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Original Article

Association between *BRAF* and *RAS* mutations, and *RET* rearrangements and the clinical features of papillary thyroid cancer

Jie Ming[1*], Zeming Liu[1*], Wen Zeng[3], Yusufu Maimaiti[1], Yawen Guo[1], Xiu Nie[2], Chen Chen[1], Xiangwang Zhao[1], Lan Shi[1], Chunping Liu[1], Tao Huang[1]

[1]Department of Breast and Thyroid Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China; [2]Department of Pathology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China; [3]Department of Ophthalmology, Zhongnan Hospital, Wuhan University, Wuhan, Hubei, China. [*]Equal contributors.

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Abstract: Objective: To evaluate the significance of *BRAF*^{V600E} and *Ras* mutations, and *RET* rearrangements in papillary thyroid cancer (PTC) in the South central region of China. Methods: We included patients from Union hospital's pathology archive diagnosed with PTC and meeting the criteria for *BRAF* mutation, *RAS* mutation, and *RET* rearrangement testing. Medical records were analyzed for *BRAF* and *RAS* mutation status, *RET* rearrangements (positive or negative), and a list of standardized clinicopathologic features. Results: Positive *BRAF* mutation was found to be significantly associated with age and extrathyroidal extension (P=0.011 and P=0.013, respectively). However, there was no significant association between *BRAF* mutation and sex, tumor size, histological subtype, multifocality, or accompanying nodular goiter and Hashimoto's. On the other hand, none of these characteristics of PTC were been found to be associated with *RAS* mutation. Additionally, the frequency of *RET* rearrangements was higher in patients ≤45 years old than that in patients >45 years old. Conclusions: We demonstrated that the *BRAF*^{V600E} mutation slightly correlated with the clinicopathological characteristics of PTC in the Han population. Furthermore, neither *RAS* mutation nor *RET* rearrangements were found to be associated with the clinicopathological characteristics of PTCs. Our work provides useful information on somatic mutations to predict the risk of PTC in different ethnic groups.

Keywords: Papillary thyroid cancer, BRAF, RAS, RET

Introduction

Thyroid carcinoma is the most common endocrine malignancy. Papillary thyroid cancer (PTC) accounts for majority of the thyroid carcinomas and its incidence are still on the rise [1-3]. The typical treatment for PTC includes surgery, TSH suppressive therapy, and radioactive Iodine (RAI) administration. PTC has an excellent prognosis with an average 10-year survival rate in more than 90% cases. However, disease recurrence rates remain roughly at 20% after resection, at 10-year follow-up, which is associated with increased mortality [4]. Therefore, it is important for clinicians to focus on thyroid cancer patients displaying more aggressive phenotype marked by disease recurrence or death, so that perform individual treatment. Efforts have been made to identify patients at risk of disease recurrence and develop suitable markers, such as for the detection of somatic mutations, which might aid in predicting good versus poor prognosis.

Recently, *BRAF* and *RAS* mutations as well as *RET* rearrangements have been given a great deal of attention as novel prognostic markers for thyroid carcinoma [5-8]. The V600E somatic mutation of the *BRAF* gene is the most frequent genetic alteration in patients with PTC, and plays a fundamental role in tumorigenesis of various thyroid tumors [1]. Initially it was demonstrated that BRAF-V600E maintained tumor growth in a xenograft tumor model [9]. Since

then, numerous clinical studies have suggested a strong association between BRAF-V600E and poor clinicopathological outcomes of PTC, including aggressive pathological features, increased recurrence, and loss of radioiodine avidity [10]. However, some studies have demonstrated inconsistent results perhaps owing to variations in study design and differences in ethnic groups examined [11, 12].

In thyroid cancer, *RAS* mutations are second in prevalence to *BRAF* mutations. *RAS* mutations seem to preferentially activate the PI3K-AKT pathway in thyroid tumorigenesis, as suggested by the preferential association of *RAS* mutations with AKT phosphorylation in thyroid cancers [13]. Activated RAS may have a role in early follicular thyroid cell tumorigenesis and RAS mutations are a common occurrence in follicular thyroid adenoma (FTA) [1]. However, there are limited studies that focus on RAS mutations in papillary thyroid cancer [13-15].

RET-PTC is the best example of gene translocation resulting in oncogenic rearrangements [1]. *RET* is a proto-oncogene encoding an RTK. *RET-PTC* occurs as a consequence of genetic recombination between the 3' tyrosine kinase portion of *RET* and the 5'portion of a partner gene. Sapio et al. found a correlation between the presence of *RET-PTC* and a high growth rate of benign thyroid tumors [16]. However, the role of *RET-PTC* in early thyroid tumorigenesis remains unclear.

Currently, there is still controversy over the association between *BRAF* and *RAS* mutations, and *RET* rearrangements with the poor clinicopathological features of PTC. Furthermore, previous work often studied these gene mutations in PTCs separately. In our study, we attempted to analyze the relationship between these three gene mutations, together, with the clinicopathological characteristics of PTCs in the Han population to demonstrate the association between somatic mutation and clinicopathological features.

Materials and methods

The department of pathology at Union Hospital began to perform routine $BRAF^{V600E}$ testing of PTC tumor specimens larger than 0.4 cm in July 2014. We identified 64 patients as having had *BRAF* testing of thyroid specimens. We included all patients with PTC who underwent total thyroidectomy with routine central lymph node dissection between July 2014 and July 2015. This retrospective study was approved by the ethics committees of the Union Hospital.

Medical records of all patients were reviewed for *BRAF*, *RAS*, and *RET* status, age, sex, tumor size, extrathyroidal extension, infiltration, distant metastasis, multicentricity, accompanying disease (Hashimoto's, simple goiter), lymph node status, and final TNM stage.

BRAF testing

DNA was extracted from each sample using a commercial kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A portion of *BRAF* exon 15 encompassing codon 600 was amplified using polymerase chain reaction (PCR) with specific primers, and amplification of codon 600 was analyzed with fluorescence-labeled hybridization probes in a real-time LightCycler 480 PCR (Applied Biosystems) melting curve assay A melting temperature of approximately 65°C corresponded with the wild-type sequence, while melting at approximately 60°C indicates the T to A transversion at nucleotide 1799 that results in the V600E mutation. This assay was validated to have sensitivity for V600E mutation detection down to a minimum of at least 25% tumor cells in the specimen.

RAS testing

Mutational analysis of *RAS (HRAS, KRAS,* and *NRAS)* in codons 12, 13, and 61, respectively, was performed using a loop-hybrid mobility shift assay (LH-MSA). DNA was extracted from thinly-sliced formalin-fixed and paraffin-embedded tumor samples using a Pinpoint slide DNA isolation system. Briefly, the deparaffinized tissues were digested with proteinase-K followed by heat inactivation at 95°C for 10 minutes, and directly subjected to polymerase chain reaction (PCR) with primers designed to amplify genomic DNA, including the site to be examined. At the end of 45 PCR cycles, the 72-merloop-hybrid generator, specific to each mutation, was added to the reaction mixture at a final concentration of 500 nM and processed using heat-denaturation followed by an annealing step to generate loop-hybrids. The final PCR products were separated on native 10% poly-

Table 1. Clinicopathologic characteristics of the 64 patients with papillary thyroid carcinoma in the study population

Characteristic	No. (%)
Age at diagnosis, y	
<45	39 (60.9%)
≥45	25 (39.1%)
Sex	
Female	42 (65.5%)
Male	22 (34.4%)
Tumor size, cm	
≤1	45 (70.3%)
>2	19 (29.7%)
Histologic subtype	
Classic type	38 (59.4%)
Follicular variant or other	26 (40.6%)
Multifocality	
Absent	30 (46.9%)
Present	34 (53.1%)
Extrathyroidal extension	
Absent	19 (29.7%)
Present	45 (70.3%)
Lymph node involvement	
Absent	34 (53.1%)
Present	30 (46.9%)
Infiltration	
Absent	53 (82.8%)
Present	11 (17.2%)
Distant metastasis	
Absent	57 (89.1%)
Present	7 (10.9%)
TNM stage[a]	
I-II	49 (76.6%)
III-IV	15 (23.4%)
Accompanying nodular goiter	
Absent	52 (81.3%)
Present	12 (18.3%)
Accompanying Hashimoto	
Absent	49 (76.6%)
Present	15 (23.4%)

[a]Based on criteria established by American Joint Cancer Committee-Union. Internationale Contre le Cancer, Seventh Edition, Staging System.

acrylamide gels (ATTO, Inc.). After electrophoresis, the gels were stained with SYBRGreen I (Cambrex Bio Science), and the DNA bands were detected using a Storm 860 laser-scanning imager (GE Health Care BioScience). The gene mutations were identified as mobility-shifted loop-hybrid bands. Each LH-G is a single-strand oligonucleotide with an artificial internal 10-nucleotide deletion that generates a small loop in the hybridized complementary strand adjacent to the mutated nucleotides. The PCR products found to contain mutated loop-hybrid bands, as determined by LH-MSA, were further confirmed by direct sequencing with the 3130 Genetic Analyzer (Applied Biosystems/Life Technologies).

Table 2. Mutation status of the 64 patients with papillary thyroid carcinoma in the study population

Mutation types	No. (%)
BRAF mutation	
Absent	31 (48.4%)
Present	33 (51.6%)
RAS mutation	
Absent	62 (96.9%)
Present	2 (3.1%)
RET rearrangements	
Absent	55 (85.9%)
Present	9 (14.1%)

Table 3. Details about the three gene mutations

BRAF mutation	RAS mutation	RET rearrangements	Number, %
+	+	+	1 (1.6%)
+	+	-	1 (1.6%)
+	-	+	1 (1.6%)
-	+	+	0 (0%)
+	-	-	30 (46.9%)
-	+	-	0 (0%)
-	-	+	7 (10.9%)
-	-	-	24 (37.5%)

Detection of RET/PTC rearrangements

Total RNA was extracted from the tumor specimens using the guanidine thiocyanate-phenol-chloroform method. The integrity of the RNA was verified by denaturing gel electrophoresis. Total RNA was reverse-transcribed into cDNA using the Promega reverse transcription (RT) system (Promega, Madison, WI). Nested RT-PCR was used to amplify transcripts of *RET/PTC* rearrangements. The resulting PCR products were analyzed by gel electrophoresis and directly sequenced with the 3130 Genetic

Table 4. Clinicopathologic features of papillary thyroid carcinoma with and without the BRAF V600E mutation, RAS mutation, RET rearrangements

	BRAF mutation, No. (%)			RAS mutation, No. (%)			RET rearrangements		
Characteristics	Negative	Positive	*P* value	Negative	Positive	*P* value	Negative	Positive	*P* value
Age at diagnosis, y									
≤45	24	15	0.011	38	1	1.00	30	9	0.009
>45	7	18		24	1		25	0	
Sex									
Female	22	20	0.438	40	2	0.542	35	7	0.707
Male	9	13		22	0		20	2	
Tumor size, cm									
≤1	21	24	0.786	43	5	1.000	39	6	1.000
>1	10	9		19	0		16	3	
Histologic subtype									
Classic type	19	19	0.803	36	2	0.510	31	7	0.291
Follicular variant or other	12	14		26	0		24	2	
Multifocality									
Absent	16	14	0.462	29	1	1.000	24	6	0.285
Present	15	19		33	1		31	3	
Extrathyroidal extension									
Absent	13	6	0.013	19	0	1.000	18	1	0.260
Present	18	27		43	2		37	8	
Lymph node involvement									
Absent	19	15	0.222	33	1	1.000	32	2	0.071
Present	12	18		29	1		23	7	
Infiltration									
Absent	28	25	0.186	52	1	0.316	47	6	0.177
Present	3	8		10	1		8	3	
Distant metastasis									
Absent	28	29	1.000	55	2	1.000	50	7	0.253
Present	3	4		7	0		5	2	
TNM stage[a]									
I-II	23	26	0.665	47	2	1.000	42	7	1.000
III-IV	8	7		15	0		13	2	
Accompanying nodular goiter									
Absent	25	27	0.904	51	1	0.342	44	8	0.679
Present	6	6		11	1		11	1	
Accompanying Hashimoto									
Absent	22	27	0.382	47	2	1.000	43	6	0.427
Present	9	6		15	0		12	3	

[a]Based on criteria established by American Joint Cancer Committee-Union. Internationale Contre le Cancer, Seventh Edition, Staging System.

Analyzer (Applied Biosystems/Life Technologies). GAPDH cDNA was used as an internal control for RNA quality.

Statistical analysis

Continuous variables were presented as mean ± standard deviation (SD); discrete variables were reported as a proportion and analyzed by the chi-square test or Fisher's exact test where appropriate.

Variables associated with *BRAF*^{V600E} mutation at P<.10 were used in a multivariate logistic regression model for *BRAF* mutation positivity. All statistical analyses were performed using

Table 5. Multivariate analysis of the association of BRAF V600E mutation and clinicopathologic features of papillary thyroid cancer

Characteristic	Odds Ratio (95% CI)	*P* value
Age		
Male	0.183 (0.050-0.669)	0.010
Female	1 [Reference]	
Extrathyroidal extension		
Present	0.208 (0.063-0.683)	0.010
Absent	1 [Reference]	

SPSS software, version 13.0 (SPSS, Chicago, IL). All *p* values were 2-tailed, and P<.05 was considered statistically significant.

Results

Distribution of BRAF mutation, RAS mutation and RET rearrangements

A total of 64 patients with PTC from our institution met the inclusion criteria (**Table 1**). A total of 33 (51.6%) were positive for the *BRAF* mutation, and 31 (48.4%) tested negative. Only 2 (3.1%) of the patients were positive for *RAS* mutation, and 62 (96.9%) tested negative. There were 9 (14.1%) cases that tested positive for *RET* rearrangement, while most of the cases (85.9%) were negative (**Table 2**).

When we listed the details of these three gene mutations, we found that the $BRAF^{+}RAS^{-}RET^{-}$ group occupied the highest proportion at 30%. The $BRAF^{-}RAS^{-}RET^{-}$ group was about 24%, while the other subgroup was 0%-7% (**Table 3**).

Demographic and tumor characteristics

A significantly higher proportion of elderly patients (>45 years) was found to be *BRAF* mutation positive (P=0.011). A similar outcome was see for the association between *BRAF* mutation and extrathyroidal extension (P=0.013) (**Table 4**), and these associations remained significant in multivariate analysis (both P=0.010) (**Table 5**). However, there was no significant association between *BRAF* mutation and sex, tumor size, histological subtype, multifocality, accompanying nodular goiter or Hashimoto's. On the other hand, none of the characteristics of PTC were found to be associated with *RAS* mutation. Furthermore, patients ≤45 years old were found to have more frequent *RET* rearrangements than in the patients >45 years old.

Lymph node features and metastasis

Lymph node metastasis, infiltration, distant metastasis, and TNM stage were not significantly associated with the *BRAF* mutation-positive group or *BRAF* mutation-negative group. Moreover, in our study, these tumor characteristics were not significantly associated with *RAS* mutation or *RET* rearrangements (**Table 4**).

Discussion

The BRAF-V600E mutant occurs in approximately 45% of PTCs [1]. The significance of *BRAF* and *RAS* mutations, and RET rearrangements in the management of PTC remains unclear and controversial [7, 8, 11, 12, 17, 18]. As a result of these ambiguous results, further research on the presence of genetic mutations in papillary thyroid cancer with respect to different ethnicities and regions is necessary.

The BRAF-V600E mutation causes its constitutive activation as a serine/threonine kinase. $BRAF^{V600E}$-transgenic mice were observed to developed aggressive papillary thyroid cancer [19], and a large meta-analysis uniformly support the aggressive role of the BRAF-V600E mutation in PTC [20]. However, Henke et al. illustrated that *BRAF* mutation was not a predictive factor of long-term outcome such as recurrence and survival in papillary thyroid carcinoma [17]. Additionally, in different ethnicities, $BRAF^{V600E}$ analysis presented different outcomes; Liu et al. reported that there was no correlation between $BRAF^{V600E}$ and any clinicopathological characteristics of papillary thyroid carcinoma in Taiwan [11]. Our findings draw a similar conclusion that *BRAF* mutation was not strongly correlated with the clinicopathological characteristics of papillary thyroid carcinoma.

RAS in its active state is bound with GTP. There are three isoforms of RAS: HRAS, KRAS and NRAS, and the predominantly mutated form in thyroid nodules were NRAS, which mainly involves codons 12 and 61. The intrinsic GTPase terminates RAS signaling by hydrolyzing GTP and converting RAS into an inactive GDP-bound state. RAS mutation can lead to the loss of its GTPase activity so that RAS remains

in its active GTP-bound state. RAS mutations have previously been reported in follicular thyroid carcinoma or follicular thyroid adenoma [21]. However, Frattini et al. did not detect any RAS in a series of 60 PTCs [14].

In this study, *RAS* mutation was detected in a low proportion (3.1%) of PTCs which is similar to Frattini's findings. The only significant finding was the extrathyroid extension and a younger age correlated with positivity for *BRAF* mutation. Therefore, using *RAS* mutation status to predict poorer clinical features of PTC should be cautiously considered.

The *RET* gene, which is expressed preferentially in neuroendocrine tissues such as parafollicular C cells in adults, is mapped to 10q11.2 and organized in 21 exons. PTCs that displayed oncogenic rearrangements of *RET* were detected in up to 35% of multicenter studies and 65% of post-Chernobyl tumors had *RET* rearrangements [22]. However, studies on the genotype-phenotype of *RET* rearrangements reported ambiguous results. Previously, *RET* rearrangements have been reported as an adjuvant prognostic marker useful for risk stratification of patients with medullary thyroid carcinoma [18]. While in PTC, patients with RET/PTC chimeras had no statistically significant tendency towards clinical features such as lower recurrence rate or improved survival. In this study, only age appeared to be associated with *RET* rearrangements. After the radioactive fallout in Chernobyl, childhood thyroid tumors in Belarus were frequency observed to be positive for *RET* rearrangements. Investigators generally presumed that the thyroid gland was prone to radiation-induced breaks of double-stranded DNA [23]. Therefore, even in the Han population, a history of radiation exposure may still be a significant factor to predict the risk of papillary thyroid cancer.

Sugg et al. demonstrated that RET/PTC rearrangements in young patients (<45 yr of age) with small thyroid carcinomas demonstrated a predisposition for lymphatic involvement, suggesting a possible role in the development of this subgroup of tumors [24]. In this study, we found that RET/PTC rearrangements were present in a high proportion of patients with PTCs who were less than or equal to 45 years old which was similar to Sugg's findings. Further work focusing on *RET* rearrangements in younger patients in larger population with PTC should be performed in the future to verify this observation.

Some limitations, however, must be considered for our work. First, our research was a retrospective analysis where samples were received from a single center. Secondly, the number of patients with benign thyroid nodules was relatively small. In addition, long-term outcome such as recurrence and survival should still be collected for further analysis. Therefore, multicentre research, long-term follow-up, and prospective research are required to establish that these three gene mutations value for PTCs.

Conclusion

In conclusion, we demonstrated that BRAF-V600E slightly correlated with extrathyroid extension in PTC in the Han population. Furthermore, neither *RAS* mutation nor *RET* rearrangements was found out to be associated with any clinicopathological characteristics of PTCs. Our data provides useful information on somatic mutation that can aid in predicting the risk of papillary thyroid carcinoma among different ethnicities.

Disclosure of conflict of interest

None.

Address correspondence to: Tao Huang and Chunping Liu, Department of Breast and Thyroid Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. E-mail: huangtaowh@163.com (TH); lcp191@163.com (CPL)

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