High BRAF^{V600E} mutation frequency in Chinese patients with papillary thyroid carcinoma increases diagnostic efficacy in cytologically indeterminate thyroid nodules

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1. Introduction

Thyroid nodule has become a frequently-occurring clinical disease worldwide.[1] Currently, serum thyrotropin measurement, neck ultrasound, and fine-needle aspiration cytology (FNAC) still remain the cornerstones for the differentiation of thyroid nodules. For nodules with suspicious features of malignancy by ultrasound, FNAC is usually supplemented to decide their nature and direct the following treatment. The Bethesda system for reporting thyroid cytopathology (TBSRTC) has been widely used. Ever since its birth in October 2007, TBSRTC has been recommended twice by American Thyroid Association (ATA) management guidelines for patients with thyroid nodules and differentiated thyroid cancer, in 2009[2] and 2016,[3] respectively. These recommendations provided a strategic methodology for thyroid nodule management. When FNAC is hard to confirm the diagnosis, molecular detection on BRAFV600E mutation, or other abnormalities may be effective ancillary testing to identify cancerous nodules. The present study was designed to estimate the BRAFV600E mutation frequency in Chinese patient with papillary thyroid carcinoma (PTC), and the diagnostic value of BRAFV600E mutation status in thyroid nodules with indeterminate TBSRTC categories.

2. Materials and methods

2.1. Patients and samples

A total of 4875 consecutive samples for thyroid ultrasound-guided FNAC and BRAFV600E mutation testing were collected from patients at Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine. Among those samples, 353 nodules of 314 patients (70 males and 244 females, ages 10 to 66 years, mean of 43.29 ± 12.05 years) were examined. All the patients have voluntarily signed the consent forms for confirmation, and the research was approved by the ethics committee of the hospital (2017LWKYZ004).

2.2. Find-needle aspirate and TBSRTC categories

Using locally available disposable syringes (5ml and 10 ml) and TWLB syringe needles (0.8 mm × 38.0 mm), FNA guided by ultrasound (HITACHI HVISION Preirus) was implemented on each patient. FNA samples were manually smeared, fixed with 95% ethanol, and HE-stained. According to TBSRTC,[3] the cytology of each nodule were reported as 1 of 6 categories: I, nondiagnostic or unsatisfactory (ND/UNS); II, benign (B); III, atypia of undetermined significance (AUS/FLUS); IV, follicular neoplasm suspicious or suspicious for a follicular neoplasm (FN/SFN); V, suspicious for malignancy (SM); VI, malignancy (M).

2.3. BRAFV600E mutation testing

For FNA samples or cancer tissues obtained after thyroidectomy, the genomic DNA was extracted with nucleic acid extraction kits (AmoyDx Diagnostics, Co., Ltd., Xiamen, China). Then the fresh DNA was diluted to 0.4–1 ng/μl, and paraffin-fixed DNA to 2–3 ng/μl. According to the instructions of human BRAFV600E mutation testing kit (AmoyDx Diagnostics, Co., Ltd., Xiamen, China), the reagent was prepared in the following way: every 35 μl reagent was mixed with 0.4 μl Taq polymerase; then the mixture was transferred into PCR tubes, closed, centrifugated, and placed into a PCR instrument (Life Technologies, Quant Studio Dx) for the process of quantitative PCR: stage I (95°C, 5 minutes, 1 cycle); stage II (95°C and 25 seconds, 64°C and 20 seconds, 72°C and 20 seconds, 15 cycles); stage III (93°C and 25 seconds, 60°C and 35 seconds , 72°C and 20 seconds , 31 cycles). In the thermal cycle at 60°C at stage III, FAM, and VIC signals were collected for real-time PCR analysis. The data were saved. After the reaction, the results were reported according to the value of FAM signals: <28 was considered positive and >28 negative.

2.4. Statistical analysis

SPSS 22.0 was used for statistical analysis. All quantitative data were shown as mean ± standard deviation. The best diagnostic cutoff values of TBSRTC and BRAFV600E mutation testing were decided by receiver operating characteristic (ROC) curve. When 2 methods were combined to make a diagnosis, any positive result from 1 method indicated the nodule was considered as positivity. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were all measured. ROC was drawn, and area under curve (AUC) was calculated. All these indexes were unified to make a diagnosis. P < .05 was considered to be statistically significant.

3. Results

3.1. Demographic data and histological results

A total of 4875 samples were studied. Preoperative FNAC and BRAFV600E mutation testing provided the complete measurements of 353 nodules in 314 patients (70 males and 244 females, from ages 10 to 66 years old, with average age of 43.29 ± 12.05 years). Of 353 nodules, 333 nodules were postoperatively diagnosed as PTC. The other 20 nodules included 8 with nodular goiter, 4 with lymphocytic thyroiditis, 5 with thyroid follicular adenoma, 1 with atypical adenoma, 1 with collagen fibrous proliferation accompanied with atypical follicular epithelium hyperplasia, and 1 in normal condition.

3.2. TBSRTC categories of preoperative nodules

Totally, 4875 FNA-obtained nodules were classified into 6 categories: ND/UNS (1495, 30.67%), B (366, 7.51%), AUS/FLUS (359, 7.36%), FN/SFN (132, 2.71%), SM (1467, 30.09%), and M (1056, 21.66%) (Table 1). Among them, 353 nodules of

| TBSRTC categories | Cases | Proportions (%) | BRAFV600E mutation rate (%) |
|-------------------|-------|-----------------|----------------------------|
| I                 | 1495  | 30.67           | 434 (29.03)                |
| II                | 366   | 7.51            | 82 (22.40)                 |
| III               | 359   | 7.36            | 196 (54.60)                |
| IV                | 132   | 2.71            | 19 (14.39)                 |
| V                 | 1467  | 30.09           | 1159 (79.00)               |
| VI                | 1056  | 21.66           | 906 (85.80)                |
| Total             | 4875  | 100.0           | 2796 (57.35)               |

TBSRTC = the Bethesda system for reporting thyroid cytopathology.
314 thyroidectomized patients were pathologically examined (Table 2). The results showed that 53, 3, 24, 4, 152, and 117 nodules could be listed into the above 6 categories. The malignancy rate of these 6 categories was 73.58% (39/53), 66.67% (2/3), 95.83% (23/24), 25.00% (1/4), 99.34% (151/152), 100.00% (117/117), respectively.

3.3. Results of preoperative BRAFV600E mutation testing

BRAFV600E mutation testing was performed in 4875 FNA-obtained samples. Among them, 2796 nodules (57.35%) were found to have gene mutation. The BRAFV600E mutation rate in the 6 categories of nodules was 29.03%, 22.40%, 54.60%, 14.39%, 79.00%, and 85.80%, respectively (Table 1).

Among 353 thyroidectomized nodules, 292 nodules had BRAFV600E mutation before surgery, and were diagnosed as PTC by postoperative pathology. The BRAFV600E mutation rate in the 6 categories of nodules was 56.60%, 66.67%, 79.17%, 0.00%, 88.82%, and 90.60%, respectively (Table 2). The results showed that 53, 3, 24, 4, 152, and 117 nodules could be listed into the above 6 categories. The malignancy rate was 73.58% (39/53), 66.67% (2/3), 95.83% (23/24), 25.00% (1/4), 99.34% (151/152), 100.00% (117/117), respectively.

3.4. Comparison of the diagnostic value among TBSRTC, BRAFV600E mutation testing, and their combination

In present study, ROC statistical analysis showed that the sensitivity, specificity of TBSRTC for the diagnosis of thyroid nodules was 80.48% and 95.00%. However, the sensitivity and specificity of BRAFV600E mutation detection were significantly higher than TBSRTC (87.69% and 100.00%, respectively). There was no significant difference between the AUC of TBSRTC and BRAFV600E mutation detection (0.895 vs 0.938, \( P = .0752 \)). The combination of TBSRTC and BRAFV600E mutation testing achieved the highest AUC (0.954), which was significantly higher than TBSRTC (\( P = .0009 \)) but not (\( P = .5515 \)). The sensitivity of the combination increased to 95.80%, and the specificity was the same as the single use of TBSRTC (Table 3).

4. Discussion

FNAC is the most accurate and cost-effective tool to evaluate thyroid nodules suspicious for malignancy with ultrasound. TBSRTC, the major landmark of thyroid cytology, is the creation of a uniform system for reporting thyroid cytopathology. Due to 6 recognized diagnostic categories with an incremental risk of malignancy, TBSRTC standardized the cytological diagnosis and increased its diagnostic yield. A number of clinical studies has demonstrated that this system did not clearly answer for the heterogeneous group of nodules with indeterminate cytology, including AUS/FLUS, FN/SFN, and SM. The genetic marker BRAF V600E mutation, the most robust oncogene in PTC, has been the focus of BRAF V600E mutation testing in a large population. The analysis on thyroidectomized patients clarified that the malignancy risk of nodules in TBSRTC I, II, III apparently higher than those described by ATA guidelines.\(^{[1]}\) Nodules with indeterminate cytology were also found to have a high malignancy risk in our study. The molecular testing method was recommended to improve the diagnostic efficacy for these nodules.\(^{[2,3]}\) The preoperative detection of BRAFV600E mutation can identify most of malignancy in AUS/FLUS nodules. Only a few patients with nodules were categorized as FN/SFN were surgically treated. Among them, the malignancy rate was 25.00% and no BRAFV600E mutation were detected preoperatively. Previous researches demonstrated that BRAFV600E mutation arises in tall cell variant and conventional PTC with more possibilities, while it seldom occurs in follicular variant and follicular thyroid neo-

### Table 2

| TBSRTC categories | Cases | Proportions (%) | Malignancy (%) | Preoperative BRAFV600E mutation of PTC (%) | Total BRAFV600E mutation of PTC (%) |
|-------------------|-------|-----------------|----------------|------------------------------------------|------------------------------------|
| I                 | 53    | 15.01           | 39 (73.58)     | 30 (76.92)                               | 33 (84.62)                          |
| II                | 3     | 0.86            | 2 (66.67)      | 2 (100.00)                               | 2 (100.00)                          |
| III               | 24    | 6.80            | 23 (95.83)     | 19 (82.61)                               | 21 (91.30)                          |
| IV                | 4     | 1.13            | 1 (25.00)      | 0 (0.00)                                 | 1 (100.00)                          |
| V                 | 152   | 43.06           | 151 (99.34)    | 135 (89.40)                              | 140 (92.72)                         |
| VI                | 117   | 33.15           | 117 (100.00)   | 106 (90.60)                              | 107 (91.45)                         |
| Total             | 353   | 100.00          | 333 (94.33)    | 292 (82.69)                              | 304 (91.29)                         |

*Table* 2: Distribution of TBSRTC categories, BRAFV600E mutation and postoperatively histopathological results in 314 patients.

### Table 3

|                          | TBSRTC | BRAFV600E mutation | TBSRTC+BRAFV600E mutation |
|--------------------------|--------|--------------------|---------------------------|
| Sensitivity (95% CI)     | 80.48  | 87.69 (83.7–91.0)  | 95.80 (93.0–97.7)        |
| Specificity (95% CI)     | 95.00  | 100.00 (93.2–100.0)| 95.00 (75.1–99.9)        |
| PPV (95% CI)             | 99.6   | 100.00 (98.7–100.0)| 99.7 (98.3–100.0)        |
| NPV (95% CI)             | 22.6   | 32.8 (21.3–46.0)   | 57.6 (39.2–74.5)         |
| AUC (95% CI)             | 0.895  | 0.906 (0.858–0.925)| 0.954 (0.927–0.973)      |

*Table* 3: Comparison of the diagnostic value of TBSRTC and BRAFV600E analysis.
plasm. Also, the absence of commercial molecular detection methods in China remains an obstacle to implement the accurate diagnosis for FN/SFN nodules.

In this research, totally 87.69% of PTC were found to have BRAFV600E mutation, and all the nodules with BRAFV600E mutation were confirmed to be PTCs. The mutation rate is much close to the research of Zhang et al in Nanjing and Guo et al in Beijing, but higher than the date from some other Chinese researchers. Taken them together, the mutation rate of BRAFV600E in Chinese PTC patients seems to be higher than that in the studies of Kim et al, Kim et al and Kim et al from South Korea. Xing et al from the United states, and Beisa et al from Lithuania. A reasonable answer for the difference of our research, both the sensitivity and specificity of BRAFV600E mutation testing rise up above those of TBSRTC. Their results may originate from the inappropriate combination can significantly increase the NPV of cytological diagnosis, and rule out indeterminate nodules (especially those of category NON, B, and AUS/FLUS) caused by small sample size, low-quality sections, and scant experience of pathologists. In conclusion, Chinese patients with PTC have a high BRAF mutation frequency, and the BRAFV600E mutation testing shows a higher sensitivity and specificity. Combining TBSRTC and BRAFV600E mutation testing can improve the diagnostic sensitivity and reduce the rate of indeterminate cytological diagnosis.

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References

[1] Haugen BR, Alexander EK, Bible KC, et al. 2015 American thyroid association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American thyroid association guidelines task force on thyroid nodules and differentiated thyroid cancer. Thyroid 2016;26:1–33.
[2] Thyroid N, Differentiated Thyroid C, Cooper DS, et al. American Thyroid Association Guidelines Taskforce on, Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. Thyroid 2009;19:1167–214.
[3] Cibas ES, Ali SZ. The 2017 Bethesda system for reporting thyroid cytopathology. Thyroid 2017;27:1341–6.
[4] Xing M. Molecular pathogenesis and mechanisms of thyroid cancer. Nat Rev Cancer 2013;13:184–99.
[5] Zhang YZ, Xu T, Cui D, et al. Value of TIRADS, BSRTC and FNA-BRAF V600E mutation analysis in differentiating high-risk thyroid nodules. Sci Rep 2015;5:16927.
[6] Guo L, Ma YQ, Yao Y, et al. Role of ultrasonographic features and quantified BRAFV600E mutation in lymph node metastasis in Chinese patients with papillary thyroid carcinoma. Sci Rep 2019;9:75.
[7] Guan H, Ji M, Bao R, et al. Association of high iodine intake with the BRAF mutation and cytomorphological features for the optimization of ultrasonography, fine-needle aspiration biopsy, and braf v600e mutation testing in lymph node metastasis in Chinese patients with papillary thyroid microcarcinoma. Exp Clin Endocrinol Diabetes 2018.
[8] Kim TY, Kim WK, Song YJ, et al. The BRAF mutation is not associated with poor prognostic factors in Korean patients with conventional papillary thyroid microcarcinoma. Clin Endocrinol 2005;63:588–93.
[9] Kim DS, Kim DW, Heo YJ, et al. Utility of including BRAF mutation analysis with ultrasonographic and cytological diagnoses in ultrasonography-guided fine-needle aspiration of thyroid nodules. PloS One 2018;13:e0202687.
[10] Kim SK, Lee JH, Woo JW, et al. Prediction table and nomogram as tools for diagnosis of papillary thyroid carcinoma: combined analysis of ultrasonography, fine-needle aspiration biopsy, and braf v600e mutation. Medicine 2015;94:e760.
[11] Xing M, Liu R, Liu X, et al. BRAF V600E and TERT promoter mutations cooperatively identify the most aggressive papillary thyroid cancer with highest recurrence. J Clin Oncol 2014;32:2718–26.
[12] Beisa A, Kvetkauskas M, Beisa V, et al. Significance of BRAF V600E mutation and cytomorphological features for the optimization of papillary thyroid cancer diagnostics in cytologically indeterminate thyroid nodules. Exp Clin Endocrinol Diabetes 2018.
[13] Shan Z, Chen L, Lian X, et al. Iodine status and prevalence of thyroid disorders after introduction of mandatory universal salt iodization for 16 years in china: a Cross-sectional study in 10 cities. Thyroid 2016;26:1125–30.
[14] Wang Z, Zhang H, Zhang X, et al. Serum thyroglobulin reference intervals in regions with adequate and more than adequate iodine intake. Medicine 2016;95:e5273.
[15] Paek SH, Kim BS, Kang KH, et al. False-negative BRAF V600E mutation results on fine-needle aspiration cytology of papillary thyroid carcinoma. World J Surg Oncol 2017;15:202.