Diagnostic significance of CK19, galectin-3, CD56, TPO and Ki67 expression and BRAF mutation in papillary thyroid carcinoma

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Abstract. The aim of the present study was to examine the rate of BRAF mutation and the expression profiles of CK19, galectin-3, CD56, thyroid peroxidase (TPO) and Ki67 in papillary thyroid carcinoma (PTC) and papillary thyroid micro-carcinoma (PTMC). A total of 246 cases of thyroid disease were collected, including PTC, PTMC, nodular goiter (NG) and Hashimoto thyroiditis (HT). The results revealed that CK19 expression was 116/120 in PTC, 61/64 in PTMC, 2/34 in NG and 1/28 in HT. Galectin-3 positive expression was 115/120 in PTC, 60/64 in PTMC, 6/34 in NG and 4/28 in HT. TPO positive expression was 8/120 in PTC, 1/64 in PTMC, 30/34 in NG and 25/28 in HT. CD56-positive expression was 12/120 in PTC, 3/64 in PTMC, 33/34 in NG and 26/28 in HT. Ki67 labeling index was 2.52±0.46% in PTC (120 cases), 2.62±0.52% in PTMC (64 cases), 2.55±0.44% in NG (34 cases) and 2.58±0.48% in HT (28 cases). BRAF mutation rate was 93/120 in PTC, 47/64 in PTMC, 3/34 in NG and 2/28 in HT. These results suggested that expression patterns of CK19, galectin-3, CD56 and TPO and BRAF mutation exhibit diagnosis value in thyroid disease. However, Ki67-positive rate exhibits no notable diagnosis value in thyroid disease.

Introduction

It is well-known that the incidence of papillary thyroid carcinoma (PTC) and papillary thyroid micro-carcinoma (PTMC) is increasing each year worldwide: An ~120.85% increase in PTMC incidence and an ~58.1% increase in PTC incidence was demonstrated between 1990-2015 (1-10). PTC and PTMC are the most common types of thyroid malignancies (11-21). However, distinguishing PTC and PTMC from thyroid papillary hyperplasia is challenging due to tumor heterogeneity (22-27). Occasionally, cases of papillary thyroid hyperplasia, in particular solitary nodules with papillary change, are difficult to distinguish from PTMC (22-27). Papillary formation is frequently observed in thyroid disease (benign or malignant), but the treatment plans differ considerably (1-5,7-10). In China, nodular goiter (NG) is a common disease; it was demonstrated that the incidence of NG was 5.0-10.0% from 1990 to 2011, and it was 4 times higher in females compared with in males in 2011 (11-16). It can be difficult to distinguish papillary hyperplasia in PTMC from papillary hyperplasia nodules of NG (11-16). Therefore, it was proposed that the increasing incidence of PTC and PTMC may partly be due to misdiagnosis. Thus, in the present study, the diagnosis of cases in Wuhan Puai Hospital (Wuhan, China) and Jiangda Pathology Institute (Wuhan, China) was reviewed. The expression profiles of CK19, galectin-3, CD56 and thyroid peroxidase (TPO), as well as BRAF mutation, were used to distinguish between benign and malignant papillary formation. Although numerous previous studies have reported that CK19, galectin-3, CD56 and TPO expression, as well as BRAF mutation, are markers for PTC and PTMC diagnosis (17-49), few studies have considered them together and studied their value in the diagnosis of PTC and PTMC. In the present study, the diagnostic value of CK19, galectin-3, CD56, TPO and Ki67 expression and BRAF mutation in PTC and PTMC were investigated.

Materials and methods

Patients. A total of 246 thyroid samples from the Department of Pathology, Wuhan Puai Hospital (Wuhan, China) and the Department of Histopathology, Jiangda Pathology Institute, Jianghan University (Wuhan, China) obtained during biopsy between January 2010 and March 2016 were used in the present study. The samples included 120 cases of PTC (33 males, 87 females; mean age, 52.3 years; age range, 17-72 years), 64 cases of PTMC (22 males, 42 females; mean age, 50.7 years; age range, 21-69 years) and 62 non-malignant cases (18 males,

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44 females; mean age, 47.5 years; age range, 28-65 years). The 62 non-malignant cases included 34 cases of NG and 28 cases of Hashimoto thyroiditis (HT). The diagnosis of PTC and PTMC was based on characteristic cytological features, including nuclear irregularity, nuclear groove and pseudoinclusions and psammoma bodies (Fig. 1A) (10), and immunohistochemistry results, including CK19^{+} and galectin-3^{+}, which was performed using the immunohistochemistry methods described below (28). All resected specimens were fixed in 10% neutral buffered formalin (pH 7.4) at room temperature for 24 h, embedded in paraffin, and cut into 4-μm sections. Informed consent was obtained from all patients, and all experiments were approved by the Ethics Committee of Jianghan University.

Reagents. Anti-CK19 (keratin 19) mouse monoclonal antibody (cat. no. TA500212), anti-TPO rabbit polyclonal antibody (cat. no. TA323628), rabbit polyclonal anti-Ki67 antibody (cat. no. TA314198), rabbit polyclonal anti-galectin-3 antibody anti-galectin-3 antibody (cat. no. AP54962SU-N), and mouse monoclonal anti-CD56 antibody (cat. no. TA353710) were purchased from OriGene Technologies, Inc. (Beijing, China). The biotin-streptavidin horseradish peroxidase detection systems (SP test kit; cat. no. SP-9000) and diaminobenzidine (DAB) colorization test kit (cat. no. ZLI-9017) were purchased from Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd. (Beijing, China). The TIANamp FFPE DNA kit (DP331) was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). The Human BRAF V600E gene mutation detection kit was from Wuhan YZY Biopharma Co., Ltd. (Wuhan, China).

Histology and immunohistochemistry. Standard hematoxylin and eosin staining was performed on 4-μm paraffin sections of above specimens: Tissues that were fixed in 10% neutral buffered formalin for 12 h at room temperature, the processed and embedded in paraffin wax. Sections measuring 4-μm thickness were stained in 0.5% hematoxylin staining solution (1 g haematoxylin, 15 g aluminum potassium sulfate, 10 ml absolute ethyl alcohol and 200 ml distilled water) for 10 min at room temperature. The slides were placed under running tap water at room temperature for at least 10 min following 1% hydrochloric acid alcohol differentiation for 1 min at room temperature. Then, the samples were stained in working 1% eosin Y staining solution (1 g eosin Y, 100 ml distilled water and 1 drop glacial acetic acid) for 1 min at room temperature and dehydrated at room temperature. Then, the slides were viewed with a microscope subsequent to the addition of a coverslip. Immunostaining was performed using following appropriate antibodies on 4-μm tumor sections using a 'two-step' method. The tissue slides were deparaffinized twice with 100% xylene for 15 min at 37˚C and rehydrated gradually in an ethanol series (100, 95 and 80% ethanol) for 10 min at room temperature. The endogenous peroxidase activity was inhibited by incubation for 10 min at room temperature in a 3% hydrogen peroxide/methanol buffer. Antigen retrieval was performed by immersing the slides in an ethylenediamine tetraacetic acid buffer (pH 8.0), followed by boiling in a water bath at 100˚C for 10 min. The slides were rinsed in PBS and subsequently incubated with anti-CK19 (keratin 19) mouse monoclonal antibody (dilution 1:100; cat. no. TA500212), rabbit polyclonal anti-galectin-3 antibody (dilution 1:100; cat. no. AP54962SU-N), anti-TPO rabbit polyclonal antibody (dilution 1:100; cat. no. TA323628), mouse monoclonal anti-CD56 antibody (dilution 1:100; cat. no. TA353710) or rabbit polyclonal anti-Ki67 antibody (dilution 1:100; cat. no. TA314198) (all from OriGene Technologies, Inc.) overnight at 4˚C in a humidified chamber. Following this incubation, the slides were washed three times with PBS containing 0.05% Tween-20. The slides were then incubated with biotin-labeled goat anti-mouse/Rabbit IgG secondary antibodies antibody at a ready-to-use dilution (cat. no. SP-9000; Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd.) for 1 h at 37˚C. The slides were then washed three times and developed with DAB chromogen. The slides were then washed gently with tap water, prior to counterstaining with hematoxylin at room temperature for 10 min, and then were observed by light microscope (magnification, x400, BX51; Olympus Corporation, Tokyo, Japan).

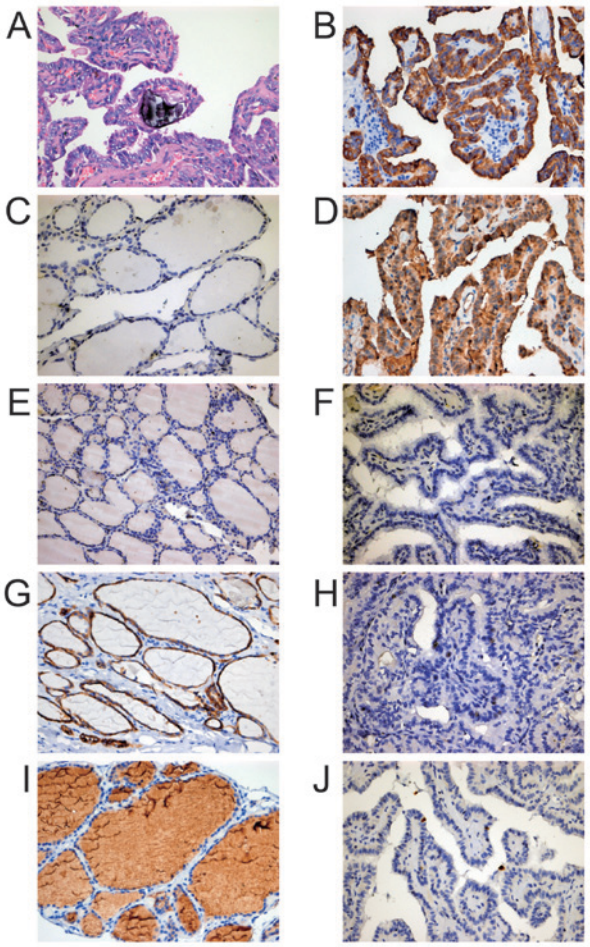

Figure 1. Immunohistochemical staining and histological morphology of thyroid samples. (A) Hematoxylin and eosin staining of PTC. Magnification, x400. (B) Representative staining for CK19 in PTC. (C) Negative expression of CK19 in NG. (D) Representative staining for galectin-3 in PTC. (E) Negative expression of galectin-3 in NG. (F) Negative expression of TPO in PTC. (G) Representative staining for TPO in NG. (H) Negative expression of CD56 in PTC. (I) Representative staining for CD56 in NG. (J) Representative staining for Ki67 in PTC. PTC, papillary thyroid carcinoma; NG, nodular goiter; TPO, thyroid peroxidase.

Evaluation of immunohistochemical staining. For staining of CK19, galectin-3, CD56 and TPO, the signals were considered positive when immunoreactivity was clearly observed in the cell membrane and/or cytoplasm by light microscope

Table I. CK19 expression.

Groups	CK19 expression		χ^2	P-value
	Positive	Negative		
PTC vs. NG			116.8	P<0.001
PTC	116	4		
NG	2	32		
PTC vs. HT			113.3	P<0.001
PTC	116	4		
HT	1	27		
PTC vs. PTMC			0.028	P=0.957
PTC	116	4		
PTMC	61	3		
PTMC vs. NG			77.5	P<0.001
PTMC	61	3		
NG	2	32		
PTMC vs. HT			70.5	P<0.001
PTMC	61	3		
HT	1	27		
NG vs. HT			0.298	P=0.863
NG	2	32		
HT	1	27		

PTC, papillary thyroid carcinoma; PTMC, papillary thyroid micro-carcinoma; NG, nodular goiter; HT, Hashimoto thyroiditis.

(magnification, x400). It was scored manually, and all fields of view that included the tumor were examined. For each antibody, with the exception of Ki67, no staining or weak staining in <10% of the cells was scored as negative, and staining in ≥10% of cells was scored as positive. The known positive or negative controls were taken at the same time; the judgement of the staining was compared with the control. If the staining was the same as the negative control or only no more than 10% cells were weakly stained, it was scored as negative. While, if the staining was the same as the positive control or >10% cells were stained, it was scored as negative. The individual cells were counted. The proportion was the number of positive cells divided by the total number of cells. For Ki67 staining, the Ki67 staining in the cell nuclei was examined in ~500 cells manually and indicated as a percentage of the total nuclei. All above experiments were scored or examined by at least two pathologists.

DNA isolation from formalin-fixed, paraffin-embedded (FFPE) tissue sections. DNA was isolated from 5-8 FFPE tissue sections from each patient using the TIANamp FFPE DNA kit, according to the manufacturer's instructions. The DNA was stored at -20˚C.

BRAF mutation detection. BRAF mutation was detected using the Human BRAF V600E gene mutation detection kit (Wuhan YZY Biopharma Co., Ltd.) with TaqMan probe, according to the manufacturer's instructions. The all primers used were included as part of the Human BRAF V600E gene mutation detection kit (Wuhan YZY Biopharma Co., Ltd.). DNA amplification was performed with the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a total volume of 25 μl. The thermocycling conditions were as follows: An initial UNG treatment for 10 min at 37˚C and pre-degeneration for 5 min at 95˚C, then 40 cycles of denaturation at 95˚C for 15 sec, an annealing step at 60˚C for 60 sec. After the reaction, according to the amplification curve, suitable fluorescence thresholds (threshold defined in the amplification curve of logarithmic exponential growth) were identified and Cq values were calculated (29).

Statistical analysis. Statistical analysis was performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. The χ^2 test and Fisher's exact test were used to compare immunohistochemistry results between the experimental groups and the control groups. A one-way analysis of variance and Dunnett's test was used to compare Ki67 immunohistochemistry results between groups. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

CK19 expression. CK19 staining was detected predominantly in the cytoplasm in PTC (Fig. 1B), but was absent in NG (Fig. 1C). Positive staining of CK19 was detected in 116/120 cases of PTC, 61/64 in PTMC, 2/34 in NG and 1/28 in HT (Table I). CK19 expression was significantly

Table II. Galectin-3 expression.

Groups	Galectin-3 expression		χ^2	P-value
	Positive	Negative		
PTC vs. NG			91.6	P<0.001
PTC	115	5		
NG	6	28		
PTC vs. HT			90.7	P<0.001
PTC	115	5		
HT	4	24		
PTC vs. PTMC			0.07	P>0.791
PTC	115	5		
PTMC	60	4		
PTMC vs. NG			55.1	P<0.001
PTMC	60	4		
NG	6	28		
PTMC vs. HT			54.4	P<0.001
PTMC	60	4		
HT	4	24		
NG vs. HT			0.13	P>0.72
NG	6	28		
HT	4	24		

PTC, papillary thyroid carcinoma; PTMC, papillary thyroid micro-carcinoma; NG, nodular goiter; HT, Hashimoto thyroiditis.

more common in PTC compared with NG (P<0.001), PTC compared with HT (P<0.001), PTMC compared with NG (P<0.001) and PTMC compared with HT (P<0.001; Table I). However, no significant differences in CK19 expression were observed between PTC and PTMC, or between NG and HT (Table I).

Galectin-3 expression. Galectin-3 expression was detected predominantly in the cytoplasm and nucleus in PTC (Fig. 1D), but was absent in NG (Fig. 1E). Galectin-3-positive staining was detected in 115/120 cases of PTC, 60/64 of PTMC, 6/34 of NG and 4/28 of HT. Galectin-3 expression was significantly more common in PTC compared with NG (P<0.001), PTC compared with HT (P<0.001), PTMC compared with NG (P<0.001) and PTMC compared with HT (P<0.001; Table II). No significant differences in galectin-3 expression were observed between PTC and PTMC, or between NG and HT (Table II).

TPO expression. TPO expression was detected predominantly in the plasma membrane in PTC (Fig. 1F), but was absent in NG (Fig. 1G). TPO-positive staining was detected in 8/120 cases of PTC, 1/64 in PTMC, 30/34 in NG and 25/28 in HT. TPO expression was significantly less common in PTC compared with NG (P<0.001), PTC compared with HT (P<0.001), PTMC compared with NG (P<0.001) and PTMC compared with HT (P<0.001; Table III). However, no significant differences were observed between PTC and PTMC, or between NG and HT (Table III).

CD56 expression. CD56 expression was detected predominantly in the cytoplasm in PTC (Fig. 1H), but was absent in NG (Fig. 1I). CD56 positive staining was detected in 12/120 cases of PTC, 3/64 in PTMC, 33/34 in NG and 26/28 in HT. CD56 expression level was significantly less common in PTC compared with NG (P<0.001), PTC compared with HT (P<0.001), PTMC compared with NG (P<0.001) and PTMC compared with HT (P<0.001; Table IV). No significant differences in CD56 expression were observed between PTC and PTMC, or between NG and HT (Table IV).

Ki67 expression. Ki67 expression was detected predominantly in the cell nucleus (Fig. 1J). Ki67-positive index was 2.52±0.46% in PTC (120 cases), 2.62±0.52% in PTMC (64 cases), 2.55±0.44% in NG (34 cases) and 2.58±0.48% in HT (28 cases). Ki67-positive index was not significantly different between PTC and NG, PTC and HT, PTC and PTMC, PTMC and NG, PTMC and HT or NG and HT (Table V).

BRAF mutation rate. The BRAF mutation rate was identified to be 77.5% (93/120) in PTC, 73.4% (47/64) in PTMC, 8.8% (3/34) in NG and 7.1% (2/28) in HT. The BRAF mutation rate was significantly higher in PTC compared with NG (P<0.001), PTC compared with HT (P<0.001), PTMC compared with NG (P<0.001) and PTMC compared with HT (P<0.001; Table VI). However, no significant differences in BRAF mutation rate were observed between PTC and PTMC, or between NG and HT (Table VI).

Table III. TPO expression.

Groups	TPO expression		χ^2	P-value
	Positive	Negative		
PTC vs. NG			90.5	P<0.001
PTC	8	112		
NG	30	4		
PTC vs. HT			84.7	P<0.001
PTC	8	112		
HT	25	3		
PTC vs. PTMC			1.36	P>0.242
PTC	8	112		
PTMC	1	63		
PTMC vs. NG			73.2	P<0.001
PTMC	1	63		
NG	30	4		
PTMC vs. HT			69.7	P<0.001
PTMC	1	63		
HT	25	3		
NG vs. HT			0.07	P>0.785
NG	30	4		
HT	25	3		

PTC, papillary thyroid carcinoma; PTMC, papillary thyroid micro-carcinoma; NG, nodular goiter; HT, Hashimoto thyroiditis; TPO, thyroid peroxidase.

Table IV. CD56 expression.

Groups	CD56 expression		χ^2	P-value
	Positive	Negative		
PTC vs. NG			92.9	P<0.001
PTC	12	108		
NG	33	1		
PTC vs. HT			77.3	P<0.001
PTC	12	108		
HT	26	2		
PTC vs. PTMC			0.97	P>0.331
PTC	12	108		
PTMC	3	61		
PTMC vs. NG			77.6	P<0.001
PTMC	3	61		
NG	33	1		
PTMC vs. HT			66.1	P<0.001
PTMC	3	61		
HT	26	2		
NG vs. HT			0.03	P>0.863
NG	33	1		
HT	26	2		

PTC, papillary thyroid carcinoma; PTMC, papillary thyroid micro-carcinoma; NG, nodular goiter; HT, Hashimoto thyroiditis.

Table V. Ki67 expression.

	Ki67, %			
Groups	Mean	SD	F-value	P-value
PTC	2.52	0.46	0.521	P=0.669
PTMC	2.62	0.52		
NG	2.55	0.44		
HT	2.58	0.48		

PTC, papillary thyroid carcinoma; PTMC, papillary thyroid micro-carcinoma; NG, nodular goiter; HT, Hashimoto thyroiditis; SD, standard deviation.

Table VI. Mutation rate of BRAF.

	BRAF			
Groups	Mutation	No mutation	χ^2	P-value
PTC vs. NG			50.3	P<0.001
PTC	93	27		
NG	3	31		
PTC vs. HT			48.9	P<0.001
PTC	93	27		
HT	2	26		
PTC vs. PTMC			0.19	P>0.664
PTC	93	27		
PTMC	47	17		
PTMC vs. NG			34.5	P<0.001
PTMC	47	17		
NG	3	31		
PTMC vs. HT			31.7	P<0.001
PTMC	47	17		
HT	2	26		
NG vs. HT			0.05	P>0.821
NG	3	31		
HT	2	26		

PTC, papillary thyroid carcinoma; PTMC, papillary thyroid micro-carcinoma; NG, nodular goiter; HT, Hashimoto thyroiditis.

Discussion

Papillary formation is often observed in benign and malignant thyroid diseases (30-32), meaning that it is difficult to distinguish between benign and malignant lesions (30-32). The pathological morphological characteristics, such as papillary architecture with typically complex branching, nuclear features including nuclear irregularity, nuclear groove and pseudoinclusion and psammoma bodies, are widely used in the diagnosis of thyroid diseases. However, to distinguish PTC from thyroid papillary hyperplasia and solitary nodules with papillary change is challenging due to tumor heterogeneity. Thus, immunohistochemistry is also essential in the diagnosis (30-32).

CK19 is a member of the keratin family that is an intermediate filament protein in epithelial cells (33). CK19 is highly expressed in papillary carcinoma, but not in benign follicular nodules, which is useful in diagnosis (33). In previous studies, the CK19 positive rate was reported to be 84-100% in PTC, 59-84% in PTMC, 26.80% in NG and 20% in HT (33-38). In the present study, CK19 expression was detected in 96.7% (116/120) of PTC, 95.3% (61/64) of PTMC, 5.9% (2/34) of NG and 3.6% (1/28) of HT. Thus, CK19 expression is indicated to be valuable in the diagnosis of thyroid carcinoma.

Galectin-3 is a member of the β-galactoside-binding mammalian family of lectins that serves functions in metastasis, angiogenesis, proliferation and apoptosis of multiple tumor types, including thyroid carcinoma (39). In previous

studies, the galectin-3 expression rate was reported to be 64.86-100% in PTC, 45.7-98% in PTMC, 0-52.6% in NG and 19-38.9% in HT (33,38,40-45). In the present study, galectin-3 positive rate was 95.8% (115/120) in PTC, 93.8% (60/64) of PTMC, 17.6% (6/34) of NG and 14.3% (4/28) in HT. Thus, galectin-3 has the potential to be used as a diagnostic marker for thyroid cancer.

TPO is a membrane-bound protein essential for the production of thyroid hormones. It catalyzes the iodination and coupling of tyrosyl residues to thyroglobulin to form thyroid hormones T3 and T4 (46). TPO expression is used as a thyroid differentiation marker (46) and is decreased in thyroid carcinoma (46). In previous studies, TPO expression rate was reported to be 3-29.4% in PTC, 9.4% in PTMC and 100% in benign thyroid disease (34,46-48). In the present study, TPO expression was 6.7% (8/120) in PTC, 1.6% (1/64) in PTMC, 88.2% (30/34) in NG and 89.3% (25/28) in HT. These results indicated that the downregulation of TPO expression may be used for diagnosis of thyroid carcinoma.

CD56 is a member of the immunoglobulin superfamily that has five extracellular immunoglobulin domains and two fibronectin type III domains (44,45,49,50). CD56 is expressed in thyroid follicular cells (43). Downregulation or lack of CD56 expression is often observed in PTC, thyroid follicular carcinoma and thyroid anaplastic carcinoma (49). In previous studies, CD56 expression rate was reported to be 0-24.3% in PTC, 0-60% in PTMC and 91.7-100% in benign thyroid diseases (43,45,49,50). In the present study, CD56 expression was 10% (12/120) in PTC, 4.7% (3/64) in PTMC, 97.1% (33/34) in NG and 92.8% (26/28) in HT. These results indicated that the downregulation of CD56 has value in the diagnosis of thyroid tumors.

Ki67 is a widely used marker of cell proliferation and is used for differential diagnosis between malignant and benign human neoplasms (32). However, no significant difference in Ki67 labeling index was observed between malignant and benign thyroid neoplasms in a previous study (32). Ki67 labeling index was previously reported to be 0-20% in PTC, 0-20% in PTMC and 0-1% in benign thyroid disease (37,51-53). It was proposed that Ki67 may be used as a marker for cell proliferation and prognosis in PTC (54). However, in the present study, Ki67 labeling index was 2.52±0.46% in PTC (120 cases), 2.62±0.52% in PTMC (64 cases), 2.55±0.44% in NG (34 cases) and 2.58±0.48% in HT (28 cases). Thus, Ki67 labeling index has little value in the diagnosis of malignant and benign thyroid neoplasms. This is likely to be due the fact that PTC and PTMC are low proliferation tumors (37,51-53).

BRAF belongs to the family of RAF proteins and is an intracellular effector of the mitogen-activated protein kinase-signaling cascade (55). The BRAF V600E mutation is a molecular marker that has been reported in thyroid carcinoma (56-64). BRAF mutation rate was previously reported to be 29-83% in PTC, 37.5-77% in PTMC and 0.2-5.7% in benign thyroid disease (19,33-35,65-68). In the present study, BRAF mutation rate was 77.5% (93/120) in PTC, 73.4% (47/64) in PTMC, 8.8% (3/34) in NG and 7.1% (2/28) in HT. In addition, the present results suggested that BRAF mutation was associated with the expression of CK19 and galectin-3 in PTC and PTMC. These results suggested that BRAF mutation may be valuable in the diagnosis of thyroid carcinoma.

The incidence of NG is high in China compared with papillary formation (11-16). However, it can be difficult to distinguish NG from malignant papillary formation (11-16). The present study presents evidence to support the view that analysis of expression patterns of CK19, galectin-3, TPO and CD56, together with BRAF mutation, will be useful in the diagnosis of thyroid carcinoma. It was demonstrated that CK19 and galectin-3 were often positively expressed, and TPO and CD56 were often negatively expressed, in PTC and PTMC, and it was revealed that the BRAF mutation rate was high in PTC and PTMC. However, not all PTC and PTMC cases indicated that CK19 and galectin-3 were completely positively expressed. Therefore, these negative cases require additional analysis.

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