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ORIGINAL ARTICLE

Association of XIAP and $P2X_7$ receptor expression with lymph node metastasis in papillary thyroid carcinoma

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Abstract The expression of X-linked inhibitor of apoptosis (XIAP) and the $P2X_7$ receptor were demonstrated in a variety of tumors. The purpose of the present study was to investigate the associations of XIAP and $P2X_7$ receptor expression with the clinicopathological features of patients with papillary thyroid carcinoma (PTC). In this cross-sectional study, a total of 62 cases were examined, including 43 patients with PTCs and 19 with benign nodular goiters. XIAP and $P2X_7$ receptor expression were examined by immunohistochemical methods on formalin-fixed, paraffin-embedded thyroid tissues. The staining intensity and extent were evaluated and scored using a semi-quantitative method. The immunohistochemical staining score integrating the intensity and extent of XIAP and $P2X_7$ receptors in PTCs was higher than in nodular goiters. XIAP (OR: 5.6, 95% CI: 1.5–21.1, $P = 0.009$) and $P2X_7$ receptor (OR: 6.1, 95% CI: 1.5–24.4, $P = 0.007$) expression were associated with lymph node metastasis in PTCs. In logistic regression analysis, $P2X_7$ receptor expression, tumor size, and capsular infiltration were predictors for lymph node metastasis ($P = 0.001$). Our results suggested that XIAP and $P2X_7$ receptor expression may predict the aggressiveness of PTC.

Keywords XIAP · $P2X_7$ receptor · Lymph node metastasis · Papillary thyroid carcinoma (PTC)

Introduction

Papillary thyroid carcinoma (PTC) accounts for more than 80% of thyroid cancers, which in turn constitute the most prevalent endocrine malignancy; moreover, the incidence of PTC has increased dramatically over the last 30 years [1]. Although PTC is biologically a relatively indolent disease with a low mortality, patients with some classical risk factors, including an older age at diagnosis, a larger tumor size, lymph node metastasis, genetic markers such as BRAF mutations and so forth, still have a poor prognosis [2, 3]. The results of immunohistochemistry examination on surgically excised thyroid tissues can also provide important clues regarding the invasiveness of the tumor and help clinicians to manage the disease appropriately. Thus, identifying novel biomarkers indicating the progression and aggressiveness of PTC is always the focus of research.

X-linked inhibitor of apoptosis (XIAP) belongs to the IAP family and mediates cell survival by modulating both extrinsic and intrinsic death signaling pathways [4, 5]. Overexpression of XIAP has been observed in colorectal, bladder, esophageal, and renal cancer as well as PTC [6–8].

Similarly, $P2X_7$ receptor plays a critical role in cell survival and growth. The $P2X_7$ receptor is one of the seven subtypes in P2X receptor family of cation-selective

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channels. Its natural ligand is adenosine triphosphate (ATP), and increased concentrations of ATP in the interstitium of tumors have been shown to be responsible for tumor invasion and metastasis [9]. Activation of $P2X_7$ receptors either by ATP or other stimuli may lead to the release of several kinds of pro-inflammatory cytokines including IL-1β, TNF-α and IL-6, as well as increasing the permeability of cell membrane and potentially resulting in apoptosis in several cell types [10–13]. The $P2X_7$ receptor is normally expressed in immune cells; however, its overexpression has been observed in malignant tumors, such as breast and prostate cancer [14, 15]. Recently, the overexpression and the functional role of the $P2X_7$ receptor were demonstrated in both the cell lines and human tissues of papillary thyroid carcinoma [16]. However, the relevance of $P2X_7$ receptor expression, in conjunction with XIAP expression, to the clinicopathological features of PTC has not been described.

Thus, the purpose of the present study was to evaluate the association between XIAP and $P2X_7$ receptor expression and the clinicopathological parameters of PTC.

Materials and methods

Thyroid samples

A total of 62 cases were studied, including 43 patients with PTCs and 19 with benign nodular goiters.

All formalin-fixed and paraffin-embedded thyroid sections stained by hematoxylin and eosin were reviewed by two pathologists, blinded to each other's results, to confirm the diagnosis and to sub-classify the histological variants. Forty-three papillary thyroid carcinomas were subdivided into the following categories according to the WHO histological classification of thyroid tumors: 21 classic variant, 12 follicular variant, 5 capsulated, and 5 tall cell variant. The presence of metastasis was determined by reviewing the patients' medical records. The cancer stage was defined according to the tumor node metastasis (TNM) staging system from the sixth edition of the "American Joint Committee on Cancer" Cancer Staging Manual [17].

Immunohistochemistry

XIAP and $P2X_7$ receptor expression were examined in 43 PTCs and 19 nodular goiters by immunohistochemical methods on formalin-fixed, paraffin-embedded thyroid tissues. The 5 μm-thick tissue sections were deparaffinized and heated in 0.01 mol/l citric acid (pH 6.0) for 15 min at 95°C followed by slow cooling for antigen retrieval. Slides were treated with 3% hydrogen peroxide to block endogenous peroxidase activity and then incubated for 60 min at 37°C with anti-XIAP (clone 2F1, mouse IgG1, concentration 1 mg/ml, Medical & Biological Laboratories Co.) and anti-$P2X_7$ (rabbit IgG, concentration 2 mg/ml, Abcam) which was used at a dilution of 1:100. Controls were incubated with PBS in place of the primary antibody, and no positive staining was observed.

The intensity and extent of staining for XIAP and the $P2X_7$ receptor were evaluated as previous reported [8, 16, 18]. The staining intensity was semi-quantitatively evaluated on a scale of 0–3, with a score of 0 corresponding to negative staining, 1 to weak staining, 2 for moderate staining and 3 for strong staining. The staining extent was also scored semi-quantitatively according to number of positive stained cells in the specimen, as follows: 0 (less than 10%), 1 (10–25%), 2 (25–75%), or 3 (greater than 75%). The score for intensity was multiplied by the score for extent to obtain an immunohistochemical score for the expression of XIAP and $P2X_7$ receptors for each specimen. The score for XIAP and $P2X_7$ receptor expression for each individual was then added to get a final total score. Cases with a staining intensity score ≥ 2 and extent score ≥ 1 were considered to have positive expression, while a complete absence of staining or weak staining with less than 10% of positive cells were defined as negative.

Two of the authors independently rated the level of intensity and extent of the staining of each slide. The discordance rate of the evaluation was 6%. The final decision of the discordant cases was made after discussing with each other.

Images of the histochemical stainings were taken with an Olympus BX51 microscope plus an Olympus DP71 CCD camera (Olympus Corporation, Japan).

Statistical analysis

Data were expressed as mean ± SEM. Comparisons between groups were performed using the Student's t test for continuous data. Differences in the frequency of single variables were tested using a χ^2 test. Univariate analyses were used to estimate the influence of XIAP or $P2X_7$ receptor expression on clinicopathological parameters. Factor found to be significant was then selected as dependent variable for multivariate forward stepwise conditional logistic regression analyses. A P value < 0.05 denoted the presence of a significant difference. All tests were performed using the Statistical Package for Social Sciences for Windows (SPSS Inc., Chicago, IL).

Results

XIAP and $P2X_7$ receptor expression in PTCs and nodular goiters

Figure 1 illustrates the immunohistochemical expression of XIAP and the $P2X_7$ receptor in PTCs.

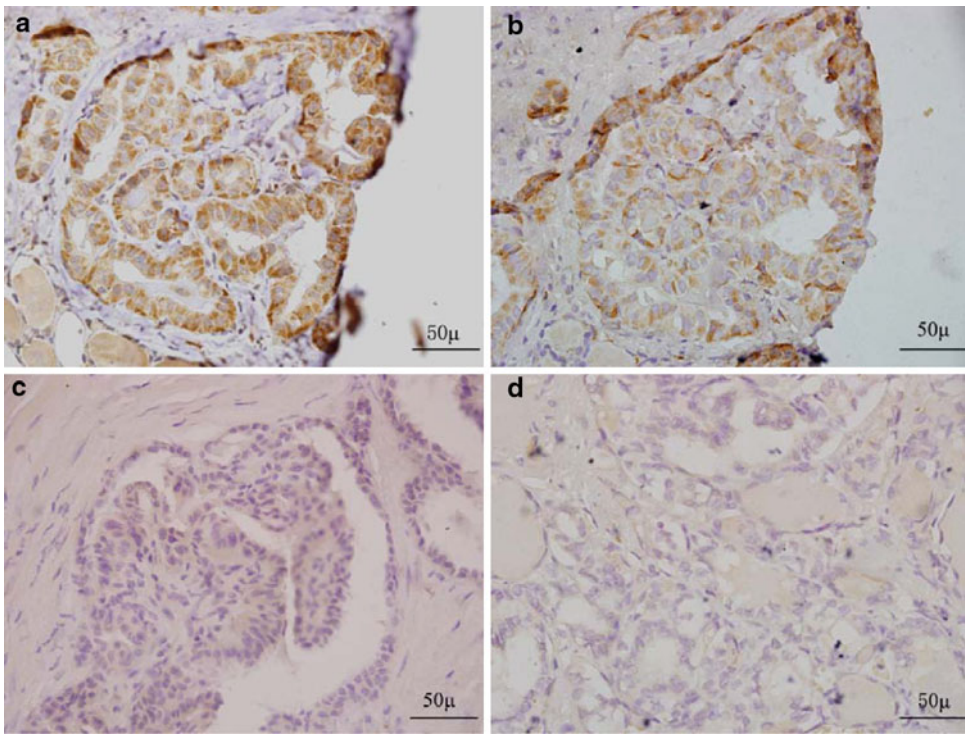

Fig. 1 Immunohistochemical expression of XIAP and $P2X_7$ receptor in PTCs **a** $P2X_7$ receptor-positive staining; **b** XIAP-positive staining; **c** $P2X_7$ receptor-negative staining; **d** XIAP-negative staining

The intensity and extent of XIAP and $P2X_7$ receptor expression in PTCs and nodular goiters are shown in Tables 1 and 2. The scores for XIAP (4.21 ± 0.51 vs. 0.79 ± 0.34, $P = 0.000$) and $P2X_7$ receptor (4.35 ± 0.56 vs. 0.47 ± 0.29, $P = 0.000$) expression in PTCs were significantly higher than those in nodular goiters (Fig. 2). The final total score of these two markers was also markedly increased in PTCs (8.56 ± 0.95 vs. 1.26 ± 0.59, $P = 0.000$).

In PTCs, XIAP and $P2X_7$ receptor expression were also significantly correlated with each other even after adjusting for age, gender, tumor size, multifocality, capsular invasion, lymph node metastasis, and tumor subtype ($r = 0.580$, $P = 0.001$).

Association of XIAP and $P2X_7$ receptor expression with clinicopathological parameters of PTC

In our PTC specimens, there were 19 (44.2%) cases with multifocal lesions, 18 (41.9%) with capsular invasion and 21 (48.8%) that showed lymph node involvement. Among these patients, 18 expressed both XIAP and the $P2X_7$ receptor (duel-positive expression), 14 expressed only one of the two markers (single-positive expression), and 11 were negative for both markers (duel-negative expression).

The correlations between XIAP and $P2X_7$ receptor expression and clinicopathological features of PTCs are shown in Table 3. PTC patients with $P2X_7$ receptor-positive staining were more likely to be female. It was also observed that PTC patients with XIAP-positive expression (OR: 5.6, 95% CI: 1.5–21.1, $P = 0.009$) and $P2X_7$ receptor-positive expression (OR: 6.1, 95% CI: 1.5–24.4, $P = 0.007$) were more likely to have lymph node metastasis compared to those who were negative expression, respectively. The duel-positive expression of XIAP and the $P2X_7$ receptor was associated with an even higher risk of lymph node metastasis as compared with duel-negative expression (OR: 15.8, 95% CI: 2.4–104.5, $P = 0.002$).

Age, gender, tumor size, capsular infiltration, histopathological variants, XIAP expression, and $P2X_7$ receptor expression were included in a multivariate forward stepwise conditional logistic regression analysis to identify independent predictors of lymph node metastasis. It was demonstrated that $P2X_7$ receptor expression, tumor size, and capsular infiltration were significantly associated with lymph node metastasis ($P = 0.001$). A group comparison between PTC patients with and without lymph node metastasis showed that both the XIAP (5.33 ± 0.70 vs. 3.14 ± 0.68, $P = 0.03$) and $P2X_7$ receptor (5.62 ± 0.65 vs. 3.14 ± 0.82, $P = 0.023$) immunohistochemical scores were significantly elevated in the former (Fig. 3). The final total score was also significantly higher in patients with lymph node metastasis (10.95 ± 1.17 vs. 6.27 ± 1.35, $P = 0.012$).

Additionally, the tumor size in PTC patients with lymph node involvement was marginally larger than in patients without lymph node metastasis (1.29 ± 0.40 vs. 0.99 ± 0.51 cm, $P = 0.055$).

Table 1 XIAP expression in PTCs and nodular goiters

	XIAP intensity				XIAP extent			
	0	1	2	3	0	1	2	3
Nodular Goiter ($n = 19$)	13 (68.4%)	3 (15.8%)	3 (15.8%)	0	13 (68.4%)	3 (15.8%)	3 (15.8%)	0
PTC ($n = 43$)	7 (16.3%)	12 (27.9%)	11 (25.6%)	13 (30.2%)	7 (16.3%)	7 (16.3%)	11 (25.6%)	18 (41.9%)
Classic ($n = 21$)	3 (14.3%)	6 (28.6%)	3 (14.3%)	9 (42.9%)	3 (14.3%)	3 (14.3%)	5 (23.8%)	10 (47.6%)
Follicular ($n = 12$)	3 (25%)	3 (25%)	4 (33.3%)	2 (16.7%)	3 (25%)	3 (25%)	3 (25%)	3 (25%)
Capsulated ($n = 5$)	1 (20%)	2 (40%)	0	2 (40%)	1 (20%)	1 (20%)	1 (20%)	2 (40%)
Tall cell ($n = 5$)	0	1 (20%)	4 (80%)	0	0	0	2 (40%)	3 (60%)

Table 2 $P2X_7$ receptor expression in PTCs and nodular goiters

	$P2X_7$ receptor intensity				$P2X_7$ receptor extent			
	0	1	2	3	0	1	2	3
Nodular Goiter ($n = 19$)	16 (84.2%)	1 (5.3%)	2 (10.5%)	0	16 (84.2%)	1 (5.3%)	2 (10.5%)	0
PTC ($n = 43$)	12 (27.9%)	5 (11.6%)	9 (20.9%)	17 (39.5%)	12 (27.9%)	4 (9.3%)	8 (18.6%)	19 (44.2%)
Classic ($n = 21$)	5 (23.8%)	2 (9.5%)	1 (4.8%)	13 (61.9%)	5 (23.8%)	1 (4.8%)	4 (19.0%)	11 (52.4%)
Follicular ($n = 12$)	6 (50%)	0	4 (33.3%)	2 (16.7%)	6 (50%)	1 (8.3%)	2 (16.7%)	3 (25%)
Capsulated ($n = 5$)	1 (20%)	0	2 (40%)	2 (40%)	1 (20%)	1 (20%)	0	3 (60%)
Tall cell ($n = 5$)	0	3 (60%)	2 (40%)	0	0	1 (20%)	2 (40%)	2 (40%)

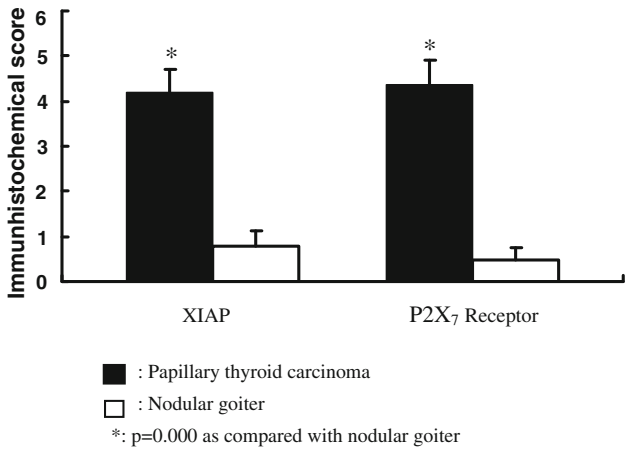

Fig. 2 Immunohistochemical score for XIAP and $P2X_7$ receptor expression in patients with papillary thyroid carcinoma and nodular goiter

Discussion

In the present study, it was demonstrated that the XIAP and the $P2X_7$ receptor expression were associated with lymph node metastasis in PTCs.

Inhibitors of apoptosis (IAPs) are a family of proteins with multiple biological activities that include the inhibition of cell death by binding and suppressing caspase activity and the promotion of survival signaling pathways, which are important for tumor maintenance. The X-linked inhibitor of apoptosis (XIAP) is the most potent caspase inhibitor in the IAP family, playing an important role in apoptotic signaling [19, 20].

Overexpression of XIAP was reported in several human malignancies including PTC [6–8]. Similar results were obtained from the present study, with higher immunohistochemical scores for XIAP observed in PTCs. Furthermore, XIAP expression was also associated with lymph node metastasis. It has also been reported that increased XIAP expression was responsible for tumor recurrence, therapeutic failure and poor prognosis in some, but not all, studies [21–23]. Although still inconclusive, the predictive value of XIAP expression for the invasiveness of PTC needs further investigation.

$P2X_7$ receptor now becomes a focus of increasing attention in cell growth, apoptosis, cytokine secretion, and neoplastic transformation [12, 24]. The $P2X_7$ receptor was weakly expressed in normal human primary thyrocytes [13]; however, it was overexpressed in cell lines of human thyroid cancer and thyroid specimens from PTC patients [16]. Similarly, enhanced expression of the $P2X_7$ receptor was observed in our PTC histological samples, and it was also predominantly expressed in patients with lymph node metastasis.

Although the expression of XIAP and that of the $P2X_7$ receptor in PTC are closely related to each other, the underlying mechanism for this relationship is not clear. XIAP, as a strong endogenous caspase inhibitor, can block the apoptotic pathway [5], while activation of the $P2X_7$ receptor may also have an anti-apoptotic or even growth-promoting effect [25, 26]. It was demonstrated that the

Table 3 Correlations between XIAP and $P2X_7$ receptor expression with clinicopathological features of PTCs

	XIAP			$P2X_7$ receptor		
	Negative ($n = 19$)	Positive ($n = 24$)	P value	Negative ($n = 17$)	Positive ($n = 26$)	P value
Age at diagnosis (years)	46.7 ± 3.2	44.9 ± 1.9	0.619	48.1 ± 3.5	44.1 ± 1.9	0.271
Male/Female	6/13	7/17	0.864	9/8	4/22	0.009
Tumor size (cm)	1.04 ± 0.14	1.20 ± 0.09	0.317	1.16 ± 0.17	1.11 ± 0.08	0.792
Multifocality			0.708			0.350
No	10 (52.6%)	14 (58.3%)		8 (47.1%)	16 (61.5%)	
Yes	9 (47.4%)	10 (41.7%)		9 (52.9%)	10 (38.5%)	
Capsule infiltration			0.066			0.480
No	14 (73.7%)	11 (45.8%)		11 (64.7%)	14 (53.8%)	
Yes	5 (26.3%)	13 (54.2%)		6 (35.3%)	12 (46.2%)	
LN metastases			0.009			0.007
No	14 (73.7%)	8 (33.3%)		13 (76.5%)	9 (34.6%)	
Yes	5 (26.3%)	16 (66.7%)		4 (23.5%)	17 (65.4%)	
TNM stage			0.115			0.272
I/II	14 (85.7%)	12 (58.3%)		12 (70.5%)	14 (53.8%)	
III/IV	5 (14.3%)	12 (41.7%)		5 (29.5%)	12 (46.2%)	
Histological variant			0.599			0.464
Classic	9 (47.4%)	12 (50%)		7 (41.2%)	14 (53.8%)	
Follicular	6 (31.6%)	6 (25%)		6 (35.3%)	6 (23.1%)	
Capsulated	3 (15.8%)	2 (8.3%)		1 (5.9%)	4 (15.4%)	
Tall cell	1 (5.2%)	4 (16.7%)		3 (17.6%)	2 (7.7%)	

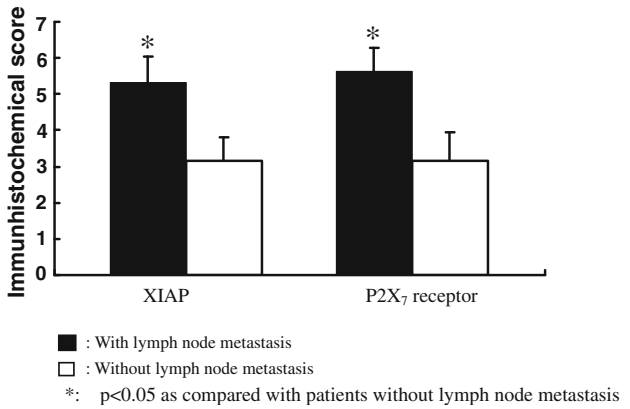

Fig. 3 Immunohistochemical scores for XIAP and $P2X_7$ receptor expression in PTC patients with and without lymph node metastasis

$P2X_7$ receptor played a specific role in mediating the release of IL-6 and substance P, the trophic factors for human thyroid cancer cells and neuroblastoma cells, respectively [16, 27].

Expression of the $P2X_7$ receptor in various cancers has been extensively investigated, but its association with the clinicopathological features of PTCs has not been described. Age at diagnosis, gender, tumor size, multifocality, and histologic variants of PTC are traditional prognostic factors for thyroid carcinoma [2]. In our study, higher rates of $P2X_7$ receptor expression were observed in female patients and in those with lymph node metastasis. The clinical significance of $P2X_7$ receptor overexpression in female PTC patients is not clear. Male gender was generally considered to be a poor prognostic factor, but it was recently demonstrated that gender has no influence on recurrence or survival in a series of 950 PTC patients [28]. In the current study, $P2X_7$ receptor expression, tumor size, and capsular invasion were significantly associated with lymph node metastasis. Our data also showed that in PTC patients with lymph node metastasis, the staining scores for XIAP and the $P2X_7$ receptor were significantly higher than in those without lymph node involvement, and that these patients also had a marginal significant larger tumor size. Although lymph node metastasis does not seem to affect mortality, it is seen in 20–90% of PTCs at the time of presentation and even in micro-PTCs [29], and it is the most important risk factor for PTC persistence and recurrence [2]. The role of XIAP and the $P2X_7$ receptor overexpression in lymph node metastases of PTC need to be further explored.

The biological significance of the $P2X_7$ receptor in tumorigenesis and development is now under thorough investigation and heated discussion, but still remains unknown. An enhanced or decreased expression of the $P2X_7$ receptor has been reported in various kinds of

malignant cell lines or tissue specimens [30]. The $P2X_7$ receptor is well known for its cytotoxic, pro-inflammatory, and pro-apoptotic effects [31]. However, the anti-apoptotic or even growth-promoting activity of the $P2X_7$ receptor has recently been proposed [25, 26, 30]. Overexpression of the $P2X_7$ receptor was a consistent finding in previous two studies performed in PTCs [16, 30] as well as in ours. The massive increases in intracellular Ca^{2+} and the secretion of IL-6, as well as the elevated intracellular ATP concentration mediated by the $P2X_7$ receptor, may be responsible for thyroid cancer cell growth [16]. Genetic variations in the $P2X_7$ receptor gene might alter its function [32] and have been shown to be related to some clinicopathological parameters of PTC, such as tumor size, TNM staging, and follicular subtype [33]. However, an immunohistochemical analysis of the $P2X_7$ receptor was not performed in these studies.

In the present study, the duel-positive expression of XIAP and the $P2X_7$ receptor was associated with a higher risk of lymph node metastasis in PTCs. The underlying mechanism for this observation is not clear. As discussed above, $P2X_7$ receptors are responsible for the release of IL-6 in human thyroid cancer cell lines [16]. It was revealed that the local expression of IL-6 is related to the aggressiveness of PTCs [34]. IL-6 could also increase mRNA and protein levels of XIAP in ovarian cancer cells, promoting chemoresistance by activating multiple signaling pathways including the PI3K/Akt pathway [35]. However, the PI3K/Akt survival pathway is regulated not only by XIAP [36, 37] but also by the $P2X_7$ receptor [38]. Thus, it is important to explore a common pathway, such as the IL-6/XIAP/PI3K/Akt pathway, and to evaluate its direct role in the pathogenesis or biological behavior of PTC.

The expression of XIAP and the $P2X_7$ receptor was not related to other clinicopathological features of PTCs, including their histological subtypes, although nearly half of the classic variants expressed these two markers, possibly due to the relatively larger number of such cases (48.8%, or 21/43) in the present cohort.

There were several limitations in this study. First, it was a cross-sectional study, and the sample size was small. Thus, the question of whether the expression of XIAP or the $P2X_7$ receptor is related to the long-term outcomes of PTC, such as remission and mortality, should be further investigated. Second, the protein and mRNA expression levels of XIAP and the $P2X_7$ receptor were not analyzed in the thyroid tissues. Third, the study was not performed in the metastases of the tumors and the mechanisms by which XIAP and the $P2X_7$ receptor contribute to the aggressiveness of PTCs were not investigated. Finally, since clinical data on thyroid auto-antibodies was not available, the influence of the inflammatory state on the expression of the $P2X_7$ receptor could not be evaluated in the present study. It has been suggested that the $P2X_7$ receptor may involve in the immune response and autoimmune diseases [32, 39]. However, the presence of lymphocytic thyroiditis in papillary thyroid microcarcinomas did not influence the frequency of lymph node metastasis [40].

In summary, our study revealed that XIAP and $P2X_7$ receptor expression were correlated with lymph node metastasis in PTCs, which may support their potential clinical value as novel prognostic markers for papillary thyroid carcinomas. Further studies are required to explore the mechanism underlying the up-regulation of XIAP and $P2X_7$ receptor expression in thyroid cancer and the resulting influences on recurrence and survival in PTC patients.

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