

# Age and $BRAF^{V600E}$ Mutation Stratified Patients with Cytologically Benign Thyroid Nodules

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**Purpose:** Our objective was to evaluate the diagnostic performance of  $BRAF^{V600E}$  mutation for malignant, and to identify clinical characteristics associated with positive  $BRAF^{V600E}$  mutation in low-risk cytological and ultrasound diagnostic thyroid nodules. This aims to identify patients who may benefit from  $BRAF^{V600E}$  mutation testing and subsequent surgical intervention.

**Patients and Methods:** We analysis the clinical characteristics correlated with  $BRAF^{V600E}$  mutation in our detection cohort, including 204 patients with 217 thyroid nodules, and separate analyses were performed in 103 thyroid nodules with benign cytological result. Signaling pathway and immune response associated with age and  $BRAF^{V600E}$  mutation status were also evaluated in Asian patients with thyroid cancer from the Cancer Genome Atlas (TCGA) dataset.

**Results:** The positive  $BRAF^{V600E}$  mutation was significantly associated with higher Ultrasound (US) classification ( $p < 0.001$ ) and fine-needle aspiration (FNA) categories ( $p < 0.001$ ).  $BRAF^{V600E}$  mutation as a risk factor for malignancy, showing the optimal diagnostic efficacy for malignancy combined with FNA categories, with the AUC was 0.923. Otherwise,  $BRAF^{V600E}$  mutation is a risk factor in screening malignancy in low-risk FNA and US classification, which is significant correlation with patients age. Patients over 50 years old exhibiting a higher percentage of positive  $BRAF^{V600E}$  mutation when both ultrasound and FNA results indicate benign conditions, with higher risk of malignancy.

**Conclusion:**  $BRAF^{V600E}$  mutation is an accurate adjunctive diagnostic marker on FNA to screen malignancy. In low risk of both ultrasound and FNA results, the positive  $BRAF^{V600E}$  was significant increased in patients over 50 years old, which have higher risk of malignancy. Thus, the  $BRAF^{V600E}$  mutation detection and further surgery should be strengthened in older patients with benign cytological and US results thyroid nodules.

**Keywords:** thyroid nodule,  $BRAF^{V600E}$  mutation, benign cytology, older, diagnosis

## Introduction

The thyroid gland is one of the largest endocrine glands in the human body. Thyroid lesions are often found in the gland, with a prevalence of 4% to 7%. Most of them are asymptomatic, and thyroid hormone secretion remains normal.<sup>1</sup> Bernet et al have reported that thyroid nodules are associated with a 7–15% risk of malignancy.<sup>2</sup> Ultrasound (US) is the most sensitive model to assess thyroid nodule morphology, aiding in identifying high-risk cases for malignancy.<sup>3</sup> Certain sonographic features like hypoechogenicity, microcalcifications, irregular margins, absent halo sign, and increased intramodular blood flow are associated with a higher risk of malignancy.<sup>1</sup> One of the newest radiology systems, the Chinese Thyroid Imaging Reporting and Data System (C-TIRADS), established by Chinese experts using a counting method, is suitable for Chinese clinical practices, and easy for clinical application and promotion.<sup>4</sup> Nodules with higher C-TIRADS score are required to perform cytological analysis through fine-needle aspiration (FNA) to determine the risk of malignancy.<sup>5</sup>

Ultrasound-guided FNA (US-FNA) is a reliable and effective approach for evaluating thyroid nodules before surgery, providing cytopathological assessment with high sensitivity and specificity for malignancy.<sup>6</sup> Otherwise, the false-negative rate of US-FNA diagnosis is approximately 12%. Based on the literature, approximately 70% of FNA are categorized as Bethesda II, with an anticipated 3% being false negatives.<sup>7</sup> It is necessary to provide definitive diagnoses to distinguish malignant ones from benign cytological results for better management of patients.

Molecular analysis can be used to enhance the diagnostic accuracy of cytological diagnosis and as a marker for targeted therapy.<sup>8</sup> The main known genetic causes of thyroid cancer include point mutations in the *BRAF*, *RAS*, *TERT*, *RET*, and *TP53* genes and the fusion genes *RET/PTC*, *PAX8/PPAR-γ*, and *NTRK*.<sup>9</sup> Fusion gene positive PTCs were associated with more aggressive behavior disease than fusion gene negative PTCs.<sup>10</sup> A comprehensive study reported by Pekova et al recently identified that *RET* fusion was associated with a 100% probability of malignancy.<sup>11</sup> *BRAF*<sup>V600E</sup> is the most common genetic mutation in thyroid cancer, with a prevalence ranging from 37% to 83% in papillary thyroid cancer (PTC),<sup>12</sup> and has shown a strong association with lymph node metastasis, extra thyroidal extension, disease recurrence, and lymph node metastasis-associated mortality.<sup>13,14</sup> Several studies have proved *BRAF*<sup>V600E</sup> mutation is a valuable and accurate adjunctive diagnostic marker on US-FNA to screen malignancy in non-diagnostic and indeterminate thyroid nodules.<sup>15,16</sup> Few studies have focused on the influence of *BRAF*<sup>V600E</sup> mutation in benign cytological analysis on malignancy, and the clinical characteristics associated with *BRAF*<sup>V600E</sup> mutation in the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) II remained unclear.<sup>17–19</sup>

In this study, we evaluated the diagnostic efficacy of *BRAF*<sup>V600E</sup> mutation in thyroid nodules for screening out malignancy, and the association with progression of thyroid cancer. On the other hand, in order to avoid overdiagnosis and overtreatment, we further focused on clinical characteristics and US features associated with positive *BRAF*<sup>V600E</sup> mutation in patients in low-risk cytology and US diagnosis to accurately locate the risk of *BRAF*<sup>V600E</sup> mutation and malignant risk.

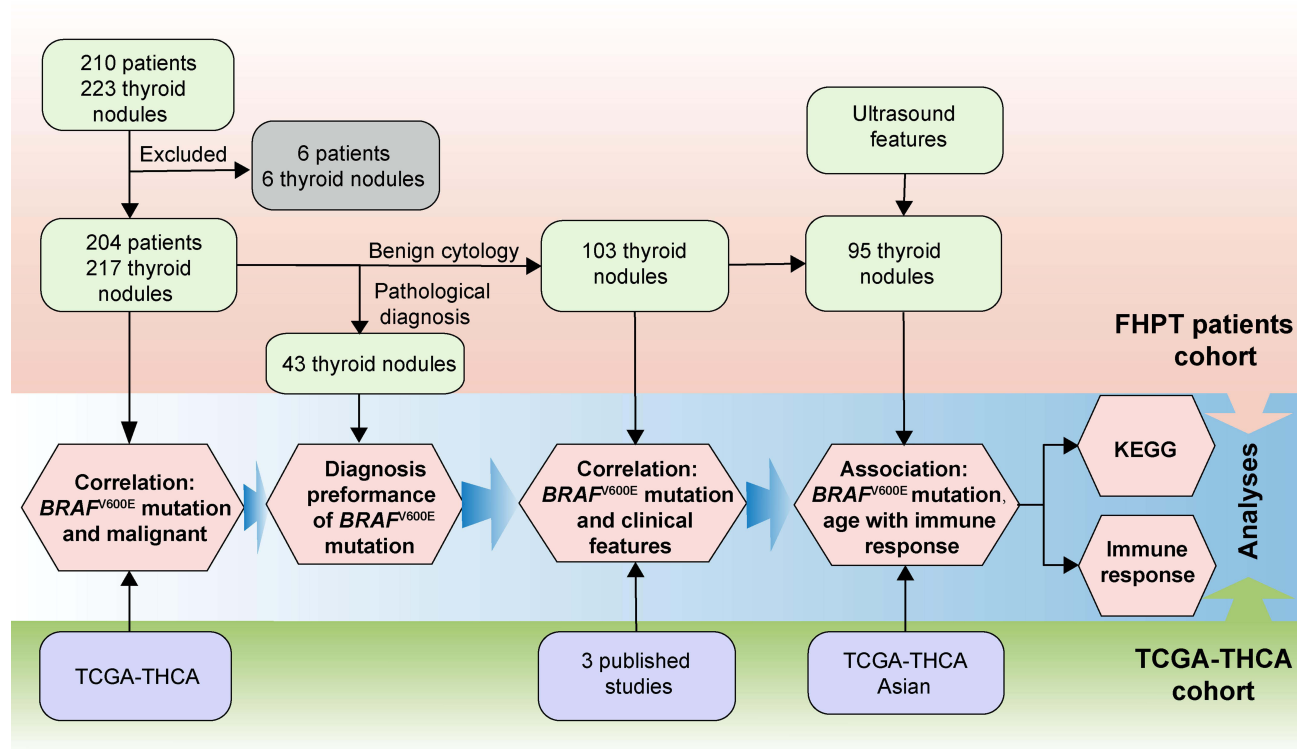
## Methods

### Patients and Data

A total of 223 thyroid nodules in 210 patients underwent US and US-FNA with an additional *BRAF*<sup>V600E</sup> mutation detection in the Department of Thyroid and Breast Surgery of the First Hospital of Putian City (FHPT patients cohort) in Fujian Province from January 2021 to May 2022. Six patients with 6 nodules were excluded because of an incomplete cytopathological diagnosis or insufficient sonography diagnosis. As a result, 204 patients with 217 nodules were included in the analysis. The patients included 152 females and 52 males, aged from 17 to 91 years, mean age was 47.25±12.16 years. All the patients experienced clinical follow-up after six months, and no progression occurred during this period. Among all the samples, 44 thyroid nodules were treated with thyroidectomy, and 43 thyroid nodules had been diagnosed pathologically by postoperative paraffin sections, of which 38 were PTC and 1 was medullary thyroid cancer (MTC), three cases with nodular goiter, and 1 with thyroiditis. Patients characteristics including *BRAF*<sup>V600E</sup> mutation, the classification of US and FNA, sonography diagnosis information, gender, age, drinking, smoking, location, multiplicity, size, and pathological diagnosis were also recorded, the detail information was shown in [Supplementary Tables 1 and 2](#). Both clinical data and *BRAF*<sup>V600E</sup> mutation detection results of 507 patients with thyroid cancer from the Cancer Genome Atlas (TCGA) were obtained from UCSC Xena (<https://xenabrowser.net/datapages/>), 501 patients among them with the RNA-seq expression data and 491 patients with mutation data. The diagram of the study group is shown in [Figure 1](#).

### Ultrasound Examination

US examinations were performed by an experienced US radiologist (GH) used Siemens S2000 ultrasound diagnostic instrument equipped with a 5–12-MHz linear-array transducer. The thyroid glands were scanned in the supine position to determine the location, size (at their longest diameter), number, internal echo, morphology, margin, shape, and calcification. Suspicious US features of malignancy include microcalcification, irregular margins, taller-than-wide shape, hypoechogenicity and marked hypoechogenicity, irregular thick hole, and evidence of extrathyroidal growth.<sup>20</sup> Two radiologists with more than ten years' experience in thyroid ultrasonography classified all nodules into four category



**Figure 1** The diagram of the study. The sample used for analysis and the analysis diagram of the study.

according to C-TIRADS: 3 (probably benign), 4A (low suspicion malignant), 4B (moderate suspicion malignant) and 4C (high suspicion malignant).<sup>4</sup>

## FNA of Thyroid Nodules

FNA was performed under the guideline of US by experienced radiologists with a 0.7 mm syringe. Each lesion was aspirated three or four passes in different directions to complete the sampling. Two or three of them were smeared on glass slides and fixed for cytopathological analysis. Based on TBSRTC, the results of FNA were categorized into six classifications, I–VI were non-diagnostic, benign, atypia undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), suspicious for follicular neoplasm or suspicious for a Hurthle cell neoplasm, suspicious for malignancy and malignancy,<sup>21</sup> respectively.

## *BRAF*<sup>V600E</sup> Mutation Test

The specimens for *BRAF*<sup>V600E</sup> mutation analysis were from Formalin-Fixed Paraffin-Embedded (FFPE) tissues, thyroid FNA samples or serum samples. Genomic DNA extraction from FFPE tissues and thyroid FNA samples were used TargetingOne<sup>®</sup> FFPE TNA-P1, TargetingOne<sup>®</sup> FFPE TNA-P2 (TargetingOne<sup>®</sup> Biotech. Beijing, China), respectively. CfDNA from serum samples were used TargetingOne<sup>®</sup> cfDNA-B1 (TargetingOne<sup>®</sup> Biotech. Co. Ltd. Beijing, China). The quantity of isolated DNA was assessed by Nanodrop, all samples were adequate for *BRAF*<sup>V600E</sup> detection.

The *BRAF*<sup>V600E</sup> mutation was detected by human *BRAF*<sup>V600E</sup> mutation detection kit (TargetingOne<sup>®</sup> Biotech. Beijing, China) according to the manufacturer's instructions. The reaction components were prepared as follows: 7.5  $\mu$ L of PCR reagent A (*BRAF*<sup>V600E</sup>), 7.5  $\mu$ L of PCR reagent B (*BRAF*<sup>V600E</sup>), and 20–50 ng of DNA, sterilized water was used to make a volume of up to 30  $\mu$ L. The optimal annealing temperature is determined to be 60°C. Each experiment included a negative and positive (*BRAF*<sup>V600E</sup>) control. The ddPCR platform system (TargetingOne<sup>®</sup> Biotech. Beijing, China) was utilized for evaluation. Droplets were generated and analyzed using the Drop Maker M1 and the Chip Reader R1. The

quantitative results were estimated by modeling as a Poisson distribution using chip Analyzer-V1 (TargetingOne<sup>®</sup> Biotech, Beijing, China).

## Different Expression Genes (DEGs) Identified and Immune Response Analysis

The R package “limma” was applied to calculate differentially expressed genes (DEGs) between older and younger and positive and negative *BRAF*<sup>V600E</sup> mutation, the threshold was set to  $p < 0.05$  and  $|\log\text{FC}| > 1$ .<sup>22</sup> Then, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis were performed on the DEGs within older and younger groups, and the intersection DEGs of positive and negative *BRAF*<sup>V600E</sup> mutation groups and younger and older age groups, respectively. The R package “clusterProfiler” (v.4.7.1.2) was used for functional annotation of the DEGs. An immune-related gene set (immune inhibitors and immunostimulators) was collected from the online tool TISIDB.<sup>23</sup> The results were exhibited by bubble diagram and box plot drawn by “ggplot2” (v.3.4.1) package.

## Somatic Mutation Analysis

We performed somatic mutation analysis based on thyroid carcinoma (THCA) samples. To reduce the false-positive rates, we deleted synonymous mutations. Mutational spectrum and mutational signature were depicted via “maftools” (v.2.12.0) R packages which provides a multiple of analysis modules to perform the visualization process. The mutation portrait matrix was factorized into two nonnegative matrices, “signatures” and “contributions” by the ExtractSignatures function of “maftools”, where “signatures” represented mutational processes and “contributions” reflected the associated mutational activities. This approach can automatically estimate the ideal number of retrieved mutational signatures because it is based on Bayesian variant nonnegative matrix factorization.

## Statistical Analysis

The statistical analysis was performed with SPSS version 26.0 and R version 4.2.1. Fisher’s exact test or Pearson’s chi-squared test was used for categorical variables analysis. Normally distributed data were expressed as mean  $\pm$  standard deviation and analyzed with a two-sample *t*-test, and non-normally distributed variables were presented as medians and interquartile range (IQR) using the Wilcoxon-Mann-Whitney test. The “ggalluvial” (v.0.12.3) R package was used to produce the alluvial diagrams. To measure the specificity and sensitivity of the prognostic capability of C-TIRADS, *BRAF*<sup>V600E</sup>, TBSRTC category, and the combined methods, we calculated the area under the curve (AUC) using the R “pROC” (v.1.18.0) package. A *p*-value of  $<0.05$  was considered statistical difference and *p*-value of  $<0.01$  was considered statistically significant difference.

## Results

### *BRAF*<sup>V600E</sup> Mutation Was Associated with Malignancy of Thyroid Nodules and Progression of Thyroid Cancer

To evaluate the effect of *BRAF*<sup>V600E</sup> mutation on malignant progression of thyroid nodules, the *BRAF*<sup>V600E</sup> mutation detection was performed in all 204 patients with 217 thyroid nodules, the positive *BRAF*<sup>V600E</sup> mutation was detected in 89 (41.0%) thyroid nodules, and the other 128 nodules (59.0%) were negative. In TBSRTC II, the incidence of negative *BRAF*<sup>V600E</sup> mutation significantly higher than that of positive *BRAF*<sup>V600E</sup> mutation. Conversely, the positive *BRAF*<sup>V600E</sup> mutation was statistically significantly associated with TBSRTC III–IV ( $p=0.007$ ) and TBSRTC V–VI ( $p<0.001$ ), which poses a higher risk of malignancy. Patients in C-TIRADS 4b–4c had more frequent positive *BRAF*<sup>V600E</sup> mutation than patients with low-risk US classification ( $p<0.001$ ) (Table 1). Notably, a substantial majority of thyroid nodules exhibiting positive *BRAF*<sup>V600E</sup> mutation were found to be situated in high-risk FNA and US categories (Figure 2A). We further investigated the correlation of *BRAF*<sup>V600E</sup> mutation with different pathological stages of thyroid cancers based on TCGA database. The *BRAF*<sup>V600E</sup> mutation was increased in advanced stages (stage III–IV) compared with early stages (stage I–II) ( $p<0.001$ ) (Figure 2B), similarly, the proportion of positive *BRAF*<sup>V600E</sup> mutation was higher in advanced pathological T stage (T3–T4) ( $p<0.001$ ) and N1 stage ( $p<0.001$ ) (Figure 2C and D). With the development of M stage, there was no statistically significant difference between positive and negative *BRAF*<sup>V600E</sup> mutation (Figure 2E). When separated from Asian patients, the *BRAF*<sup>V600E</sup> mutation increased as the pathological stage and

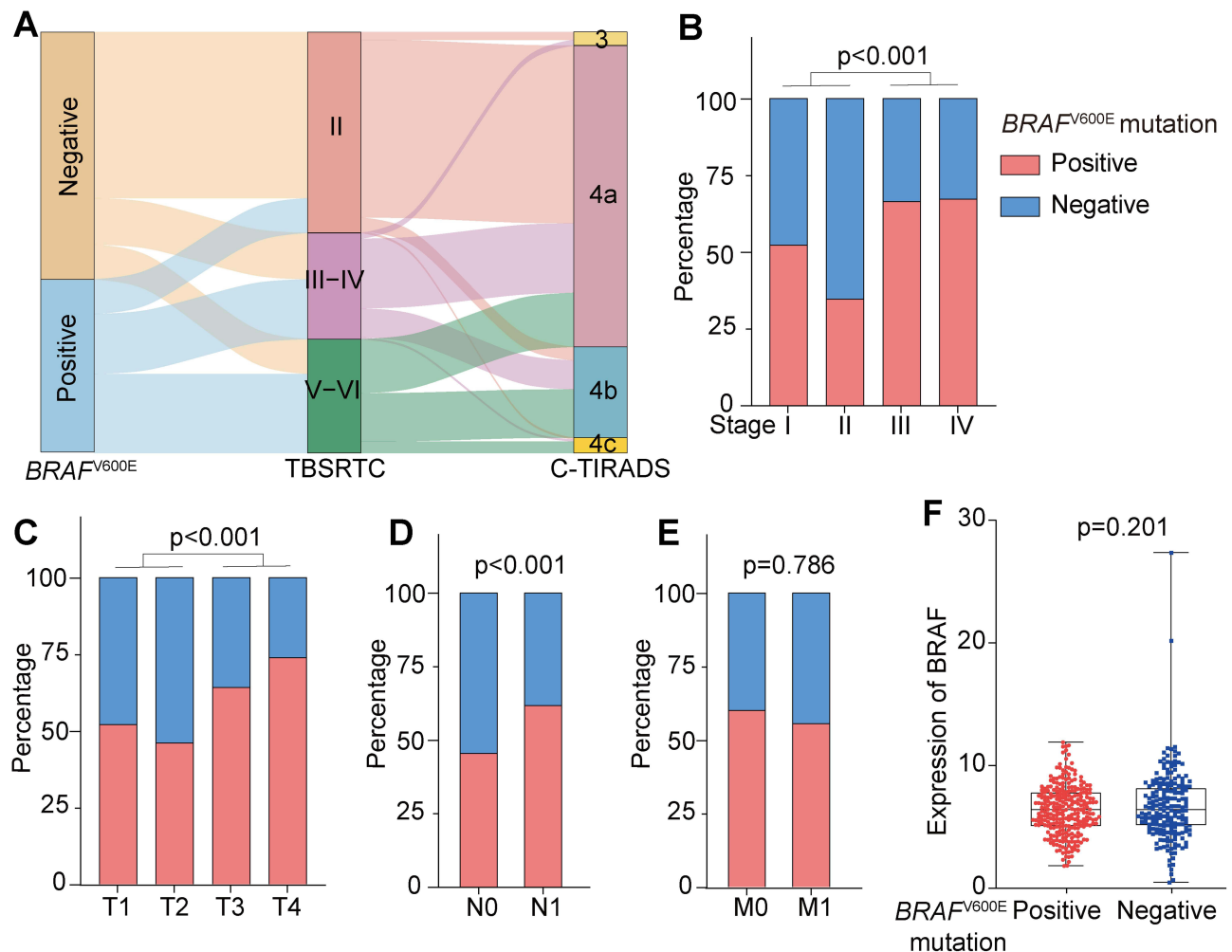
**Table 1** Correlation Between Clinical Characteristics and *BRAF*<sup>V600E</sup> Mutation in All Thyroid Nodules

Characteristics	<i>BRAF</i> <sup>V600E</sup>		P-value
	Positive (89)	Negative (128)	
Gender (Males)	23/89(25.8%)	33/89(25.7%)	0.992
Age (mean ± S.D.)	46.01±12.640	48.11±11.783	0.233
Drinking	5/89 (5.6%)	6/128 (4.7%)	0.496
Smoking	7/89 (7.9%)	14/128 (10.9%)	0.451
TBSRTC II	17/89 (19.1%)	86/128 (67.2%)	<0.001***
TBSRTC III-IV	31/89 (34.8%)	24/128 (18.8%)	0.007**
TBSRTC V-VI	41/89 (46.1%)	18/128 (14.1%)	<0.001***
C-TIRADS 4b-4c	37/89 (41.6%)	18/128 (14.1%)	<0.001***

Notes: \*\*\*P<0.001, \*\*P<0.01.

Abbreviations: mean±S.D., mean ± standard deviation; TBSRTC, the Bethesda System for Reporting Thyroid Cytopathology; C-TIRADS, Chinese Thyroid imaging reporting and data system.

T stage increased (Supplementary Figure 1A and B), but the results were not statistically significant. No differences were found in M stage and N stage (Supplementary Figure 1C and D). The expression of *BRAF* has no statistically significant difference between positive and negative *BRAF*<sup>V600E</sup> mutation (Figure 2F and Supplementary Figure 1E), indicated that the *BRAF*<sup>V600E</sup>



**Figure 2** Associations of *BRAF*<sup>V600E</sup> mutation with thyroid nodule classifications and clinicopathological stages. (A) Alluvial diagram of the *BRAF*<sup>V600E</sup> mutation with different classification of US classification C-TIRADS, TBSRTC categories, and Pathological diagnosis; (B–E) Correlation between *BRAF*<sup>V600E</sup> mutation status and (B) tumor stages, (C) T stages, (D) N stages, (E) M stages in TCGA-THCA cohort, red: positive *BRAF*<sup>V600E</sup> mutation, blue: negative *BRAF*<sup>V600E</sup> mutation; (F) The correlation between *BRAF*<sup>V600E</sup> mutation status and the expression of *BRAF* in TCGA-THCA cohort.

mutation has no influence in expression of BRAF, associated with malignant in thyroid nodules directly. We further analyzed the molecular features of  $BRAF^{V600E}$  mutation in thyroid cancer using TCGA-THCA cohort, we found that  $BRAF^{V600E}$  was the most commonly mutated gene. Interestingly, most patients with positive  $BRAF^{V600E}$  mutation did not have any other mutations in the top 30 most frequently mutated genes (Supplementary Figure 2A). And the coincident and exclusive associations across mutated genes showed that the top 30 genes with the highest mutation frequency were mutual exclusivity with positive  $BRAF^{V600E}$  mutation (Supplementary Figure 2B). The results indicate that positive  $BRAF^{V600E}$  mutation is associated with higher risk of malignancy in thyroid nodules.

## $BRAF^{V600E}$ Was an Adjunctive Diagnostic Marker on FNA to Screen Malignancy in Benign Cytology

Among all the thyroid nodules, 43 of them have postoperative pathological diagnosis, of which 4 cases with nodular goiter or thyroiditis were benign, and the other 39 were malignant, including 38 PTC and 1 MTC. As shown in Table 2, the percentage of positive  $BRAF^{V600E}$  mutation is 74.4% (29/39) in PTC cases in our study, which was significantly associated malignant of thyroid nodules ( $p=0.008$ ). All the thyroid nodules belonged to TBSRTC V–VI were confirmed malignant, however, the results were not statistically significant ( $p=0.072$ ). Seven (17.9%) samples classified as TBSRTC II and 18 (47.2%) samples classified as C-TIRADS 3–4a confirmed to be malignant by pathological diagnosis, indicating that the sensitivity for malignancy diagnosis of the TBSRTC and C-TIRADS classification was low in our study. No statistically significant differences were observed in gender, age, drinking, and smoking between the benign and malignant thyroid nodules. Noteworthy, the median age in malignancy is  $45.92\pm 12.55$ , which was younger than those in benign, but there was no statistical significance between them ( $p=0.664$ ).

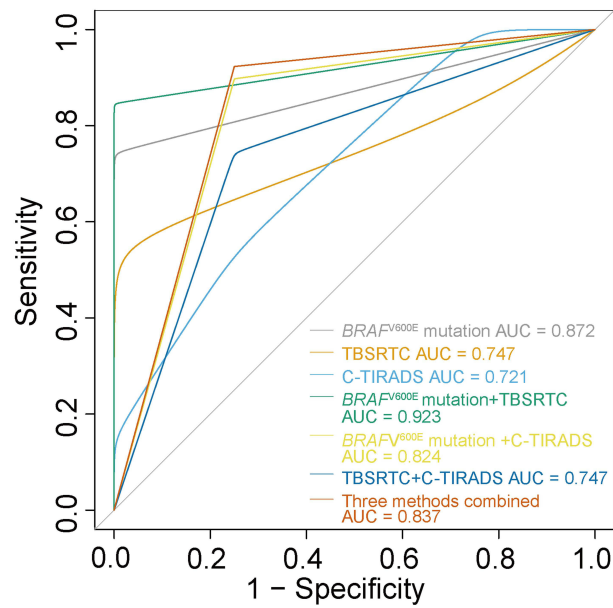
We analyzed the sensitivity and specificity of TBSRTC,  $BRAF^{V600E}$  mutation, and C-TIRADS along with the combination of both or three methods. The results revealed that the specificity of TBSRTC was as high as 100%; however, the sensitivity was lower compared with  $BRAF^{V600E}$  mutation and C-TIRADS classification. The combination of TBSRTC and  $BRAF^{V600E}$  mutation greatly increase the sensitivity of TBSRTC, and showed the highest AUC, which was 0.923 (Figure 3 and Table 3). All samples with positive  $BRAF^{V600E}$  mutation were malignant by pathological diagnosis, including benign cytology diagnosis TBSRTC II (Table 4). Several studies had assessed the risk of malignancy in patients with benign cytology,<sup>17–19</sup> the results revealed that the majority thyroid nodules with positive  $BRAF^{V600E}$  mutation confirmed malignant (Table 5). The results above indicated that there was a risk of malignant in benign cytological results, which may be associated with positive  $BRAF^{V600E}$  mutation.

**Table 2** Clinical Characteristics Associated with Malignant in Thyroid Nodules

Characteristics	Malignant (39)	Benign (4)	P-value
Gender (Male)	14/39(35.9%)	0/4(0%)	0.192
Age (mean±S.D.)	45.92±12.55	48.75±8.26	0.664
Drinking	6/39(15.4%)	0/4(0%)	0.535
Smoking	8/39(20.5%)	0/4(0%)	0.315
Positive $BRAF^{V600E}$	29/39 (74.4%)	0/4 (0%)	0.008**
TBSRTC II	7/39(17.9%)	1/4(25%)	0.576
TBSRTC III–IV	12/39(30.8%)	3/4(75%)	0.114
TBSRTC V–VI	20/39(51.3%)	0(0%)	0.072
C-TIRADS 3–4a	18/39(46.2%)	3/4(75%)	0.286

Notes: \*\* $P<0.01$ .

Abbreviations: mean±S.D., mean ± standard deviation; TBSRTC, the Bethesda System for Reporting Thyroid Cytopathology; C-TIRADS, Chinese Thyroid imaging reporting and data system.



**Figure 3** ROC analysis of diagnosis methods for malignancy. The ROC analysis of TBSRTC, *BRAF*<sup>V600E</sup> mutation status, and C-TIRADS for malignancy in thyroid nodules.

### The Prediction of Clinicopathologic and US Characteristics to *BRAF*<sup>V600E</sup> Mutation in Patients with Low-Risk Cytological and US Classification

Based on the results described above, the positive *BRAF*<sup>V600E</sup> mutation was a risk factor of malignancy in patients with benign cytology. However, according to the 2015 American Thyroid Association (ATA) guideline, the *BRAF*<sup>V600E</sup> mutation detection was un-practical and not required immediately in benign cytology,<sup>24</sup> and the clinicopathologic and

**Table 3** Diagnostic Efficacy of Different Methods for Discrimination Malignant in Thyroid Nodules

Diagnosis Methods	AUC	Sensitivity	Specificity
<i>BRAF</i> <sup>V600E</sup>	0.872	74.4%	100.0%
TBSRTC	0.747	51.3%	100.0%
C-TIRADS	0.721	53.8%	75.0%
<i>BRAF</i> <sup>V600E</sup> + TBSRTC	0.923	84.6%	100.0%
<i>BRAF</i> <sup>V600E</sup> +C-TIRADS	0.824	89.7%	75.0%
TBSRTC +C-TIRADS	0.747	74.4%	75.0%
Three methods combined	0.837	92.3%	75.0%

**Abbreviations:** AUC, area under the curve; TBSRTC, the Bethesda System for Reporting Thyroid Cytopathology; C-TIRADS, Chinese Thyroid imaging reporting and data system.

**Table 4** Correlations of BSRTC Categories with *BRAF*<sup>V600E</sup> Mutation and Pathological Results

TBSRTC	all	<i>BRAF</i> <sup>V600E</sup> Mutation	The Rate of <i>BRAF</i> <sup>V600E</sup> Mutation	Malignant Rate
II	8	Positive	3(37.5%)	3/3(100%)
		Negative	5(62.5%)	4/5(80%)
III-IV	15	Positive	10(66.7%)	10/10(100%)
		Negative	5(33.3%)	2/5(25%)
V-VI	20	Positive	16(80%)	16/16(100%)
		Negative	4(20%)	4/4(100%)

**Abbreviation:** TBSRTC, the Bethesda System for Reporting Thyroid Cytopathology.

**Table 5** The Prediction of  $BRAF^{V600E}$  Mutation on Malignancy in Thyroid Nodules with Benign Cytology

Studies	Number of Cases in Benign FNA	Malignant in $BRAF^{V600E}$ Mutation
Kim et al, 2014 <sup>18</sup>	4028	15/17
Zhang et al, 2022 <sup>19</sup>	49	20/21
Chen et al, 2018 <sup>17</sup>	292	31/36

**Abbreviation:** FNA, fine needle aspiration.

US characteristics associated with  $BRAF^{V600E}$  mutation in patients with benign cytology were unclear. In this study, all the cases classified TBSRTC II had  $BRAF^{V600E}$  mutation detection, 18 of those were positive. The mean age of patients with positive  $BRAF^{V600E}$  mutation was  $56.35 \pm 11.89$  years, significantly higher than that of negative  $BRAF^{V600E}$  mutation, which was  $47.67 \pm 9.38$  years ( $p=0.0012$ ), other clinicopathologic and US features had no statistically significant differences between positive and negative  $BRAF^{V600E}$  mutation. Meanwhile, higher US classification C-TIRADS 4b-4c was not significantly correlated with positive  $BRAF^{V600E}$  mutation (Table 6). Considering the majority samples with benign cytology belonged to C-TIRADS 3–4a, we further analyzed the clinical and US features associated with positive  $BRAF^{V600E}$  mutation in patients with C-TIRADS 3–4a in TBSRTC II results. Consisted with the result of Table 6, positive  $BRAF^{V600E}$  mutation was upregulated in older patients ( $p=0.001$ ), multiple nodules and right location also showed difference between positive and negative  $BRAF^{V600E}$  mutation, with p value were 0.031 and 0.036, respectively (Table 7). Other suspicious malignant US features, such as hypoechogenicity, irregular morphology, unclear boundary and larger thyroid nodules size have no significant association with  $BRAF^{V600E}$  mutation.

## $BRAF^{V600E}$ Mutation Was Upregulated as Age Increased in Patients with Low Risk FNA and US Diagnosis

Patients of older age exhibit a higher proportion of  $BRAF^{V600E}$  mutations in benign cytology TBSRTC II ( $p=0.0012$ ) and in C-TIRADS 3–4a patients of benign cytology ( $p=0.001$ ) (Figure 4A and B), but no significant difference was found in the entire population in our cohort (Figure 4C). We then conducted a statistical analysis using the TCGA database on thyroid cancer, there was no correlation between age and  $BRAF^{V600E}$  mutation in all the patients ( $p=0.343$ ) and separated Asian patients ( $p=0.08$ ) (Figure 4D and E). Graphing the positive probability of the three thyroid groups, including patients in benign cytology, patients in C-TIRADS 3–4a of benign cytology, and thyroid cancer in TCGA Asian patients,

**Table 6** Correlation Between  $BRAF^{V600E}$  Mutation and Clinical Characteristics in Thyroid Nodules with Benign Cytology

Characteristics	$BRAF^{V600E}$		P-value
	Positive (%)	Negative (%)	
<b>Gender (Males)</b>	4(23.5%)	22(25.6%)	0.564
<b>Age (mean±S.D.)</b>	$47.67 \pm 9.38$	$56.35 \pm 11.89$	0.0012**
<b>Drinking</b>	1(5.9%)	3(3.5%)	0.52
<b>Smoking</b>	1(5.9%)	7(8.1%)	0.607
<b>C-TIRADS 4b-4c</b>	6(7%)	2(11.8%)	0.393
<b>Multifocality</b>	13(76.5%)	45(53.2%)	0.056
<b>Right Location</b>	10(58.8)	34(39.5)	0.142
<b>Irregular Morphology</b>	14(82.4)	56(65.1)	0.164
<b>Hypoechoic</b>	16(94.1%)	80(93%)	0.675
<b>Unclear Boundary</b>	12(70.6)	55(64%)	0.41
<b>Size (mm) median (IQR)</b>	8(4.6–14.5)	8(6–12.25)	0.879

**Note:** \*\* $P < 0.01$ .

**Abbreviations:** mean±S.D., mean ± standard deviation; C-TIRADS, Chinese Thyroid imaging reporting and data system; IQR, interquartile range.

**Table 7** The Relationship Between  $BRAF^{V600E}$  Mutation and Clinical Characteristics in C-TIRADS 3–4a Patients with Benign Cytology

Characteristics	$BRAF^{V600E}$		P-value
	Negative	Positive	
Gender (Male)	3(20%)	21(26%)	0.441
Age (mean±S.D.)	56.87±12.55	47.28±9.31	0.001**
Drinking	1(6.7%)	3(3.8%)	0.503
Smoking	1(6.7%)	6(7.5%)	0.695
Multifocality	12(80%)	40(50%)	0.032*
Location (Right)	10(66.7%)	30(37.5%)	0.036*
Irregular Morphology	12(80%)	52(65%)	0.254
Hypoechoic	14(93.3%)	74(92.5%)	0.695
Unclear Boundary	10(66.7%)	51(63.7%)	0.829
Size (mm) median (IQR)	7.0(4.4–15.0)	8.0(6.0–13.0)	0.786

Notes: \*P<0.05, \*\*P<0.01.

Abbreviations: C-TIRADS, Chinese Thyroid imaging reporting and data system; mean±S.D., mean ± standard deviation; IQR, interquartile range.

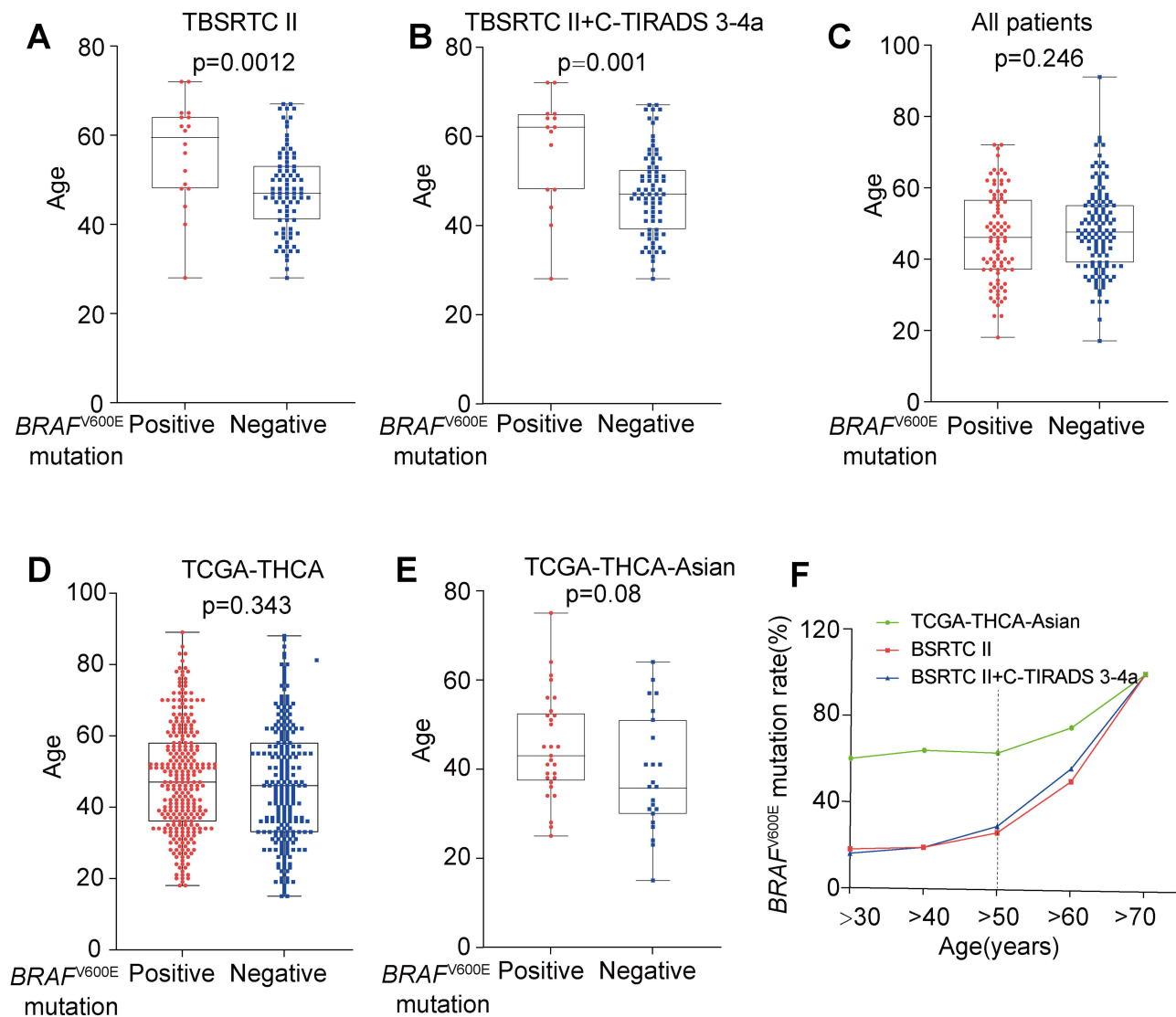
we found that the probability of  $BRAF^{V600E}$  mutations was significantly higher in thyroid cancer patients than in other two groups, regardless of age. In all three groups, the probability of positive  $BRAF^{V600E}$  mutations in the thyroid significantly increased with age over 50 years (Figure 4F). Therefore, greater attention should be paid to patients older than 50 years in benign cytological results, and  $BRAF^{V600E}$  testing should be conducted under appropriate conditions for screening high risk of malignancy, followed by surgical treatment.

## The Molecular Mechanisms Underlying the Increased Frequency of $BRAF^{V600E}$ Mutations in Older Patients

Differential gene expression analysis was performed between patient age groups ( $\leq 50$  years vs  $> 50$  years) in TCGA-THCA-Asian cohort, 277 differentially expressed genes were identified, which were significantly enriched in Ras signaling pathway (Figure 5A and B), closing interconnected with  $BRAF$  mutation. 1174 genes between the  $BRAF^{V600E}$  mutation groups were also identified (Figure 5C). The intersection differential genes of  $BRAF^{V600E}$  mutations and age were significantly enriched in immune-related signaling pathways (Figure 5D). Therefore, we hypothesized that the association between age and  $BRAF^{V600E}$  mutations may be immune-related. Furthermore, we investigated the correlation of immune response with patients age and  $BRAF^{V600E}$  mutation, we demonstrated the majority of immunostimulators were down regulated in older patients and patients with positive  $BRAF^{V600E}$  mutation, *BTNL2*, *CXCR4*, *TNFRSF14* and *TNFRSF25* were significant higher in younger patients and *CXCR4* was significant higher in patients with negative  $BRAF^{V600E}$  mutation (Figure 5E). No statistic differences in immunoinhibitors were obtained between patients age groups ( $\leq 50$  years vs  $> 50$  years) (Supplementary Figure 3).

## Discussion

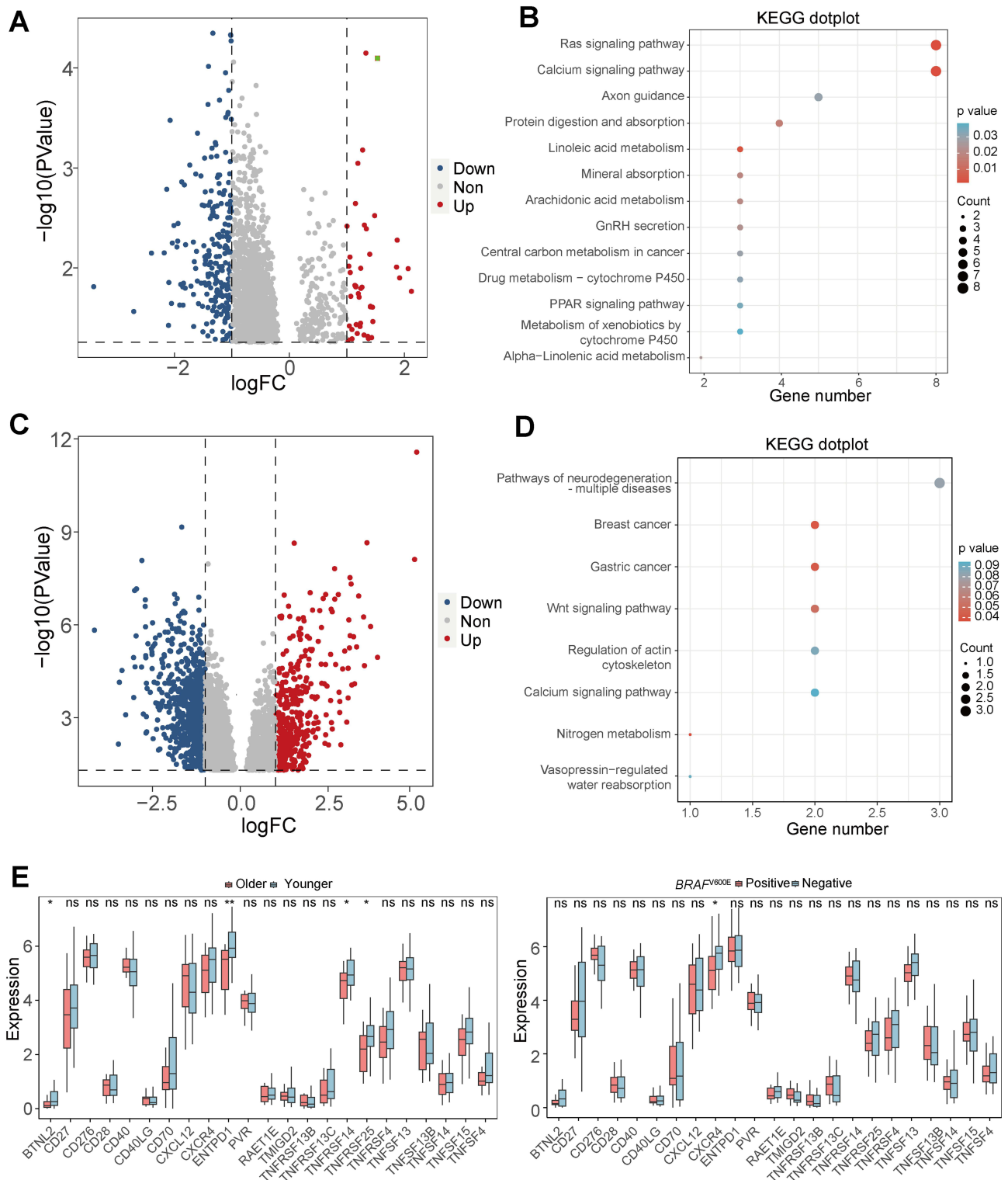
Accurately distinguishing between benign and malignant thyroid nodules is crucial to prevent patient overtreatment and tailor personalized treatment strategies. High-risk malignant thyroid nodules typically require surgical excision. Regarding surgical approaches, studies suggest no significant difference in early postoperative complications between total thyroidectomy (TT) and subtotal thyroidectomy (STT).<sup>25</sup> Similarly, LigaSure vessel (LS) or harmonic scalpel (HS) exhibit comparable safety in thyroidectomy.<sup>26</sup> For low-risk malignant thyroid nodules, repetitive ultrasound examinations and follow-ups can mitigate the discomfort associated with surgery. Ultrasound examination, widely utilized for assessing thyroid nodules, remains the most prevalent method. A study by Grimmichova et al indicates an 18% malignancy rate in thyroid nodules classified as low-risk by ultrasound.<sup>27</sup> In China, the C-TIRADS is extensively employed for thyroid ultrasound classification, utilizing a counting method based on suspicious malignant features.



**Figure 4** The correlation of  $BRAF^{V600E}$  mutation status and patient age. (A–E) Comparison of patient age between positive and negative  $BRAF^{V600E}$  mutation (A) in benign cytology TBSRTC II, (B) in C-TIRADS 3–4a with benign cytology, (C) in all the patients of our cohort, (D) in TCGA-THCA cohort, and (E) in TCGA-THCA-Asian cohort (E). (F) Linear correlation between patient age and  $BRAF^{V600E}$  mutation rate in different cohorts. Green: TCGA-THCA Asian cohort, red: FHPT cohort in TBSRTC II, blue: FHPT cohort in C-TIRADS 3–4a with TBSRTC II.

Nodules classified as 4b-6 are recommended for FNA testing.<sup>4,28</sup> FNA has traditionally been considered the gold standard diagnostic method for thyroid nodules before surgery, demonstrating high sensitivity and specificity in diagnosing thyroid malignancy. However, in cases of indeterminate FNA results, both sensitivity and specificity for malignancy significantly decrease.<sup>27</sup> In Mulita et al studies, the malignant of thyroid nodules that fall within Bethesda categories II and III–IV was 1.58% and 15–40%, respectively.<sup>7,29</sup> The  $BRAF^{V600E}$  mutation detection has proved can be used as a marker in FNA evaluation to diagnosis malignant in thyroid nodules.<sup>30,31</sup>

In our study, 46.2% of thyroid nodules classified as C-TIRADS 3–4a and 17.9% categorized as BSRTC II were diagnosed as PTC, a proportion significantly higher than previously reported data. This discrepancy may be attributed to our comprehensive integration of ultrasound, FNA, and  $BRAF^{V600E}$  mutation detection results and selected high-risk thyroid nodules for surgery and pathological diagnosis, contributing to a notable increase in the overall malignancy rate. Our finding also revealed the sensitivity and specificity of C-TIRADS and TBSRTC has significant improved when combined with  $BRAF^{V600E}$  mutation detection, and the combination of  $BRAF^{V600E}$  mutation and TBSRTC categories had optimal diagnostic performance of thyroid nodules, with the AUC, sensitivity, and specificity being 0.923, 84.6%, and



**Figure 5** The association in immune response with  $BRAF^{V600E}$  and patients age. **(A)** The volcano plot of differentially expressed genes (DEGs) between older and younger patients in TCGA-THCA-Asian, younger:  $\leq 50$  years vs.older:  $>50$  years. **(B)** KEGG analysis of DEGs between younger and older patients in TCGA-THCA-Asian. **(C)** The volcano plot of DEGs between patients with positive and negative  $BRAF^{V600E}$  mutation in TCGA-THCA-Asian. **(D)** KEGG analysis intersection DEGs between  $BRAF^{V600E}$  mutations groups and age groups. **(E)** The proportion of immunosuppressors in younger and older patients, positive and negative  $BRAF^{V600E}$  mutation in TCGA-THCA-Asian. Younger:  $\leq 50$  years vs.older:  $>50$  years \* $p < 0.05$ , \*\* $p < 0.01$ .

100%, respectively. A large number of studies have reported that  $BRAF^{V600E}$  mutation was significantly associated with PTC or papillary thyroid microcarcinoma (PTMC),<sup>13,32,33</sup> and the specificity of  $BRAF^{V600E}$  mutation for PTC was nearly 100%.<sup>34</sup> Consistent with the report, in pathological diagnosed group, all the patients with positive  $BRAF^{V600E}$  mutation were confirmed malignant, including those belonged to low-risk cytological categories TBSRTC II and C-TIRADS 3–4a.

Positive  $BRAF^{V600E}$  mutation could provide prognostic information for thyroid cancer, showed significantly higher risk of mortality and recurrence.<sup>35,36</sup> In addition, the  $BRAF^{V600E}$  mutation has an additive effect with various clinical features, such as older age, male gender, and lymph node metastasis, significantly increasing the risk of mortality in PTC.<sup>14,37,38</sup> Actually, positive  $BRAF^{V600E}$  mutation was significantly associated with high-risk FNA and US classification, along with advanced pathological T stage and lymph node metastases in thyroid cancer in our study. Thus, the thyroid nodules with positive  $BRAF^{V600E}$  mutation should take more attention for repeating US examination to make the final decision on whether to proceed with surgical intervention and remove thyroid nodules.

Few studies have focused on  $BRAF^{V600E}$  mutation analysis in thyroid nodules with benign cytology, and all three literature reports we retrieved have confirmed that the majority thyroid nodules in benign cytology with positive  $BRAF^{V600E}$  mutation confirmed malignant.<sup>17–19</sup> Consistent with the reports, three patients in TBSRTC II with positive  $BRAF^{V600E}$  mutation confirmed malignant by pathological diagnosis, confirming that there is a malignant risk even in the low-risk category of BSRTC II, and the  $BRAF^{V600E}$  mutation can identify it. However, for thyroid nodules with benign cytologic evaluation, in order to avoid overdiagnosis and overtreatment, the  $BRAF^{V600E}$  mutation detection was unpractical and not required immediately according to the 2015 ATA guideline.<sup>24</sup> Using clinical and ultrasound features to identify positive  $BRAF^{V600E}$  mutation, and including the  $BRAF^{V600E}$  mutation status in the prognostic risk stratification can lead to more precise management of patients with low-risk cytologic category.

Recently, a study revealed that more than two suspicious US features in benign cytologic analysis should consider further treatment of  $BRAF^{V600E}$  mutation detection.<sup>17</sup> Several US features, such as microcalcification, marked hypoechogenicity and irregular margins were more frequently observed in thyroid nodules harbouring positive  $BRAF^{V600E}$  mutation than negative  $BRAF^{V600E}$  mutation.<sup>30,39</sup> In our study, there were no significant differences in C-TIRADS classification, along with US features between positive and negative  $BRAF^{V600E}$  mutation in benign cytologic results. The reason may be that the majority of cases were classified as 3–4a, so the result of C-TIRADS classification was not referential. Surprisingly, the positive  $BRAF^{V600E}$  mutation was increased in older patients in benign cytology and in the nodules classified both C-TIRADS 3–4a and TBSRTC II.

For thyroid cancer, The American Joint Committee on Cancer (AJCC) staging system has emphasized that the age as the part of progression for malignant, treated as a dichotomous variable into risk stratification and treatment,<sup>40,41</sup> and the mortality risk of PTC was increased as increasing of patients age.<sup>42</sup> A study has reported that in patients older  $\geq 60$  years, the papillary microcarcinomas (PMCs) were most likely to enlarge or show clinical node metastases.<sup>43</sup> Shen et al has reported that the patient age exhibits a linear correlation with mortality rates in individuals with the positive  $BRAF^{V600E}$  mutation, conversely, among patients with the negative  $BRAF^{V600E}$  mutation, mortality rates remain relatively low and stable with increasing age.<sup>37</sup> In our study, we observed a significant upregulation of the  $BRAF^{V600E}$  mutation in patients aged over 50, particularly in the context of low-risk FNA and US diagnoses. Specifically, when dealing with thyroid nodules exhibiting lower suspicious US features and negative FNA findings, older patients warrant heightened attention for additional  $BRAF^{V600E}$  testing and further surgery.

We identified the different expression genes between two age groups ( $\leq 50$  years vs  $> 50$  years) were significantly enriched in Ras signaling pathway, which are closely interconnected with  $BRAF$  mutation.<sup>44,45</sup> The signaling pathway enriched in the different genes intersection of age groups and  $BRAF^{V600E}$  mutation played a crucial role in modulating immune response. Previous study has shown that in thyroid cancer,  $BRAF^{V600E}$  mutation exhibits a largely immunosuppressive profile and demonstrates disruptions in the immune surveillance.<sup>46</sup> Consistent with the study, our results showed a decrease expression of most immunostimulators in the patients with positive  $BRAF^{V600E}$  mutation and older age. However, immunoinhibitors had no significant difference between patients age groups. More samples are necessary for further analysis.

There are several limitations of this study. Firstly, the malignancy rate in our study was higher than other studies before, the reason may be that the nodules with the pathological diagnosis in our study were at high risk of malignant,

such as higher TBSRTC category, more suspicious US features, and positive  $BRAF^{V600E}$  mutation, which made the proportion of malignant was high. Additionally, all the thyroid nodules were followed up by regular clinic visits for half of the year, which is shorter and inadequate for detecting the progression. On the other hand, for the correlation of  $BRAF^{V600E}$  mutation and patient age, the results were obtained by Asian patients in TCGA-THCA, the number of samples was limited and there was no clinical data for analysis and validation. Subsequently, more prospective data were required for a deeper analysis. Otherwise, this is a retrospective study of single-institution analysis, we need more cases and prospective studies to verify our results in future studies.

## Conclusions

The  $BRAF^{V600E}$  mutation serves as a risk factor in screening malignancy in thyroid nodules, and its combination with TBSRTC classification can enhance diagnostic sensitivity. In cases of TBSRTC II and C-TIRADS 3–4a in benign cytology, thyroid nodules with the positive  $BRAF^{V600E}$  mutation exhibit an increased malignant risk. Additionally, the proportion of patients aged 50 and above with the  $BRAF^{V600E}$  mutation is significantly higher than those below 50 years old. The new results may help to make a better decision to screen thyroid nodules in low-risk FNA and US diagnostic results to have further  $BRAF^{V600E}$  mutation detection, which have higher risk of malignancy, and further surgery should be considered.

## Abbreviations

PTC, Papillary thyroid cancer; C-TIRADS, Chinese Thyroid imaging reporting and data system; TCGA, The Cancer Genome Atlas; FNA, Fine needle aspiration; US, Ultrasound; US-FNA, Ultrasound-guided fine needle aspiration; TBSRTC, The Bethesda System for Reporting Thyroid Cytopathology; MTC, Medullary thyroid cancer; DEGs, Different expression genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; THCA, Thyroid carcinoma; AUC, Area under the curve; ATA, American Thyroid Association; PTMC, Papillary thyroid microcarcinoma; DOR, Diagnostic odds ratio; AJCC, American Joint Committee on Cancer.

## Data Sharing Statement

The datasets supporting the conclusions of this article are included within the [Supplementary Tables 1](#) and [2](#). The data generated in the present study may be requested from the corresponding author.

## Ethics Approval and Consent to Participate

This study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was approved by the Ethics Institutional Review Board of The First Hospital of Putian City (approval number: 2023-066). The patients/participants provided their written informed consent to participate in this study.

## Consent to Publication

The details of any images and tables can be published, and that the persons providing consent have been shown the article contents to be published, all authors agree with the publication.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The Authors declare that there is no conflict of interests.

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