

RESEARCH ARTICLE

Combined Application of Fine-Needle Aspiration Cytology with BRAFV600E Mutation Screening in the Detection of Different Grades of Papillary Thyroid Carcinoma

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Abstract: The present study aimed to evaluate the application of fine-needle aspiration cytology (FNAC) of the thyroid coupled with the screening of serine/threonine-protein kinase B-Raf (BRAF) gene mutation in the detection of papillary thyroid carcinoma (PTC). From October 2016 to June 2019, 1244, liquid-based specimens originated from the patients diagnosed with PTC and benign lesions were collected from the Pathology Department of First Central Hospital of Baoding. BRAFV600E mutation was screened in all the liquid-based specimens using quantitative fluorescence polymerase chain reaction. Combined with the pathological results after surgery, the results of FNAC alone and FNAC combined with BRAFV600E screening results in PTC of different grades were compared and analyzed. Of the 1244 cases, there were 818 cases with definite cytological diagnosis and 426 cases with uncertain diagnoses. Combined with BRAFV600E screening, 36 out of 90 cases with atypical cells and 150 out of 223 suspected PTC were diagnosed as PTC. In conclusion, coupled with BRAFV600E mutation screening, the pre-operative detection rate of PTC can be increased in patients with atypical cells and suspected PTC which cannot be confirmed by pre-operative FNAC.

Keywords: Papillary thyroid carcinoma, Fine-needle aspiration cytology, BRAFV600E

1. Introduction

Papillary thyroid carcinoma (PTC) is the most common malignant tumor in thyroid cancer. In recent years, the incidence of PTC has been increasing. Currently, surgery is the principal mode of treatment of PTC. Therefore, it is important to accurately determine the benign and malignant thyroid nodules before surgery^[1,2].

Ultrasound-guided fine-needle aspiration cytology (FNAC) is currently the most accurate and sensitive method for diagnosing PTC. In general, the diagnosis of PTC is not difficult since it can be done according to the characteristics of the nucleus. However, sometimes due to the atypical characteristics of the nucleus, or too few liquid-based specimens, missed diagnosis or misdiagnosis can be easily resulted^[3-5]. The diagnosis of PTC cannot be established by relying on a single diagnostic method. To further improve the pre-operative diagnostic accuracy of PTC and reduce the rates of missed diagnosis and misdiagnosis, new diagnostic methods are needed.

In recent years, most literature have reported that the BRAFV600E mutation of the

serine/threonine-protein kinase B-Raf (BRAF) gene is closely related to the occurrence and progression of thyroid cancer^[6], and screening the mutation in combination with other methods can improve the diagnostic accuracy of PTC. At present, the combined application of thyroid FNAC with BRAFV600E mutation screening has been put into clinical use. However, reports on the use of BRAFV600E mutation screening in the detection of different grades of PTC are scarce.

The present study aimed to evaluate the combined application of pre-operative thyroid FNAC with BRAFV600E mutation screening and to study its significance in the diagnosis of thyroid cytology by combining with post-operative pathology to improve the accuracy of pre-operative diagnosis of PTC and to reduce the misdiagnosis and missed diagnosis.

2. Materials and methods

2.1. Study participants

A total of 1244 retrospective specimens of thyroid liquid-based specimens which were diagnosed as PTC and benign mutation from October 2016 to June 2019, were obtained from the Pathology Department of the First Central Hospital of Baoding. There were 442 males and 802 females who aged from 20 to 74 years old, with a median age of 38.9 ± 13.9 years old.

2.2. Cytological smear staining

All specimens used in this study were the remaining liquid-based specimens collected using fine-needle aspiration of the thyroid. The number of cells in each smear was more than 100. The volume of the liquid-based specimens was more than 10 ml. The collected specimens were preserved at 4°C and used within 4 months.

According to The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC), there are six diagnostic categories for thyroid cytology reporting: Category I, unsatisfactory specimen; Category II, benign; Category III, atypical cells with unknown significance; Category IV, follicular tumor; Category V, suspected malignancy; and Category VI, malignancy.

In TBSRTC, classification results that fall into Categories III, IV, and V are considered an uncertain diagnosis, whereas results in Categories II and VI are considered definite diagnosis.

2.3. DNA extraction

The cells in the liquid-based specimens of fine-needle aspiration were centrifuged, fixed using formalin, and embedded using paraffin wax. Subsequently, DNA was extracted from the paraffin-embedded cell sections using QIAamp DNA FFPE Tissue Kit (Qiagen).

2.4. BRAFV600E mutation screening

In the mutation screening experiment, the genomic DNA of each sample was added to the reaction tube containing two different polymerase chain reaction (PCR) reaction mixtures. The experiment was carried out along with weak positive control and non-template control. The recommended concentration of the genomic DNA of the samples to be tested is 5 – 15 ng/L. Two microliters of the sample mixture were then loaded to each well. PCR amplification was carried out.

The value of the cycle threshold (Ct) was calculated using the formulae of Ct value of mutation detection and Ct value of quality control detection. The value of $\Delta Ct \geq 28$ was positive and the value of $\Delta Ct < 28$ was negative. The results were analyzed according to the specification, and the mutation of BRAFV600E was calculated.

At the same time, 5 uL of each DNA sample was used to verify the specificity of PCR amplification results using agarose gel electrophoresis.

2.5 Histopathological observation

Surgical resection specimens were fixed with 4% formaldehyde (neutral) and embedded in paraffin. The tissue block was trimmed into sections of about 4 – 6 μ m thick, which were stained with hematoxylin and eosin. The tissue sections were observed under a microscope.

2.6. Observation indicators

The diagnosis results of FNAC and the BRAFV600E mutation were analyzed. The number of cases of true positive, false positive, true negative, and false negative was determined to assess the statistical measures such as sensitivity, specificity, and accuracy of FNAC and combined application of FNAC and BRAFV600E mutation screening for diagnosing PTC. The interpretation of these diagnostic criteria is given in Table 1.

The equations for calculating sensitivity, specificity, and accuracy of two PTC detection methods are given below:

$$\text{Sensitivity (\%)} = \frac{TP}{TP+FN} \times 100$$

$$\text{Specificity (\%)} = \frac{TN}{TN+FP} \times 100$$

$$\text{Accuracy (\%)} = \frac{TP+TN}{TP+FP+FN+TN} \times 100$$

where TP, number of true-positive cases; FP, number of false-positive cases; FN, number of false-negative cases; TN, number of true negative cases.

2.7. Statistical analysis

The statistical analyses were performed using Statistical

Package for the Social Sciences (SPSS), version 20 (SPSS Inc., Chicago, IL, USA). Chi-squared (χ^2) test was used to calculate the sensitivity, specificity, and accuracy of two PTC detection methods that either uses FNAC results only or employing the results of both FNAC and BRAFV600E mutation screening. $P < 0.05$ is considered statistically significant.

3. Results

3.1. Baseline characteristics and BRAFV600E mutation screening in different diagnostic categories of thyroid cytology

Among the 1,244 retrospective specimens, the post-operative pathological results revealed that there were 996 PTC cases and 248 benign lesion cases. The average age of the PTC cases was 38.9 ± 12.8 years, while the benign cases were 38.9 ± 14.2 years. There was no significant difference in the average age between PTC cases and benign cases, as determined by the post-operative pathology test (Chi-square statistic = 0.062).

As shown in **Table 2**, the pre-operative detection rate of PTC can be increased in patients with atypical cells and suspected PTC, if BRAFV600E mutation screening was used in combination with FNAC results. Combined with the BRAFV600E detection approach, 36 out of 90 cases of atypical cells and 150 out of 223 cases of suspected PTC could be diagnosed as PTC. BRAFV600E mutation could not be detected in any of the benign cases identified by post-operative pathology test.

Regardless of the post-operative pathological results, there were 818 cases with a definite diagnosis, comprising 208 benign cases (Category II) and 610 PTC cases (Category VI), according to cytological diagnostic criteria. In the 818 cases

with definite cytological diagnosis, 610 PTC cases had been confirmed to be PTC in the post-operative pathology test. On the other hand, 198 out of 208 benign cases had been confirmed to be benign in post-operative pathology test, while the remaining ten benign cases had been diagnosed otherwise as PTC in post-operative pathology test. The remaining 426 cases were of uncertain diagnosis, comprising 108 cases of atypical cells (Category III), 75 cases of follicular tumors (Category IV), and 243 suspected PTC cases (Category VI). In the 426 cases with uncertain cytological diagnosis, 50 cases with atypical cells, follicular tumors, and suspected PTC were diagnosed as benign, whereas 376 cases with atypical cells, follicular tumors, and suspected PTC were diagnosed as PTC, in the post-operative pathology test.

Different thyroid cytological categories that were diagnosed using FNAC method and post-operative pathological diagnosis of PTC are shown in **Figure 1**.

3.2. Significance of FNAC combined BRAFV600E detection in the auxiliary diagnosis of PTC

The current study evaluated and compared two methods for the detection of PTC. The sensitivity, specificity, and accuracy of using FNAC only and combined application of FNAC and BRAFV600E mutation screening for detecting PTC were calculated. We found that the combined application of FNAC with BRAFV600E mutation screening in the detection of different grades of PTC can also improve the sensitivity, specificity, and accuracy of PTC diagnosis (**Table 3**).

The sensitivity, specificity, and accuracy of simple cytology in the diagnosis of PTC are 61.24%, 79.83%, and 64.95%, respectively. On the other hand, the sensitivity,

Table 1. Diagnostic test interpretation of true-positive, false-positive, true-native, and false-negative cases assessing papillary thyroid carcinoma detection methods

	PTC detection method	
	FNAC only	FNAC+BRAFV600E screening
True-positive case	Cases which were identified as PTC in both post-operative pathological results and cytological diagnosis	Cases in which BRAFV600E mutation was detected, and determined as PTC in post-operative pathological results
False-positive case	Non-benign cases according to cytological diagnosis but determined as benign in post-operative pathological results	Non-benign cases according to cytological diagnosis but determined as benign in post-operative pathological results
True-native case	Cases which were identified as benign in both post-operative pathological results and cytological diagnosis	Cases which were identified as benign in both post-operative pathological results and cytological diagnosis
False-negative case	Non-PTC cases according to the cytological diagnosis but determined as PTC in post-operative pathological results	Cases in which BRAFV600E mutation was not detected, but determined as PTC in post-operative pathological results

PTC, papillary thyroid carcinoma; FNAC, fine-needle aspiration cytology.

Table 2. Detection of BRAFV600E mutation in six diagnostic categories for thyroid cytology reporting^a

Post-operative pathology (n)	Cytological diagnosis	Number of cases	BRAFV600E mutation (%)
PTC (n=996)	Unsatisfactory specimen (Category I)	0	0
	Benign (Category II)	10	0
	Atypical (Category III)	90	36 (40)
	Follicular tumors (Category IV)	63	0
	Suspected PTC (Category V)	223	150 (67)
	PTC (Category VI)	610	580 (95)
Benign (n=248)	Unsatisfactory specimen (Category I)	0	0
	Benign (Category II)	198	0
	Atypical (Category III)	18	0
	Follicular tumors (Category IV)	12	0
	Suspected PTC (Category V)	20	0
	PTC (Category VI)	0	0

^aAccording to The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC); PTC, papillary thyroid carcinoma.

specificity, and accuracy of cytology combined with BRAFV600E mutation screening in the diagnosis of PTC are 76.90%, 79.83%, and 77.49%, respectively. Although the specificity of the two methods was similar, the sensitivity and accuracy of FNAC combined with BRAFV600E mutation screening in the detection or diagnosis of PTC were significantly higher ($P < 0.01$).

4 Discussion

In recent years, the global incidence of thyroid cancer has shown a rising trend, of which more than 80% of thyroid cancer is PTC^[7]. Therefore, how to correctly evaluate the nature of thyroid nodules before surgery and to develop reasonable treatment plans and surgical methods have become the focus of current research^[8]. With the development of ultrasound-guided FNAC, the detection rate of PTC is increasing. FNAC is safe, less invasive, and highly sensitive. It is the gold standard for pre-operative diagnosis of thyroid cancer. However, the diagnosis of thyroid cancer using FNAC is limited by the atypical characteristics of the nucleus and the small number of cells that are not amenable to clear diagnosis.

In the clinical setting, the molecular markers of thyroid nodules can help aid in the preoperative diagnosis of PTC and selecting the pre-operative surgical procedures, as well as choosing a more suitable diagnosis and treatment plan. In recent years, genetic and immunohistochemical techniques have gradually developed into an indispensable means for clinicopathological diagnosis^[9-12]. Most literature reported that the combination of FNAC and BRAFV600E mutation screening can achieve higher accuracy and sensitivity in the detection of PTC, which effectively improve the

discriminatory accuracy of suspected malignant results, and promote the rationalization of clinical treatment mode, thus greatly enhancing its future therapeutic effect^[13,14].

The BRAFV600E mutation is the most common genetic alteration in PTC. It is a point mutation (T1799A) that is located at the 1799th position of the 15th exon of the BRAF gene. The mutation resulted in the substitution of valine (V) at the 600th codons in its protein product with glutamic acid (E)^[15]. The mutation rate of BRAFV600E in PTC was as high as 65.1 – 84.0% and secondary mutations do not generally occur in PTC cases^[16]. Therefore, BRAFV600E mutation is an important indicator in assisting the diagnosis of PTC using thyroid FNAC^[17].

Zatelli *et al.*^[18] found that the BRAFV600E mutation rate was 64% out of the 469 thyroid nodule puncture being analyzed, and the sensitivity of PTC cytology increased from 77% to 87% after BRAFV600E mutation was used. Another study Zhao *et al.*^[19] reported that, in combination with the cytology approach, the BRAFV600E screening test helped diagnose more PTC cases. The analysis of 88 studies concerning thyroid FNAC and BRAFV600E mutation revealed that the combination of cytology and BRAFV600E screening assays increased the sensitivity from 81.4% to 87.4% and reduced the false-negative rate from 8.0% to 5.2%^[14]. Furthermore, BRAFV600E mutation detection can improve the sensitivity of FNAC by 10 – 15.3%^[13,18], which can effectively improve the discriminatory accuracy of suspected malignancy results, and promote the rationalization and advancement of clinical treatment mode, thus greatly enhancing the effect of treatment in the future^[20,21].

According to TBSRTC, the categories of atypical cell, follicular tumor, and suspected papillary carcinoma are

Table 3. Sensitivity, specificity, and accuracy of using FNAC only and combined application of FNAC with BRAFV600E mutation screening for detecting papillary thyroid carcinoma

PTC detection method	TP	FP	TN	FN	Total number of cases	Sensitivity (%)	Specificity (%)	Accuracy (%)
FNAC	610	50	198	386	1,244	61.24	79.83	64.95
FNAC+BRAFV600E screening	766	50	198	230	1,244	76.90*	79.83	77.49*

PTC, papillary thyroid carcinoma; TP, number of true-positive cases; FP, number of false-positive cases, TN, number of true-negative cases; FN, number of false-negative cases; FNAC, fine-needle aspiration cytology. * $P < 0.01$ compared with FNAC.

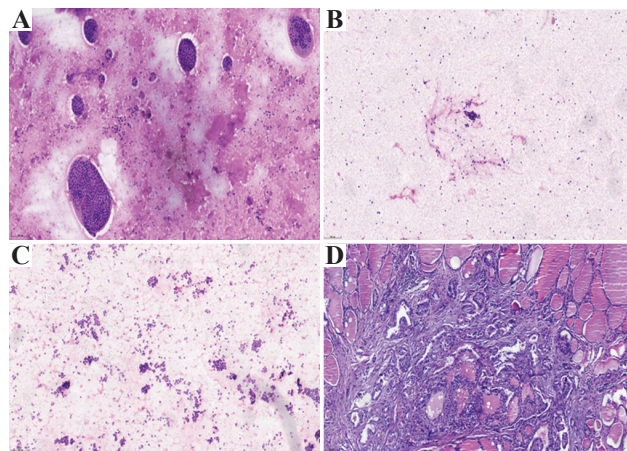


Figure 1. Cytological diagnosis of different thyroid cytological categories by fine-needle aspiration cytology and post-operative pathological diagnosis of papillary thyroid carcinoma (PTC). (A) Benign lesions; (B) suspected PTC; (C) PTC; (D) post-operative pathological diagnosis of PTC. All images were pictured at $\times 20$ power.

considered the gray regions of the cytological diagnosis, accounting for 20–40% of all the diagnoses^[22]. In this experiment, the significance of combined BRAFV600E mutation screening in the detection of different grades of PTC was studied. It is suggested that the detection of atypical cells and suspected PTC along with BRAFV600E mutation can improve the pre-operative detection rate of PTC. In addition, no BRAFV600E mutation was detected in patients with benign lesions who were diagnosed by the cytological methods in this study. This may be due to the inadequate amount of cells acquired during fine-needle aspiration, which was not enough to be used in morphological and genetic tests. Therefore, we do not think it is necessary to detect BRAFV600E mutation in patients with benign lesions who were diagnosed before a surgical operation. If malignant lesions are suspected, the cells can be aspirated again or re-examined regularly.

In this experiment, there were fewer cases of atypical cells and follicular tumors that were diagnosed by cytology. This may be caused by the insufficient amount of cells needed for the genetic test as a result of disqualified remaining liquid-based specimens of fine-needle aspiration that did not meet the inclusion criteria or fewer

malignant tumor cells drawn by aspiration. In addition, the existing detection system is not sensitive enough to detect BRAFV600E mutation when there are a large number of normal follicular epithelial cells and lymphocytes in the background, and when the proportion of diseased cells in nuclear cells is $<10\%$.

5. Conclusion

Thyroid fine-needle aspiration combined with BRAFV600E mutation screening can improve the accuracy and sensitivity of pre-operative PTC diagnosis, thereby providing a theoretical basis for clinical treatment.

Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

R.H. and J.Z. conceived the ideas of the paper. R.H. wrote the paper. R.H., J.Z., Y.J., and B.Z. revised the paper.

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