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 FTMC 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 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 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 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 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 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 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 thyroid peroxidase (TPO), as well as BRAF mutation, were used to distinguish between benign and malignant papillary formation.
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 pseudo-inclusions 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. APS4962SU-N), and mouse monoclonal anti-CD56 antibody (cat. no. TA353710) were purchased from OriGene Technologies, Inc. (Beijing, China). The Human BRAF V600E gene mutation detection kit was from Wuhan YZY Biopharma Co., Ltd. (Wuhan, China). The TIANamp FFPE DNA kit (DP331) was purchased from Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd. (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).

**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.
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 \(\geq10\%\) 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.). 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 \(\chi^2\) 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

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

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, 25/28 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, 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 II. Galectin-3 expression.

| Groups          | Positive | Negative | $\chi^2$ | P-value |
|-----------------|----------|----------|----------|----------|
| 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.
Table III. TPO expression.

| Groups       | Positive | Negative | \( \chi^2 \) | P-value |
|--------------|----------|----------|---------------|---------|
| 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       | Positive | Negative | \( \chi^2 \) | P-value |
|--------------|----------|----------|---------------|---------|
| 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.
**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...
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, galecin-3, TPO and CD56, together with BRAF mutation, will be useful in the diagnosis of thyroid carcinoma. It was demonstrated that CK19 and galecin-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 galecin-3 were completely positively expressed. Therefore, these negative cases require additional analysis.

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