118	0	
Nodular hyperplasia	61	61	0	-	61	0	-	61	0	-
PTC	129	57	72	< 0.001[a] < 0.001[b]	64	65	< 0.001[a] < 0.001[b]	68	61	< 0.001[a] < 0.001[b]
Classic PTC	80	36	44	0.817	39	41	0.972	42	38	0.890
Follicular variant of PTC	21	8	13		11	10		10	11	
Tall cell variant of PTC	15	6	9		8	7		8	7	
Oncocytic variant of PTC	13	7	6		6	7		8	5	
Age (years)										
< 45	69	29	40	0.597	35	34	0.786	37	32	0.824
≥ 45	60	28	32		29	31		31	29	
Gender										
Male	32	15	17	0.724	18	14	0.386	16	16	0.867
Female	97	42	55		46	51		42	45	
Tumor size (cm)										
< 2.3	83	42	41	0.049	46	37	0.076	47	36	0.232
≥ 2.3	46	15	31		18	28		21	25	
TNM stage										
I-II	69	36	33	0.050	40	29	0.042	40	29	0.200
III-IV	60	21	39		24	36		28	32	
Lymph node metastasis										
Absent	61	36	25	0.001	40	21	0.001	43	18	< 0.001
Present	68	21	47		24	44		25	43	

P-values derived using Chi-square test to compare the expression of GPER1, EGFR and CXCR1 between subgroups defined by each clinicopathological parameter; [a]stands for significant difference between PTCs and normal thyroid tissues; [b]stands for significant difference between PTCs and nodular hyperplasia. P < 0.05 stands for significant difference.

Table 4. Correlation of GPER1, EGFR and CXCR1 protein expression with one another in 129 PTCs

Proteins	GPER1				CXCR1			
	Low	High	r_s	*P* value	Low	High	r_s	*P* value
EGFR								
Low	37	27	0.272	0.002	41	23	0.226	0.010
High	20	45			27	38		
CXCR1								
Low	38	30	0.249	0.004				
High	19	42						

P-values for Spearman rank test; GPER1, EGFR and CXCR1 were tested pairwise; P < 0.05 stands for significant difference.

ngshan Golden Bridge Biotechnology, China) for 30 min, peroxidase-labeled streptavidin (ZB-2404; Zhongshan Golden Bridge Biotechnology, China) for 20 min and diaminobenzidine chromogen substrate (Sigma, USA) for 5 min. Slides were counterstained with hematoxylin, dehydrated, and mounted.

Immunohistochemical scoring

A semiquantitative assessment of immunohistochemical (IHC) scoring was performed by two observers blinded to the diagnosis. The IHC score was assigned based on staining intensity and percentage of positive cells. The intensity score was assigned as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The proportion score was assigned as 0 (< 5% positive cells), 1 (6-25% positive cells), 2 (26-50% positive cells), 3 (51-75% positive cells), and 4 (> 75% positive cells). Multiplication of the intensity and percentage scores gave rise to the final staining score: 0 (negative), + (1-4), ++ (5-8), and +++ (9-12). For statistical analysis, a final staining score of negative or + was combined into the low expression group, and a final staining score of ++ or +++ was combined into the high expression group.

RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted from frozen thyroid tissues using TriZol reagent (Invitrogen, Camarillo, CA, USA), and residual genomic DNA was eliminated by DNase I digestion (Ambion, USA). RNA purity was confirmed by spectrophotometry. Total RNA was reverse transcribed to cDNA by using SuperScript III Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's protocol. The final cDNA product was amounted to 25 μL and stored at -80°C. Real-time PCR was performed by using SYBR-Green real-time PCR method on the ABI-Prism 7000 sequence detector (Applied Biosystems, USA). The primers are shown in **Table 1**. The predicated product size of the primers for GPER1, EGFR and CXCR1 was 240 bp, 106 bp, and 214 bp, respectively. Quantities of gene specific mRNA expression were determined by the CT method. Samples were analyzed in triplicate. Average threshold cycle (CT) values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal calibrator. The $2^{-\Delta\Delta ct}$ method was used for relative quantitation [22]. Results are presented as the mean ± standard deviation of three independent experiments. The real-time PCR mix was made on the basis of the prescription from the supplier: 6 μL sterile water, 1 μL sense and 1 μL antisense primers, 10 μL Platinum SYBR Green qPCR SuperMix-UDG w/ROX (Invitrogen, USA) and 2 μL target cDNA in a total volume of 20 μL. Run conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 statistical software. Data are presented as percentages and mean and standard deviation, according to the distribution. Significance was assessed using Chi-square, Spearman rank and Mann-Whitney U test as appropriate, to compare the groups. *P* value < 0.05 was considered statistically significant.

Results

Immunohistochemical expression of GPER1, EGFR and CXCR1 in PTCs, nodular hyperplasia and normal thyroid tissues

GPER1, EGFR and CXCR1 protein expression were examined by immunohistochemistry and illustrated in **Figure 1**. The immunoreactivities of GPER1, EGFR and CXCR1 were detected in the cytoplasm and cell membrane. In nodular hyperplasia tissues, there were only a few follicular cells with weak staining for GPER1 (A), EGFR (B) and CXCR1 (C). However, in PTCs, some cases had quite a few tumor cells with

Table 5. Correlation of concomitant expression of GPER1, EGFR and CXCR1 with LNM

	LNM		
	Absent	Present	*P* value
GPER1/EGFR			< 0.001[a]
(1) Both GPER1/EGFR low expression	38 (84.4)	7 (15.6)	
(2) One of GPER1/EGFR high expression	18 (41.9)	25 (58.1)	0.002[b]
(3) Both GPER1/EGFR high expression	5 (12.2)	36 (87.8)	
GPER1/CXCR1			< 0.001[a]
(1) Both GPER1/CXCR1 low expression	36 (90.0)	4 (10.0)	
(2) One of GPER1/CXCR1 high expression	19 (38.1)	30 (61.2)	0.013[b]
(3) Both GPER1/CXCR1 high expression	6 (15.0)	34 (85.0)	
EGFR/CXCR1			< 0.001[a]
(1) Both EGFR/CXCR1 low expression	33 (84.6)	6 (15.4)	
(2) One of EGFR/CXCR1 high expression	21 (41.2)	30 (58.8)	0.018[b]
(3) Both EGFR/CXCR1 high expression	7 (17.9)	32 (82.1)	
GPER1/EGFR/CXCR1			
Not all of GPER1/EGFR/CXCR1 high expression	57 (62.6)	34 (37.4)	< 0.001[c]
All of GPER1/EGFR/CXCR1 high expression	4 (10.5)	34 (89.5)	

Correlation of concomitant expression of GPER1, EGFR and CXCR1 with LNM was measured by Chi-square test; [a]stands for significant difference among the three groups; [b]stands for significant difference between group (2) and group (3); [c]stands for significant difference between groups with and without concomitant expression of all three molecules.

moderate staining for these three molecules (D-F), and some cases had a lot of tumor cells with strong staining for the three molecules (G-I). As shown in **Tables 2** and **3**, like the normal thyroid tissues, the majority of nodular hyperplasia tissues have negative or 1 IHC score, no cases showed high expression (≥ 5) of these three molecules. However, in PTCs, the majority of cases have ≥ 3 IHC score, high expression (≥ 5) was present in 72 (55.8%), 65 (50.4%) and 61 (47.3%) of 129 cases for GPER1, EGFR and CXCR1, respectively. The differences in GPER1, EGFR and CXCR1 protein expression levels between PTCs and normal thyroid tissues as well nodular hyperplasia tissues were statistically significant (P < 0.001).

Correlation of GPER1, EGFR and CXCR1 protein expression with clinicopathological features in PTCs

The correlation of GPER1, EGFR and CXCR1 protein expression with clinicopathological data was assessed by Chi-square test and summarized in **Table 3**. There were not significant differences in GPER1, EGFR and CXCR1 protein expression between patients with different histologic subtype of PTC (*P* = 0.817, *P* = 0.972, *P* = 0.890, respectively), between older (> 45) and younger (≤ 45) patients (*P* = 0.597, *P* = 0.786, *P* = 0.824, respectively), between male and female patients (*P* = 0.724, *P* = 0.386, *P* = 0.867, respectively). However, GPER1 protein expression was associated with tumor size (*P* = 0.049) and marginally with TNM stage (*P* = 0.050), and EGFR protein expression were correlated with TNM stage (*P* = 0.042) and marginally with tumor size (*P* = 0.076), while no correlation was found to be present between CXCR1 protein expression and tumor size (*P* = 0.232) as well TNM stage (*P* = 0.200). Notably, GPER1, EGFR and CXCR1 protein expression were significantly correlated with LNM, PTCs with LNM showed significantly higher protein expression of these three molecules than those without LNM (*P* ≤ 0.001 for all the three molecules).

Correlation of GPER1, EGFR and CXCR1 protein expression with one another in PTCs

The correlation of GPER1, EGFR and CXCR1 protein expression with one another was assessed by Spearman rank test. As shown in **Table 4**, 45/129 PTCs showed high expression and 37/129 displayed low expression for both GPER1 and EGFR. The correlation between GPER1 and EGFR expression was statistically significant (r_s = 0.272, *P* = 0.002). Similarly, there was a statistically significant correlation between expression of GPER1 and CXCR1 (r_s =0.249, *P* = 0.004). For both GPER1 and CXCR1, 42/129 PTCs showed high expression. In addition, high expression for both EGFR and CXCR1 was present in 38/129 PTCs. A significantly positive correlation (r_s = 0.226, *P* = 0.010) was also present between expression of EGFR and CXCR1.

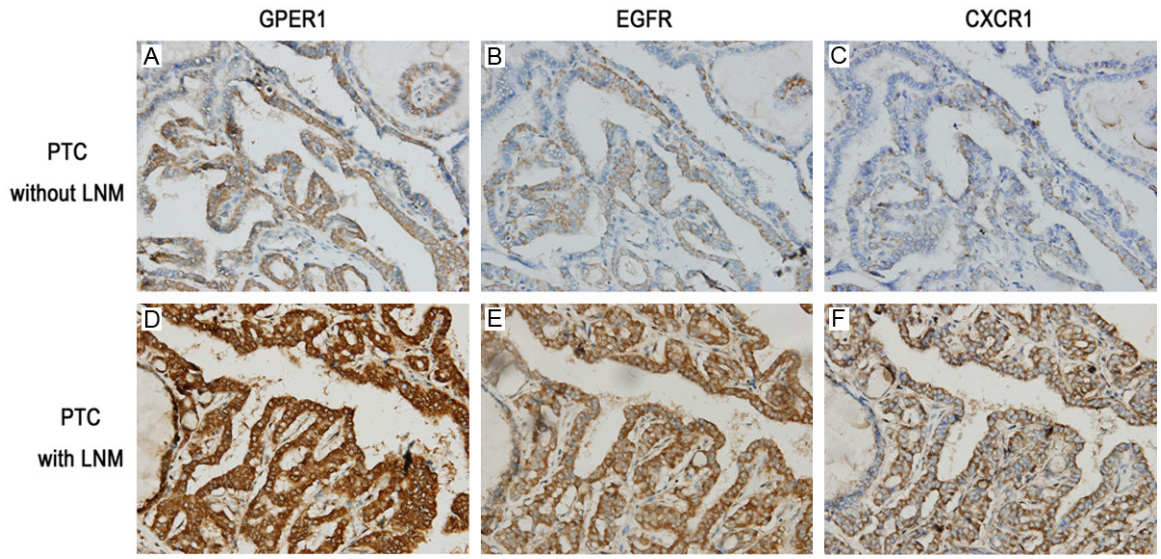

Figure 2. Association of concomitant GPER1, EGFR and CXCR1 high expression with LNM in PTCs. Columns correspond to immunostaining for GPER1, EGFR and CXCR1, respectively. The first row is the immunostaining of a representative of PTC without LNM showing only GPER1 high expression (A) and low expression of EGFR (B) and CXCR1 (C); the second row is the immunostaining of a representative of PTC with LNM showing high expression of GPER1 (D), EGFR (E) and CXCR1 (F). All the pictures are in high-power fields (×400).

Table 6. mRNA expression of GPER1, EGFR and CXCR1 in PTCs, nodular hyperplasia and normal thyroid tissues

Groups	*n*	GPER1		EGFR		CXCR1	
		ΔCT, mean ± SD	*P* value	ΔCT, mean ± SD	*P* value	ΔCT, mean ± SD	*P* value
Normal thyroid tissue	10	3.47 ± 0.91	-	2.49 ± 0.76	-	2.19 ± 0.49	-
Nodular hyperplasia	10	4.12 ± 1.16	0.106	3.01 ± 1.04	0.153	2.81 ± 1.08	0.203
PTC without LNM	15	21.15 ± 7.74	< 0.001[a]	17.36 ± 4.76	< 0.001[a]	12.81 ± 5.11	< 0.001[a]
PTC with LNM	15	33.46 ± 8.69	< 0.001[b]	28.53 ± 5.97	< 0.001[b]	24.09 ± 6.92	< 0.001[b]

Mean ± SD of GPER1, EGFR and CXCR1 mRNA expression in normal thyroid and nodular hyperplasia tissues, PTCs without LNM and PTCs with LNM after normalized to GAPDH (Mann-Whitney U test, [a]stands for significant difference between PTCs without LNM and nodular hyperplasia; [b]stands for significant difference in GPER1, EGFR and CXCR1 mRNA expression between PTCs with LNM and PTCs without LNM).

Association of concomitant GPER1, EGFR and CXCR1 high expression with LNM in PTCs

Given that GPER1, EGFR and CXCR1 protein expression were correlated with one another, and statistical analysis showed that the incidence of LNM tends to be higher in PTCs with high protein expression of GPER1, EGFR and CXCR1, we further evaluated the correlation of LNM with concomitant high expression of GPER1/EGFR, GPER1/CXCR1, or EGFR/CXCR1. As shown in **Table 5**, the incidence of LNM is significantly higher in patients (87.8%) with high expression of GPER1/EGFR than in those patients (58.1%) with high expression of only one of these two molecules, or in those patients (15.6%) without high expression for either of these two molecules. Similar results were observed in PTCs with high expression of GPER1/CXCR1 and EGFR/CXCR1. There were statistically significant differences in the incidence of LNM between patients with high expression of only one and any two of the three molecules (P = 0.002 for GPER1/EGFR, P = 0.013 for GPER1/CXCR1, and P = 0.018 for EGFR/CXCR1). In addition, statistical analysis showed that concomitant high expression of all the three molecules is significantly associated with LNM as compared with cases not showing such expression (P < 0.001). As demonstrated in **Figure 2**, A-C is a representative of PTC without LNM showing only GPER1 high expression and low expression of the other two molecules, EGFR and CXCR1; D-F is a representative of PTC with LNM showing high expression of all the three molecules, GPER1, EGFR and CXCR1, respectively.

Correlation of GPER1, EGFR and CXCR1 mRNA expression with LNM in PTCs

To compare gene expression of GPER1, EGFR and CXCR1 in PTCs without and with LNM, fifteen PTCs without LNM, and another fifteen PTCs with LNM were collected to analysize GPER1, EGFR and CXCR1 mRNA levels using real-time RT-PCR. Ten nodular hyperplasia and ten normal thyroid tissues were used for comparison and as a control. As shown in **Table 6**, GPER1, EGFR and CXCR1 mRNA levels were significantly higher in PTCs compared with nodular hyperplasia ($P < 0.001$ for all the three molecules), while there were not statistically significant differences in GPER1, EGFR and CXCR1 mRNA levels between nodular hyperplasia and normal thyroid tissues ($P = 0.106$, $P = 0.153$ and $P = 0.203$, respectively). Moreover, PTCs with LNM showed to have increased mRNA levels of GPER1, EGFR and CXCR1 compared with those without LNM. The differences in GPER1, EGFR and CXCR1 mRNA levels between PTCs with and without LNM were statistically significant ($P < 0.001$ for all the three molecules).

Discussion

Clinical and epidemiological studies have suggested that estrogen may be involved in the development and progression of PTC [1, 2], as largely demonstrated in breast, endometrial and ovarian carcinoma [3]. It is widely accepted that estrogen manifests its physiologic and pathophysiologic actions through its interaction with two estrogen receptors, ERα and ERβ, which belong to the nuclear steroid hormone receptor family, and function indisputably as hormone-dependent transcription factors to induce estrogen-dependent gene transactivation [23]. However, in the past 10 years, GPER1 has been identified as a new member of the ER family which binds estrogen with high affinity and mediates response to estrogen through transactivation of EGFR [4, 7, 13]. The activation of EGFR leads to downstream signaling events such as production of cAMP, intracellular calcium mobilization, activation of ERK1/2 and PI3K, which is strongly associated with the proliferation, invasion, metastasis, and drug resistance of various cancer cell lines [24]. CXCR1, one of two high-affinity receptors for IL-8, has been found to be associated with drug resistance, invasion, and metastasis in several types of solid tumor [19], especially, our recent study showed that the cross-talk between GPER1 and CXCR1 through transactivation of EGFR can promote the invasion and migration of nuclear estrogen receptor-negative breast cancer cells [18]. To date, studies have shown that GPER1 is overexpressed in several estrogen-related tumors such as breast, endometrial and ovarian carcinoma, which, independent of ERα and ERβ, is associated with invasion, metastasis, drug resistance and poor prognosis of these tumors [8-10]. EGFR and CXCR1 have been observed to be up-regulated in human tumors including PTC [14-16, 19, 25]. However, barely studies examined GPER1 expression in PTC, moreover, no study investigated simutaneously the expression of GPER1, EGFR and CXCR1, and assessed correlation of their expression with clinicopathological features in PTC. In our present study, we examined GPER1, EGFR and CXCR1 protein expression in PTC, nodular hyperplasia and normal thyroid tissues using immunohistochemistry. The results demonstrated that no cases of normal thyroid tissue and nodular hyperplasia show high protein expression of GPER1, EGFR and CXCR1. However, in PTCs, high protein expression was present in 55.8%, 50.4% and 47.3% of cases for GPER1, EGFR and CXCR1, respectively. The differences in GPER1, EGFR and CXCR1 protein expression between PTCs and normal thyroid tissues as well nodular hyperplasia were statistically significant ($P < 0.001$). Then we assessed the correlation of GPER1, EGFR and CXCR1 protein expression with several clinicopathological indicators. We found that GPER1, EGFR and CXCR1 protein expression were not associated with histologic subtype, gender and age. However, there was correlation or marginal correlation between GPER1 as well EGFR protein expression and tumor size as well TNM stage, while CXCR1 protein expression was not associated with tumor size and TNM stage. Remarkably, a statistically significant correlation was found to be present between LNM and single GPER1, EGFR and CXCR1 protein expression ($P \leq 0.001$). High protein expression of GPER1, EGFR and CXCR1 was associated with LNM. These results suggested that GPER1, EGFR and CXCR1 may play an important role in invasion and metastasis of PTC.

Additionally, our study, for the first time, demonstrated a significantly positive correlation between GPER1, EGFR and CXCR1 expression

in PTCs. GPER1 expression is positively correlated with EGFR expression (r_s =0.272, *P* = 0.002) and CXCR1 expression (r_s =0.249, *P* = 0.004). In addition, a significantly positive correlation (r_s =0.226, P = 0.010) was present between expression of EGFR and CXCR1. The existence of these positive correlations could be supported by the following data. Firstly, previous studies have shown that EGF and EGFR are highly expressed in PTC, which is associated with adverse pathologic features of PTC [16, 26]. Subsequently, Vivacqua A at al [27] and Albanito L et al [28] respectively reported that EGF is able to activate the GPR30 promoter and accordingly up-regulate GPR30 mRNA and protein levels via transactivation of EGFR in cancer cells. Furthermore, it was reported that stimulation of GPER1 by estrogen also transactivates EGFR which, albeit indirectly, is able to activate NF-κB via ERK and PI3K signaling [18, 29]. Then, NF-κB can serve as a transcriptional activator to up-regulate CXCR1 expression [30, 31]. Doubtlessly, it is necessary to further explore and elucidate the mechanisms underlying these correlations in PTC.

Given that GPER1, EGFR and CXCR1 protein expression were positively correlated with one another, and high expression of these single molecules was related to LNM, we subsequently evaluated the association of concomitant high expression of GPER1, EGFR and CXCR1 with LNM in PTCs. The results showed that concomitant high expression of any two of these three molecules had stronger correlation with LNM than did each alone. Concomitant high expression of all the three molecules strongly correlates with LNM.

Lastly, we analyzed GPER1, EGFR and CXCR1 mRNA expression levels in PTCs using real-time RT-PCR. The results demonstrated that GPER1, EGFR and CXCR1 mRNA levels were significantly higher in PTCs than in normal thyroid tissues as well nodular hyperplasia. Moreover, as their protein expression, GPER1, EGFR and CXCR1 mRNA expression levels were also correlated with LNM, PTCs with LNM showed to have higher mRNA levels of GPER1, EGFR and CXCR1 than those without LNM.

In summary, our results, for the first time, demonstrated a positive correlation of GPER1, EGFR and CXCR1 expression in PTCs. High expression of GPER1, EGFR and CXCR1 was associated with LNM. Concomitant high expression of any two or all of the three molecules had stronger correlation with LNM than did each alone. Consequently, our results provided a possible basis for prediction of LNM in PTC. Future studies in larger sets of patients will be necessary to determine the utility of these molecules as biomarkers of tumor diagnosis and prognosis in PTC.

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Disclosure of conflict of interest

None.

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