Significance of interstitial fibrosis and p16 in papillary thyroid carcinoma

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Abstract. We enrolled 264 patients with papillary thyroid carcinoma (PTC). We performed immunohistochemical detection of p16 and determined the degree of interstitial fibrosis (IF). The expression of p16 was associated with pathological tumor–node–metastasis (pTNM) stage and age (\(p < 0.05\)). The overall survival was longer in p16-negative patients (195.73 vs. 181.78 months, \(p = 0.007\)). p16 was significantly related to the degree of IF (\(r = 0.130, p = 0.035\)). PTC patients with no or mild fibrosis tended to have a larger tumor (\(p = 0.045\)). The degree of fibrosis was related to the proportion of papillary structure components (\(p = 0.025\)). Univariate and multivariate survival analyses showed that relapse-free survival was longer in patients with moderate/severe IF (\(p < 0.05\)). In summary, p16 was correlated with prognosis and IF of PTC. Patients with moderate/severe IF tend to have better prognosis in RFS.

Key words: Papillary thyroid carcinoma (PTC), Interstitial fibrosis, P16, Prognosis

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PAPILLARY THYROID CARCINOMA (PTC) is a common malignancy of the thyroid, occurring more frequently in women [1]. Interstitial fibrosis (IF) is a clinicopathological feature and diagnostic indicator for PTC [2]. It has been shown previously that expression of \(p16INK4A\) gene is significantly increased in type II alveolar epithelial cells in patients with idiopathic pulmonary fibrosis (IPF) and positively correlated with the severity of IPF [3]. The aging of alveolar epithelial cells contributes to the development of IPF [3]. It has been reported that p16 is involved in myocardial fibrosis [4]. However, the relationship between IF and p16 in PTC remains unclear. This study investigated the clinicopathological significance of IF, and the relationship of p16 with the development of IF in PTC, providing new insights into the treatment of PTC.

Materials and Methods

Patients

We collected the data of 264 PTC patients who underwent surgical resection from July 2005 to November 2017 in the Department of Pathology of our hospital. There were 178 women (67.4%) and 86 men (32.6%). The mean age of the patients was 43.7 years (range, 7–76 years), with a median of 44 years. There were 210 patients (79.5%) aged 7–54 years and 54 (20.5%) aged 55–76 years. There were two patients (0.8%) aged ≤10 years, 10 (3.8%) aged 11–20 years, 29 (11.0%) aged 21–30 years, 64 (24.2%) aged 31–40 years, 80 (30.3%) aged 41–50 years, 56 (21.2%) aged 51–60 years, 18 (6.8%) aged 61–70 years and five (1.9%) aged ≥71 years.

Normal adjacent tissue (NAT) was collected from 38 patients (aged 21–72 years) as controls. Five of these patients had lymphocytic thyroiditis, two had Hashimoto’s thyroiditis, and five had nodular goiter.

The maximum tumor diameter was \(\leq 1\) cm in 79 patients (29.9%); \(>1\) but \(\leq 2\) cm in 100 patients (37.9%), \(>2\) but \(\leq 4\) cm in 67 patients (25.4%), and \(>4\) cm in 18 patients (6.8%). According to the 8th edition of the American Joint Committee on Cancer (AJCC) pathological tumor–node–metastasis (pTNM) classification system, 231 cases
(87.5%) were stage I, 28 (10.6%) stage II, three (1.1%) stage III, and two (0.8%) stage IV. There were 224 cases of classical type; 19 of mixed classical and another subtype (16 mixed follicular and classical type in different foci, two partial Warthin-tumor like type, and one local squamous cell carcinoma); and 15 cases of pure follicular subtype. For statistical convenience, the other six cases were placed in other subtypes (two eosinophilic subtype, two tall cell type, one columnar cell type, and one cribriform-adenocystic arrangement).

We followed up 200 patients by telephone, and 64 patients were lost to follow-up. The deadline for follow-up was April 30, 2022. Recurrence/metastasis occurred after surgery in 64 patients (32.0%, 64/200). Fourteen patients died of PTC at 5–158 months (median 94.5 months; mean 83.6 months). The overall survival (OS) of 200 patients was 5–201 months. Relapse-free survival (RFS) was 1–198 months.

Sample collection and treatment

After the operation, the thyroid specimens were immediately placed in 10% formalin or neutral formalin and fixed for 12–24 h. Then, the pathological doctors took the tumour specimen into small pieces and put them into the automatic dehydrator for dehydration and transparent. The tissue was embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin (HE). Neutral gum was dripped, put on the cover glass, and dried in air.

Immunohistochemical examination

Both the p16 monoclonal antibody and the universal secondary antibody were purchased from ZSQB-Bio (Beijing, China). The EnVision method was used for immunohistochemistry. None of the antibodies needed to be diluted. After high-temperature and high-pressure citrate buffer retrieval, the slides were incubated in 3% H2O2 deionized water at room temperature to eliminate endogenous peroxidase activity. After the p16 monoclonal antibody was added, the mixture was incubated overnight in the refrigerator at 4°C. The p16 antibody was washed out with phosphate buffer saline (PBS), and 1–3 drops of secondary antibody were added, and the mixture was incubated. After color development with diaminobenzidine (DAB) and restaining with hematoxylin for 3–5 min, the mixture was sealed with neutral gum and dried. p16 was expressed in both the nucleus and cytoplasm of tumor cells, and ≥10% positive tumor cells were considered positive [5]. Weak positive staining was considered negative.

Degree of IF in cancer tissues

Observing under a microscope, the degree of fibrosis was determined in accordance with previous classification [6-9]: no/mild IF often had a fibrosis area of up to one tenth of the entire tumor area (up to one seventh in a few cases). Fibrosis area >15% was regarded as moderate/severe IF. Loose papillary axis with edema was not defined as fibrosis, whereas the papillary axis showing obvious fibrosis (patchy and diffuse) was defined as IF.

Statistical analysis

SPSS 17.0 statistical software was used. Both Chi-square test and Spearman’s correlation analysis were used. Kaplan–Meier survival function was used for univariate survival analysis, during which the log-rank p values were calculated. A Cox regression model was used for multivariate survival analysis; we used forward LR (Linear regression, LR; forward algorithm based on partial maximum likelihood estimation). p < 0.05 was considered statistically significant.

Results

Expression of p16

The positive rate of p16 was 35.2% (93/264) in PTC, which was significantly higher than that in NAT (2.6%, 1/38) (p < 0.001) (Fig. 1). The positive rate of p16
Increased with the degree of IF. p16 was positively expressed in some mesenchymal fibrocytes or fibroblasts of PTC (Fig. 1A, B). p16 was positively correlated with pTNM stage ($r = 0.225$, $p < 0.001$) and age ($r = 0.157$, $p = 0.011$). The relationship of p16 expression with other clinicopathological parameters lacked significance ($p > 0.05$) except IF.

Univariate survival analysis showed that p16-negative patients had longer OS than p16-positive patients had (195.73 vs. 181.78 months, $p = 0.007$) (Fig. 2), but there was no significant difference in RFS (148.29 vs. 145.87 months, $p = 0.448$).

**Analysis of degree of IF**

IF was no/mild in 108 cases (40.9%) (Fig. 3A–C) and moderate/severe in 156 (59.1%) (Fig. 3D–F). The relationships between the degree of IF in cancer tissues and other clinicopathological parameters are shown in Table 1. Univariate survival analysis showed that RFS was significantly longer in the moderate/severe IF group than in the no/mild IF group (158.37 vs. 129.23 months, $p = 0.020$, Fig. 4). OS was not significant different between the moderate/severe IF group and the no/mild IF group ($p = 0.707$).

**Multivariate Cox regression analysis of degree of IF, p16 and clinicopathological parameters**

IF, p16 and clinicopathological parameters (Excluding recurrence and metastasis. If it was included, the coefficient did not converge) were included in the multivariate Cox regression analysis, which showed that only pTNM stage and gender affected OS. pTNM stage, lymph node metastasis and IF affected RFS (Table 2), and moderate/severe IF was a protective factor for RFS.

**Discussion**

We found that the positive rate of p16 increased with the degree of IF, and there was a positive correlation; in addition, p16 expression was detected in some interstitial fibrocytes or fibroblasts.
fibrocytes or fibroblasts. It has been reported that p16 expression is higher in skin hyperplastic scars than in normal tissues and keloid [10]. Expression of p16 and p21 and the activity of aging-related β-galactosidase are increased in alveolar epithelium of IPF [3]. Expression of these three proteins also significantly increases in the perivascular fibrotic areas of aortic arch stenosis lesions [4]. The increased p16 expression is related to the fibrosis

Table 1  Comparison of degree of interstitial fibrosis and clinicopathological parameters

| Degree of fibrosis | Chi-square test | Spearman’s correlation |
|--------------------|-----------------|------------------------|
| None/mild          | Moderate/severe |                        |
| Solitary           | 58 (38.7%)      | 92 (61.3%)             | 0.395 | −0.052 | 0.397 |
| ≥2 foci            | 50 (43.9%)      | 64 (56.1%)             |       |        |      |
| TNM stage          |                 |                        |
| I                  | 94 (40.7%)      | 137 (59.3%)            | 0.850 | −0.012 | 0.851 |
| II–IV              | 14 (42.4%)      | 19 (57.6%)             |       |        |      |
| Maximum tumor diameter |               |                        |
| ≤1 cm              | 25 (31.6%)      | 54 (68.4%)             | 0.045 | −0.123 | 0.046 |
| >1 cm              | 83 (44.9%)      | 102 (55.1%)            |       |        |      |
| Lymph node metastasis |               |                        |
| No                 | 60 (37.5%)      | 100 (62.5%)            | 0.162 | −0.086 | 0.164 |
| Yes                | 48 (46.2%)      | 56 (53.8%)             |       |        |      |
| Age (yr)           |                 |                        |
| <55                | 86 (41.0%)      | 124 (59.0%)            | 0.977 | 0.002  | 0.978 |
| ≥55                | 22 (40.7%)      | 32 (59.3%)             |       |        |      |
| Gender             |                 |                        |
| Female             | 77 (43.3%)      | 101 (56.7%)            | 0.264 | 0.069  | 0.266 |
| Male               | 31 (36.0%)      | 55 (64.0%)             |       |        |      |
| Invasion capsule   |                 |                        |
| Without capsular invasion | 4 (30.8%) | 9 (69.2%) | 0.464 | 0.108 | 0.081 |
| Only/just penetrating capsule | 22 (50.0%) | 22 (50.0%) |       |        |      |
| Invading the extracapsular fibroadipose tissue and deeper | 25 (43.9%) | 32 (56.1%) |       |        |      |
| Papillary structure |                 |                        |
| <50%               | 26 (31.0%)      | 58 (69.0%)             | 0.025 | −0.138 | 0.025 |
| ≥50%               | 82 (45.6%)      | 98 (54.4%)             |       |        |      |
| p16                |                 |                        |
| −                  | 78 (45.6%)      | 93 (54.4%)             | 0.035 | 0.130  | 0.035 |
| +                  | 30 (32.3%)      | 63 (67.7%)             |       |        |      |
| Recurrence and metastasis* |         |                        |
| No                 | 49 (36.0%)      | 87 (64.0%)             | 0.060 | −0.133 | 0.061 |
| Yes                | 32 (50.0%)      | 32 (50.0%)             |       |        |      |
| Subtype            |                 |                        |
| Classic type       | 91 (40.6%)      | 133 (59.4%)            | 0.663 | −0.007 | 0.905 |
| Classical type and its mixed type* | 10 (52.6%) | 9 (47.4%) |       |        |      |
| Follicular variant | 5 (33.3%)       | 10 (66.7%)             |       |        |      |
| Other subtypes**   | 2 (33.3%)       | 4 (66.7%)              |       |        |      |

* 64 patients were lost to follow-up.
# The expected count of two cells (25.0%) was <5. The minimum expected count was 2.45. The statistical results are for reference.
¥ 16 cases of mixed follicular type and classical type, two cases of partial Warthin-tumor-like type, one case of local squamous cell carcinoma.
※ There were two tall cell subtype, one columnar cell subtype, one cribriform-morular subtype and two eosinophilic subtype.
and thickness of the ligamentum flavum, suggesting that p16 expression is associated with hypertrophy of the ligamentum flavum in patients with lumbar spinal stenosis [11]. p16 may have been related to development of IF in PTC in our study, and this needs further study.

p16 may also be related to the occurrence of tumors. As a tumor suppressor in normal tissues, p16 is also a cyclin-dependent kinase inhibitor that modulates cell cycle progression and cell senescence by binding to cyclin-dependent kinase. Similar to p53, it can also cause tumor development when a mutation occurs. A meta-analysis [12] including 734 PTC patients showed that the frequency of p16 promoter methylation in cancer tissues was significantly higher than that in normal and benign tissues. Lam et al. [13] reported that p16 promoter methylation and p16 overexpression were common in PTC, and p16 promoter methylation was associated with high AMES (age, distant metastasis, extrathyroid infiltration, and size) risk and advanced pTNM stage. Alterations in p16 gene were closely related to the histological characteristics and biological aggressiveness of PTC. Mohammadi-asl et al. [14] also demonstrated that the average methylation level of p16 in PTC was significantly higher than that in benign tumors. Therefore, p16 gene promoter methylation may play an important role in the pathogenesis of PTC [12-14] and thus may be used as a potential biomarker for PTC [14]. In the present study, p16 expression was high in PTC tissues and low in NAT and nodular goiters, which was consistent with previous findings [12]. p16 expression was related to older age and advanced clinical stage, which was similar to the findings of Lam et al. [13]. p16-positive patients had shorter OS in our study. In summary, p16 was highly expressed in PTC and was probably involved in the pathogenesis and prognosis of PTC.

IF is a clinicopathological feature and diagnostic indicator of PTC. Visual evaluation of the area ratio of fiber area was mentioned in a previous study [6]. We referred to the risk classification of collagen proportionate area (CPA) of liver fibrosis by Huang et al. [7] (C1: 0%–5%, C2: >5%–10%, C3: >10%–20%, C4: >20%). Standish et al. [8] showed that ≥4 points ≥13.7% CPA in the pathological liver tissue of chronic hepatitis C according to Ishak score of 0–6. Ma et al. showed that stage S3 and above was ≥15% CPA according to the stages of chronic hepatitis B fibrosis in China [9]. Based on the above studies and our study, we set less than one seventh as mild IF and >15% as moderate and severe IF. Visual assessment under the microscope was simple and practical. Other methods were complex and cumbersome. In our study, inactive fibrosis was dominated in the group of moderate and severe fibrosis.

Univariate survival analysis and multivariate Cox regression analysis both showed that RFS was significantly longer in the moderate/severe IF group than in the no/mild IF group. The effect of IF on OS lacked statistical significance. The results also showed that there was a

| Step | Risk Factor          | B   | SE  | Wald  | df | Sig.   | Exp (B) | 95.0% Exp (B) Confidence Interval |
|------|----------------------|-----|-----|-------|----|--------|---------|----------------------------------|
| Step 1 | Lymph node metastasis | 1.411 | .273 | 26.797 | 1  | .000   | 4.099    | 2.403 6.994                     |
| Step 2 | pTNM                 | .970 | .287 | 11.449 | 1  | .001   | 2.639    | 1.504 4.629                     |
|       | Lymph node metastasis | 1.146 | .279 | 16.872 | 1  | .000   | 3.147    | 1.821 5.438                     |
| Step 3 | pTNM                 | .989 | .285 | 12.072 | 1  | .001   | 2.689    | 1.539 4.698                     |
|       | Lymph node metastasis | 1.148 | .277 | 17.187 | 1  | .000   | 3.151    | 1.832 5.421                     |
|       | Degree of fibrosis   | −.554 | .252 | 4.821  | 1  | .028   | .575     | .351  .942                      |

Abbreviations: RFS, relapse-free survival; pTNM, pathological tumor-node-metastasis.
negative correlation between the degree of IF and tumor size. With the increase of tumor size, the incidence of no/mild fibrosis increased. Thus, IF was associated with RFS after surgery, and also tended to be related to recurrence. This can be explained that when moderate/severe IF was present in PTC, especially with collagen or vitreous degeneration, both the epithelial components and vascular components were reduced and the proliferative activity of tumor cells decreased; at the same time, IF can also prevent tumor diffusion and wrap tumor to a certain extent, which may have beneficial effects on survival. It has been reported that, after the Chernobyl nuclear accident in 1986, PTC with a short latent period had significantly less peritumoral fibrosis, more invasive spread, and lower degree of structural differentiation [15]. In our study, the impact of fibrosis on prognosis was limited. Moreover, this was a single center study. The subtypes were mainly classical and follicular. The results need to be confirmed in future experiments.

The degree of IF in PTC was also negatively correlated with papillary structural components. The results showed that the incidence of no/mild IF was higher when there were more papillary components in PTC. We further subdivided the groups and found that the incidence of no/mild IF was the highest (45.6%) in the group with ≥50% papillary components, followed by the group with ≤10% papillary components (37.2%), and the lowest group with >10% and <50% papillary components (24.4%).

It has been reported that IF is an independent prognostic factor for the recurrence of papillary thyroid microcarcinoma [16], which is different from our results. The difference may be because IF was not graded in the previous study [16], and it was considered that papillary thyroid microcarcinoma ≤1 cm had unique biological behavior [16]. In our study, there were 79 cases (29.9%) with tumor size ≤1 cm and only four with tumor size <0.5 cm, compared with 44.62% (228 cases) <0.5 cm in the study of Liu et al. [16]. In our study, moderate/severe IF was seen in 68.4% of cases with tumor size ≤1 cm. In the >1 cm group, with the increase of tumor size, the rate of moderate/severe IF gradually decreased, but lack of statistical significance in our study, which needs to be studied in the future.

PTC is different from other invasive carcinomas because it is inert, stromal fibroblasts are usually inactive, often featured as glassy degeneration and collagenization. Therefore, our conclusions on IF are only applicable to PTC.

**Disclosure**

All the authors declare that there was no conflict of interest.

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