

CLINICAL STUDY

Expressions of miRNAs in papillary thyroid carcinoma and their associations with the *BRAF*^{V600E} mutation

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Abstract

Objective: Alterations in microRNA (miRNA) expression have been described in thyroid tumors, suggesting a role for miRNAs in thyroid carcinogenesis. *BRAF*^{V600E} is the most frequently identified genetic alteration in papillary thyroid carcinoma (PTC). We investigated the link between *BRAF*^{V600E} status and the expression of miRNAs in PTC and analyzed the associations of these factors with clinicopathological characteristics.

Design and methods: Prospective study of patients who underwent thyroid surgery between October 8, 2008 and November 1, 2010. *BRAF*^{V600E} status was determined by mutant allele-specific amplification PCR and direct sequencing of exon 15 of the *BRAF* gene in 69 PTC tissues and 69 respective paracancerous normal thyroid tissues. Initially, miRNA expression was analyzed in 12 PTC tissues and three associated paracancerous tissues using a miRNA microarray. miRNAs differentially expressed between *BRAF*^{V600E}-positive and -negative PTC tissues were then validated by real-time quantitative PCR on 69 PTC tissues and 69 paracancerous tissues. We also explored the associations between *BRAF*^{V600E} status or differential miRNA expression and clinicopathological characteristics.

Results: The mutation rate of *BRAF*^{V600E} in PTC was 47.8%. Twelve miRNAs were upregulated and six were downregulated in PTC tissues, among which miR-15a, 15a*, 34a*, 34b*, 551b, 873, 876-3p, and 1274a were first identified. miR-21* and 203 were significantly dysregulated ($P < 0.05$) in PTC tissues with *BRAF*^{V600E}. Additionally, there were significant associations ($P < 0.05$) between *BRAF*^{V600E} and a higher tumor–node–metastasis staging (III/IV), and between miR-21* over-expression and lymph node metastasis.

Conclusions: We identified two miRNAs that are differentially expressed in PTC tissues with *BRAF*^{V600E} and revealed their associations with clinicopathological features. These findings may lead to the development of a potential diagnostic biomarker or prognostic indicator of PTC.

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Introduction

Thyroid carcinoma is one of the most common endocrine malignancies, accounting for ~1% of all newly diagnosed cancers (1). It consists of four major histological types – papillary thyroid carcinoma (PTC), follicular thyroid carcinoma, medullary thyroid carcinoma, and anaplastic thyroid carcinoma – of which PTC accounts for more than 80% (2). The biological behavior and growth patterns of PTC are variable: most have a low potential for malignancy and a favorable prognosis, while others present with clinicopathological characteristics associated with a poor prognosis; these characteristics include an older

onset age, a larger tumor diameter, extrathyroidal invasion, distant metastases, and aggressive histological variants (3, 4). Surgery (sometimes with adjuvant radiotherapy) is still the most effective approach for patients with PTC.

Previous studies (1, 5) have demonstrated genetic alterations in PTC: *RET* gene rearrangements (referred to as *RET*/PTC), *NTRK1* gene rearrangements (referred to as TRK), and *RAS* or *BRAF* gene mutations, which are collectively involved in the *RET*/PTC (TRK)–*RAS*–*BRAF*–*MEK*–*MAPK* signaling pathway. V600E in *BRAF*, a thymine-to-adenine transversion at nucleotide 1799, is a hot spot for mutations in patients with PTC. It affects exon 15 of the *BRAF* gene and is present in 29–83% of

PTC cases (6, 7, 8, 9). Nucera *et al.* (10) revealed that $BRAF^{V600E}$ is required to maintain and promote thyroid cancer progression. Some other studies (8, 11, 12, 13, 14) have indicated that $BRAF^{V600E}$ is associated with an increased incidence of poor clinicopathological parameters in PTC.

MicroRNAs (miRNAs), short RNAs that are usually 21–25 nucleotides in length and are generated from single-stranded miRNA gene transcripts, represent a recently identified class of endogenous noncoding RNAs. Their regulatory influence is mediated by their interactions with a protein complex called the RNA-induced silencing complex, which delivers them to their mRNA targets (15). Previous studies (16, 17, 18, 19) have demonstrated that miRNAs play a role not only in individual growth and development but also in regulating the expression of well-known oncogenes and tumor suppressor genes, and increasingly, dysregulated miRNAs have been identified in various malignancies. Recently, using miRNA microarrays and miRNA real-time quantitative PCR (qPCR), several studies (18, 19, 20, 21, 22, 23, 24, 25, 26) have reported dysregulated miRNAs (such as miR-7, 21, 30, 31, 34a, 144, 146, 146b, 155, 181b, 187, 200, 200b, 221, and 222) in PTC, suggesting that these are promising targets for studying the genesis and development of PTC.

The association between $BRAF^{V600E}$ status and miRNA expression in PTC has not been thoroughly examined. Cahill *et al.* (27) and Nikiforova *et al.* (18) showed dysregulated miRNAs (miR-127, 130a, 141, 144, 146b, 155, 187, 200a, 200b, 221, and 222) in thyroid-derived cell lines and tissues with $BRAF^{V600E}$ respectively. Additionally, resulting from the rapid development of miRNA microarray, more and more human miRNAs have been identified. In this study, we i) compared miRNA expression between PTC tissues and paracancerous normal thyroid tissues using an up-to-date miRNA microarray and determined the differentially expressed miRNA in PTC tissues with $BRAF^{V600E}$ and ii) explored the associations between $BRAF^{V600E}$ status or differential miRNA expression and the clinicopathological characteristics of PTC.

Materials and methods

This study complies with the Declaration of Helsinki and was approved by the Local Ethics Committee. Informed consent was obtained from each subject or his/her guardian.

Selection of cases and controls

The current study was conducted on patients of Asian descent who had undergone thyroid surgery between October 8, 2008 and November 1, 2010. All patients were from southeast China and were admitted to the

Thyroid Department of The First Affiliated Hospital of Sun Yat-Sen University. Lesions and paracancerous thyroid tissues (the latter ones were resected from the margin of the post-resection samples and confirmed by both naked eye and hematoxylin and eosin staining to contain no apparent abnormal tissues) were successfully obtained from each of 69 patients with PTC (confirmed by hematoxylin and eosin staining, according to the widely accepted histological diagnostic criteria (28)). All the tissues were collected from surgically resected tissues and stored immediately at -80°C until analysis. The 69 patients (male: female = 1:4.3) were aged 14–75 years (median, 41 years; interquartile range, 23 years); 59 had classical PTC while the other 10 had the follicular variant of PTC. The tumor size was 2.48 ± 1.31 cm (mean \pm s.d.); 31 cases (44.9%) were solitary lesions, 36 cases (52.2%) presented with lymph node metastasis, 25 cases (36.2%) presented with extrathyroidal invasion, and 48 cases (69.6%) were tumor–node–metastasis (TNM) staging (29) I/II.

RNA and DNA extraction

Genomic DNA and RNA were extracted from frozen specimens by standard techniques. Total RNA and DNA were extracted using TRIzol reagents (Invitrogen) and a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) respectively according to the manufacturer's instructions. The RNA and DNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only RNA samples that showed intact 18S and 28S ribosomal bands upon 1% agarose gel electrophoresis, and for which the A260/A280 ratio was 1.9–2.1 and the 28S/18S ratio was ≥ 2.0 , were deemed acceptable.

Detection of $BRAF^{V600E}$

Mutant allele-specific amplification PCR was conducted essentially as described previously (30) to detect $BRAF^{V600E}$ mutation in the 69 PTC tissues and the 69 paracancerous thyroid tissues. KTC-1 (a thyroid carcinoma cell line with $BRAF^{V600E}$) and WRO (a thyroid carcinoma cell line without $BRAF^{V600E}$) were used as positive and negative controls respectively. PCR products were analyzed by 2% agarose gel electrophoresis. At the same time, according to a previous study (30), exon 15 of the $BRAF$ gene was PCR amplified from genomic DNA from the 69 PTC tissues and the 69 paracancerous thyroid tissues. PCR products were sequenced by Invitrogen. The data were analyzed using Lasergene Software (version 7.1.0; DNASTAR, Madison, WI, USA), comparing the sequences with the wild type human sequence obtained from the National Center for Biotechnology Information Gene database (<http://www.ncbi.nlm.nih.gov/gene/673>). PCR primers were synthesized by Invitrogen China.

miRNA microarray analysis

miRNA expression was analyzed using a Human miRNA Microarray System Version 3 (Agilent Technologies) according to the manufacturer's instructions, with probe sets for 866 human miRNAs and 89 viral miRNAs (Sanger miRBase release 12.0). In brief, using a miRNA Complete Labeling and Hyb Kit (Agilent Technologies), 120 ng total RNA was fluorescently labeled with Cy3 in a hybridization oven (Agilent Technologies) for 20 h at 55 °C, with rotation at 20 rounds per minute. Microarray hybridization, data generation, and normalization were performed by the Shanghai Biochip Corporation (Shanghai, China), following standard Agilent protocols. MeV 4.7.4 (<http://www.tm4.org/mev/>) was used for bioinformatic analysis and visualization of microarray data.

miRNA qPCR

cDNA was synthesized from 10 ng total RNA and extracted from 69 PTC tissues and 69 associated paracancerous thyroid tissues, using the TaqMan miRNA RT Kit (Applied Biosystems, Inc.). qPCR was performed to assess the expression of differentially expressed miRNAs, which were identified in *BRAF*^{V600E}-positive PTC tissues compared with *BRAF*^{V600E}-negative PTC tissues by the miRNA microarray. The expression levels of miRNAs were quantified using the miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems). According to the manufacturer's instructions, qPCR was performed on the Applied Biosystems 7500 Sequence Detection system. The expression of miRNAs was defined based on the threshold of amplification cycle number (Ct), and the quantity of each miRNA was normalized to the expression of U6 small nuclear RNA using the equation of $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{\text{miRNA}} - Ct_{\text{U6}}$).

Statistical analysis

The miRNA microarray data were analyzed using Significance Analysis of Microarrays (SAM) version 3.0 (<http://www-stat.stanford.edu/~tibs/SAM>). miRNAs were considered to be significantly differentially expressed only under the condition that the false discovery rate=0. Other data were analyzed using SPSS version 17.0 (SPSS, Inc.); $P < 0.05$ was considered statistically significant. For the qPCR data, the Wilcoxon rank sum test was used to compare PTC tissues with and without *BRAF*^{V600E}, and the paired *t*-test was used to compare *BRAF*^{V600E}-positive PTC tissues with their associated paracancerous thyroid tissues. Moreover, the χ^2 test (for age at diagnosis, gender, number of lesions, lymph node metastasis, TNM staging, and metastasis or recurrence) and the Wilcoxon rank sum test (for tumor size) were also used.

Results

miRNA expression profiles

Initially, miRNA expression was analyzed in 12 continuously PTC tissues plus paracancerous thyroid tissues from three out of 12 PTC patients. Figure 1 shows 12 upregulated miRNAs (fold-change > 2) and six downregulated miRNAs (fold-change < 0.5 for all except miR-30a) in PTC tissues, with statistical differences ($P < 0.05$).

Detection of *BRAF*^{V600E}

Combining mutant allele-specific amplification PCR and sequencing of *BRAF* exon 15, we determined that *BRAF*^{V600E} was present in 47.8% (33/69) of 69 PTC tissues (Fig. 2) and was absent from all 69 paracancerous thyroid tissues.

Expression of miRNAs relative to *BRAF*^{V600E} status

Two miRNAs (miR-21* and 203) were upregulated in seven *BRAF*^{V600E}-positive PTC tissues compared with five *BRAF*^{V600E}-negative PTC tissues (Fig. 3A), with statistical differences ($P < 0.05$). These findings were confirmed by qPCR (Fig. 3B and C). To further validate the association between *BRAF*^{V600E} and these two miRNAs, the expressions of miR-21* and 203 were tested by qPCR on the other 57 PTC tissues and all 33

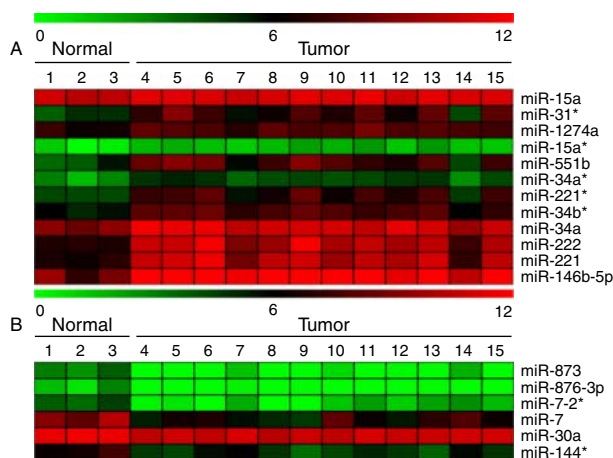


Figure 1 Heat maps of miRNA expression in papillary thyroid carcinomas and normal thyroid tissues by SAM analysis. Green bars indicate low expression and red bars indicate high expression. Nos 1–3 are normal thyroid tissues adjacent to the papillary thyroid carcinomas of Nos 7, 8, and 12 respectively. Twelve significantly upregulated ($P < 0.05$) miRNAs (A) and six significantly downregulated ($P < 0.05$) miRNAs (B) were found in 12 papillary thyroid carcinomas (Nos 4–15), compared with those expressed in three normal thyroid tissues (Nos 1–3). Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-1029>.

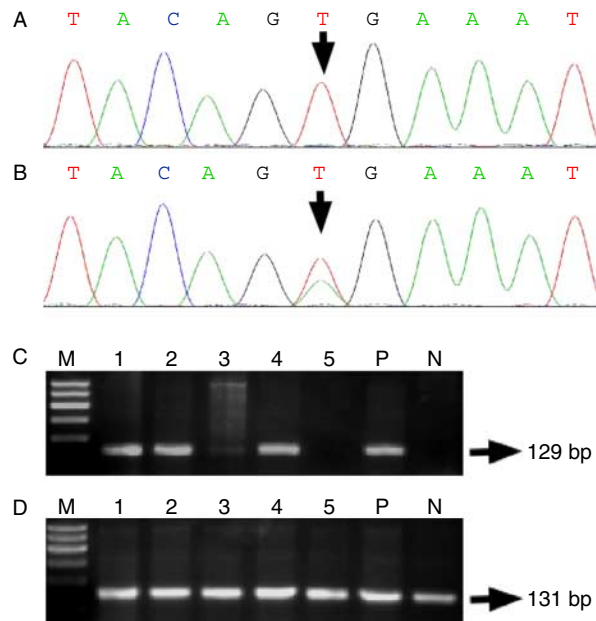


Figure 2 Detection of $BRAF^{V600E}$ in papillary thyroid carcinomas by DNA sequencing and mutant allele-specific amplification PCR. (A) A $BRAF^{V600E}$ -negative DNA sample shows a single peak at nucleotide 1799 (arrow); (B) a $BRAF^{V600E}$ -positive (1799 T>A) DNA sample shows a double peak at nucleotide 1799 (arrow); (C) arrow shows the PCR products obtained by primers specific for mutant; (D) arrow shows the PCR products obtained by primers specific for wild type. M, marker (100–600 bp); P, positive control KTC-1 cell line (a thyroid carcinoma cell line with $BRAF^{V600E}$); N, negative control MRO cell line (a thyroid carcinoma cell line without $BRAF^{V600E}$). Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-1029>.

paracancerous thyroid tissues from $BRAF^{V600E}$ -positive PTC patients. miR-21* and 203 were upregulated in 45 and 41 PTC tissues respectively. And both of them were significantly dysregulated ($P<0.05$) in $BRAF^{V600E}$ -positive PTC tissues compared with $BRAF^{V600E}$ -negative PTC tissues or the associated paracancerous thyroid tissues, with coherence of changing trend (Fig. 4).

Relationships between $BRAF^{V600E}$ status or differential miRNA expression and the clinicopathological characteristics

Significant associations ($P<0.05$) were identified for the 69 PTC patients between $BRAF^{V600E}$ positivity and a higher TNM stage (III/IV), and between miR-21* over-expression and lymph node metastasis (Table 1). By contrast, there was no significant association ($P>0.05$) between miR-203 expression and the clinicopathological characteristics (Table 1).

Discussion

Although PTC is the most common type of thyroid carcinoma, and also has the best prognosis, local

recurrence or distant metastasis can occur (3, 4). Moreover, some studies (8, 11, 12, 13) have pointed out the increased incidence of poor clinicopathological features in PTC with $BRAF^{V600E}$. Owing to the great progress in the field of miRNA, it has become a hot research topic in kinds of diseases (including PTC). Limited in PTC and highlighting the paracancerous normal thyroid tissues as control group, the current study provided new insights into the following by up-to-date miRNA microarray and $BRAF^{V600E}$ detection: i) besides some previously reported (18, 19, 20, 21, 22, 23, 24, 25, 26) miRNAs, a set of miRNAs were dysregulated in PTC; ii) the expressions of miR-21* and 203 were significantly associated ($P<0.05$) with $BRAF^{V600E}$ status; and iii) over-expression of miR-21* was significantly associated ($P<0.05$) with poor clinicopathological prognostic feature.

According to recent reports (31, 32, 33), the differential expression of miRNAs in different thyroid nodules, sampled by fine-needle aspiration, may improve the preciseness of preoperative diagnosis. As early as 2005, He *et al.* (20) demonstrated that three miRNAs (miR-146, 221, and 222) were significantly upregulated in PTC, using a miRNA microarray. Similar studies (18, 19, 21, 22, 23, 24, 25, 26) have appeared in the later literature. Because of the rapid development of miRNA microarray technology (there are now more than 1000 identified human miRNAs), using up-to-date miRNA microarray may help to enrich the knowledge about miRNA expression in PTC. In the current study, we found 18 dysregulated miRNAs, among which miR-15a, 15a*, 34a*, 34b*, 551b, 873, 876-3p, and 1274a were first found in PTC.

$BRAF^{V600E}$, formerly named $BRAF^{V599E}$, was first discovered by Davies *et al.* (34) in malignant melanoma.

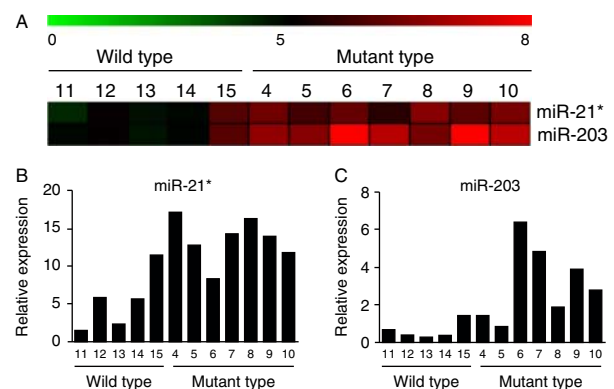


Figure 3 Differential expressions of miR-21* and 203 between $BRAF^{V600E}$ -positive and -negative papillary thyroid carcinomas (PTC). (A, B and C) Expressions of miR-21* and 203 in five $BRAF^{V600E}$ -negative (wild type, Nos 11–15) PTC tissues and seven $BRAF^{V600E}$ -positive (mutant type, Nos 4–10) PTC tissues using miRNA microarray (A) and real-time quantitative PCR (B and C). Green bars indicate low expression and red bars indicate high expression. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-1029>.

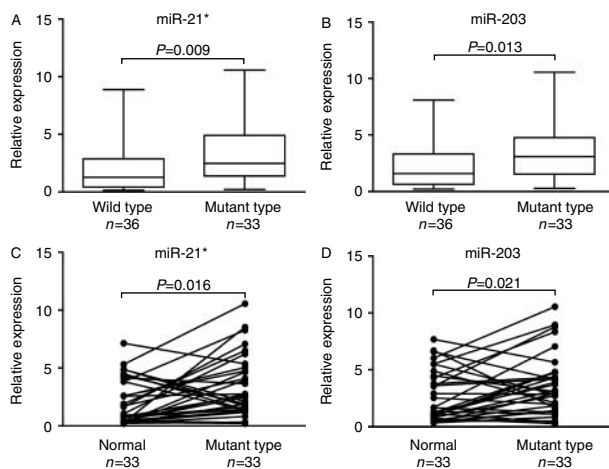


Figure 4 miR-21* and 203 expressions in papillary thyroid carcinomas (PTC) and paracancerous normal thyroid tissues by real-time quantitative PCR. (A and B) The expressions of miR-21* and 203 in 36 *BRAF*^{V600E}-negative (wild type) PTC tissues and in 33 *BRAF*^{V600E}-positive (mutant type) PTC tissues; (C and D) the expressions of miR-21* and 203 in 33 *BRAF*^{V600E}-positive PTC tissues and in 33 associated paracancerous normal thyroid tissues (normal).

It is believed that in thyroid tissues (including normal tissue, benign tumor tissue, and malignant tumor tissue), *BRAF*^{V600E} is restricted to PTC and poorly differentiated or anaplastic carcinomas arising from PTC (30, 35, 36). We detected *BRAF*^{V600E} in nearly half

of the PTC tissues but in none of the associated paracancerous thyroid tissues, implying that it was a somatic mutation. There is a wide range of reported (6, 7, 8, 9) *BRAF*^{V600E} mutation rates, from 29 to 83%; this might be attributable to ethnic heterogeneity and different sample sizes.

Few studies have examined the association between *BRAF*^{V600E} and miRNA expression in PTC. On the one hand, *BRAF*^{V600E} has an effect on activating BRAF–MEK–MAPK signaling pathway (37); on the other hand, miRNAs are posttranscriptional regulators that bind to complementary sequences on target mRNAs, usually resulting in target degradation, translational repression, or gene silencing (15). Cahill *et al.* (27) investigated the expression of 160 miRNAs in a normal thyroid cell line and two thyroid cell lines containing *BRAF*^{V600E}; three miRNAs (miR-141, 200a, and 200b) were significantly upregulated and three (miR-127, 130a, and 144) were significantly downregulated in the mutation-carrying cell lines. Nikiforova *et al.* (18) selected six out of the 158 most highly expressed human miRNAs in PTC tissue and further explored their expressions in 23 PTC tissues (with/without *BRAF* or *RAS* gene mutations, or *RET*/PTC), revealing that five miRNAs (miR-146b, 155, 187, 221, and 222) were differentially expressed among the groups. Sheu *et al.* (38) assessed the expressions of five selected miRNAs (miR-21, 146b, 181b, 221, and 222) in 56 PTC tissues and concluded that they were not associated with

Table 1 Relationships between clinicopathological characteristics and *BRAF*^{V600E} status, miR-21* expression, or miR-203 expression in patients with papillary thyroid carcinoma (*n*=69).

Parameter	<i>BRAF</i> ^{V600E} status		<i>P</i>	miR-21*		<i>P</i>	miR-203		<i>P</i>
	Mutant type (%; <i>n</i> 1 = 33, <i>n</i> 2 = 31)	Wild type (%; <i>n</i> 1 = 36, <i>n</i> 2 = 35)		Up ^a (%; <i>n</i> 1 = 45, <i>n</i> 2 = 43)	Down ^b (%; <i>n</i> 1 = 24, <i>n</i> 2 = 23)		Up ^a (%; <i>n</i> 1 = 41, <i>n</i> 2 = 39)	Down ^b (%; <i>n</i> 1 = 28, <i>n</i> 2 = 27)	
Age			0.124			0.408			0.436
≥45 years	18 (55)	13 (36)		23 (51)	10 (42)		20 (49)	11 (39)	
<45 years	15 (45)	23 (64)		22 (49)	14 (58)		21 (51)	17 (61)	
Gender			0.828			0.935			0.863
Male	6 (18)	7 (19)		9 (20)	4 (17)		8 (20)	5 (18)	
Female	27 (82)	29 (81)		36 (80)	20 (83)		33 (80)	23 (82)	
Number of lesions			0.570			0.260			0.837
Single	16 (48)	15 (42)		18 (40)	13 (54)		20 (49)	11 (39)	
Multiple	17 (52)	21 (58)		27 (60)	11 (46)		21 (51)	17 (61)	
Lymph node			0.390			0.005			0.765
N0	14 (42)	19 (53)		16 (36)	17 (71)		19 (46)	14 (50)	
N1	19 (58)	17 (47)		29 (64)	7 (29)		22 (54)	14 (50)	
TNM staging ^c			0.043			0.715			0.941
I and II	17 (52)	27 (75)		28 (62)	16 (67)		26 (63)	18 (64)	
III and IV	16 (48)	9 (25)		17 (38)	8 (33)		15 (37)	10 (36)	
Metastasis or recurrence ^d			0.597			1.000			1.000
Yes	2 (6)	1 (3)		2 (5)	1 (4)		2 (5)	1 (4)	
No	29 (94)	34 (97)		41 (95)	22 (96)		37 (95)	26 (96)	

*n*1 is the total number of patients available for the data of age at diagnosis, gender, number of lesion, and tumor–node–metastasis (TNM) staging. *n*2 is the total number of patients available for the data of metastasis or recurrence by 20–45 months of follow-up (median, 32 months; interquartile range, 11 months).

^aExpression of the miRNA in tumor tissue was higher than that in associated paracancerous normal thyroid tissue.

^bExpression of the miRNA in tumor tissue was lower than that in associated paracancerous normal thyroid tissue.

^cTNM classification is defined according to the American Joint Committee on Cancer (2010).

^dConfirmed by color Doppler ultrasonography or computed tomography during the follow-up period.

BRAF^{V600E} status. In our study, we initially identified two differentially expressed miRNAs (miR-21* and 203) between 12 *BRAF*^{V600E}-positive and -negative PTC tissues by both miRNA microarray and qPCR. This was further validated by qPCR in a total of 69 PTC tissues and 33 paracancerous thyroid tissues from *BRAF*^{V600E}-positive PTC patients. Previous studies have revealed that: activator protein 1 (AP-1) activated the miR-21 transcription in a human promyelocytic cell line (39) and increased the expression of miR-203 in human keratinocytes (40); MAPK signaling pathway had an effect on activating AP-1 (41); and the effect of *BRAF*^{V600E} was mediated by the BRAF–MAPK signaling pathway (37). Taken together, we hypothesize that the relationship between *BRAF*^{V600E} and over-expression of miR-21* or 203 may be mediated by the MAPK signaling pathway and AP-1, and both miR-21* and 203 have the effect on PTC aggressiveness. However, the absence of differential expressions of miR-21* and 203 between PTC tissues and paracancerous thyroid tissues on the miRNA microarray were probably due to the following: i) the small sample sizes of PTC tissues and paracancerous thyroid tissues for miRNA microarray analysis in the study; ii) the genotypic status (with/without *BRAF* or *RAS* gene mutations, *RET*/PTC, *TRK*, etc.) might be different among the 12 PTC tissues; and iii) these two miRNAs might be differentially expressed among different genotypic groups, while this hypothesis is needed to be further tested.

There are discordant findings in the literature in the relationship between *BRAF*^{V600E} status and clinicopathological parameters: numerous studies (8, 11, 12, 13, 14) showed that *BRAF*^{V600E} was significantly associated with poor clinicopathological parameters, while a small number of studies (5, 42) did not. In this study, we revealed significant relationships between poor clinicopathological features and the following: *BRAF*^{V600E} positivity (using *BRAF*^{V600E}-negative PTC tissues as the control group) and miR-21* over-expression (using paracancerous thyroid tissues as the control group). Interestingly, we also identified a significant association between *BRAF*^{V600E} positivity and miR-21* over-expression. The molecular basis of this association should be explored in future studies.

In conclusion, our study has shed new light on the relationships between *BRAF*^{V600E} status and miRNA expression, and between differentially expressed miRNA and clinicopathological feature in PTC. Although our findings need to be confirmed in studies of larger sample size, they may lay the foundation for the identification of a potential diagnostic biomarker or prognostic indicator of PTC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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