

Original Article

Association of *BRAF*^{V600E} mutation with clinicopathological features of papillary thyroid carcinoma: a study on a Chinese population

Shu Liu, Bingfei Zhang, Yanru Zhao, Pu Chen, Meiju Ji, Peng Hou, Bingyin Shi

Department of Endocrinology, The First Affiliated Hospital of Xi'an Jiaotong University Health Science Center, Xi'an 710061, P. R. China

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Abstract: Background: The new finding of the heterogeneous distribution of *BRAF*^{V600E} mutation in primary papillary thyroid carcinoma suggested the percentage of *BRAF*^{V600E} alleles should be taken into consideration when evaluating its association with clinicopathological features of papillary thyroid carcinoma. The aim of this study was to detect both the presence and the percentage of *BRAF*^{V600E} alleles in fine-needle aspiration biopsy samples and to assess its association with clinicopathological characteristics of papillary thyroid carcinoma in a Chinese population. Materials and methods: Fine needle aspiration samples were collected in a total of 182 patients (132 conventional papillary thyroid carcinomas and 50 goiters). The associations of the presence and percentage of *BRAF*^{V600E} alleles genotyped by pyrosequencing with clinicopathological characteristics were evaluated in papillary thyroid carcinomas. Results: 80 (60.61%) of papillary thyroid carcinomas exhibited *BRAF*^{V600E} mutation in a range of 7.7% to 46.3% of the total *BRAF* alleles. The presence of *BRAF*^{V600E} mutation was significantly associated with extrathyroidal invasion. There was no significant difference between the presence of *BRAF*^{V600E} mutation and other clinicopathological features. It was not found that the significant relationship between percentage of *BRAF*^{V600E} alleles and clinicopathological characteristics. Conclusion: We concluded that the presence of *BRAF*^{V600E} could be preoperatively predictive of extrathyroidal invasion in a Chinese population.

Keywords: Papillary thyroid carcinoma (PTC), percentage of *BRAF*^{V600E} alleles, pyrosequencing, preoperative risk stratification

Introduction

Papillary thyroid carcinoma (PTC) was characterized by mutations or rearrangements involved in MAPK signaling cascade [1, 2]. The most common genetic mutation is *BRAF*^{V600E} mutation, which exhibits high specificity for PTC [3]. The associations of *BRAF*^{V600E} mutation with the clinicopathological characteristics of PTC patients and predictive value of this mutation for aggressive behaviors have been extensively investigated [4-13]. However, unexplained controversial results are present in the literature. A number of studies [4-8] demonstrated that *BRAF*^{V600E} mutation was closely associated with aggressive pathological behaviors of PTC, such as extrathyroidal extension, lymph node metastasis, and high TNM stages. All of these aggressive pathological features were identified as major clinicopathological risk factors of

increased rates of recurrence and mortality of PTC [14]. However, some studies [9-13] failed to verify the relationship between *BRAF*^{V600E} mutation and aggressive pathological features in PTC. The recent study [15] on the clonal status of *BRAF*^{V600E} in PTC offered a new point of view on this issue. They demonstrated that most of *BRAF*^{V600E}-positive PTC tumors harbored this mutation in less than 50%, suggesting that only a part of PTC cells of the tumor harbored this mutation, whereas the remaining PTC cells harbored the wild-type *BRAF*. Thus, the heterogeneous distribution of *BRAF*^{V600E} mutation in primary tumors indicated that this mutation was a secondary genetic event and the percentage of *BRAF*^{V600E} alleles should be taken into consideration to analyze the association of *BRAF*^{V600E} mutation with clinicopathological features. A later study [16] from the same group took a further step to demonstrate that a high percent-

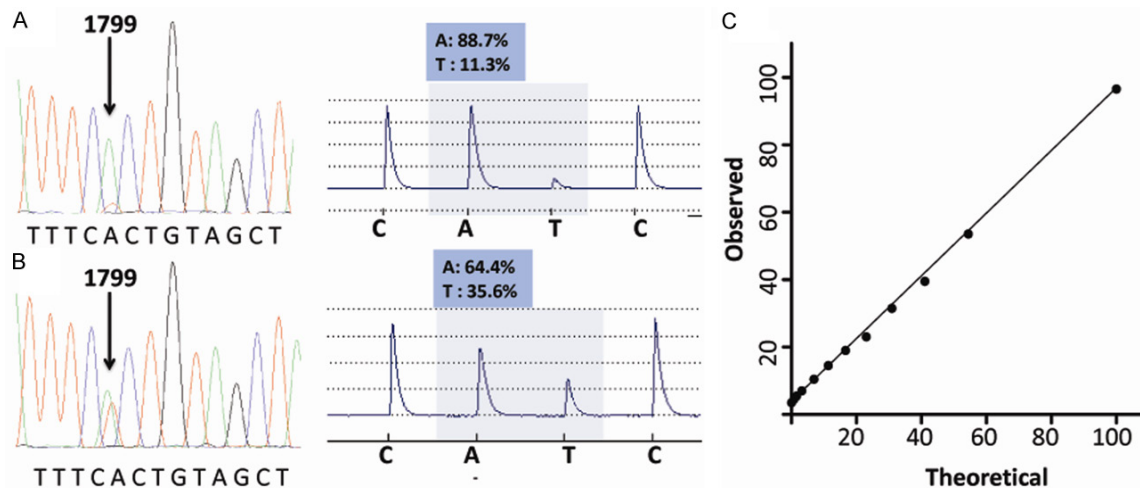


Figure 1. *BRAF*^{V600E} mutation analyses of two FNAB samples of PTC patients by dideoxy sequencing and pyrosequencing. A and B. the pyrogram demonstrates the differential peak heights of T and A that show the percentage of *BRAF*^{V600E} alleles. C. observed/theoretical percentage of mutant alleles at given dilutions obtained by pyrosequencing.

age of *BRAF*^{V600E} alleles predicted a poorer outcome. In this study, several approaches were taken to minimize the normal cell contamination and ensure the purity of PTC cells, including laser-captured harvest of PTC cells in a series of primary PTC tissues, exclusion of patients with concomitant Hashimoto's thyroiditis or samples with the presence of evident lymphoid infiltration. The preoperative knowledge of the quantitative *BRAF* mutation status may be more important to tailor the optimal extent of initial thyroidectomy and prophylactic central neck dissection, as well as guiding the postoperative medical treatments including ¹³¹I remnant ablation and long-term thyrotropin suppression [17].

Fine needle aspiration biopsy (FNAB) is the method choice for obtaining samples of thyroid tissue and the procedure is technically quite simple [18-20]. In the present study, we try to detect the presence and percentage of *BRAF*^{V600E} alleles in a large series of FNAB samples from conventional PTC patients and to evaluate its association with clinicopathological features of papillary thyroid carcinoma.

Methods

Participants and clinicopathological characteristics collection

From April 2011 to April 2014, a total of 182 patients (132 conventional PTC and 50 goiters)

were consecutively enrolled in the Department of Endocrinology, the First Affiliated Hospital of Xi'an Jiaotong University Health Science Center. All of these patients underwent fine-needle aspiration by an experienced endocrinologist under ultrasound-guidance. All PTC patients underwent total thyroidectomy and pretracheal and paratracheal lymph node dissection. The clinicopathological characteristics included: age at diagnosis, gender, tumor volume, extrathyroidal extension, multifocality, lymph node metastasis and TNM stages were carefully recorded. Extracapsular extension was defined as a tumor infiltrating thyroid capsule with invasion of perithyroidal soft tissues or muscle. All goiter patients were subjected to surgery and confirmed by histological pathology. Histological slides from thyroid tumors stained by hematoxylin and eosin stain were reviewed by an experienced pathologist, who were blinded to *BRAF* status

DNA extraction from FNAB samples

The FNAB material was obtained from a nodule in a 3 to 4 passes with a 23 gauge needle. The FNAB aspirates were harvested and divided into two parts: one part was smeared on the glass slide for HE stain; the remaining in the needle and the washed with 1 ml TRI reagent (Invitrogen, Carlsbad, CA) was collected into a 1.5 ml eppendorf tube for DNA isolation following the suggested protocol. Samples with concomitant Hashimoto's thyroiditis or with the

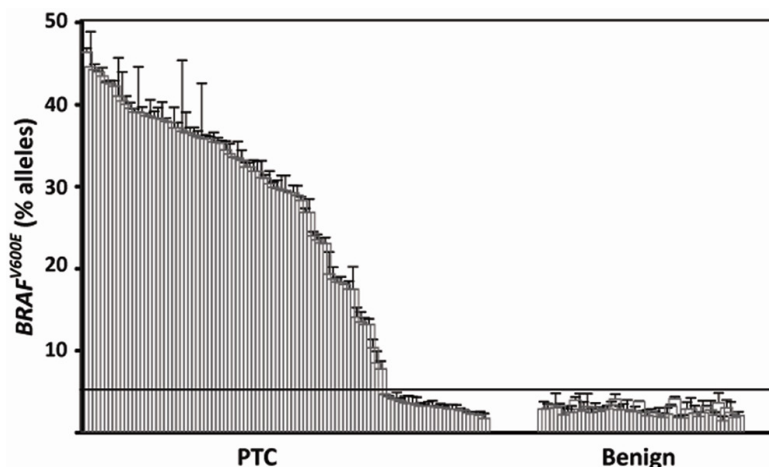


Figure 2. Pyrosequencing analysis of *BRAF*^{V600E} mutation in FNAB samples. The cutoff was set at 5%. The FNAB samples from benign nodules (n = 50) was used as negative controls, displayed a signal less than 5%.

presence of evident lymphoreticular cells infiltration were excluded.

DNA mixing studies

DNA from thyroid cancer cell line with homozygous *BRAF*^{V600E} mutation (BCPAP) was diluted with thyroid normal cell line with wild-type *BRAF* (HTori3) to generate mixtures with mutant DNA percentages. The percentage of mutant allele was determined by pyrosequencing. The linear relationship between observed and theoretical percentages was calculated by the Pearson correlation.

BRAF^{V600E} mutation detection by pyrosequencing and dideoxy sequencing

A total of 25 µl polymerase chain reaction (PCR) mix contained 5'-biotinylated forward and reverse primer (each 0.2 µM), dNTP (0.5 µl), MgCl₂ (1.5 mM), 1×PCR buffer, Taq polymerase (0.2 IU) (Invitrogen), and 50-100 ng DNA extraction. PCR was performed as follows: 95°C for 5 min, 35 cycles consisting of 95°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, followed by 10 minutes extension at 72°C. The PCR products were electrophoresed in a 1.5% of agarose gel to confirm the successful amplification of the PCR products. Biotinylated PCR products (20 µl) were immobilized on streptavidin-coated Sepharose beads (GE healthcare, Piscataway, NJ) according to standard protocol, by incubating in binding buffer with agitating at room temperature for 15 minutes. The beads containing immobilized templates were captured and DNA

strands were separated according to the instructions using the PyroMark Q24 vacuum workstation. Separated DNA strands were immediately released in 25 µl annealing buffer containing sequencing primer (0.3 µM) in the PyroMark Q24 plate and incubated at 80°C for 2 minutes followed by cooling to room temperature. Pyrosequencing was performed with the AQ assay using PyroMark Gold Q24 reagent (Qiagen, Hilden, Germany). PCR primer and sequencing primer sequences were as follows: 5'-biotin-CTTCATAATGCTTGCTCTGATAGG-3', reverse primer 5'-GGCCAAAATTTAATCAGTGGAA-3', sequencing reverse primer 5'-CCACTCCATCGAGATT-3'.

Pyrosequencing reactions were performed in triplicate for each sample and the mean of the triplicates were used in subsequent analyses. 50 FNAB samples from goiter nodules were used as negative control. In addition, Two FNAB samples from conventional PTC patients were also subjected to dideoxy sequencing.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 for Windows (SPSS Chicago, IL). Statistical procedures included ANONA, non-parametric test, χ^2 analysis, and binary logistic regression analysis. Categorical data were presented as the prevalence and continuous data were presented as median or mean, as appropriate. The linear relationship was calculated by the Pearson correlation. *P* value less than 0.05 is considered to be statistically significant.

Results

Analyses of DNA mixtures

Mixtures of homozygous *BRAF*^{V600E} cell DNA and *BRAF* wild-type cell DNA were prepared and subjected to detect the actual percentage of *BRAF*^{V600E} alleles by pyrosequencing. The linear relationship between the observed and theoretical percentage of *BRAF*^{V600E} alleles in the mixture was demonstrated (Pearson correlation-0.99, *P* < 0.001, **Figure 1C**). When we

Table 1. *BRAF*^{V600E} mutation and clinicopathological characteristics of conventional papillary thyroid carcinoma

	<i>BRAF</i> ^{V600E} -positive	Mutation-negative	P-value	
Number of patients	80	52	*Uni-	**Multi-
Age (mean ± SD)	40.57±11.62	39.25±13.78	n.s.	n.s.
< 45 ys	54 (67.5)	37 (71.15)	n.s.	n.s.
≥ 45 ys	26 (32.5)	15 (28.85)		
Female	62 (77.50)	41 (78.85)	n.s.	n.s.
Male	18 (22.50)	11 (21.15)		
Nodule size (median cm ³)	2.16	2.38	n.s.	n.s.
Lymph node metastasis	46 (57.50)	32 (61.54)	n.s.	n.s.
Multifocal	24 (30.00)	11 (21.15)	n.s.	n.s.
Extrathyroidal invasion	49 (61.25)	22 (42.31)	0.033	0.040
TNM stage			n.s.	n.s.
I/II	61 (76.25)	41 (78.84)		
III/IV	19 (23.75)	11 (21.15)		

*univariate analysis, continuous variables were compared by ANOVA or non-parametric test, as appropriate. Category variables were compared by χ^2 analysis.

**multivariate analysis (binary logistic regression analysis).

detected DNA from wild-type *BRAF* cell in triplicate, the results showed 2.9%, 4.5%, and 3.6%, respectively (mean = 3.6%, SD = 0.8).

Detection of BRAF^{V600E} mutation by pyrosequencing and dideoxy sequencing

The *BRAF* status of 182 samples was successfully analyzed by pyrosequencing (Figure 2). By setting a 5% cut off as previously described [15, 21], corresponding to the mean percentage of normal tissues plus 2 SD, none of benign nodules had *BRAF*^{V600E} mutation but 80 (60.61%) of PTC samples exhibited a peak representing the mutant nucleotide on the program. Two FNAB samples from conventional PTC patients, with 11.3% and 35.6% mutant *BRAF* alleles, respectively, were confirmed by dideoxy sequencing (Figure 1A, 1B). The *BRAF*^{V600E} alleles accounted for 7.7-46.3% of the total *BRAF* alleles, with the mean and median of 31.2% and 33.5%, respectively. 47 (58.75%) of 80 PTC with *BRAF*^{V600E} mutation harbored the higher percentage ($\geq 30\%$) of *BRAF*^{V600E} allele and even in 11 PTC samples *BRAF*^{V600E} allele was in the range of 40.0-46.3%.

Presence of BRAF^{V600E} mutation and clinicopathological characteristics at diagnosis

The *BRAF*^{V600E} mutation was significantly associated with extrathyroidal invasion ($P < 0.05$).

However, there was no significant association of age at diagnosis, sex, tumor volume, multifocality, lymph node metastasis, and advanced tumor stages with *BRAF*^{V600E} mutation (Table 1).

Percentage of BRAF^{V600E} alleles and clinicopathological characteristics at diagnosis

According to the percentage of *BRAF*^{V600E} alleles detected by pyrosequencing, all PTC patients were arbitrarily divided into three groups: less than 5% (group 1: without *BRAF*^{V600E} mutation), 5%-29% (group 2), 30% or greater (group 3) (Table 2). No significant differences of sex, age at diagnosis, tumor volume,

and lymph node metastasis were found among groups. Extrathyroidal invasion, multifocality and advanced tumor stages in group 3 were more frequent than those in group 1; however, there was no significant difference.

Discussion

BRAF^{V600E} mutation has been characterized as the risk factor for poor prognosis, extrathyroidal invasion, lymph node metastasis and advanced disease stages and a poorer clinical outcome [17]. Nevertheless, the association of *BRAF*^{V600E} mutation with aggressive behaviors and the prognostic value of *BRAF*^{V600E} mutation were still debated. Some studies failed to demonstrate that *BRAF* status is associated with clinicopathological features [9-13, 22, 23]. Xing [24] pointed out that many factors, including variability of the histological subtype, age distribution of patients, mean follow-up time, the incompleteness of patient records, could bias the conclusion about the prognostic role of *BRAF*^{V600E} mutation. It was also mentioned that some factors potentially affecting *BRAF*^{V600E} mutation were not identified, but there is no definitive explanation for these inconsistent results. Recently, the study [15] on the clonal status of *BRAF*^{V600E} mutation in PTC may be a good explanation on this issue. They demonstrated the heterogeneous distribution of *BRAF*^{V600E} in primary tumors indicated that this

Table 2. Clinicopathological characteristics at diagnosis among three groups based on the level of the percentage of *BRAF*^{V600E} alleles

	Quantitative classification of <i>BRAF</i> ^{V600E} allele			P-value
	Group 1 < 5%	5% ≤ Group 2 < 30%	Group 3 ≥ 30%	
Number of patient	52	33	47	
Age (mean)	39.25±13.78	41.45±10.52	40±12.34	n.s.
< 45 ys	37 (71.15)	24 (72.72)	30 (63.83)	
≥ 45 ys (%)	15 (28.85)	9 (27.27)	17 (35.29)	
Female (%)	41 (78.85)	26 (78.79)	36 (76.60)	n.s.
Male	11 (21.15)	7 (21.21)	11 (23.40)	
Nodule size (median cm ³)	2.38	1.80	3.00	n.s.
Lymph node metastasis	32 (61.54)	19 (57.58)	27 (57.44)	n.s.
Multifocal	11 (21.15)	8 (24.24)	16 (34.04)	n.s.
Extrathyroidal invasion	22 (42.31)	21 (63.64)	28 (59.57)	0.075
TNM stage				
I/II	41 (78.84)	27 (81.82)	34 (72.34)	n.s.
III/IV	11 (21.15)	6 (18.18)	13 (27.66)	

Continuous variables were compared by ANOVA or non-parametric test, as appropriate. Category variables were compared by χ^2 analysis.

mutation was a secondary genetic event and the percentage of *BRAF*^{V600E} alleles could impact on the analysis of the association of *BRAF*^{V600E} mutation and clinicopathological features. A further study was taken to certify that a high percentage of *BRAF*^{V600E} alleles predicted a poorer outcome. Moreover, a retrospective multicenter study [25] evidenced that the *BRAF*^{V600E} mutation was significantly associated with increased cancer-related mortality in patients with PTC.

Our data indicated the presence of *BRAF*^{V600E} mutation was significantly associated with extrathyroidal invasion and not correlated with age at diagnosis, gender, tumor volume, multifocality, lymph node metastasis, TNM stages of PTC patients. Moreover, extrathyroidal invasion, multifocality and advanced tumor stages were more frequent in tumors with a high percentage (≥ 30%) of *BRAF*^{V600E} mutant alleles than in those harboring the *BRAF*^{wild-type} allele, although there is no significant difference. This data suggested that *BRAF*^{V600E} mutation didn't drive the development of lymph node metastasis in PTC, it could be involved in the aggressive behaviors of PTC by promoting extrathyroidal extension of tumor cells in Chinese population.

There are some limitations to this study, it didn't include the association of the presence and percentage of *BRAF*^{V600E} alleles with recurrence and survival data. Because most of PTC

patients had a better prognosis, a further well-designed study with long-term follow up will be needed to classify if a higher percentage of *BRAF*^{V600E} alleles could be an indicator for poor prognosis.

In conclusion, *BRAF*^{V600E} mutation was significantly associated extrathyroidal invasion and there was no significant difference between the percentage of *BRAF*^{V600E} alleles and clinicopathological features of PTC in Chinese population.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bingyin Shi, Department of Endocrinology, The First Affiliated Hospital of Xi'an Jiaotong University Health Science Center, Xi'an 710061, P. R. China. Tel: +86-29-85323974; Fax: 86-29-85323974; E-mail: shibingy@126.com

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