Nrf2 as a novel diagnostic biomarker for papillary thyroid carcinoma

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ABSTRACT

Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy. However, it is very difficult to distinguish PTC from benign carcinoma. Thus, specific diagnostic biomarkers are actively pursued. Previous studies observed that Nrf2 was highly expressed in PTC. Based on this research, we hypothesized that Nrf2 may serve as a novel specific diagnostic biomarker. A single-center retrospective study, including 60 patients with PTC and 60 patients with nodular goiter, who underwent thyroidectomy at the Central Theater General Hospital from 2018 to July 2020, was conducted. The clinical data of the patients were collected. Nrf2, BRAF V600E, CK-19, and Gal-3 proteins were compared from paraffin samples of the patients. Through this study, we obtained the following results: i) Nrf2 exhibits high abundance expression in PTC, but not in adjacent to PTC and nodular goiter; increased Nrf2 expression could serve as a valuable biomarker for PTC diagnosis; the sensitivity and specificity for the diagnosis of PTC were 96.70% and 89.40%, respectively. ii) Nrf2 also shows higher expression in PTC with lymph node metastasis, but not adjacent to PTC and nodular goiter, thus the increased Nrf2 expression might serve as a valuable predictor for lymph node metastasis in PTC patients; the sensitivity and specificity for the prediction in lymph node metastasis were 96.00% and 88.57%, respectively; excellent diagnostic agreements were found between Nrf2 and other routine parameters including HO-1, NQO1 and BRAF V600E. iii) The downstream molecular expression of Nrf2 including HO-1 and NQO1 consistently increased. In conclusion, Nrf2 displays a high abundance expression in human PTC, which leads to the higher expression of downstream transcriptional proteins: HO-1 and NQO1. Moreover, Nrf2 can be used as an extra biomarker for differential diagnosis of PTC and a predictive biomarker for lymph node metastasis of PTC.

Key words: Nrf2; predictive biomarker; papillary thyroid carcinoma; nodular goiter.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common thyroid cancer subtype, accounting for approximately 80% of all thyroid cancers worldwide. However, by some estimates, the incidence of PTC, particularly in the Chinese population, could be as high as 95.1% of all thyroid cancer diagnoses.[1] Most of the patients with PTC have latent onset and lack specific clinical symptoms, and only show nodular changes in the cervical thyroid region. The differential diagnosis of benign and malignant thyroid nodules is difficult. It was reported that patients with lymph node metastasis (LNM) might be more likely to have distant metastasis and poorer survival than those without LNM.[1] Therefore, it is critical to find effective biomarkers for prompt diagnosis and good prognosis. Pathological examination including immunohistochemistry (IHC) has been a gold standard for PTC diagnosis and prognosis. Currently, BRAF V600E, Cytokeratin 19 (CK-19), and Galectin-3 (Gal-3) proteins' expressions, as determined by IHC, are approved as diagnostic or prognostic biomarkers for PTC. Nevertheless, BRAF V600E mutation only accounts for 60% of PTC.[2] A large number of studies have confirmed that the expression of CK-19 was generally increased in malignant thyroid conditions.[3,4] However, the predictive value of CK-19 in PTC with LNM has not been confirmed. Gal-3 has a diagnostic sensitivity and specificity of 78.75% and 66.67%, for papillary thyroid cancer, respectively,[5] which is far from adequate for accurate diagnosis. Therefore, it is essential to identify novel valuable biomarkers for the treatment, diagnosis, and prognosis of PTC.

Numerous studies have shown that oxidative stress was associated with the occurrence and development of PTC.[6] Simultaneously, plenty of studies have identified that nuclear factor erythroid 2–related factor 2 (Nrf2) is implicated as a master regulator in the cellular response to oxidative stress.[7] Nrf2 modulates the cytoprotective response through the induction of its target genes, such as heme oxygenase 1 (HO-1) and encoding NAD(P)H-quinone oxidoreductase 1 (NQO1).[8] Recent studies indicated that cancer cells hijack Nrf2 to survive under oxidative or electrophilic conditions. Higher expressions of Nrf2 were presented in a broad range of tumors, including neuroendocrine carcinomas (approximately 32%), head and neck cancers (approximately 30%), lung cancers (approximately 28%), uterine cancers (approximately 21%), esophageal cancers (approximately 25%), and bladder cancers (approximately 15%).[9] It was also reported that Nrf2 was highly expressed in PTC. Luzón-Toro *et al*.[10] detected the expression levels of 84 lncRNAs in 61 PTC tissues and their adjacent non-tumor tissues by PCR, a significant decrease was observed on *Nrf2* gene. Stuchi *et al*.[11] examined tissue samples from colloid goiter, papillary thyroid cancer (PTC), and normal thyroids, and found higher levels of Nrf2 transcripts. It is not clear whether Nrf2 can serve as a diagnostic marker for PTC and a predictor for LNM in PTC patients. Therefore, the aim of the present study is to explore the diagnostic implications of PTC, and the predictive value for LNM in PTC patients.

Materials and Methods

Study design and participant selection

A single-center retrospective study was conducted at the Department of Pathology, the Central Theater General Hospital. From January 2018 to July 2020, a total of 60 PTC and 60 nodular goiters (NG) postoperative patients, who were undergoing thyroid surgery in this hospital, were collected. Based on the results of the pathology of nodules, patients were divided into two groups: PTC and NG. The clinical characteristics and pathological data were collected from medical records.

Inclusion criteria were i) normal thyroid function and normal thyroid-related antibodies; ii) confirmed PTC by pathological results. The inclusion criteria of the NG group were as follows: i) normal thyroid function and normal thyroid-related antibodies; ii) pathologically diagnosed benign thyroid nodules. Exclusion criteria were i) recent use of Nrf2 agonists: sulforaphane and analogues, curcumin, resveratrol, dithiocyclopentadiene copper sulfide, *etc*.; ii) recent use of Nrf2 inhibitors: all-trans visual sulfonic acid, bruceopicol, *etc*.; iii) use of iodine contrast agents such as coronary angiography and ERCP within half a year before operation; iv) use of amiodarone drugs; v) hematologic diseases, endocrine disorders, liver or renal dysfunction, and tumors other than PTC at the same time. In addition, patients with missing data on thyroid function and whose outcomes were temporarily unclassified were excluded. This study was approved by the Research Ethics Committee of the General Hospital of Central Theater Command in China. [2021]005-02.

Specimens

Venous blood sampling was obtained on admission for the determination of thyroid, renal, liver function, lipid profile, and fasting plasma glucose levels. Tumor and goiter tissue samples, along with adjacent tissues were collected from 120 patients, as follows: 60 PTC patients and 60 NG patients. Tumor and goiter samples, along with adjacent tissue samples, were sent to the Pathology Service of General Hospital of Central Theater Command for diagnosis and microdissection. The tumors were classified according to the parameters of The American Joint Committee for Cancer (AJCC).[12]

Immunohistochemistry

IHC was accomplished at the Pathology Department of General Hospital of Central Theater Command. Four μm paraffin embedded sections were prepared for IHC. The paraffin sections were de-paraffinized with xylene followed by rehydration. Each slide was autoclaved for 20 min at 121°C in antigen repair solution for EDTA antigen retrieval. The slides were then washed with PBS and incubated in 0.3% H_2O_2/methanol for 30 min at 20°C to inactivate endogenous peroxidase. After washing three times with PBS, the slides were blocked with 2% normal goat serum/PBS for 30 min at 20°C and incubated with primary antibodies in the blocking solution for 1 h at 20°C. After washing three times with PBS, the slides were incubated with HRP-conjugated secondary antibodies in the blocking solution for 1 h at 20°C. After washing three times with PBS, the slides were incubated in 0.1 mg/mL DAB/0.03% H_2O_2/50 mM Tris-HCl (pH 7.6) for 3-5 min at 20°C. The negative control for all proteins was to replace the primary antibody with goat non-immune serum. Finally, after hematoxylin counterstaining and hydrophobization, the sections were mounted with mounting medium (MP500, Matsunami). IHC staining was evaluated by NIH Image software Image J according to the average optical density (AOD) value.

The antibodies and reagents used in this study are listed in Table 1.

Statistical analysis

Continuous variables with normal distribution were presented as mean with standard deviation (SD), and the *t*-test was used to compare the between-group difference. Continuous variables with skewed distribution were presented as median with interquartile range (IQR), and Mann-Whitney U test was used to compare the between-group difference. Categorical variables were presented as

frequencies with percentages, and Chi-square test and Fisher's exact test were used to compare the between-group difference. For the comparison between groups greater than two, we adjusted the significance level according to Bonferroni's method to reduce the risk of type I errors. Evaluating the diagnostic value of Nrf2 in PTC using ROC curves. Intra-class correlation coefficients (ICC) were adopted to assess consistency in diagnosis, with ICC >0.75 defined as good consistency. All data were analyzed with IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, N.Y., USA). The differences were considered significant when $p<0.05$ (*) or $p<0.01$ (**). GraphPad Prism v7 (GraphPad Software, Inc., La Jolla, CA, USA) was used for graph preparations.

Results

Increased expression of Nrf2 and other biomarkers in PTC

Clinical data of the patients

A total of 120 patients were included in this study, 60 with PTC and 60 with nodular goiter (NG). There is no significant difference in basic clinical data between the two groups ($p>0.05$) (Table 2). It confirmed that there are no distinctive features in clinical presentation between patients with PTC and NG.

Expression of Nrf2 protein

To determine if changes in Nrf2 protein abundance correlated with difference among the tissues, we performed IHC to compare Nrf2 expression levels in PTC, NG and their adjacent tissues. The expression of Nrf2 protein in PTC group was higher than that in adjacent tissue of PTC, NG group and adjacent tissue of NG group ($p<0.001$), The expression in adjacent to PTC group was significantly higher than that in NG group and adjacent to NG group ($p<0.001$). There was no significant difference in expression between NG group and adjacent to NG group ($p>0.05$) (Figure 1).

Expression of HO-1 protein

To determine if changes in HO-1 protein abundance correlated with differences among the tissues, we performed immunohistochemistry to compare HO-1 expression level in PTC, NG and its adjacent tissues. The expression of HO-1 protein in the PTC group was higher than that in adjacent to the PTC group, NG group and adjacent to NG group ($p<0.001$); the expression of HO-1 protein in adjacent to PTC group was higher than that in adjacent to NG group ($p<0.001$); there was no statistical difference between adjacent to PTC group and NG group ($p>0.05$); the expression of NG group was higher than adjacent to NG group ($p=0.028$) (Figure 2).

Expression of NQO1 protein

To determine if changes in NQO1 protein abundance correlated with differences among the tissues, we performed IHC to compare NQO1 expression level in PTC, NG and its adjacent tissues. The expression of NQO1 in PTC group was significantly higher than that in adjacent to the PTC group, NG group and adjacent to NG group ($p<0.001$); the expression of NQO1 in adjacent to PTC group was lower than that in NG group ($p<0.001$); there was no statistical difference between adjacent to PTC group and adjacent to NG group ($p>0.05$). The expression in NG group was significantly higher than that in adjacent to NG paracenter ($p<0.001$) (Figure 3).

Expression of BRAF V600E protein

To determine if changes in BRAF V600E protein abundance correlated with differences among the tissues, we performed IHC to compare BRAF V600E expression level in PTC, NG and its adjacent tissues. The expression of BRAF V600E in the PTC group was significantly higher than that in adjacent to PTC group, NG group and adjacent to NG group ($p<0.001$); there was no significant difference between adjacent to the PTC group and NG group ($p>0.05$); the expression of adjacent to PTC group was significantly higher than adjacent to NG group; there was no significant dif-

Table 1. Antibodies and reagents used.

Reagent	Manufacturer	Article number	Dilution ratio	Positive control
Anhydrous ethanol	Sinopharm Group Chemical Reagent Co., Ltd	100092683		
Xylene	Sinopharm Group Chemical Reagent Co., Ltd	10023418		
Antibody dilution	Dako Denmark A/S	S2022		
EDTA antigen repair solution	Dako Denmark A/S	JYF-9062	1:50	
PBS buffer	Fuzhou Maixin Biotechnology Development Co., Ltd	PBS-0060		
3% hydrogen peroxide	Sinopharm Group Chemical Reagent Co., Ltd	10011218		
DAB color development kit	Dako Denmark A/S	K5007		
Hematoxylin dye	Self-prepared reagent			
Hydrochloric acid ethanol solution	Sinopharm Group Chemical Reagent Co., Ltd			
Ammonia solution	Sinopharm Group Chemical Reagent Co., Ltd	10002118		
Neutral gum	Sinopharm Group Chemical Reagent Co., Ltd	10004160		
Nrf2 primary antibody	Wuhan Bode Biological Co., Ltd	PB9290	1:200	Human breast carcinoma tissue
HO-1 primary antibody	Wuhan Bode Biological Co., Ltd	BA0605	1:200	Human spleen tissue
NQO1 primary antibody	Wuhan Bode Biological Co., Ltd	BM4978	1:50	Human breast adenocarcinoma tissue sections
BRAF V600E primary antibody	Beijing Zhongshan Jinqiao Biotechnology Co., Ltd	ZA-0668	Working fluid	Human melanoma tissue with B-RAF V600E mutation
CK-19 primary antibody	Fuzhou Maixin Biotechnology Development Co., Ltd	Kit-0030	Working fluid	Human stomach adenocarcinoma tissue
Gal-3 primary antibody	Fuzhou Maixin Biotechnology Development Co., Ltd	MAB-0572	Working fluid	Human colon cancer tissues
Secondary antibody	DAKO REALTMEnVision	K5007		
Goat non-immune serum	Fuzhou Maixin Biotechnology Development Co., Ltd	SP KIT-B3		

ference between NG group and adjacent to NG group (p>0.05) (Figure 4).

Expression of CK-19 protein

To determine if changes in CK-19 protein abundance correlated with difference among the tissues, we performed IHC to compare CK-19 expression level in PTC, NG and its adjacent tissues. The expression of CK-19 protein in PTC group was significantly higher than that in adjacent to PTC group, NG group and adjacent to NG group (p<0.001); there were no significant differences between adjacent to PTC group and NG group (p=0.867), adjacent to PTC group and adjacent to NG group (p=0.675), NG group and adjacent to NG group (p>0.05) (Figure 5).

Expression of Gal-3 protein

To determine if changes in Gal-3 protein abundance correlated with differences among the tissues, we performed IHC to compare Gal-3 expression level in PTC, NG and its adjacent tissues. The expression of Gal-3 protein in PTC group was higher than that in adjacent to the PTC group, NG group and adjacent to NG group (p<0.001); the expression of Gal-3 protein in adjacent to PTC group was higher than that in NG group and adjacent to NG group (p<0.001); there was no significant difference between NG group and adjacent to NG group (p=0.329) (Figure 6).

Increased Nrf2 expression serves as a valuable biomarker for diagnosis in PTC

In order to confirm increased Nrf2 expression serves as a valuable biomarker for diagnosis in PTC, we constructed some tests for evaluating Nrf2 and other biomarkers to compare their diagnostic value.

Correlation analysis of Nrf2 protein expression

We examined a possible association between Nrf2 and each of the proteins under the current study by performing a correlation analysis among these proteins. The expression of Nrf2 protein was positively correlated with the expression of HO-1, NQO1, BRAF V600E, CK-19 and Gal-3 protein (Figure 7).

Area under curve of Nrf2 protein

The area under curve (AUC)of Nrf2 protein was 0.977, which had a significant diagnostic value. The Yoden index is 0.8611. The diagnostic truncation value is AOD = 0.2966. Sensitivity: 96.70%; specificity: 89.40%. To determine the diagnostic capacity of the selected biomarkers, we calculated the AUC. BRAF V600E, CK-19 and Gal-3 are commonly used proteins to distinguish benign and malignant thyroid nodules in pathology departments, while HO-1 and NQO1 are not classical proteins to distinguish benign and malignant thyroid nodules in previous studies. Therefore, we compared the area under curve values of Nrf2 with BRAF V600E, CK-19 and Gal-3. The AUC of BRAF V600E, CK-19 and Gal-3 were 0.978, 0.999 and 0.918, respectively. The AUC of the Nrf2 protein is significantly smaller than that of the CK-19 protein, but significantly larger than the Gal-3 protein (Nrf2 *vs* CK19 p=0.0027; Nrf2 *vs* Gal-3 p=0.0002). The AUC of Nrf2 protein was less than that of BRAF V600E, but no statistical significance (Nrf2 *vs* BRAF V600E p=0.8498) (Figure 8).

Table 2. **Basal clinical data of the patients.**

	Total	PTC group	NG group	p
Age (years)	48.16±1.17	47.60±1.63	50.72±1.62	0.438
Gender (male)	46 (38.3%)	25 (41.7%)	21 (35.0%)	0.453
BMI	24.09±0.30	24.61±0.40	23.57±0.45	0.086
Systolic blood pressure (mmHg)	122.94±1.37	122.20±1.96	125.68±1.86	0.056
Diastolic pressure (mmHg)	77.41±1.04	77.43±1.28	77.38±1.66	0.981
TRAb (IU/ml)	0.30 (0.30, 0.45)	0.30 (0.30, 0.42)	0.31 (0.30, 0.68)	0.053
TSH (μIU/ml)	1.94±0.08	1.91±0.10	1.98±0.13	0.654
T3 (nmol/l)	1.70±0.03	1.75±0.04	1.65±0.05	0.098
T4 (nmol/l)	95.41±1.27	94.01±1.82	96.80±1.76	0.272
FT3 (pmol/l)	4.83±0.06	4.86±0.07	4.80±0.09	0.578
FT4 (pmol/l)	15.83±0.22	15.92±0.30	15.73±0.34	0.673
TGAb (IU/ml)	10.00 (10.00, 12.07)	10.00 (10.00, 11.79)	12.10 (10.00, 16.88)	0.070
TPOAb (IU/ml)	12.28 (9.36, 15.53)	11.35 (8.58, 14.54)	13.87 (8.94, 17.37)	0.705
Parathyroid hormone (pg/ml)	44.13±1.79	42.49±2.33	45.64±2.69	0.381
Alanine transaminase (U/L)	20.00 (14.00, 25.00)	21.00 (15.00, 28.00)	16.00 (12.75, 24.25)	0.580
Glutaminase (U/L)	22.00 (18.00, 27.00)	25.00 (19.00, 29.00)	19.50 (17.00, 25.25)	0.521
Urea (mmol/l)	4.79±0.10	4.81±0.15	4.77±0.14	0.877
Creatinine (μmol/l)	55.00 (47.00, 66.00)	52.00 (46.00, 64.00)	52.50 (45.75, 58.25)	0.478
Uric acid (μmol/l)	318.28±9.24	332.34±12.31	303.98±13.66	0.126
Blood glucose (mmol/l)	5.06 (4.84, 5.50)	5.03 (4.76, 5.43)	5.15 (4.87, 5.60)	0.178
Total cholesterol (mmol/l)	4.64±0.14	4.61±0.16	4.68±0.21	0.802
Triglycerides (mmol/l)	1.31 (1.14, 1.74)	1.28 (1.14, 1.59)	1.43 (1.18, 1.82)	0.403
High density lipoprotein (mmol/l)	1.20 (1.06, 1.47)	1.20 (1.06, 1.42)	1.18 (1.06, 1.51)	0.834
Low density lipoprotein (mmol/l)	2.58±0.09	2.53±0.11	2.62±0.14	0.645

BMI, body weight index; TRAb: thyrotropin receptor antibody; TSH, thyrotropin; TGAb,anti-thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; p, comparison between PTC group and NG group.

Diagnostic efficacy of Nrf2

In order to examine consistency, we have employed the ICC coefficient defined from the linear mixed model (LMM). The ICC coefficients of Nrf2, BRAF V600E, CK-19 and Gal-3 were all higher than 0.75, suggesting good consistency (Table 3).

Increased expression of Nrf2 and other biomarkers in PTC with LNM

In PTC, cervical LNM often affects patients' treatment and prognosis. Conventional pathology may fail to detect occult nodal metastasis. Thus, we conducted experiments to verify whether Nrf2 can be promising for the detection of occult LNM.

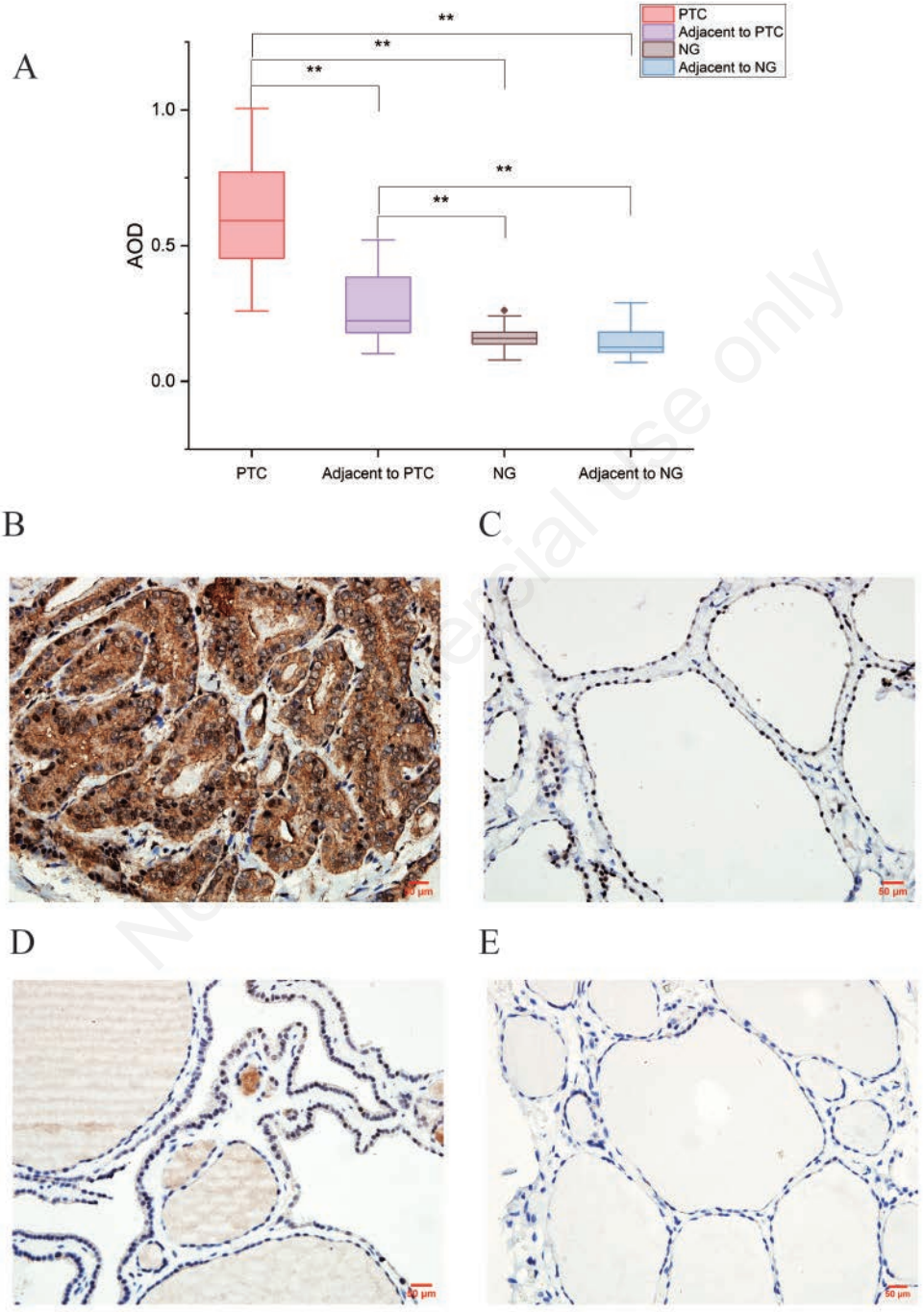

Figure 1. Expression of Nrf2 protein in thyroid papillary carcinoma, nodular goiter and its adjacent tissues. A) Quantitative analysis of the average optical density (AOD) of Nrf2 protein in papillary thyroid carcinoma, nodular goiter and adjacent tissue; these data represent the median and quartile range of average optical density (IQR); *p<0.05; **p<0.01. B) Papillary carcinoma of the thyroid. C) Tissue adjacent to papillary thyroid carcinoma. D) Nodular goiter. E) Tissue adjacent to the nodular goiter. Section thickness: 4 μm; microscope magnification: ×400.

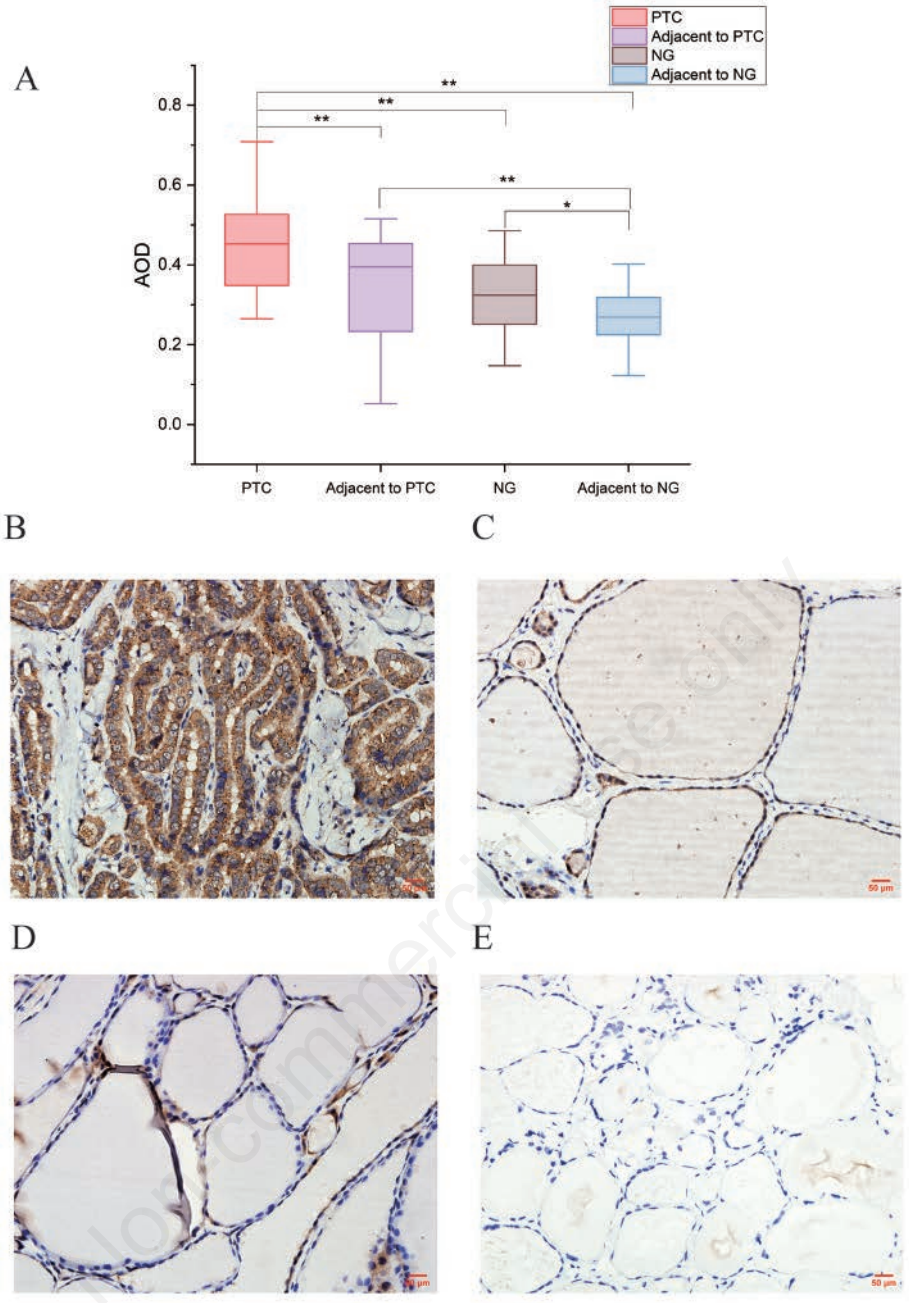

Figure 2. Expression of HO-1 protein in thyroid papillary carcinoma, nodular goiter and its adjacent tissues. A) Quantitative analysis of the average optical density (AOD) of HO-1 protein in papillary thyroid carcinoma, nodular goiter and adjacent tissue; these data represent the median and quartile range of average optical density (IQR); *p<0.05; **p<0.01. B) Papillary carcinoma of the thyroid. C) Tissue adjacent to papillary thyroid carcinoma. D) Nodular goiter. E) Tissue adjacent to the nodular goiter. Section thickness: 4 μm; microscope magnification: ×400.

Table 3. Diagnostic consistency analysis in papillary thyroid carcinoma of Nrf2 protein expression with BRAF V600E, CK-19 and Gal-3 protein.

	Intraclass correlation	95% Confidence Interval		p
		Lower bound	Upper bound	
BRAF V600E	0.863	0.824	0.894	<0.001
CK-19	0.804	0.747	0.848	<0.001
Gal-3	0.870	0.832	0.899	<0.001

The intra-group correlation coefficient (ICC) can be used to evaluate the consistency of different measurement methods for the same quantitative measurement. ICC value between 0 and 1, generally considered: ICC.

Basic clinical data of the patients of PTC with LMN

Among 60 PTC patients, 25 had LNM. The patients were divided into two groups according to the presence or absence of LNM. In comparison of the two groups of basic clinical data, the PTC patients with LNM were younger, and their results of TSH, total T3, free T3 and uric acid were higher; however, total cholesterol and low-density lipoprotein were lower (Table 4).

Expression of Nrf2 and other biomarkers in PTC with lymph node metastasis

The expression of Nrf2, HO-1, NQO1, BRAF V600E and Gal-3 protein in the metastasis group was significantly higher than that in the non-metastasis group ($p<0.01$), but there was no significant difference in the expression of CK-19 between the two groups ($p>0.05$) (Figure 9).

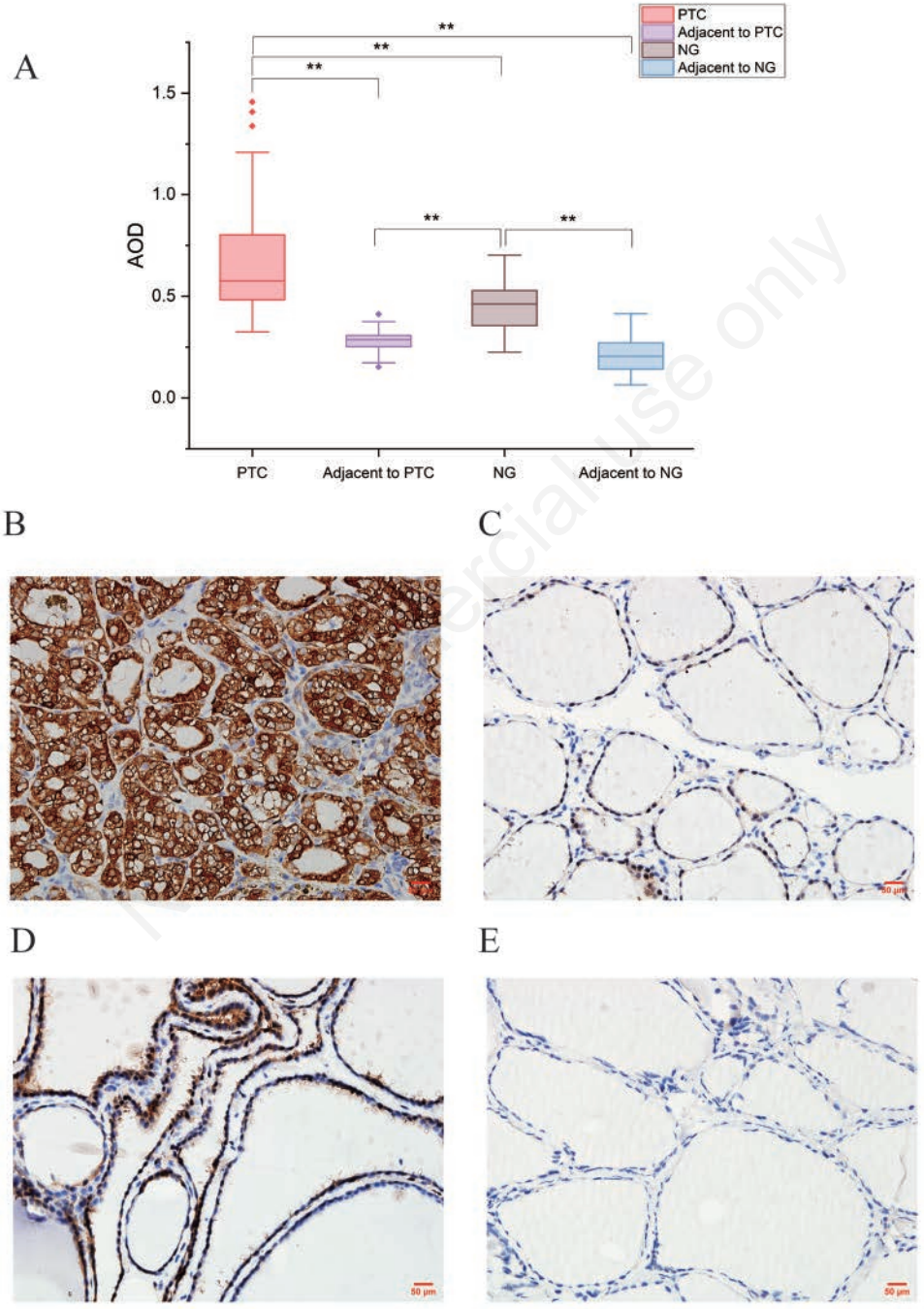

Figure 3. Expression of NQO1 protein in thyroid papillary carcinoma, nodular goiter and its adjacent tissues. A) Quantitative analysis of the average optical density (AOD) of NQO1 protein; these data represent the median and quartile range of average optical density (IQR); *p<0.05; **p<0.01. B) Papillary carcinoma of the thyroid. C) Tissue adjacent to papillary thyroid carcinoma.(D) Nodular goiter. E) Tissue adjacent to the nodular goiter. Section thickness: 4 µm; microscope magnification: ×400.

AUC of Nrf2 protein in PTC

The AUC of Nrf2 protein in PTC was 0.951, which had a significant diagnostic value. The Youden index was 0.8457. The diagnostic truncation value is AOD 0.5986, sensitivity: 96.00%; specificity: 88.57%. BRAF V600E, CK-19 and Gal-3 are commonly used proteins in pathology to distinguish benign and malignant thyroid nodules. This experiment also aims to compare the diagnostic ability of these three proteins with Nrf2 in distinguishing LNM of papillary thyroid cancer. HO-1 and NQO1 proteins, as downstream gene expression proteins of Nrf2, have been proven to be more expressed in lymph node metastatic cancer in many previous studies, so these two proteins are also included in the study. The AUC of HO-1, NQO1 and BRAF V600E protein was 0.984, 0.989 and 0.984, respectively, which had a significant diagnostic

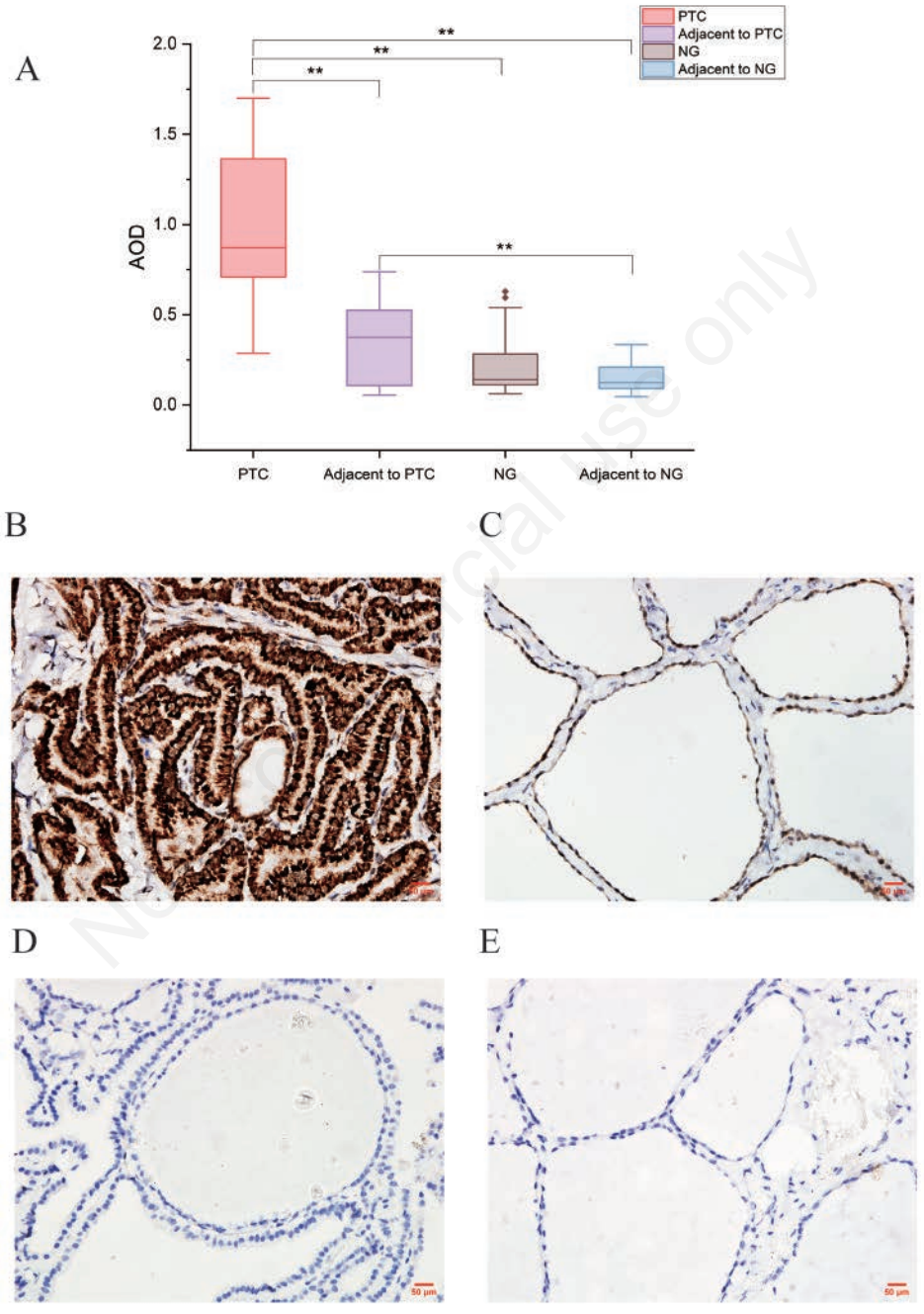

Figure 4. Expression of BRAF V600E protein in thyroid papillary carcinoma, nodular goiter and its adjacent tissues. A) Quantitative analysis of the average optical density (AOD) of BRAF V600E protein in papillary thyroid carcinoma, nodular goiter and adjacent tissue; these data represent the median and quartile range of average optical density (IQR); *p<0.05; **p<0.01. B) Papillary carcinoma of the thyroid. C) Tissue adjacent to papillary thyroid carcinoma. (D) Nodular goiter. E) Tissue adjacent to the nodular goiter. Section thickness: 4 μm; microscope magnification: ×400.

value. The AUC of CK-19 and Gal-3 protein was 0.566 and 0.760 respectively, which had no diagnostic value. The AUC of Nrf2 protein is significantly smaller than that of NQO1 protein, but significantly larger than that of HO-1 protein (Nrf2 *vs* NQO1 p=0.0027; Nrf2 *vs* HO-1 p=0.0002). The AUC of Nrf2 protein was smaller than that of BRAF V600E, but no statistical significance (Nrf2 *vs* BRAF V600E p=0.8498) (Figure 10).

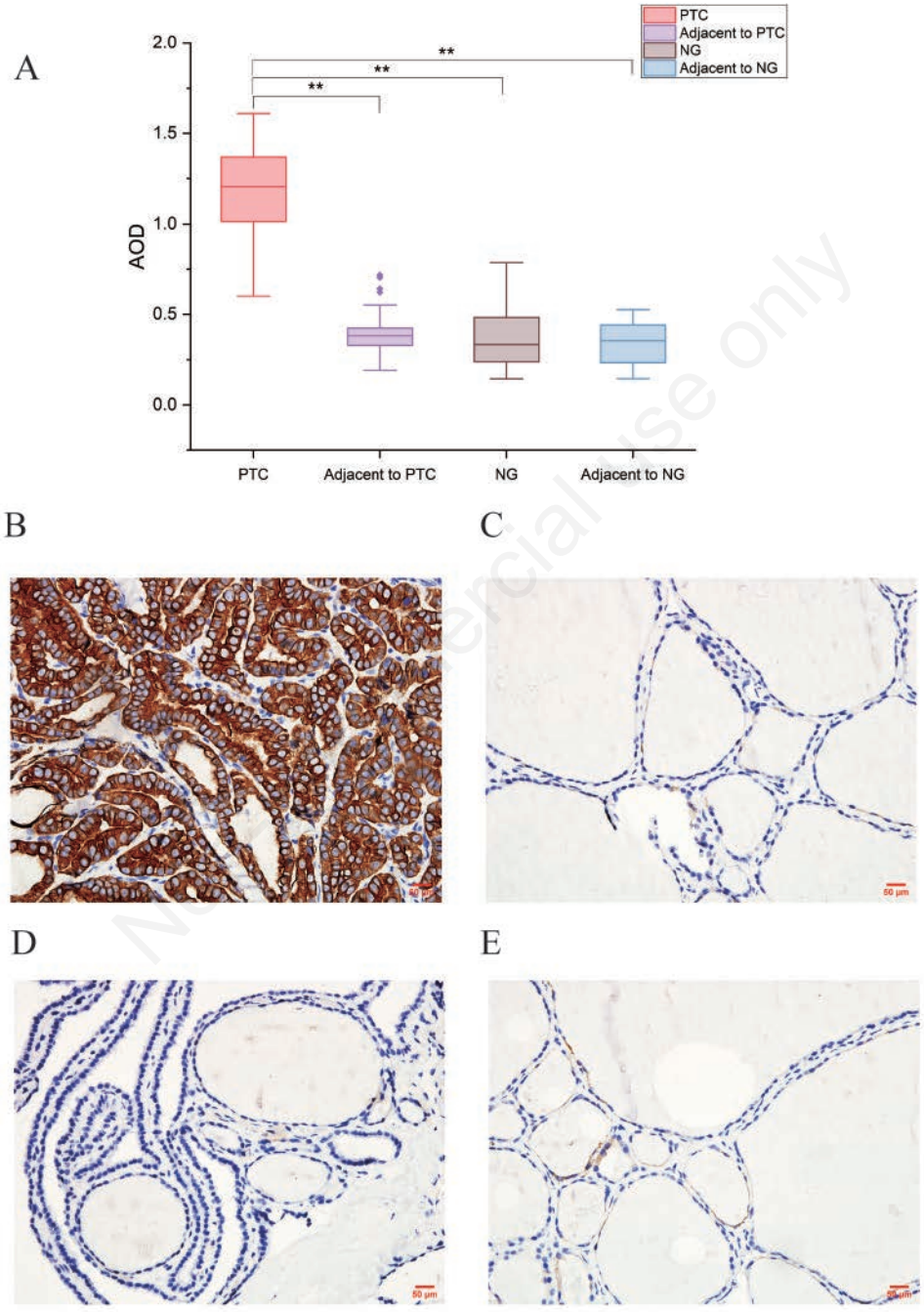

Figure 5. Expression of CK-19 protein in thyroid papillary carcinoma, nodular goiter and its adjacent tissues. A) Quantitative analysis of the average optical density (AOD) of CK-19 protein in papillary thyroid carcinoma, nodular goiter and adjacent tissue; these data represent the median and quartile range of average optical density (IQR); *p<0.05; **p<0.01. B) Papillary carcinoma of the thyroid. C) Tissue adjacent to papillary thyroid carcinoma (D) Nodular goiter. E) Tissue adjacent to the nodular goiter. Section thickness: 4 µm; microscope magnification: ×400.

Diagnostic efficacy of Nrf2 in PTC with LNM

The ICC coefficients of Nrf2, HO-1, NQO1 and BRAF V600E were all higher than 0.75, and the diagnostic consistency was good. The diagnostic consistency of Nrf2 and CK-19 was less than 0.4, and the diagnostic consistency was poor. The consistency ICC coefficient of Nrf2 and Gal-3 was 0.510, and the consistency of diagnosis was general (Table 5).

Discussion

PTC is characterized by its indolent growth. In recent years, there has been debate about the overtreatment of PTC. Overmedication can be costly for patients and healthcare systems, and it can result in permanent hypothyroidism or other surgical complications. As a result, preoperative detection of the nodule's

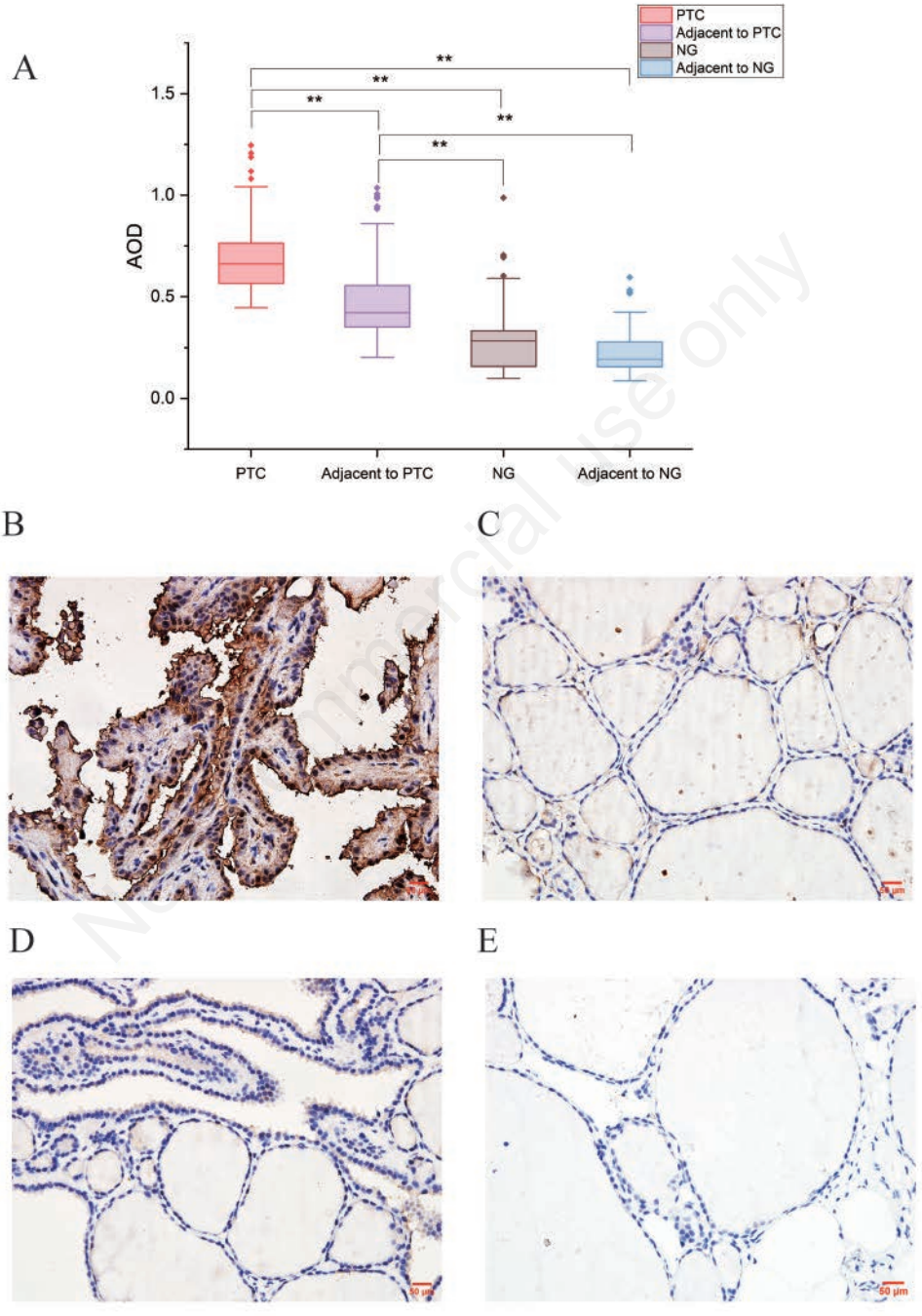

Figure 6. Expression of Gal-3 protein in thyroid papillary carcinoma, nodular goiter and its adjacent tissues. A) Quantitative analysis of the average optical density (AOD) of Gal-3 protein in papillary thyroid carcinoma, nodular goiter and adjacent tissue; these data represent the median and quartile range of average optical density (IQR); *p<0.05; **p<0.01. B) Papillary carcinoma of the thyroid. C) Tissue adjacent to papillary thyroid carcinoma. D) Nodular goiter. E) Tissue adjacent to the nodular goiter. Section thickness: 4 μm; microscope magnification: ×400.

benignity or malignancy is critical. Thyroidectomy is especially important in patients who have thyroid nodules.

Many PTCs do not differ from benign thyroid nodules in terms of clinical presentation, signs and serum laboratory parameters. Although many diagnostic markers for thyroid cancer have been studied so far, such as circulating miRNA,[13] specific gene mutations,[14] CTCs,[15] and CECs.[16] However, these diagnostic techniques are expensive and operate in a limited environment. Pathological examination is the gold standard for identifying benign and malignant thyroid nodules, of which IHC is the best choice because it is cheap and convenient. However, the currently used IHC-related markers are deficient, so we need to find markers with higher specificity and sensitivity for early prediction of LNM.

. Nuclear transcription factor E2 related factor 2 (Nrf2) protein is an important antioxidant stress protein in cells. It is associated with oxidative damage and thyroid cancer. Nrf2 inhibitors are expected to serve as chemosensitizers in combination with anticancer agents.[17] Therefore, this study focuses on the possibility of Nrf2 as a molecular marker.

Nrf2 can encode antioxidase and detoxifying protein (HO-1, NQO1, *etc*.),[18] maintains redox equilibrium and plays a key role in the redox reaction. Aberrant expression of Nrf2 plays many roles in the pathogenesis of tumors, metabolic diseases and inflammation. Overexpression of Nrf2 lowers the level of oxidative stress. Low levels of oxidative stress lower the level of autophagy. Autophagic substrate p62 is overexpressed, and competes with Nrf2 for autophagic degradation of Keap1, Keap1, which further activates Nrf2, forming a positive feedback loop of antioxidant reactions that protects tumor cells.[19-21] Previous studies[9] have reported high expression of Nrf2 in a variety of tumors, including neuroendocrine carcinoma (approximately 32%), head and neck cancer (approximately 30%), lung cancer (approximately 28%), uterine cancer (approximately 21%), esophageal cancer (approximately 25%) and bladder cancer (approximately 15%). Nrf2 protein is not only highly expressed in the cancer tissues studied above. It was also found to be highly expressed in PTC in our study. Experiments by other researchers have the same results. Stuchi *et al*.[11] detected VEGFA and Nrf2 transcription levels and VEGFA protein levels in goiter, PTC and normal thyroid tissue samples, and found that VEGFA and Nrf2 transcription levels and

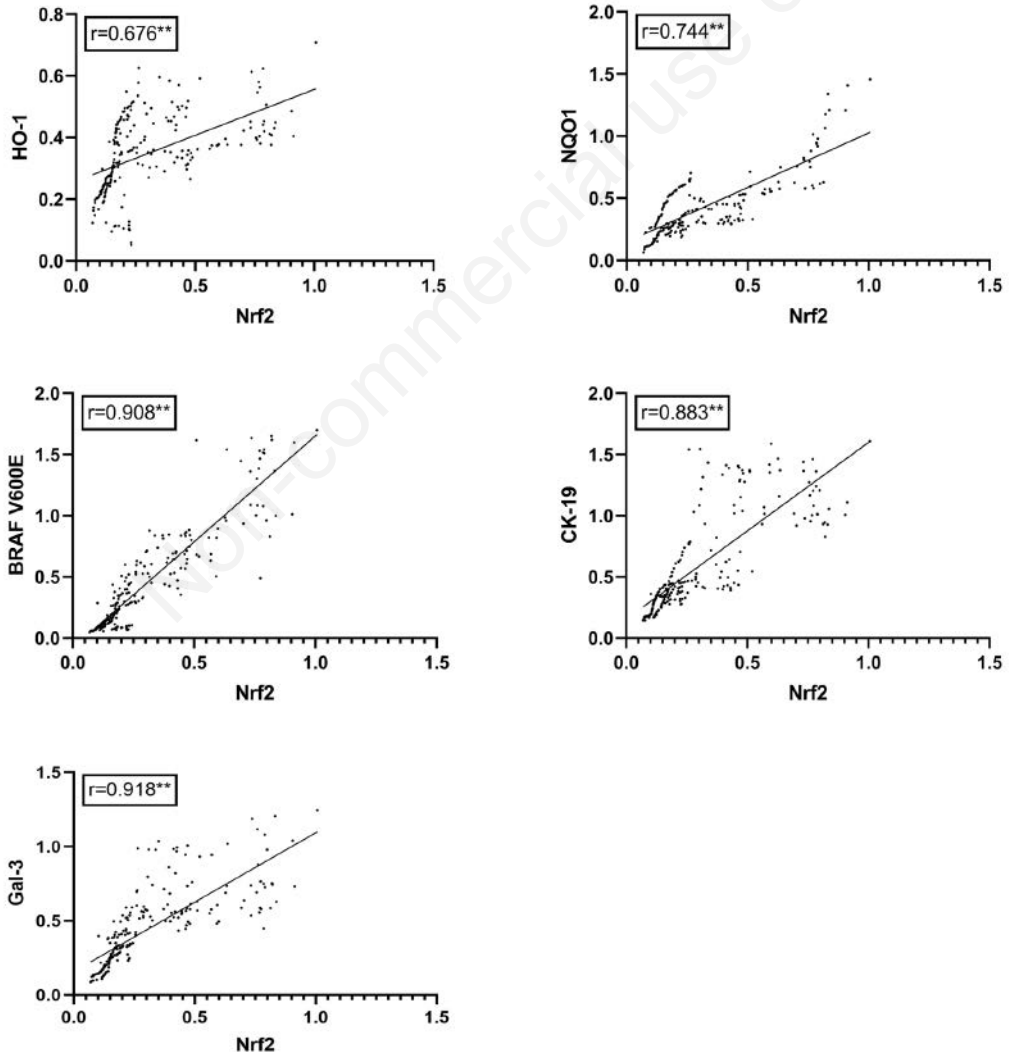

Figure 7. Correlation analysis between expression of Nrf2 and HO-1, NQO1, BRAF V600E, CK-19, Gal-3 protein. r, Spearman's rank correlation coefficient; *p<0.05; **p<0.01.

VEGFA protein levels were higher in PTC samples than in goiter and normal tissue. Ziros *et al*.[22] immunohistochemical analysis of PTC and benign nodules showed that Nrf2 and NQO1 were highly expressed in tumor tissues of PTC patients, and PTC cells promoted the proliferation of tumor cells through Nrf2/ARE/NQO1 signaling pathway. However, these studies only explored the expression level of Nrf2 protein between PTC and benign thyroid nodules, and did not include adjacent and adjacent tissues in the comparison. Our study found that the expression of Nrf2 in PTC tissues, tissue adjacent to PTC, NG and tissue adjacent to goiters decreased gradually and significantly. This may be related to the decreasing oxidative stress response in these four tissues.

After oxidative stress, Nrf2 is transferred to the nucleus, where it binds to the ARE sequence of the gene promoter to regulate the transcription of its target gene. Its regulatory genes include HO-1 and NQO1. Chen *et al*.'s[23] studies on the regulatory effect of HO-1 on apoptosis of human PTC cells (KAT5) showed that cells with elevated HO-1 levels are more resistant to apoptotic stimuli than cells with normal HO-1 levels, suggesting that HO-1 protects thyroid carcinoma cells from apoptosis induced by external stimuli. NQO1 is highly expressed in most solid human tumors, including colon, breast, pancreas, ovary, and thyroid.[24-26] In our study, high

Table 4. Basal clinical data of papillary thyroid carcinoma patients with lymph node metastasis.

	PTC	PTC with lymph node metastasis	PTC without lymph node metastasis	p
Age (years)	47.60±1.63	41.64±2.68	48.43±1.92	0.038
Gender (male)	25 (41.7%)	14 (56.0%)	11 (31.4%)	0.057
BMI	24.61±0.40	25.16±0.64	24.22±0.51	0.255
Systolic blood pressure (mmHg)	122.20±1.96	122.68±3.49	118.43±2.26	0.289
Diastolic pressure (mmHg)	77.43±1.28	77.52±1.83	77.37±1.78	0.955
TRAb (IU/ml)	0.30 (0.30, 0.42)	0.30 (0.30, 0.43)	0.30 (0.30, 0.40)	0.835
TSH (μIU/ml)	1.91±0.10	2.16±0.13	1.73±0.13	0.025
T3 (nmol/l)	1.75±0.04	1.87±0.06	1.66±0.04	0.007
T4 (nmol/l)	94.01±1.82	95.67±2.81	92.81±2.40	0.440
FT3 (pmol/l)	4.86±0.07	5.05±0.11	4.73±0.09	0.027
FT4 (pmol/l)	15.92±0.30	16.30±0.44	15.65±0.40	0.285
TGAb (IU/ml)	10.00 (10.00, 11.79)	10.00 (10.00, 11.85)	10.00 (10.00, 12.42)	0.943
TPOAb (IU/ml)	11.35 (8.58, 14.54)	12.97 (10.34, 16.04)	9.27 (6.60, 12.09)	0.595
Parathyroid hormone (pg/ml)	42.49±2.33	40.87±3.04	43.74±3.44	0.547
Alanine transaminase (U/L)	21.00 (15.00, 28.00)	22.50 (14.25, 28.25)	21.00 (13.50, 37.00)	0.088
Glutaminase (U/L)	25.00 (19.00, 29.00)	22.00 (18.00, 33.50)	27.00 (18.00, 28.50)	0.384
Urea (mmol/l)	4.81±0.15	4.60±0.19	4.95±0.23	0.274
Creatinine (μmol/l)	52.00 (46.00, 64.00)	57.00 (45.00, 65.25)	47.00 (45.00, 65.00)	0.179
Uric acid (μmol/l)	332.34±12.31	364.60±17.43	308.62±16.11	0.023
Blood glucose (mmol/l)	5.03 (4.76, 5.43)	5.19 (4.78, 6.78)	4.98 (4.34, 5.16)	0.942
Total cholesterol (mmol/l)	4.61±0.16	4.28±0.20	4.97±0.20	0.027
Triglycerides (mmol/l)	1.28 (1.14, 1.59)	1.22 (1.07, 1.64)	1.28 (1.21, 1.79)	0.549
High density lipoprotein (mmol/l)	1.20 (1.06, 1.42)	1.18 (1.03, 1.49)	1.21 (1.06, 1.49)	0.720
Low density lipoprotein (mmol/l)	2.53±0.11	2.31±0.14	2.79±0.12	0.021

PTC, papillary thyroid carcinoma; BMI, body weight index; TRAb, thyrotropin receptor antibody; TSH, thyrotropin; TGAb, anti-thyroglobulin antibody; TPOAb, thyroid peroxidase antibody. p, comparison between PTC group and NG group.

Table 5. Diagnostic consistency analysis in papillary thyroid carcinoma with lymph node metastasis of Nrf2 protein expression with HO-1, NQO1,BRAF V600E, CK-19 and Gal-3 protein.

	Intraclass correlation	95% Confidence interval		p
		Lower bound	Upper bound	
HO-1	0.836	0.726	0.902	<0.001
NQO1	0.868	0.779	0.921	<0.001
BRAF V600E	0.789	0.546	0.838	<0.001
CK-19	-0.374	-1.300	0.179	0.887
Gal-3	0.510	0.180	0.707	0.003

The intra-group correlation coefficient (ICC) can be used to evaluate the consistency of different measurement methods for the same quantitative measurement. ICC value between 0 and 1, generally considered: ICC 0.75 consistency is good, 0.40~0.75 as a general, <0.40 poor.

expression of HO-1 and NQO1 was also observed in PTC tissues. However, the expression trend of HO-1 and NQO1 in PTC tissues, tissue adjacent to PTC, NG and tissue adjacent to goiters was slightly different from that of Nrf2. This may be that HO-1, NQO1 is regulated by other factors in addition to Nrf2.

LNM is a significant factor associated with disease recurrence in patients with PTC.[27-29] Ho *et al*.[30] used data from 2000 to 2016 from hospital-based and population-based US cancer registries to analyze differences in prognosis between invasive PTC and non-invasive PTC. They found that long-term survival outcomes for aggressive PTC subgroups exhibit heterogeneous clinical behavior and a wide range of mortality risk. Nf2 has been reported to influence tumor metastasis, so we suspect that Nf2 can serve as molecular markers to predict lymphatic metastasis in PTC patients. Prolonged activation of Nrf2 facilitates transcription of genes associated with migration and invasion, resulting in enhanced migration and invasion. In addition, the high expression of Nrf2 promotes the growth, metastasis and angiogenesis of some tumor cells.[31,32] Nrf2 also regulates MMP2 and VEGF to mediate invasion and metastasis of colon, breast and hepatocellular carcinoma cells.[33,34] Fan *et al*.[35] found that Nrf2 overexpression increases the proliferation, migration and invasion of oral squamous cell carcinoma by modulating the Notch signal. Danilovic *et al*.[36] sequenced NFE2L2 and KEAP1 coding regions in 131 patients with PTC, analyzed clinical and histopathological features, and detected expression of Nrf2 in mutant carcinoma tissues by IHC. Expression of Nrf2 in the nuclei of all mutant carcinoma cells tended to increase and showed poor prognostic characteristics in histopathology. In this study, the expression of Nrf2 in PTC tissues with LNM was significantly higher than that in PTC tissues without LNM. Our experiments have come to the same conclusion.

Many studies have shown that HO-1 promote proliferation and migration of endothelial cells, and promote angiogenesis.[37] Angiogenesis is an important process for the continuous growth and invasion of solid tumors. In addition, NQO1 is also highly expressed in cancers with LNM. Mikami *et al*.[38] reported higher levels of NQO1 protein in colon cancer tumors with LNM than those without metastases. Our study also found high expression of HO-1 and NQO1 in PTC tissues with lymphatic metastasis. However, studies have reported that HO-1 activation may inhibit

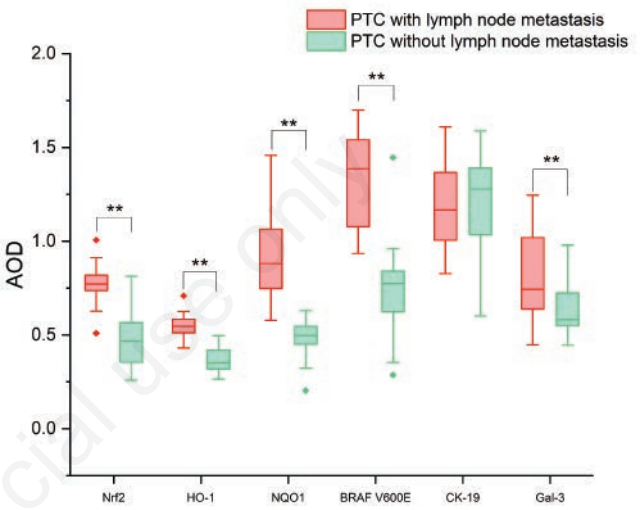

Figure 9. Quantitative average optical density (mean light density) analysis of Nrf2, HO-1, NQO1, BRAF V600E, CK-19 and Gal-3 proteins in papillary thyroid carcinoma with and without lymph node metastasis; these data represent the median and quartile ranges of average optical density (IQR). *p<0.05; **p<0.01.

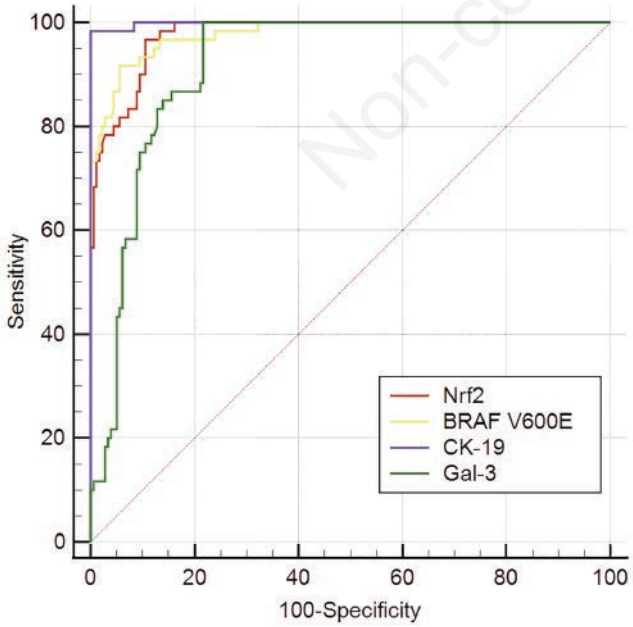

Figure 8. Area under curve of Nrf2, BRAF V600E, CK-19 and Gal-3 protein expression.

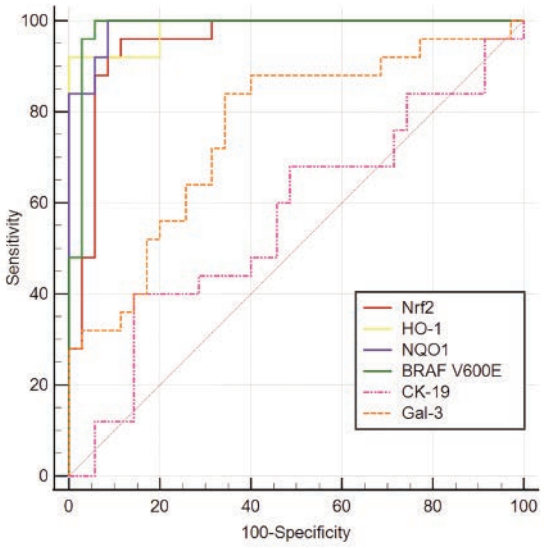

Figure 10. ROC curve area of Nrf2, HO-1, NQO1, BRAF V600E, CK-19 and Gal-3 protein expression.

breast cancer proliferation and prostate cancer angiogenesis.[39] These contradictory effects may be related to the different metabolic pathway crosstalk of HO-1 in different tumor types.

The common immunohistochemical markers in clinical use are BRAF V600E, CK-19 and Gal-3. Zheng *et al*.[40] performed an observational cohort study to identify molecular characteristics of PTC and a prognosis comparison of PTC with different genetic mutations. This study showed that the main mutation is common BRAFV600E (66.2%, 43/65) in PTC, which is associated with tall-cell variant, extrathyroidal invasion and advanced tumor stage (III/IV). Ivković *et al*.[41] conducted a retrospective study in which they analyzed the BRAF V600E mutation as prognostic markers on archival tissue samples of 49 patients without (control group) and 97 with (study group) PTC metastases in the cervical lymph nodes at the time of initial diagnosis. They found that patients who presented with the BRAF V600E mutation had shorter disease-free survival (log-rank test; 105.0 months vs. 146.6 months; $p<0.001$; HR 8.32, 95% CI: 2.91-23.83), and are at an increased risk for recurrence and require more intensive monitoring (Cox proportional hazards regression model; X =17.5, d =10, p=0.025). BRAF V600E protein was also found to be highly expressed in PTC as well as metastatic PTC in our experiments. Xin *et al*.[42] performed the quality assessment by using diagnostic accuracy studies scoring tool. The pooled result of CK-19 showed that sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR) and diagnostic odds ratio (DOR) were 0.816 (95% CI: 0.799-0.832), 0.872 (95% CI: 0.855-0.888), 5.900 (95% CI: 5.193-6.703), 0.205 (95% CI: 0.185-0.228), respectively. For Galectin-3, the pooled sensitivity, specificity, PLR, NLR and DOR were 0.842 (95% CI: 0.825-0.858), 0.833 (95% CI: 0.814-0.851), 5.057 (95% CI: 4.494-5.690), 0.176 (95% CI: 0.154-0.200) and 33.312 (95% CI: 26.403-42.029).

The AUC values in the summary receiver operating characteristic curve of CK-19 and Galectin-3 were 0.9134 (95% CI: 0.877-0.950) and 0.8452 (95% CI: 0.809-0.882), respectively. Our experiment showed similar results. In addition, we concluded that Gal-3 was highly expressed in PTC with LNM, while CK-19 expression was not significantly different. Searching for recent studies also did not find an association between CK-19 and LNM. It has been shown that Gal-3 promotes angiogenesis and neovascularization,[43] which may lead to its high expression in PTC with LNM.

The above-mentioned previous experiments can show that Nrf2 is highly expressed in PTC like BRAF V600E, CK-19, and Gal-3. Like HO-1, NQO1, and BRAF V600E, it is strongly expressed in papillary thyroid carcinoma with lymphatic metastasis. To find out the difference of Nrf2 from these proteins, we experimentally compared the ability of Nrf2 and other common diagnostic markers to diagnose PTC. We found that Nrf2 was more effective than BRAF V600E and Gal-3 and non-inferior to CK-19. We made ROC curves also indicating the high sensitivity and specificity of Nrf2. Therefore, we can conclude that Nrf2 can be used as a marker for the diagnosis of PTC. When we focused on papillary thyroid cancer LNM, we found that our experiments yielded a non-inferior diagnostic ability of Nrf2 over BRAFV600E for papillary thyroid cancer LNM. BRAF V600E is currently the most useful diagnostic and prognostic molecular marker for predicting occult LNM in patients with PTC.[44] Therefore, Nrf2 can be used as a marker not only for papillary thyroid cancer, but also for LNM of papillary thyroid cancer. This is very helpful for the diagnosis and prediction of the prognosis of papillary thyroid cancer.

Nrf2 can play a direct or indirect role in every cancer feature described so far, including carcinogenesis, sustained proliferation, evasion of apoptosis, metabolic reprogramming, altered redox homeostasis, metastasis formation, and treatment resistance.[43] Therefore, there is a growing interest in the development of effective therapeutic strategies that might disrupt the oncogenic functions of Nrf2. Therefore, testing for Nrf2 in patients with thyroid nodules may also be able to guide treatment.

Although we have gained some new discoveries, our research still has some limitations. The first limitation is the retrospective and single-center nature of our study. Secondly, the expression of Nrf2 protein in normal thyroid tissue was not analyzed in our study and compared with tumor tissue and goiter. Since the tissue adjacent to the tumor may not represent the normality of the tissue in the true sense and, in fact, may present some of the cellular alterations that precede these diseases. In view of the above limitations, larger multicenter and preferably prospective studies are warranted to validate our findings in the future.

In summary, the expression of Nrf2 protein in PTC is higher than that in nodular goiter and paracancerous tissue, and its diagnostic value is not inferior to that of immunohistochemical differential proteins BRAF V600E, CK-19 and Gal-3 in PTC. Therefore, Nrf2 protein can be used as a biomarker in the differential diagnosis of PTC. Nrf2 protein is highly expressed in patients with LNM from PTC and should be paid close attention to during postoperative follow-up. This study can be further promoted, combined with serum metabolism and fine needle biopsy of thyroid, for the preoperative diagnosis of PTC to provide a new program.

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