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Expression of Foxp3 and TLR4 in human papillary thyroid carcinoma and its clinical significance

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Key words: papillary thyroid carcinoma; clinicopathological characteristics; Foxp3; TLR4; lymph node metastasis; high risk

Tables: Table 1 to 2
Figures: Fig. 1 to 3

Short title: Expression of Foxp3 and TLR4 in thyroid carcinoma
Abstract

Objective This study aimed to explore the association of Foxp3 and TLR4 with clinical pathological characteristics in papillary thyroid carcinoma (PTC) patients. Methods 78 cases of PTC were used as experimental group and 20 cases of normal thyroid tissue were used as control group. The expression of Foxp3 and TLR4 in thyroid tissue from the two groups was detected by immunohistochemistry, and the experimental group was divided into several groups on the basis of different clinicopathological indicators. The association between Foxp3 and TLR4 expression and clinicopathological parameters was statistically analyzed. Results Foxp3 and TLR4 were expressed in higher levels in PTC than in normal thyroid tissue (P < 0.05). Foxp3 was mainly localized in the cytoplasm and nucleus of PTC cells, while TLR4 was found in the cytoplasm and cell membrane of cancer cells. The expression of both proteins associated with lymph node metastasis and TNM clinical stage (P < 0.05). The expression of Foxp3 correlated with the expression of TLR4 in tested PTC tissues (P < 0.05). In addition, the result of confocal fluorescence microscopy showed that Foxp3 and TLR4 co-localized in PTC cells. Conclusion Foxp3 and TLR4 were upregulated and associated with lymph node metastasis and advanced TNM stage in PTC tissues. Together they may act as valuable factors for the identification of high-risk PTC patients.

[Key words] papillary thyroid carcinoma; clinicopathological characteristics; Foxp3; TLR4; lymph node metastasis; high risk

Introduction

Foxp3 (Forkhead box protein 3) is a functional determinant of CD4+Tregs (Regulatory T cells). Under normal physiological conditions, Foxp3 expressed by CD4+Tregs inhibits the immune response by regulating the expression of various inhibitory molecules to prevent autoimmune diseases. When the immune function of the body is excessively inhibited, the immune escape of the tumor occurs, resulting in the development of the tumor. In recent years, studies have confirmed that a variety of malignant tumor cells express Foxp3 (Hinz et al., 2007; Zuo et al., 2007; Wang et al., 2009; Merlo et al., 2009; Ladoire et al., 2011; Fu et al., 2013; Kato et al., 2018), but its regulation mechanism is not clear.
TLR4 is a very important member of the Toll like receptors (TLRs) family. TLR4 was first discovered by Medzhitov et al. (Medzhitov et al., 1997) and confirmed to be involved in the regulation of Foxp3 expression in CD4+ Treg cells. Recent studies have found that it is also expressed in tumor cells (Li et al., 2014), but its role is contrary to the role in immune cells, promoting tumor immune escape (Huang et al., 2005; He et al., 2007; Goto et al., 2008; Kim et al., 2012; Wang et al., 2013). Studies found that TLR4 is highly expressed in various tumor tissues such as lung cancer, ovarian cancer, colorectal cancer, and gastric cancer, and is closely related to tumor progression and poor prognosis (Schmausser et al., 2005; Zhou et al., 2009; Wang et al., 2010, 2013; Zhang et al., 2010; Kim et al., 2012; Fu et al., 2013; Li et al., 2014). It can be used as an independent risk factor for predicting poor prognosis in some tumors (Wang et al., 2010; Li et al., 2014).

Papillary thyroid carcinoma has a high degree of differentiation and low malignancy, but the proportion of recurrence and metastasis is large and is the major factor affecting the prognosis of patients (Huang et al., 2014). Studies have shown that an increased proportion of CD4+ Tregs is closely related to the development of thyroid papillary carcinoma, but there is still no relevant research on the expression of Foxp3 itself in PTC and the role of TLR4 on its expression. To investigate the significance of Foxp3 and TLR4 expression in the pathogenesis and development of PTC and the relationship between them, PTC was selected as the research object to detect the expression of Foxp3 and TLR4 in pathological tissues of PTC patients. The relationship of Foxp3 and TLR4 expression and PTC clinical pathological indicators were analyzed, and the action of Foxp3 and TLR4 expression in the development of PTC was further elucidated.
Materials and Methods

Patients and tissue samples

The experimental specimens of 78 patients (20 males and 58 females) were obtained from the surgical specimens of bilateral thyroidectomy in the thyroid surgery of the China-Japan Union Hospital of Jilin University from 2011 to 2012. The average age of patients was 42.8 (range 18-67 years). In the control group, 20 specimens (5 males and 15 females) were normal thyroid tissues at the margin of the specimens of the nodular goiter patients without PTC. The average age of patients was 45.7 (range 32-72 years).

All patients had no other systemic malignant tumors by imaging examination and hematological examination before operation. No radiotherapy, chemotherapy or radiofrequency treatment was performed before operation. No thyroid surgery was performed before admission. The postoperative pathology was clearly diagnosed as PTC. All patients were grouped according to age, tumor size, gender, lymph node metastasis, and TNM staging (NCCN guidelines, 2011). All patients signed a written informed consent form prior to surgery to allow their surgical specimens to be used in medical scientific research. The entire experiment was approved by the Ethics Committee of the China-Japan Union Hospital of Jilin University.

Immunohistochemical analysis

The immunohistochemical SP method was used to detect the expression of Foxp3 and TLR4 in paraffin specimens. All procedures were performed according to the procedures provided in the kit. Foxp3 was detected by streptavidin-peroxidase (SP) using mouse anti-human Foxp3 monoclonal antibody, mouse anti-human TLR4 monoclonal antibody, immunohistochemical hypersensitive SP kit (Santa). The PTC tissues were made into 3-5 μm sections. The sections were then treated with antigen retrieval, peroxidase blocking, and incubation with blocking serum. Tissue sections were subsequently incubated with anti-human Foxp3 monoclonal antibody (1:100) or anti-human TLR4 monoclonal antibody (1:200) at 4 °C overnight, followed by detection with SP kit according to the manufacturer’s instruction. The cytoplasm and nucleus of the cells showed that the brownish yellow particles were positive cells. A
double-blind experimental procedure was used, using a bright field microscope (CX31; Olympus Corp., Tokyo, Japan). The results of the experiment were judged by two experienced pathologists, and the average of the results of the two doctors was calculated. Ten high-powered (×200) fields were randomly selected from each section, and 100 tumor cells were counted in each field. The immunohistochemical staining results of Foxp3 and TLR4 were determined by the combination of staining intensity and percentage of positive area.

**Immunofluorescence analysis**

Immunofluorescence confocal assay was used to detect the co-localization of Foxp3 and TLR4 in the PTC. The preparation of paraffin sections is the same as those in immunohistochemistry. The tissue sections were blocked with 10% normal goat serum at 37 °C for 45 min, then incubated with mouse anti-human Foxp3 monoclonal antibody (1:50) and rabbit anti-human TLR4 polyclonal antibody (1:100) at 4 °C overnight. The FITC-labeled rat anti-mouse secondary antibody (1:100) and APC-labeled goat anti-rabbit secondary antibody (1:100) were subsequently added onto the sections and incubated at 37 °C for 1 hour, followed by rinsing with PBS and adding anti-fluorescence quenching sealer to seal. The results were observed with a laser confocal microscope (Olympus FV1000).

**Statistical analysis**

Data were presented as count/count (percentage). Foxp3 and TLR4 were compared by Chi-square test or Fisher’s exact test between the tumor group and the normal group. The correlation analysis between Foxp3 and TLR4 was performed by Spearman correlation analysis. P < 0.05 was considered a statistically significant difference. All statistical analyses were performed using SPSS 26.0 software (SPSS, Inc., Chicago, IL, USA).
Results

Expression of Foxp3 and TLR4 in PTC Tissue

In order to know the expression of Foxp3 and TLR4 in PTC tissues, immunohistochemistry was used to detect the expression of Foxp3 and TLR4. Foxp3 was expressed in PTC tissue tumor cells, the positive expression of Foxp3 in tumor cells was 28 cases among 78 PTC patients, the positive rate was 35.90%. The positive expression of Foxp3 in 20 normal thyroid tissue was 2 cases, the positive rate was 10.00%, and the difference between the tumor group and the normal group was statistically significant (P = 0.029), as shown in Table 1. The expression of Foxp3 is present in PTC tissue tumor cells. Most Foxp3 proteins are scattered, and a few are focally expressed. The Foxp3 expression is mainly located in the cytoplasm and nucleus, and the staining intensity is different (see Figure 1A). Foxp3 positive expression of thyroid cancer specimens was in 15 cases of cytoplasmic expression, 9 cases of nucleoplasm co-expression, nuclear expression in 4 cases. In the stroma, a strong positive expression of Foxp3 in a small amount of infiltrating lymphocytes was observed, clustered or scattered around the cancer nest (see Figure 1B). The expression of Foxp3 protein in normal thyroid tissue was mostly negative, and a few were weakly positively expressed in the cytoplasm of epithelial cells (see Figure 1C).

The expression of TLR4 was detected by immunohistochemical staining. The positive expression of TLR4 in PTC tissues was 48 cases, the positive rate was 61.54%. The positive expression of TLR4 in 20 normal thyroid tissues was 7 cases, the positive rate was 35.00%. The difference between the tumor group and the normal group was statistically significant (P = 0.044), see Table 1. The expression of TLR4 in PTC tissues was diffusely distributed, and the subcellular localization was in the cytoplasm or cell membrane of tumor cells (see Figure 2A). TLR4 protein was mostly negative or weakly positive in normal thyroid tissue, and mostly expressed in cytoplasmic or cell membrane of thyroid follicular epithelial cells (see Figure 2B).

In addition, 25 of the 28 PTC tissues positive for Foxp3 expressed TLR4, and 23 of the 50 PTC tissues that were negative expressed TLR4. Foxp3 and TLR4 were significantly positively correlated in papillary thyroid carcinoma (r = 0.508, P = 0.007), and the results from confocal fluorescence microscopy also showed that Foxp3 expressing PTC cells simultaneously expressed TLR4 (see Figure 3), suggesting that
there may be close interaction between the two molecules.

**Association between expression of Foxp3 and TLR4 and clinicopathological parameters in PTC tissues**

To further investigate the role and clinical significance of high expression of Foxp3 and TLR4 in PTC tumor cells, we performed statistical analysis of the expression of Foxp3 and TLR4 proteins and the clinical pathology of patients (see Table 2). The results showed that the expression of Foxp3 protein in tumor cells of PTC tissue was significantly positively associated with lymph node metastasis and TNM clinical stage. There were 17 cases of Foxp3 positive expression in 30 tumor tissues with lymph node metastasis, the positive rate was 56.67%. In the 48 tumor tissues without lymph node metastasis, 11 cases were positive for Foxp3, and the positive rate was 22.92%. The difference between the two groups was statistically significant (P = 0.003). In 36 cases of TNMII+III stage specimens, 22 cases were positive for Foxp3, the positive rate was 61.11%; 6 cases were positive for Foxp3 in 42 cases of TNMII, the positive rate was 14.29%. The difference between the two groups was statistically significant (P = 0.000). There was no significant association between Foxp3 protein expression and age, sex and tumor size (P > 0.05). See Table 2 for details.

The statistical analysis of TLR4 protein expression and clinicopathological parameters (see Table 2) in PTC tissues revealed that the expression of TLR4 protein was significantly associated with lymph node metastasis of tumor and TNM stage. TLR4 showed positive expression in 24 specimens of lymph node metastasis in 24 cases. The positive rate was 80.00%; 24 cases of TLR4 positive expression in 48 cases without lymph node metastasis, the positive rate was 50.00%, the difference between the two groups was statistically significant (P = 0.008). In 36 cases of TNMII+III stage specimens, 28 cases were positive for TLR4, the positive rate was 77.78%; 20 cases were positive for TLR4 in 42 cases of TNMII, the positive rate was 47.62%. The difference between the two groups was statistically significant (P = 0.006). The expression of TLR4 protein in tumor tissues was not associated with gender, age and tumor size (P > 0.05), as shown in Table 2.
Discussion

PTC is the most common pathological type of thyroid cancer, accounting for 70%-80% of thyroid cancers (Jankovic et al., 2013). Papillary thyroid carcinoma has a low degree of malignancy, and most patients are early treated because of the improvements in detection methods. However, there are still a large number of patients with lymph node metastasis, which is one of the main factors affecting the prognosis of patients (Modi et al., 2003). Therefore, the study of the molecular mechanisms involved in the occurrence, development, invasion and metastasis of PTC is of great significance for improving the prognosis of PTC patients. Our study examined the expression of Foxp3 and TLR4 by observing the histomorphology of pathological sections of papillary thyroid carcinoma, and analyzed the relationship between them and clinicopathological parameters and the relationship between them to further explore their role in the development of PTC.

Studies on PTC have found that Tregs cell-mediated immunosuppression has an increasing infiltration of PTC tumor cells and is closely related to the clinical progression of thyroid cancer (French et al., 2010). It can be seen that Foxp3+Tregs plays an important role in the development and progression of PTC through its immunosuppressive function, affecting tumor progression and patient prognosis. Foxp3 (Forkhead box protein 3) is a determinant of Tregs and plays an important role in maintaining the body's own immune function (Ziegler, 2006). Our study found that Tregs' characteristic gene Foxp3 is expressed not only in Tregs but also in PTC tumor cells directly.

We used immunohistochemical SP method to detect the expression of Foxp3 in 78 surgical specimens with pathologically confirmed PTC. The results showed that 35.90% of PTC tissues expressed Foxp3, and Foxp3 was highly expressed in tumor cells, while only 10.00% normal thyroid tissue had low Foxp3 expression. Its expression in papillary thyroid carcinoma was significantly higher than that in normal thyroid tissue (P < 0.05), suggesting that the expression of Foxp3 in PTC cells may be closely related to the development of PTC. Immunohistochemical results also confirmed that in the PTC tumor tissue, the increase of Foxp3 protein expression is composed of two aspects: First, the expression of a large number of infiltrating Tregs;
Second, the expression of PTC tumor cells. Both may play the same immunosuppressive role in the development of PTC. Cunha et al., using immunohistochemical staining of 253 PTCs, 13 follicular carcinomas, 114 benign diseases, and 5 normal thyroid tissues confirmed that 71% of benign thyroid tumors and 91.9% of malignant thyroid tumor cells expressed Foxp3. Our findings are similar to Cunha et al., but the positive expression rates are not consistent (Cunha et al., 2012). It may be due to the difference of tissue source of the tumor and the reagents of the experiment. In addition, we found that the localization expression of Foxp3 in PTC tumor cells is different from Tregs. The subcellular localization pattern of Foxp3 in PTC cells is mainly in the cytoplasm of tumor cells, followed by nucleoplasmic coexpression and nuclear expression. Cunha et al (Cunha et al., 2012) suggested that the expression of Foxp3 in differentiated thyroid cancer cells was more expressed in nucleus than in cytoplasm, and nuclear expression could be used as an indicator of tumor malignancy, but could not accurately assess the prognosis of patients. In addition, although cytoplasmic expression of Foxp3 in tumor cells is lower than their nuclear expression, its cytoplasmic expression is significantly higher in malignant tumor than that in benign tumor, which is thought to be related to chromosomal variation (Lopes et al., 2006; Zuo et al., 2007; Ladoire et al., 2011). The expression of Foxp3 in tumor cells has also been reported in other studies. Hinz et al. (Hinz et al., 2007) concluded that Foxp3 protein is expressed in the nucleus and cytoplasm of pancreatic cancer cells. Merlo et al. (Merlo et al., 2009) confirmed that Foxp3 protein is mainly expressed in the cytoplasm of breast cancer cells, partially in both the cytoplasm and nucleus, and a few in nucleus. Our findings are inconsistent with Cunha et al., and are similar to those of Merlo et al. in breast cancer cells. Except for non-specific staining caused by different staining methods or reagent selection, different sources of tumors may lead to different localization of Foxp3 cell expression and this may be one of the reasons. The localization of proteins in cells is the basis of their biological functions. Foxp3 of CD4+ Tregs is localized in the nucleus and interacts with transcription factors such as NFAT to exert its transcriptional inhibition (Wu et al., 2006). It has been suggested that the cytoplasmic expression of Foxp3 is due to Foxp3 mutation, and the key domain of Foxp3, FKH, determines the nuclear localization of Foxp3 and DNA binding. When the two lysine residues at the C-
terminus of the FKH domain are mutated into glutamate, Foxp3 is expressed in the cytoplasm (Ziegler, 2006). In addition, studies on Tregs have found that when cells are activated by the TCR signaling pathway, the intracellular Foxp3 may translocate from cytoplasm to nucleus, which may be due to post-translational modification of Foxp3 (Chen et al., 2006). For the reasons for the different subcellular localization of Foxp3 in PTC cells in this study, we have three hypotheses. First, Foxp3 expressed in PTC cells has the FKH domain variation as described above. Second, tumor cells in PTC could not express Foxp3 persistently and stably. Foxp3 is primarily expressed in the nucleus and transported to the cytoplasm. Third, in the process of PTC tumor onset and development, tumor metabolism produces various cytokines and oncogenes which affect the post-translational modification of Foxp3 in PTC cells, and this post-translational modification results in a difference in its distribution within the cell. At present, the research on the subcellular localization of Foxp3 in different tumors is still not consistent. The PTC sample is not large in our study, the number of Foxp3 nuclear expression and cytoplasmic expression is limited, and the different subcellular localization and clinical pathology of Foxp3 in PTC are required to confirm. No significant association was found between the indicators. Therefore, it is possible to further expand the sample size or conduct further research on genes in subsequent studies to reveal the cause and significance of different cell localization of Foxp3 in PTC cells.

To further investigate the clinical significance of high expression of Foxp3 in PTC cells, we performed Spearman analysis on the expression of Foxp3 in PTC tumor cells and related clinicopathological parameters. The results showed that the expression of Foxp3 in tumor cells was closely related to lymph node metastasis and TNM staging of tumor. The positive rate of Foxp3 in lymph node metastasis group (56.67%) was significantly higher than that in non-lymph node metastasis group (22.92%), and the positive rate of Foxp3 in stage II+III PTC patients (61.11%) was significantly higher than that of stage I (14.29%). The presence or absence of lymph node metastasis and TNM staging is an important indicator for assessing the prognosis and tumor-free survival of cancer patients. Therefore, our results suggest that the high expression of Foxp3 is significantly positively associated with the poor prognosis of patients with PTC. The PTC patients with high expression of Foxp3 in tumor cell are more prone to
lymph node metastasis. The likelihood is higher and the prognosis is relatively poor. Cunha et al. suggested that the high expression of Foxp3 in PTC cells may be related to the aggressive invasion ability of tumor cells, but the precise mechanism and clinical significance are unknown. Studies have shown that the high expression of Foxp3 provides an immune microenvironment at the molecular level that is more suitable for tumorigenesis (Hinz et al., 2007). Other studies have confirmed that Foxp3 expression affects the pattern of molecule expression in tumor cells and increases the invasiveness of tumor cells (Merlo et al., 2009). Merlo et al. (Merlo et al., 2009) studied breast cancer tumor cells and found that Foxp3 positive tumor cells were negatively correlated with the overall survival rate of breast cancer patients. Breast cancer patients with lymph node metastasis were significantly associated with Foxp3 expression, and Foxp3 expression indicated poor prognosis. Ladoire et al. have a diametrically opposite conclusion about the expression of Foxp3 in breast cancer, suggesting that Foxp3 positive expression suggests a longer tumor-free survival and long-term survival rate (Ladoire et al., 2011). The results of this study are similar to those of Merlo et al. in breast cancer cells. It is believed that PTC cell Foxp3 may play a similar role as Tregs, mimicking Tregs, providing a more suitable immune microenvironment at the molecular level for tumor development, participating in the malignant progression of the tumor.

TLR4 was studied earlier, first discovered in 1997 by Medzhitov et al. (Medzhitov et al., 1997), and was involved in innate and acquired immunity. Recent studies have found that it is also expressed in tumor cells, but its effect is opposite to that of immune cells (Schmausser et al., 2005; Zhou et al., 2009; Wang et al., 2010, 2013; Zhang et al., 2010; Ladoire et al., 2011; Kim et al., 2012; Dang et al., 2013). The results of this study showed higher TLR4 expression in human PTC tissues (61.54%) than in normal thyroid tissues (35.00%), suggesting that the TLR4 signaling pathway may play an important role in the development of PTC. In addition, TLR4 was found to have two expression patterns in PTC cells, cytoplasm or cell membrane, similar to the results of Dang et al. (Dang et al., 2013). Other studies have shown that (Gatti et al., 2006), TLR4 can localize to the Golgi of the cell membrane or cytoplasm, and is expressed on the cell surface when TLR4 is highly glycosylated. TLR4 can circulate between the Golgi apparatus and the cell membrane surface, and TLR4 expressed in
the cytoplasm can still respond to the stimulation of LPS. The expression of TLR4 is up-regulated after LPS stimulation and induces the production of nitric oxide synthase and various chemokines (Gatti et al., 2006).

Statistical analysis of the expression and clinical pathological parameters of local TLR4 protein in PTC tissue revealed that the expression of TLR4 in PTC was only associated with lymph node metastasis and TNM clinical stage, but there was no significant association with other clinical pathological indicators including tumor size. Dang et al. confirmed that TLR4 expression is associated with lymph node metastasis and tumor size (Dang et al., 2013). The results of this study are not completely consistent with the results of Dang et al. (Dang et al., 2013). For this reason, we consider that the first cause may be due to the number of cases selected, and the second may be due to regional differences in selected cases. Thirdly, the up-regulation of TLR4 expression in PTC is only a passive up-regulation, which is one of the multiple signaling pathways in the development of tumors.

In addition, we performed a statistically relevant analysis of the expression of Foxp3 and TLR4 in the aforementioned thyroid cancer tissues. The results confirmed that the expression of Foxp3 and TLR4 protein in PTC was positively correlated, indicating that there may be some intrinsic relationship between the high expression of Foxp3 and the high expression of TLR4 in PTC tumor cell tissue. The synergistic effect of Foxp3 and TLR4 on the development of PTC plays an important role. Zhang et al. (Zhang and Zhang, 2017) found a significant positive association between TLR4 expression and Foxp3 expression in cervical cancer tissues, which is similar to the results of this study. In addition, we confirmed by immunofluorescence confocal microscopy that Foxp3 and TLR4 can colocalize in a thyroid cancer cell. It is suggested that there may be close interaction between the two molecules. However, it is unclear what is the cause of the positive correlation between Foxp3 and TLR4 expression. We hypothesize that, first, there may be some molecules in the tumor microenvironment that regulate the expression of Foxp3 and TLR4. Second, in the process of tumor development, Foxp3 and TLR4 can be up-regulated in succession. Li et al. (Li et al., 2017) found that in lung cancer A549 cells, activation of TLR4 promotes up-regulation of Foxp3 expression, and this effect promotes the expression of KDM3A, then subsequently KDM3A binds to the Foxp3 promoter to promote the
production of downstream inhibitory cytokines, thereby promoting tumor escape. However, the expression of Foxp3 and TLR4 in this study is jointly regulated, and the relationship between upstream and downstream needs further research and interpretation.

In conclusion, the present study showed that Foxp3 and TLR4 were highly and independently upregulated and associated with lymph node metastasis and advanced TNM stage in PTC tissues. Their expressions in thyroid PTC were significantly positively correlated, suggesting that they may act as valuable factors for the identification of high-risk PTC patients. Further studies need to be done to explore Foxp3/TLR4 expression and whether their cell localizations influence PTC aggressiveness.

Acknowledgment
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A conflict of interest statement
The authors declare that there is no conflict of interest.

References
act as early sensors of infection by up-regulating TLR4 expression and proinflammatory mediators upon LPS stimulation. J. Leukoc. Biol. 79, 989-998.


Tab 1. The comparison of Foxp3 and TLR4 expression in PTC and normal thyroid tissue

<table>
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<th>Groups</th>
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<td></td>
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<td>-</td>
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<tr>
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<tr>
<td>Normal</td>
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Note: PTC: papillary thyroid carcinoma; P: probability; *: Comparison was determined by Fisher’s exact test (2-sided). P value < .05 was considered significant and marked with *.

Tab 2. Relationship of Foxp3 and TLR4 protein expression with clinicopathological factors in PTC

<table>
<thead>
<tr>
<th>Clinicopathological Factors</th>
<th>N</th>
<th>Foxp3 Positive (%)</th>
<th>P</th>
<th>df</th>
<th>TLR4 Positive (%)</th>
<th>P</th>
<th>df</th>
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<td>24 (50.00)</td>
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Note: PTC: papillary thyroid carcinoma; P: probability; *: Comparison was determined by Chi-square test. P value < .05 was considered significant and marked with *; df: degree of freedom.
Figure legend

Fig 1. Distribution of Foxp3 expression in the tumor cells and lymphocytes of PTC and control thyroid tissue specimens, observed by immunohistochemical staining. (A) The expression of Foxp3 (brown stain) was found in PTC tissue tumor cells (magnification, 200x). Most Foxp3 proteins were scattered, and a few were focally expressed. The cell localization was mainly located in cytoplasm (red arrows) and nucleus (blue arrows), and the staining was not completely consistent. (B) A strong positive expression of Foxp3 (brown stain) in a small amount of infiltrating lymphocytes (magnification, 200x), clustered or scattered around the cancer nest. (C) A representative micrograph showed Foxp3 protein expression in normal thyroid tissue (magnification, 200x) was mostly negative in the cytoplasm of epithelial cells.

Fig 2. Immunohistochemical staining was used to detect the expression of TLR4 in PTC and control thyroid tissue specimens. (A) The expression of TLR4 in PTC tissues (magnification, 200x) was diffusely distributed, and it localized in the cytoplasm or cell membrane of tumor cells (red arrows). (B) TLR4 protein was mostly negative in thyroid follicular epithelial cells of normal thyroid tissue (magnification, 200x).

Fig 3. Immunofluorescence staining was used to detect the Co-expression of Foxp3 and TLR4 protein in PTC tissues by laser confocal fluorescence microscopy (magnification, 200x). The co-localization of Foxp3 and TLR4 (white arrows) was observed by confocal microscopy. (A) Green fluorescence shows Foxp3 expression. (B) Red fluorescence shows TLR4 expression. (C) Merge of Graph A and B. (D) Light microscopy results.