

Evaluation of peripheral blood E2F3 mRNA as a potential diagnostic biomarker in human papillary thyroid cancer

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Research

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Abstract

Background: The transcription factor E2F3 plays a vital role in regulating cell cycle progression and proliferation. In addition, many reports have shown that E2F3 exerts an oncogenic role in various cancers and is a promising therapeutic target for the treatment of some human cancers. However, the value of E2F3 in the molecular diagnosis and prognosis evaluation of papillary thyroid cancer (PTC) has rarely been reported. The aim of this study was to evaluate the clinical significance of E2F3 in the early diagnosis and monitoring of PTC.

Materials and methods: Peripheral blood samples from 20 patients with PTC, 20 patients with benign thyroid lesions and 20 normal controls were collected. Peripheral blood E2F3 mRNA and thyroid sample E2F3 mRNA expression were detected by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The expression of E2F3 protein in normal thyroid tissue and thyroid tissue from benign thyroid lesions and PTC were detected by immunohistochemistry. The optimal cut-off value for differentiating PTC was obtained by using receiver operating characteristic (ROC) curve analysis.

Results: E2F3 mRNA expression levels in peripheral blood and thyroid samples were significantly higher in PTC patients than in patients with benign thyroid lesions and normal subjects ($P < 0.05$). Moreover, benign hyperplasia had higher E2F3 expression than normal tissue ($P < 0.01$).

There was no significant association between the level of E2F3 mRNA and aggressive clinicopathologic features of PTC, including TNM staging, extrathyroidal invasion, multifocality, and lymph node metastasis ($P > 0.05$). With a cut-off value of 2.3, blood E2F3 mRNA showed 90% sensitivity, 60.9% specificity, a 70.5% positive predictive value, an 85.47% negative predictive value and 74.4% accuracy for discriminating PTC from benign hyperplasia disease of the thyroid and normal tissue. In addition, immunohistochemical results showed that E2F3 protein expression was also higher in the PTC group than in the other groups.

Conclusions: The highest expression of E2F3 was found in peripheral blood and thyroid tissue of PTC patients. Moreover, blood E2F3 mRNA can be considered a promising diagnostic marker for discriminating PTC from benign thyroid diseases.

Introduction

Thyroid cancer is the most common tumor of the endocrine malignancies, accounting for 95% of all endocrine malignancies[1–4]. In 2015, the guidelines of the American Thyroid Association (ATA) pointed out that thyroid cancer was increasing year by year and the number of newly diagnosed thyroid cancer increased from 37,200 in 2009 to 63,000 in 2014[5]. The yearly incidence nearly tripled from 4.9 per 100,000 in 1975 to 14.3 per 100,000 in 2009, which was mainly attributed to the increase in papillary thyroid cancer (PTC) [6]. Moreover, in 2019, PTC became the 3rd most common cancer in women, and its national medical expenditure will reach 19 billion to 21 billion US dollars, bringing huge economic burden to families and society[5]. In view of its high incidence and recurrence rate (20%-40%) and the mortality

rate accounting for 44.8% of the total mortality rate for thyroid cancer[7], early diagnosis and individualized prognostic assessment are critical in choosing appropriate clinical treatment as well as in reducing the recurrence and mortality rate due to PTC.

At present, the commonly used methods for early diagnosis of thyroid cancer include thyroid radionuclide scanning, thyroid ultrasonography and fine needle aspiration (FNA)[8, 9]. Among them, FNA is the most important diagnostic method, but it can only be used for cytological examination, and the positive detective rate of FNA is closely related to the experience of operators and the number of specimens. FNA can even fail to discriminate benign and malignant tumours in up to one-third of cases, which has adverse effects for clinical diagnosis and treatment[10, 11]. For those with an initially indeterminate cytology, 71% ultimately demonstrated benign histopathology[12]. These data indicated the need to implement other approaches, such as the development and subsequent use of molecular markers, to improve diagnostic accuracy. Furthermore, FNA is an invasive procedure. It is very necessary to find a new noninvasive and acceptable diagnostic biomarker for the early screening of PTC, especially one that is highly correlated tumour makers at the molecular level.

With the detailed study of tumor-related genes, the molecular mechanism of PTC has been further understood. Studies have shown that the BRAF gene mutation, RAS gene mutation and RET/PTC gene rearrangement are correlated with PTC occurrence and invasiveness to varying degrees[13, 14]. BRAF gene mutation are most closely related to PTC. Studies have shown that the BRAF V600E mutation is closely related to multifocality, lymph node metastasis, extrathyroidal extension and advanced-stage of PTC, thus predictive of poor prognosis of tumours[15, 16]. However, because the mutation only exists in 40–45% of PTC patients, it has some limitations as a diagnostic and prognostic marker[15]. In addition, there are some limitations and lags in the use of tissue samples for molecular biology detection, such as the limitation of the source of specimens and the lack of continuous detection and follow-up. Hence, the detection of nucleic acid molecules in the peripheral blood of cancer patients has become a hot topic for medical practitioners. E2F3 is oncogenic in tumorigenesis, and its functions correspond with poor prognosis in many cancers[17]. Early studies showed that E2F3 overexpression is associated with the development of breast, bladder and prostate cancer in humans[18, 19]. However, the relationship between E2F3 and human PTC has rarely been reported. In the present study, we measured blood E2F3 mRNA using the quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) technique in order to evaluate its clinical significance in the early diagnosis of PTC and to discuss the relationship between E2F3 and the clinicopathological features of PTC.

Materials And Methods

Subjects and sample collection

This study was conducted on 60 subjects consecutively recruited from the characteristic medical centre of Chinese People's Armed Police Force between July 2018 and December 2018. Subjects provided informed consent, and the study protocol was approved by the institute's committee on human research.

The study included 20 newly diagnosed patients with PTC, 20 patients diagnosed with benign thyroid lesions and 20 normal controls. The diagnoses of PTC and benign thyroid lesions were confirmed by histopathological evaluation of the ultrasound-guided biopsy sample preoperatively and by samples retrieved postoperatively. The normal patients were those who came to our hospital for physical examinations. All of them underwent thyroid function tests and thyroid ultrasound tests, and the results were negative. The normal thyroid tissues were located 1cm away from the benign lesion of thyroid, which was resected during the operation. No any prior chemotherapy or radiotherapy was received by any patient preoperatively. No other malignant tumours were found in any of the patients. There was no history of microbial infection in the past three months. Blood samples were collected three days before thyroidectomy, and the specimens were immediately snap-frozen in liquid nitrogen and then refrigerated at -80°C. Patients data were collected, including age, sex, TNM staging, extrathyroidal invasion, multifocality and lymph node metastasis.

All procedures used were in accordance with the Logistics University of the Chinese People's Armed Police Force human welfare guidelines. The protocols were approved by the Ethics Review Committee of the Logistics University of the Chinese People's Armed Police Force (Tianjin, China).

RNA isolation

All of the following RNA isolation protocols were performed in an RNA-dedicated work area with RNase/DNase-free water and RNase-free labware. Total RNA was extracted from whole blood samples with Quick-RNA™ Whole Blood (ZYMO RESEARCH, USA) and from thyroid samples with Quick-RNA™ MicroPrep (ZYMO RESEARCH, USA), according to the instructions provided in the kit. The RNA was then purified and concentrated with RNA Prep Buffer and RNA Wash Buffer. The total RNA concentrations and quality of each sample were then assessed with a NanoDrop ND1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). In addition, OD260/OD280 ratios between 1.8 and 2.1 were acceptable, while OD260/OD230 ratios less than 1.8 were unacceptable.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Following RNA extraction from whole blood and thyroid samples, cDNA was synthesized with reverse transcriptase according to the instructions provided in the kit (TIAN GEN, China). PCR was conducted in a 10-µl reaction volume consisting of the following: 1 µl cDNA, 5 µl Premix, 0.3 µl primer forward, 0.3 µl primer reverse, 0.2 ul ROX and 3.2 µl H₂O. The qPCR reaction was performed on a SYBR Green 7500 Real-Time PCR System (Applied Biosystems) as follows: one 15-min cycle at 95°C, forty cycles of 10 s at 95°C and 32 s at 60°C. β-actin served as a control. PCRs were conducted in triplicate. Relative E2F3 expression was calculated with the $2^{-\Delta\Delta C_t}$ method.

HE staining & Immunohistochemistry

Formalin-fixed paraffin-embedded biopsy specimens were sectioned into sections (5 µm). After that, sections were deparaffinized with xylene, rehydrated with graded alcohol solutions and stained with hematoxylin and eosin (HE). Finally, sections were examined microscopically.

Immunohistochemical detection of E2F3 was performed with formalin-fixed paraffin-embedded biopsy specimens. Sections (5 µm) were deparaffinized with xylene and rehydrated with graded alcohol solutions. Endogenous peroxidase activity was quenched by boiling the sections in 10 mM citrate buffer (pH 6.0) for 3 min, followed by cooling at room temperature for more than 20 min. Sections were incubated with anti-E2F3 antibody (1:200; cat. no. ab50917; Abcam, Cambridge, UK) overnight at 4°C. Sections were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (PV6001, Zhongshan Goldenbridge Biotechnology Co, Ltd, Beijing, China) for 30 min at 37°C, and the colour was developed with 3, 3'-diaminobenzidine for 1min. The negative control involved the omission of primary antibody. All slides were analysed by two independent observers. The images were acquired using NIS-Elements BR 4.30.00 64-bit software by Nikon DS Ri2 Microscopy (Kodak, Rochester, NY, USA).

The expression levels of E2F3 in different groups were systematically assessed. E2F3-positive cells were identified at low magnification (×10 ocular and ×10 objective) as “hot spots”. We selected ten hot spots per section to observe at higher magnification (×10 ocular and ×40 objective, high power field). Using a grid in the ocular lens (Olympus 100×), all positively stained cells in every high-power field were counted. The mean percentage of positively stained cells was used to evaluate the expression of the protein in a section. The fraction of E2F3-positive cells was scored as 0 (0% positive cells), 1 (1%–25% positive cells), 2 (26%–50% positive cells), 3 (50%–75% positive cells) or 4 (>75% positive cells). The intensity of E2F3 immunostaining was scored as 0 (negative), 1 (weak), 2 (intermediate) or 3 (strong). The intensity score (0–3) was multiplied by the fraction score (0–4), and a final score was assigned as follows: 0 (negative, -), 1–4 (weak expression, +), 5–8 (moderate expression, ++), and 9–12 (strong expression, +++).

Statistical analysis

Quantitative data are descriptively expressed as the median and interquartile range (IQR). Comparison between the ranks of more than 2 independent groups, in case of nonparametric data, was done by Kruskal Wallis test, while that of 2 independent groups was done using the Wilcoxon rank sum Test. The chi-square test was used to compare the counting data. To assess E2F3 staining intensity, we used the nonparametric Mann-Whitney test for the comparison of groups. A receiver operator characteristic (ROC) curve was applied to assess the overall diagnostic value for discriminating PTC from benign hyperplastic diseases and normal tissue. “ $P < 0.05$ ” was used for statistical significance.

Results

Expression of E2F3 mRNA by qRT-PCR

Statistical analysis showed that there was no difference in age and sex among the three groups ($P > 0.05$, Table 1). The expression of E2F3 mRNA in blood samples and tissue samples is shown in Fig.1. The levels of peripheral blood E2F3 mRNA expression and thyroid sample E2F3 mRNA expression were higher in the PTC group than in either the benign hyperplasia group ($P = 0.001$, $P = 0.001$, Table 1) or the normal group ($P = 0.005$, $P = 0.012$, Table 1). There was no significant difference in the level of peripheral blood

E2F3 mRNA expression between the benign and normal groups ($P>0.05$, Table 1). However, benign hyperplasia had higher E2F3 expression than normal tissue ($P=0.001$, Table 1). This means that the level of E2F3 mRNA expression may help identify benign hyperplasia and normal tissue.

Correlations of E2F3 expression with clinicopathological features of PTC

We researched the correlations between peripheral blood E2F3 mRNA and clinicopathological features of PTC. The results showed that there was no statistically significant correlation among blood E2F3 mRNA and TNM staging, extrathyroidal invasion, multifocality, or lymph node metastasis in PTC ($P>0.05$, Table 2).

Peripheral blood E2F3 mRNA is a sensitive diagnostic marker in PTC

The optimal cut-off value for differentiating PTC was obtained by using receiver operating characteristic (ROC) curve analysis. Peripheral blood E2F3 mRNA, as a biomarker for discriminating PTC from benign hyperplasia diseases and normal tissue, revealed that the best diagnostic cut off for blood E2F3 mRNA ($2^{-\Delta\Delta CT}$) was 2.30. This showed 90% sensitivity, 60.9% specificity, a 70.5% positive predictive value, an 85.47% negative predictive value, and 74.4% accuracy, and the area under the curve (AUC) was 0.784 (Fig. 2).

The expression of E2F3 protein in thyroid tissue was upregulated in the PTC group and the PTC with lymphatic metastasis group

Sections were subjected to both routine Hematoxylin-eosin (HE) staining (Fig3.) and Immunohistochemistry staining (Fig4.). Positive E2F3 staining was brown and localized in the nuclear region (Fig4.). No statistically significant difference in the level of E2F3 expression was detected among normal, nodular goiter ($Z=-1.122$, $P=0.262$) and follicular adenoma ($Z=-1.826$, $P=0.068$) groups. The expression of E2F3 was upregulated in the PTC group ($Z=-5.701$, $P<0.001$) and the PTC with lymphatic metastasis group ($Z=-4.524$, $P<0.001$) compared with the normal group, the nodular goiter group and the follicular adenoma group. However, there was no statistically significant difference in the level of E2F3 expression between the PTC group and the PTC with lymphatic metastasis group ($Z=-1.420$, $P=0.156$) (Table 3).

Discussion

E2F3 plays a critical role in the transcriptional activation of genes that control the rate of proliferation of both primary and tumour cells. E2F3 contains two different isoforms, namely, E2F3a and E2F3b[20, 21]. Previous studies showed that E2F3b represses the expression of *Arf* in normal mouse embryo fibroblasts, while E2F3a may contribute to *Arf* activation in response to oncogenic stress, and the *Arf* tumour suppressor is a key component of the p53 tumour surveillance pathway[22, 23]. Moreover, E2F3 plays an important role in determining the time of the G1/S transition and the rate of DNA synthesis [24, 25]. Early studies demonstrated that E2F3 is oncogenic in various human cancers, including breast, bladder, lung

and prostate cancer[18, 19, 26]. E2F3 is essential in promoting the proliferation of ovarian cancer cells by initiating integral signaling of the EGFR-driven cell response[27]. Furthermore, Foster CS et al.[28]found that E2F3 expression may be an independent factor predicting overall and cause-specific survival in prostate cancer and advocated that the pRB-E2F3-EZH2 control axis may have a critical role in modulating the aggressiveness of individual cases of human prostate cancer. For lung cancer, E2F3 is a sensitive diagnostic marker and a promising prognostic marker for differentiating early from advanced stages[26]. These studies have also indicated that E2F3 exerts an oncogenic role in various cancers and is a promising therapeutic target for the treatment of human cancers[29]. However, the role of E2F3 in the development and prognosis of PTC has rarely been reported. In the present study, we found the highest expression of peripheral blood E2F3 mRNA in PTC patients compared to patients with benign thyroid nodules or normal controls. The alterations in E2F3 mRNA and protein expression in thyroid tissues from different groups had similar patterns to the expression of peripheral blood E2F3 mRNA in different groups. Moreover, the level of E2F3 mRNA expression in patients with benign thyroid nodules was higher than that in normal individuals. Given the previous studies in lung cancer and breast cancer[30-32], we predicted that circulating tumour cells present in the peripheral blood might contribute to increases in the peripheral blood mRNA levels of the E2F3 gene in the peripheral blood of patients with PTC. By ROC curve analysis, blood E2F3 mRNA was able to effectively distinguish PTC from benign hyperplasia diseases and normal tissue at a cut off 2.30. These findings were in close agreement with previous studies that demonstrated that peripheral blood E2F3 mRNA may be a diagnostic marker in lung, breast and prostate cancer[18, 19, 26, 31, 33, 34].

To further explore the regulatory mechanism of E2F3 in PTC, we also carried out relevant experimental studies. Pen et al reported that the hsa_circRNA_100395/ miR-200a-3p/E2F3 axis may be involved in the pathogenesis of PTC[35]. Therefore, we also detected hsa_circRNA_100395 and miR-200a-3p expression in the peripheral blood of PTC patients by using qRT-PCR in this study. However, our study showed that there were no significant differences among the PTC group, benign thyroid lesion group, and control group. Therefore, we did not expand the sample to continue the in-depth study, and the results are not shown in this paper.

Our study revealed that the tissue expression of E2F3 was upregulated in the PTC group and the PTC with lymphatic metastasis group compared with the normal group, the nodular goitre group and the follicular adenoma group. There was no statistically significant difference in the level of E2F3 expression between the PTC group and the PTC with lymphatic metastasis group. This is similar to the peripheral blood E2F3 mRNA expression alterations between the PTC group and the PTC with lymphatic metastasis group. Furthermore, the level of peripheral blood E2F3 mRNA expression had no statistically significant relationship with the clinicopathological features of PTC patients. This means that E2F3 expression may not be a prognostic biomarker for PTC, which is different from lung and prostate cancer. We speculated that the reason may be that lung cancer and prostate cancer have a higher incidence and mortality than PTC. Scientists pay more attention to them. However, PTC has a very high incidence rate in China and has been increasing rapidly in recent years, which has aroused great concern from Chinese doctors. Therefore, we studied the relationship between E2F3 expression in the peripheral blood and PTC to find a

noninvasive and effective tool for screening as early as possible to benefit people. The molecular mechanisms by which E2F3 mRNA is upregulated in the peripheral blood of PTC patients need to be studied in the future.

At present, FNA is still considered the gold standard for the diagnosis of thyroid nodules[5]. A study showed that the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of FNA were 96.9%, 81.7%, 73.8%, 98%, and 86.9%, respectively[36]. In the study of Chieng J et al., FNAC predicted malignancy with a sensitivity, specificity, PPV, NPV, false positive rate, false negative rate and total accuracy of 90.7%, 53.6%, 43.3%, 93.7%, 46.4%, 9.3% and 64.1%, respectively[37]. However, FNAC is considered to be “uninterpretable” in 10 to 20% of cases or simply “suspicious” in 9 to 38% of cases[38]. In addition, it is currently estimated that cytologically indeterminate results are diagnosed in up to 25% to 30% of thyroid biopsies[39, 40]. Therefore, the use of molecular markers is important for improving diagnostic accuracy. In this study, blood E2F3 mRNA predicted malignancy with a sensitivity, specificity, PPV, NPV, and accuracy of 90%, 60.9%, 70.5%, 85.47%, and 74.4%, respectively. Furthermore, FNA is an invasive procedure, and there is a low acceptance rate among Chinese patients, so it has some limitations. FNA also brings extra financial burden to patients, and our hospital has not yet carried out FNA tests for PTC. Patients with Bethesda III nodules will be advised to follow up regularly, while patients with Bethesda IV nodules will be operated on directly. Therefore, the PTC patients in this study were all Bethesda IV patients. Because the detection of serum E2F3 is noninvasive and less expensive than FNA detection, it may be an economic and effective way to screen PTC patients from Bethesda III and IV patients in the future. Regardless, no test is perfect. If patients can afford the expense, the combination of an FNA test and serum E2F3 test will greatly improve diagnostic accuracy. The abovementioned results demonstrated that peripheral blood E2F3 can be considered a promising molecular diagnostic biomarker but not a prognostic biomarker in PTC.

Conclusions

Our study revealed the highest expression of peripheral blood E2F3 mRNA, thyroid sample E2F3 mRNA and tissue E2F3 protein in the PTC group compared to the benign thyroid lesion group and the control group. Peripheral blood E2F3 mRNA can be considered a promising biomarker for the diagnosis of PTC. As a relatively noninvasive examination, it is more acceptable to patients than FNA. Further studies are needed to clarify the function and mechanism of E2F3 in PTC pathogenesis.

Declarations

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Authors' contributions

All authors were involved in the conception and design of the study. CRL and FYW conceived and designed the study. YZ and ZQZ wrote the manuscript, and contributed to the experiments and project management. DDW and WL involved in the subjects and samples collecting. YLG and YH were involved in the analysis and interpretation of datasets. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All patients signed an informed consent form, and the experimental protocol was approved by the Ethics Committee of the Characteristic medical center of Chinese People's Armed Police Force(no.2017-0023).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

PTC: papillary thyroid cancer; FNA: fine needle aspiration; qRT-PCR: quantitative real-time reverse transcription-polymerase chain reaction; IQR= Interquartile range; ROC: receiver operating characteristic;

AUC: area under curve; ETE: extrathyroidal extension; LNM: Lymph node metastasis.

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Tables

Table1 Descriptive and comparative statistics of the studied parameters among different groups

	PTC group	Benign hyperplasia group	Normal group	P ₁	P ₂	P ₃	P ₄
Age (Years, n)				0.299	-	-	-
<55	17	12	14				
≥55	3	8	6				
Sex (n)				0.800	-	-	-
Male	5	6	4				
Female	15	17	16				
E2F3 mRNA ^a (Median (IQR))	32.87(3.96-140.36)	1.61(0.03-19.21)	0.92(0.28-2.39)	0.001	0.005	0.001	0.349
E2F3 mRNA ^b (Median (IQR))	42.83(13.49-163.38)	4.13(2.57-22.98)	1.15(0.26-1.92)	0.001	0.012	0.001	0.002

PTC= Papillary thyroid cancer; IQR= Interquartile range; P₁: Difference among 3 groups; P₂: Difference between PTC group and benign hyperplasia group; P₃: Difference between PTC group and normal group; P₄: Difference between benign hyperplasia group and normal group; ^a blood samples; ^b thyroid samples.

Table 2 Correlations of peripheral blood E2F3 mRNA and clinicopathological features of papillary thyroid cancer

	Number	Z	P
TNM Staging		-1.217	0.223
I	17		
II	3		
T Staging		-1.528	0.127
T1/T2	15		
T3/T4	5		
N Staging			0.724
N0	11		
N1a	5		
N1b	4		
Multifocality		-0.309	0.758
Negative	12		
Positive	8		
ETE		-1.528	0.127
Negative	16		
Positive	4		
LNM		-0.190	0.849
Negative	11		
Positive	9		

ETE=Extrathyroidal extension; LNM=Lymph node metastasis

Table 3 Variations in E2F3 expression among different groups

Group	n	Intensity of staining				Z	P
		-	+	++	+++		
Normal Thyroid	20	8	12	0	0		
Nodular Goiter	14	3	11	0	0	-1.122	0.262
Follicular Adenoma	6	0	6	0	0	-1.826	0.068
PTC	20	0	0	4	16	-5.701	<0.001
PTC with lymphatic metastasis	9	0	0	0	9	-4.524	<0.001

PTC= Papillary thyroid cancer

Figures

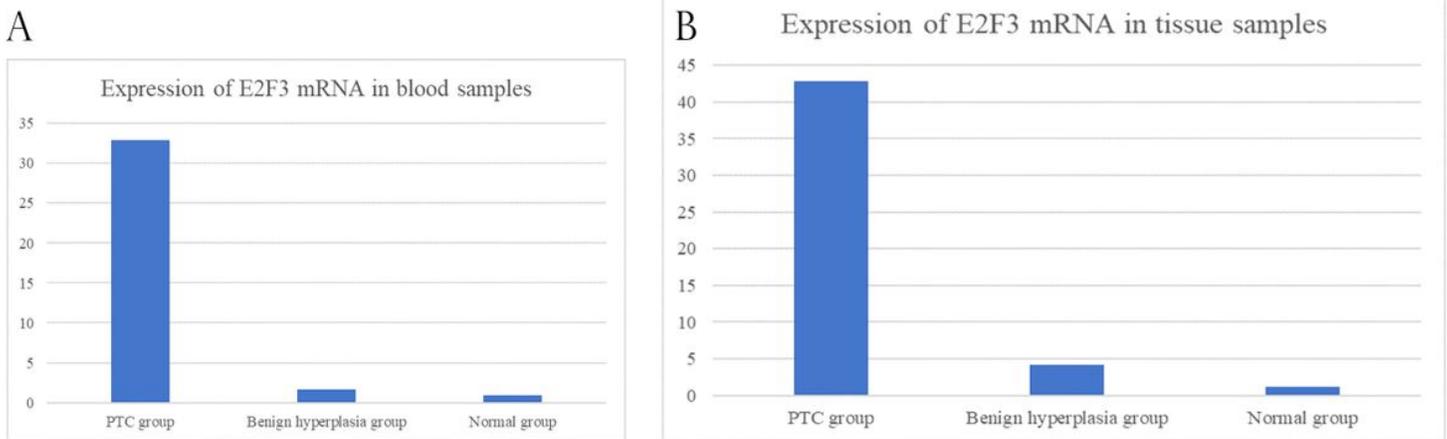


Figure 1

Expression of E2F3 mRNA in blood and tissue samples. (A) blood samples; (B) tissue samples

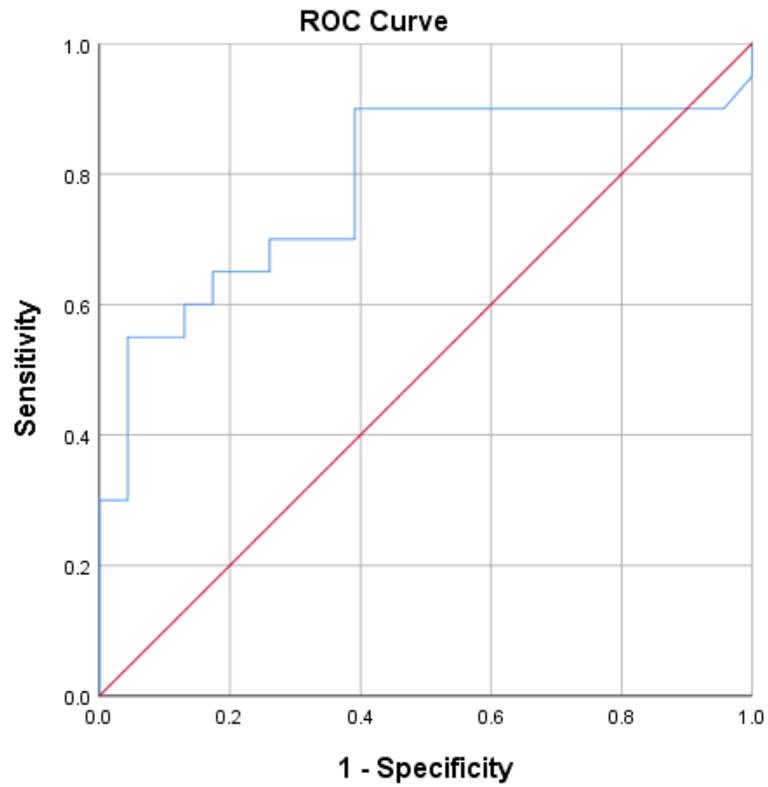


Figure 2

ROC curve analysis showing the diagnostic value of blood E2F3 mRNA for discriminating papillary thyroid cancer from benign hyperplasia diseases and normal group (AUC = 0.784).

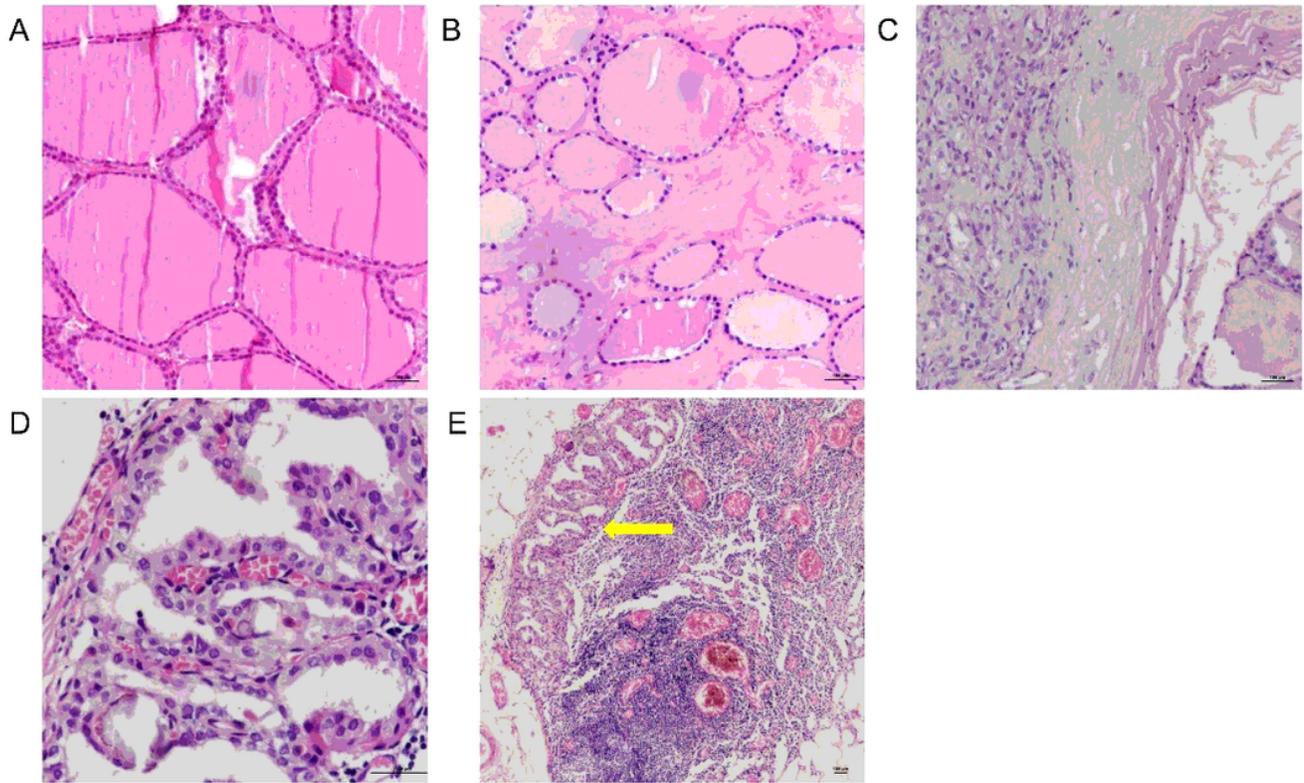


Figure 3

Microscopic examination of normal thyroid, nodular goiter, follicular adenoma, PTC and PTC with cervical lymph node metastasis HE staining. (A) normal thyroid, 100; (B) nodular goiter, 100; (C) follicular adenoma, 100; (D) PTC, 200. (E) PTC with cervical lymph node metastasis. The subcapsular sinuses distended with tumor cells. Nests of tumor cells have also invaded the subcapsular cortex (arrow), 40.

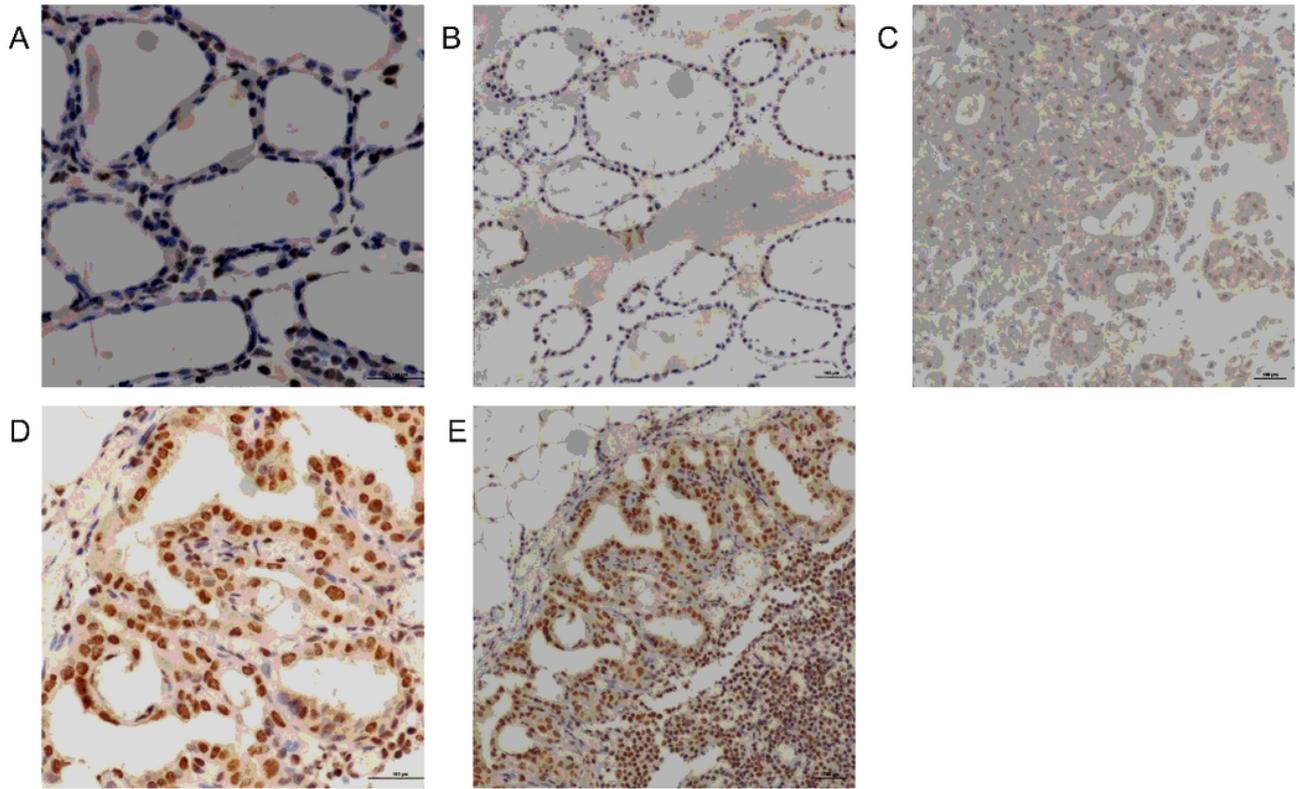


Figure 4

Representative immunohistochemical staining for E2F3 protein expression in normal thyroid, nodular goiter, follicular adenoma, PTC and PTC with cervical lymph node metastasis. (A) normal thyroid, 200; (B) nodular goiter, 100; (C) follicular adenoma, 100; (D) PTC, 200. (E) PTC with cervical lymph node metastasis, 100.