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Increased Expression of GARP in Papillary Thyroid Carcinoma

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Abstract

Regulatory T cells (Tregs) are immunosuppressive immune cells that play an important role in tumor development. Suppression of Treg function is considered to be an effective strategy for cancer therapy. Glycoprotein A repetitions predominant (GARP) has been found on the surface of activated Tregs. GARP has been recently observed in only a few solid tumors including breast, colon, lung cancers, and melanoma. However, its function in cancers remains unknown. Here, we investigated the expression of GARP in human papillary thyroid carcinoma (PTC) and its prognostic significance. In this study, immunohistochemistry was performed to examine the expression of GARP and Foxp3 in 19 human PTC tissues (including 10 cases with and 9 cases without lymph node metastasis) and 20 benign thyroid diseases (including 10 cases with nodular goiter and 10 cases with adenoma). Compared with benign thyroid diseases, we found a significant increase in the expression of GARP in PTC. Increased GARP expression in PTC was positively correlated with increased expression of Foxp3, which is very important for development of Tregs. But, there is no significant association of elevated expression of GARP with lymph node metastasis in PTC. Our results indicate that GARP is implicated in the development of PTC and might be a potential novel target for anticancer therapy. In addition, our findings further support the existence of a positive-feedback loop between GARP and Foxp3.

Keywords GARP · Foxp3 · Papillary thyroid carcinoma · Thyroid Cancer

Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence has been rapidly increasing all over the world [1–4]. Although genetic alterations and environmental factors have been found in the development of thyroid cancer [4, 5], the pathogenesis of thyroid cancer is not fully understood.

Activating or enhancing regulatory T cell (Treg) function is one of the main mechanisms by which cancer evades anti-tumor immune attacks via multiple different pathways. Tregs promote tumor development by inhibiting anti-tumor immunity. Suppression of Treg function is also considered to be an effective strategy for cancer therapy. Some studies have shown the significant association of expression of Treg-specific markers (like Foxp3, CD39), or other stromal markers (like CD73) with lymph node metastasis and poor prognosis [6–8]. Although the transcription factor Foxp3 is very important for development or function of Tregs [9], it cannot be used as a specific marker for isolation of activated or functional Tregs because of its nuclear and cellular expression in Tregs and conventional T cells [10–12]. Therefore, it is necessary to look for other specific markers of Tregs for further study.

Glycoprotein A repetitions predominant (GARP) is also known as leucine-rich repeat containing 32 (LRRC32) [13].

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It has been found that GARP can bind TGF-β/LAP on the surface of activated CD4$^+$Foxp3$^+$Tregs and megakaryocytes/platelets [14–18]. GARP seems to induce Foxp3 expression, allowing Tregs to inhibit effector cell activation [18, 19]. GARP-TGF-β complex on the surface of activated Tregs mediates AIDS lentivirus-induced T cell immunodeficiency [20]. Although GARP gene was first described in human breast cancer [13, 21], its biological significance in cancer is entirely unknown. Elevated GARP expression has been found in some types of cancers, but most of studies are focused on Tregs from cancer tissues or peripheral blood. It has been reported that GARP expression was found in only a few primary solid cancers, such as breast, colon, and lung cancers [22], and melanoma [23].

Recently, we found that the expression of protein kinase CK2α (casein kinase IIα) in thyroid carcinoma tissues was significantly higher than that in benign thyroid diseases, and the expression of CK2α was positively correlated with the degree of malignancy of thyroid cancer. Even more surprisingly, increased nuclear CK2α expression was significantly associated with lymph node metastasis [24]. As far as we know, the significance of GARP expression and prognosis in thyroid cancer is not clear. In this study, we investigated the expression of GARP and Foxp3 in PTC and benign thyroid disease tissues. The relationship between GARP expression and lymph node metastasis and Foxp3 expression in PTC was also studied.

Materials and Methods

Patients

The present study comprised 39 paraffin-embedded specimens of human PTC (classical variant) and thyroid benign diseases that were operated and diagnosed at the Department of Pathology, the First Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning, People's Republic of China, from 2011 to 2014. These tissue samples were including 19 cases with PTC (9 without and 10 with lymph node metastasis) and 20 cases with benign thyroid diseases (10 cases with nodular goiter and 10 cases with adenoma). The patients were with a mean age of 45.8 (range = 14–67) years at diagnosis, and the detailed information about the patients is described in Table 1. The study was approved by the Ethical Review Board of Jinzhou Medical University, Jinzhou, Liaoning, People's Republic of China.

Immunohistochemistry

Immunohistochemistry was performed according to the antibody manufacturer's recommended protocols and well-established methods [25]. After deparaffinization and dehydration, the sections (4 μm) of human PTC and benign thyroid diseases were microwaved for GARP immunostaining in antigen unmasking solution (Vector Laboratories, Burlingame, CA) and for Foxp3 immunostaining in EDTA buffer for 10 min and rinsed three times, each for 5 min in phosphate-buffered saline (PBS). After endogenous peroxidase was inactivated, the sections were blocked with 5% non-fat milk, 1% BSA, and 0.3% Triton X-100 in PBS for 1 h. The sections were incubated with GARP antibody (1:50, MyBioSource Inc., San Diego, CA) in blocking solution overnight at 4°C and incubated with Foxp3 antibody (1:50, Abcam Inc., Cambridge, MA) overnight at room temperature and 1 h at 37 °C, respectively. Following extensive rinsing as above, the sections were incubated in biotinylated goat anti-rabbit antibody (1:300, Vector Laboratories) for 1 h at room temperature. After incubation with the biotinylated secondary antibody, the avidin–biotin complex (ABC) (VECTASTAIN®, Vector Laboratories) and diaminobenzidine (DAB) (DAB Peroxidase Substrate Kit, Vector Laboratories) were applied to the sections for visualization of the reaction product. For negative controls, the primary antibody was omitted.

Table 1 Clinicopathological characteristics of the 39 patients

	N (%)
Gender	
Male	15 (38.46)
Female	24 (61.54)
Age	
≥ 45	22 (56.41)
< 45	17 (43.59)
Pathological type	
Nodular goiter	10 (25.64)
Adenoma	10 (25.64)
PTC without LNM	9 (23.08)
PTC with LNM	10 (25.64)

PTC papillary thyroid carcinoma, *LNM* lymph node metastasis

Evaluation of Staining

Immunohistochemical staining was evaluated and confirmed by two pathologists. According to a well-established method [24], GARP and Foxp3-positive cell counting was completed separately by two authors. The values from five random fields per section obtained by two authors were averaged and expressed as percentage of the number of GARP or Foxp3-positive cells/total cells, respectively.

Statistical Analysis

All of the following analyses were performed using SPSS 17.0. Data are expressed as mean ± standard deviation (SD) and p value. Unpaired t tests were performed for analyzing the comparison of GARP expression between males and females; between < 45 and ≥ 45 years and between clinical stages I/II and III/IV (based on the 7th edition of the AJCC/UICC TNM stage). The difference in GARP and Foxp3 expression between PTC and benign thyroid disease was also analyzed by the unpaired t test. One-way ANOVA was performed for analyzing the expression changes of GARP and Foxp3 in nodular goiter, adenoma, and PTC without and with lymph node metastasis. The correlation between increased GARP and Foxp3 expression in PTC was analyzed by Spearman correlation. P values ≤ 0.05 were considered statistically significant.

Results

GARP Was Highly Expressed in PTC Compared to Benign Thyroid Diseases

To investigate the role of GARP in the development and progression of PTC, we histologically examined GARP expression in 39 patients with PTC and benign thyroid diseases. We found GARP expression in all of the tissues of PTC and some of benign thyroid diseases (Fig. 1a–d). Increased expression of GARP was found in PTC ($n = 19$, 23.199 ± 13.186%) when compared with benign thyroid diseases ($n = 20$, 2.983 ± 3.549%, $p = 0.000$) (Fig. 1e). Relative to nodular goiter ($n = 10$, 3.209 ± 2.642%), GARP expression was increased in PTC with ($n = 10$, 27.534 ± 16.027%, $p = 0.006$) and without lymph node metastasis ($n = 9$, 18.383 ± 7.257%, $p = 0.001$). Also, higher expression of GARP was found in PTC with ($p = 0.004$) and without lymph node metastasis ($p = 0.001$) than in adenoma ($n = 10$, 2.757 ± 4.416%). However, there was no significant difference in GARP expression between nodular goiter and adenoma ($p = 1.00$). Although slightly higher GARP expression was observed in PTC with than without lymph node metastasis, no significant change was found in these two subgroups ($p = 0.559$) (Fig. 1f). These demonstrated an increase in the expression of GARP in PTC compared with benign thyroid diseases and no significant association between increased GARP expression and lymph node metastasis.

Higher Expression of Foxp3 Was Found in PTC than in Benign Thyroid Diseases

In this study, we found Foxp3 expression in all of the tissues of PTC and some of benign thyroid diseases (Fig. 2a–d) and increased Foxp3 expression was observed in PTC ($n = 19$, 4.451 ± 4.521%) compared with its expression in benign thyroid diseases ($n = 20$, 0.398 ± 0.418%, $p = 0.001$) (Fig. 2e). No significant change in the expression of Foxp3 was observed between nodular goiter and adenoma ($n = 10$, 0.514 ± 0.465% in nodular goiter; $n = 10$, 0.265 ± 0.276% in adenoma, $p = 0.884$). Similarly, there was no significant different change of Foxp3 expression in between PTC with ($n = 10$, 4.777 ± 3.982%) and without lymph node metastasis ($n = 9$, 4.089 ± 5.280%, $p = 1.000$) (Fig. 2f).

Significant Correlation of Increased GARP with Foxp3 Expression Was Found in PTC

A few studies have shown elevated GARP expression in some types of cancer tissues and cells, such as breast, colon, and lung cancers. However, the correlation of GARP with the Foxp3 expression in cancer tissues or cells is still unknown. In this study, we found that the increased expression of GARP was significantly correlated with increased Foxp3 expression in PTC compared with benign thyroid disease (Fig. 3, $r = 0.641$, $p = 0.000$).

GARP Expression Was Not Significantly Associated with Gender, Age, and Clinical Stage of the Patients with PTC

Our results indicated that there was no significant association of GARP expression with age, gender, and clinical stages of patients with PTC. However, slight increase in the expression of GARP was found in females ($n = 9$, 28.78 ± 14.33) compared with males ($n = 10$, 18.18 ± 10.30, $p = 0.079$); older patients (≥ 45 years, $n = 9$, 25.79 ± 16.65) compared with younger ones (< 45 years, $n = 10$, 20.87 ± 9.41, $p = 0.432$) and late clinical stage (III/IV, $n = 5$, 28.72 ± 21.70) relative to its expression in early clinical stage (I/II), respectively ($n = 14$, 21.23 ± 8.94, $p = 0.491$) (Table 2).

Discussion

GARP has been widely accepted to be a marker of activated Tregs, which contribute to an immunosuppressive tumor microenvironment. However, GRAP expression and function in cancers remain to be validated. Up to now, there have been a few papers published about GARP expression in primary cancer tissues or cancer cell lines. We first discovered the expression of the Treg marker GARP in PTC and benign thyroid disease tissues in this study. A significant increase in the expression of GARP was found in PTC when compared with benign thyroid diseases including nodular goiter and adenoma. But, there was no significant association of increased expression of GARP with lymph node metastasis in PTC. Our results are consistent with previous studies in other cancers. Increased expression of GARP has been observed in colon, breast, and lung cancer tissues when compared with corresponding normal ones [22]. In addition to inducing peripheral tolerance, higher GARP expression was also found in

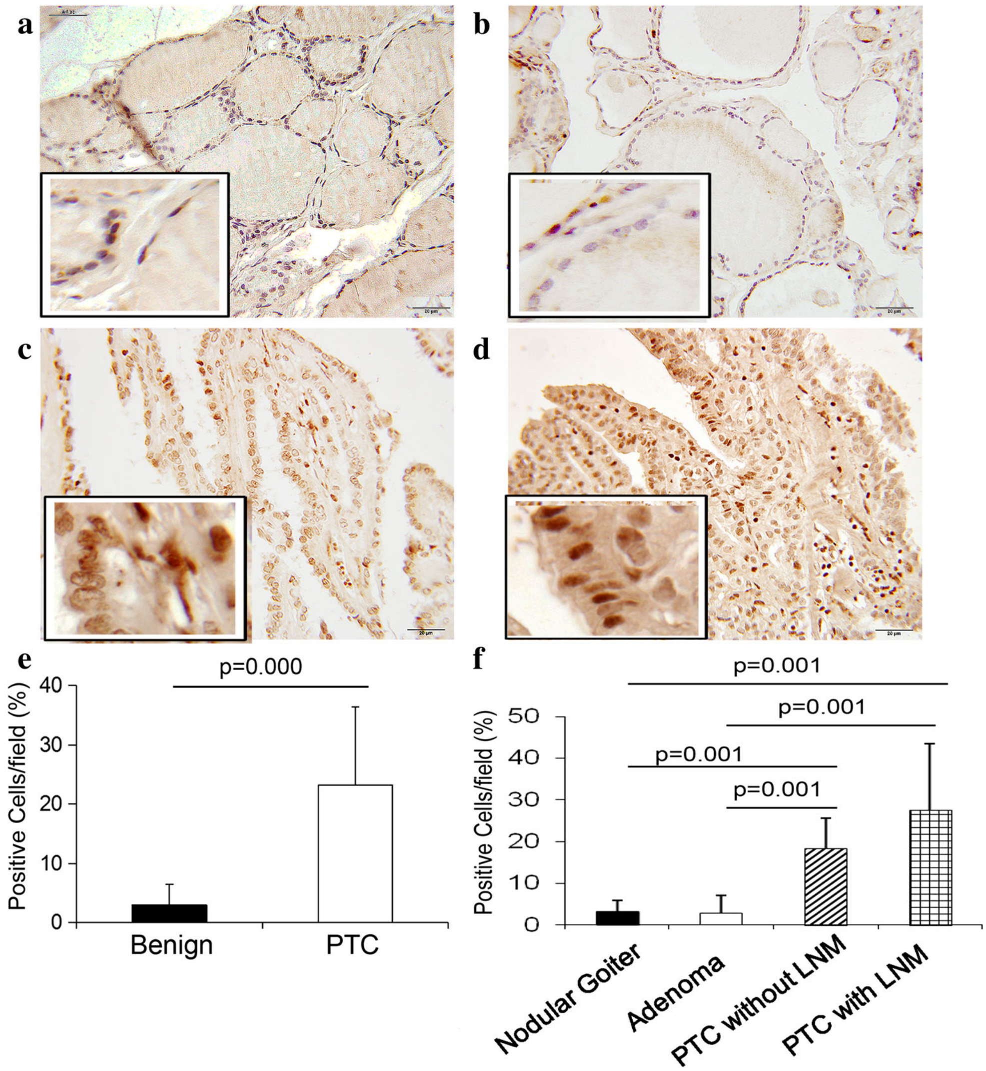

Fig. 1 Increased expression of GARP in PTC compared to its expression in benign thyroid diseases. GARP expression in the tissues of human PTC and benign thyroid diseases was analyzed using immunohistochemistry. **a**–**d** GARP was expressed in some tissues of benign thyroid diseases including nodular goiter (**a**), adenoma (**b**), and all of PTC without (**c**) and with (**d**) lymph node metastasis (LNM). **e** Increased GARP expression was found in PTC with and without LNM compared to its expression in thyroid benign diseases including nodular goiter and adenoma. All data are shown as mean ± SD (%). Significant at $p < 0.05$. **f** Elevated expression of GARP was observed in PTC with and without LNM compared to its expression in nodular goiter and adenoma, respectively. There was no significant difference in its expression between nodular goiter and adenoma. GARP expression was not significantly increased in PTC with LNM compared to its expression in PTC without LNM. All data are shown as mean ± SD (%). Significant at $p < 0.05$

melanoma and melanoma cell lines than in melanocytes [23]. Regarding GARP expression and function(s) in cancer tissues, little data is available. The immunosuppressive tumor environment may be a cause of tumor immune escape and treatment failure, where suppressive immune cells like Tregs and inhibitory factors like TGF-β secreted by tumor cells play an important role in cancer development and progression. In vitro study has shown that soluble form of GARP shed from the surfaces of Tregs and melanoma cells induces peripheral Tregs, a tumor associated (M2) macrophage phonotype and inhibits effector T ($CD4^+$ and $CD8^+$) cell functions [23]. GARP function has been also elucidated by ectopic enforced expression or downregulation of its expression. Forced expression of GARP in T helper cells with TCR stimulation induced the cells into functionally suppressive $Foxp3^+$ Tregs [26, 27]. Knocking down GARP expression on $Foxp3^+$ Tregs reduces suppressive activity [26, 28]. Importantly, enforced expression of GARP in normal murine mammary epithelial cells upregulates TGF-β bioactivity and drives oncogenesis, and its enforced expression in murine mammary cancer cells promotes TGF-β activity, tumor metastasis, and immune tolerance [22]. Especially in their study, enforced GARP expression induces EMT by reducing E-cadherin and increasing vimentin expression, consistent with our previous finding that decreased E-cadherin and increased N-cadherin expression was detected in thyroid cancer compared with benign thyroid diseases [24].

Some studies have suggested that GARP and Foxp3 might form a positive-feedback loop. In our study, the positive correlation between increased GARP and elevated Foxp3 expression provides further evidence for the existence of this loop [26, 27]. Increased proportion of tumor-infiltrating $Foxp3^+$ Tregs predicts a poor prognosis of some cancers including breast [29], ovarian [30], and hepatocellular [31]. A study using a thyroid tissue microarray has revealed significant association between nuclear Foxp3 expression and invasiveness in differentiated thyroid cancer and Foxp3 staining was more intense in malignant lesions than in benign ones. The

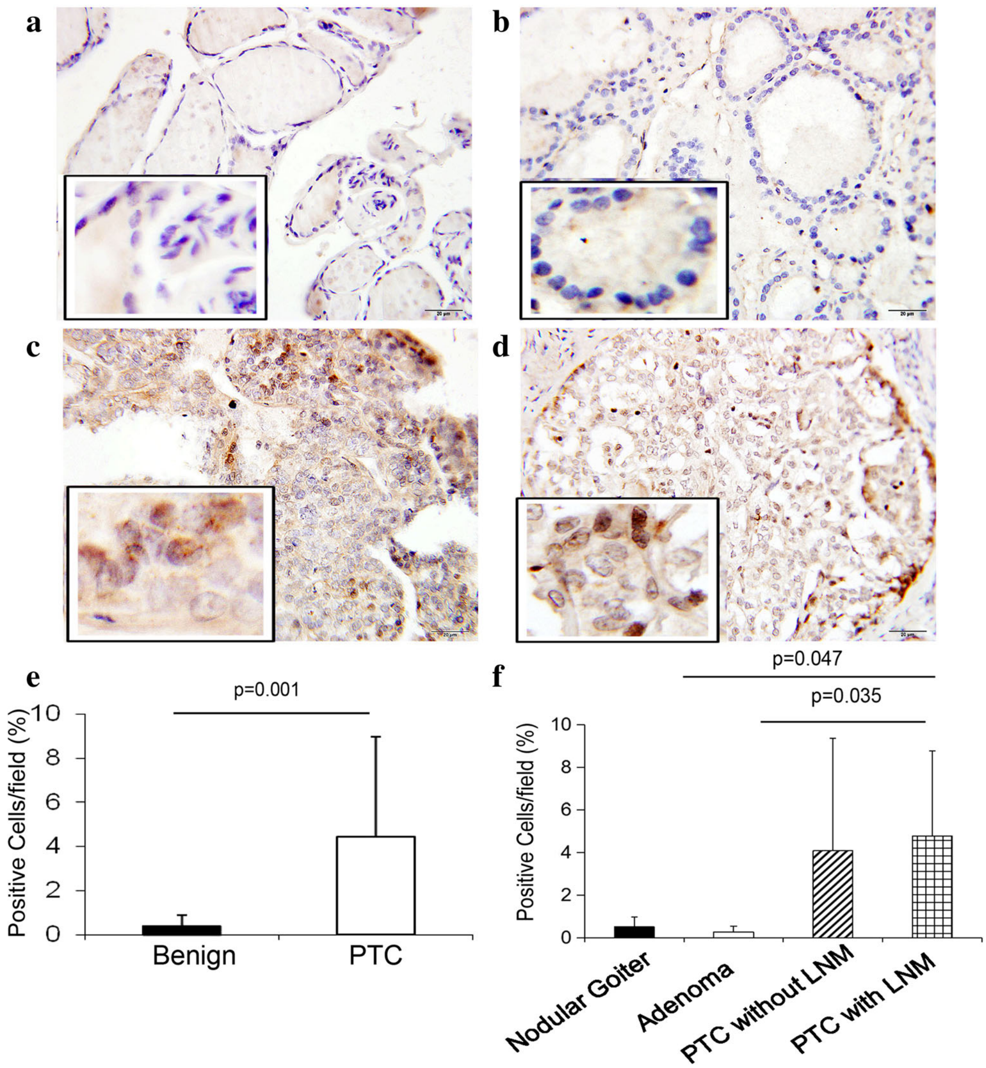

Fig. 2 Increased expression of Foxp3 in PTC compared to its expression in benign thyroid diseases. Foxp3 expression in the tissues of human PTC and benign thyroid diseases was analyzed using immunohistochemistry. **a**–**d** Foxp3 was expressed in some tissues of benign thyroid diseases including nodular goiter (**a**), adenoma (**b**), and all of PTC without (**c**) and with (**d**) LNM. **e** Increased Foxp3 expression was observed in PTC compared to benign thyroid diseases including nodular goiter and adenoma. All data are shown as mean ± SD (%). Significant at $p < 0.05$. **f** The expression of Foxp3 was significantly increased in PTC with LNM compared to its expression in nodular goiter and adenoma. However, the results did not show significantly different expression between PTC with and without LNM, as well as between nodular goiter and adenoma. All data are shown as mean ± SD (%). Significant at $p < 0.05$

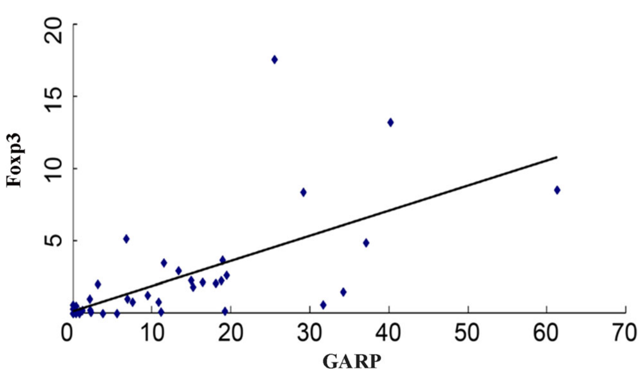

Fig. 3 Elevated GARP expression was significantly correlated with increased Foxp3 expression in PTC. Our results showed that expression of GARP and Foxp3 was increased in PTC compared to its expression in benign thyroid diseases. Here, using Spearman Correlation we analyzed whether there was correlation between elevated GARP and increased Foxp3 expression in PTC compared to their expression in benign thyroid diseases. The statistical analysis showed that elevated expression of GARP was significantly correlated with increased Foxp3 ($r = 0.641$, $p = 0.000$)

expression of cellular Foxp3 was higher in papillary and follicular carcinomas than in nodular goiter [32]. In this study, an increase in the expression of Foxp3 was observed in PTC compared with nodular goiter and adenoma. The number of samples was limited in our study. It was difficult to analyze the cellular and nuclear Foxp3 staining separately. Therefore, the positive cells counted in our study included cellular and nuclear staining. In addition, our data did not show a significant difference of Foxp3 expression between PTC with and without lymph node metastasis. Due to the limited number of samples, the results may differ slightly from the one reported above.

Our results indicated that there was no significant association of GARP expression with age, gender, and clinical stages of patients with PTC although slight increase in expression of GARP was found in females, older patients (≥ 45 years), and late clinical stage (III/IV) relative to its expression in males, younger ones (< 45 years) and early clinical stage (I/II), respectively.

Table 2 Association between the clinicopathological characteristics and expression of GARP in thyroid papillary carcinoma

	Number	GARP (%)	*p*
Gender			0.079
Male	10	18.18	
Female	9	28.78	
Age			0.432
≥ 45	10	25.79	
< 45	9	20.87	
Clinical stage			0.491
I–II	14	21.23	
III–IV	5	28.72	

Taken together, our findings suggested a potential role of GARP in PTC development. GARP expression was elevated in PTC compared with benign thyroid diseases. There was a significant correlation between elevated expression of GARP and increased Foxp3 expression in PTC compared with benign thyroid diseases. But, no significant association of increased GARP expression was observed in PTC with lymph node metastasis. This series demonstrated the potential role of GARP in the pathogenesis of PTC, and GARP might be a novel therapeutic target. Our research further supports the existence of a positive-feedback loop between GARP and Foxp3.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The study was approved by the Ethics Committee of Jinzhou Medical University.

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