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The Relationship of the BRAF^{V600E} Mutation and the Established Prognostic Factors in Papillary Thyroid Carcinomas

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Abstract It has been shown that BRAF^{V600E} mutation in papillary thyroid carcinomas (PTC) is associated both with pathogenesis and poor prognosis. In this study, we aimed to investigate the relationship of the BRAF^{V600E} mutation and the established prognostic factors in a cohort of Turkish patients with PTC. Forty-six cases of papillary thyroid carcinoma have been evaluated for the presence of BRAF^{V600E} mutation. BRAF^{V600E} has been examined by restriction fragment length polymorphism. BRAF^{V600E} mutation status has been compared with well-known histopathological and clinical prognostic parameters such as invasion of thyroid capsule, extrathyroidal extension, and the presence of lymph node and/or distant metastasis. We have found that BRAF^{V600E} mutation was present in the majority of our cases (40/46). Considering the stage of the disease, five of the negative cases were in stage 1 while the remaining one was in stage 2. Only one BRAF^{V600E} negative case has shown extrathyroidal extension and lymph node metastasis. All four patients with distant metastasis had BRAF^{V600E} mutation. Statistical analyses revealed that there are no significant relationship between the BRAF^{V600E} mutation and the established prognostic factors. We found a relatively higher BRAF^{V600E} mutation rate in classical type PTC than in other similar studies. We think that the limited number of our cases may either weaken or mask some potentially important relationship between BRAF^{V600E} mutation and the established prognostic factors.

Keywords Papillary thyroid carcinoma · BRAF^{V600E} · Prognosis · Diagnosis

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Introduction

Papillary thyroid carcinoma (PTC) is the most common endocrine organ malignancy [1]. Some mutations have been shown in one of the signaling pathways such as RET/PTC, RAS, or B-type Raf kinase (BRAF), that can create a mitogenic stimulus in most of these cancers [2]. The most common mutation in these pathways is the T1799A change in the BRAF gene that converts the V (valine) amino acid at position 600 to the E (glutamic acid) amino acid. The BRAF gene is normally on chromosome 7q24 and codes a serine–threonine kinase [3]. The BRAF oncogene is a strong activator of the mitogen-activated protein (MAP) kinase pathway [4]. Once activated by RAS, BRAF phosphorylation causes a series of events in the MAP kinase cascade. The mutant BRAF protein, which is developed as a result of the BRAF^{V600E} mutation, continuously activates the MAP kinase pathway.

This mutation has been reported at various rates (43–88 %) in PTC [5]. There are some reports in the literature mentioning a relationship between the BRAF^{V600E} mutation and an unfavorable prognosis [6]. There are also many studies reporting that, BRAF^{V600E} mutation detection can increase diagnostic accuracy of smears obtained by fine-

needle aspiration biopsy, which can also help the surgical approach to thyroid nodules [7]. A few ongoing studies aim targeted treatment by inhibiting the mutant BRAF oncoprotein [8]. However, the benefits of agents aiming to inhibit the BRAF oncoprotein are not clear yet.

Despite all these data, the presence of some tumors without any mutation which show aggressive biological features sheds doubt on the $BRAF^{V600E}$ mutation as a prognostic marker [9]. We investigated the incidence of the $BRAF^{V600E}$ mutation in PTC in this study. We also compared the mutational state with the well-known histopathological prognostic factors.

Materials and Methods

Patients and Samples

A total of 90 PTC cases diagnosed at the Gülhane Military Medical Academy and School of Medicine, Department of Pathology between the years of 1995 and 2010 were re-evaluated in this study. Forty-six of them which we could access the clinical data as well as the paraffin blocks were included. All cases were classical type PTC. The hematoxylin–eosin-stained preparations of the cases were retrieved from the archive and the most suitable blocks for DNA isolation were selected. The presence of the $BRAF^{V600E}$ mutation was then evaluated following genomic DNA isolation using the "restriction fragment length polymorphism (RFLP)" method.

DNA Extraction

DNA extraction was made in FAVOR (FMF Arthritis Vasculitis and Orphan Diseases Research) laboratories. Paraffin blocks were used for DNA extraction. Eight-micrometer-thick sections were obtained from these blocks. The tumor-containing areas of the sections were scraped into a 1.5-ml centrifuge tube from the slide. A DNA isolation kit which is developed for tissues embedded in paraffin was used for the isolation (PureLink™ Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA, USA). Extractions were realized according to the steps in the data-sheet provided by the manufacturer. Eluted samples were controlled for DNA content and the degree of purification spectrophotometrically by measuring the intensity of absorbance of the solution at wavelengths 260 and 280 nm.

PCR-RFLP

We amplified a 237 base pair (bp) length fragment in exon 15 of BRAF gene by polymerase chain reaction (PCR) using following primers: forward, 5′-GCTTGCTCTGATAGGAAAATGAG-3′; reverse, 5′-GATAGACAGCAGCATCTCAGG-3′. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 20 s and annealing at 56 °C for 20 s, and elongation at 72 °C for 20 s and final extension at 72 °C for 10 min. PCR products were restricted with endonuclease TspRI. Wild-type pattern must have three bands, 177, 87, and 33 bp, while the mutant pattern should yield only with two bands: 204 and 33 bp (Fig. 1).

Statistical Analysis

Data were represented as number of observations (n). Chi-square test was used when comparing relations between groups. All statistical analyses were performed with SPSS for Windows, version 11.0 (SPSS Inc., Chicago, IL).

Results

A total of 46 cases were evaluated for the presence of $BRAF^{V600E}$ mutation. Forty cases were positive for the $BRAF^{V600E}$ mutation and the remaining six were negative. The gender distribution of the 46 cases was 34 females and 12 males. $BRAF^{V600E}$ mutation was found in 29 of 34 females and 11 of 12 males (Table 1). No statistically significant relationship was found between $BRAF^{V600E}$ mutation and gender ($p=1.00$).

Invasion of thyroid capsule was present in half of the cases. $BRAF^{V600E}$ mutation was not present in five of 23 cases without organ capsule invasion. Only one of the 23

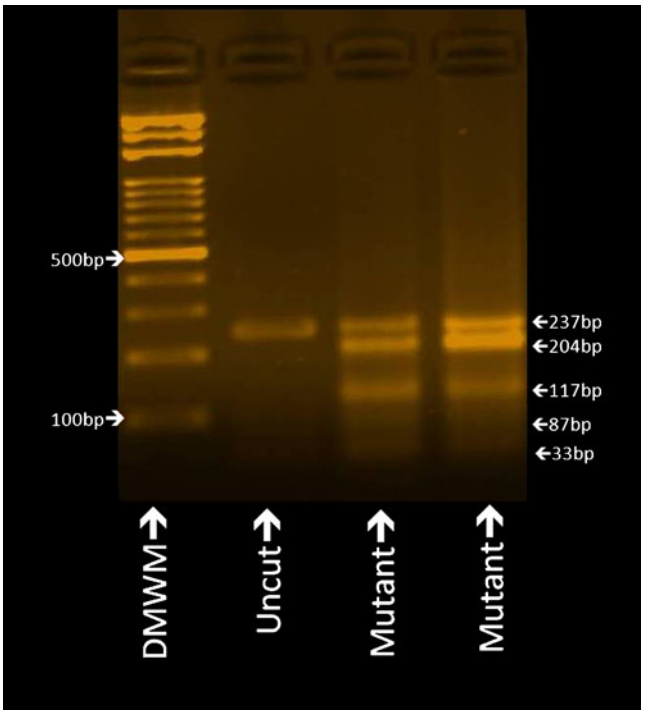

Fig. 1 Electrogel imaging of two $BRAF^{V600E}$ positive cases

Table 1 Clinicopathologic features and status of $BRAF^{V600E}$ mutation in our series

No	Age	Sex	Capsul invasion	Extrathyroidal extension	T	N	M	Stage	$BRAF^{V600E}$ mutation
1	32	F	−	−	T1	N0	M0	1	+
2	24	F	−	−	T1	N0	M0	1	+
3	70	F	−	−	T1	N0	M0	1	+
4	48	F	−	−	T1	N0	M0	1	+
5	24	M	−	−	T1	N0	M0	1	+
6	26	F	−	−	T1	N0	M0	1	+
7	44	F	−	−	T1	N0	M0	1	−
8	47	F	−	−	T2	N0	M0	2	−
9	51	F	−	−	T2	N0	M0	2	+
10	58	F	−	−	T2	N0	M0	2	+
11	14	F	−	−	T2	N0	M0	1	−
12	26	F	−	−	T2	N0	M0	1	+
13	24	F	−	−	T2	N0	M0	1	+
14	30	M	+	−	T2	N0	M0	1	+
15	54	F	+	+	T3	N1a	M0	3	+
16	51	F	+	+	T3	N1a	M0	3	+
17	46	F	+	−	T1	N1a	M0	3	+
18	51	F	+	+	T3	N0	M0	3	+
19	58	F	−	+	T3	N0	M0	3	+
20	58	F	−	−	T1	N1a	M0	3	+
21	53	M	+	−	T2	N0	M1	4b	+
22	55	M	+	−	T2	N1b	M0	4a	+
23	70	F	−	−	T2	N0	M1	4b	+
24	53	F	+	−	T1	N1b	M1	4b	+
25	59	M	+	+	T4a	N1b	M0	4a	+
26	58	F	+	+	T4a	N0	M0	4a	+
27	32	F	−	−	T1	N0	M0	1	+
28	42	F	−	−	T1	N1b	M1	2	+
29	20	M	+	+	T4a	N1b	M0	1	+
30	68	M	−	−	T1	N1b	M0	4a	+
31	24	F	+	+	T3	N1a	M0	1	+
32	41	F	+	+	T3	N1b	M0	1	−
33	25	M	+	+	T3	N1a	M0	1	+
34	50	F	+	+	T3	N1b	M0	4a	+
35	51	F	+	−	T1	N1a	M0	3	+
36	53	F	−	−	T1	N0	M0	1	−
37	43	F	−	−	T1	N0	M0	1	+
38	70	F	+	−	T2	N0	M0	2	+
39	58	M	+	−	T2	N1b	M0	4a	+
40	52	M	−	−	T1	N0	M0	1	−
41	22	F	+	−	T1	N1b	M0	1	+
42	48	F	+	−	T2	N0	M0	2	+
43	66	M	+	−	T1	N0	M0	1	+
44	48	F	+	−	T2	N0	M0	2	+
45	51	M	−	−	T2	N0	M0	2	+
46	50	F	+	+	T3	N0	M0	3	+

F Female, *M* Male, + positive, − negative

cases with thyroid capsule invasion was negative for the $BRAF^{V600E}$ mutation. However, we did not find a statistically significant relationship between the $BRAF^{V600E}$ mutation and the presence of thyroid capsule invasion (p=0.187).

Extrathyroidal extension was present in 12 cases. The $BRAF^{V600E}$ mutation was absent in 1 of these 12 cases whereas 5 of the remaining 34 cases did not have the mutation. This difference was not statistically significant, too (p=1.00).

The pT distribution was T1 in 19, T2 in 15, T3 in 9, and T4 in 3 cases. $BRAF^{V600E}$ mutation was not present in three T1 cases, two T2 cases and one T3 case. All T4 cases were positive for the $BRAF^{V600E}$ mutation. Lymph node metastases were present in 17 cases. Only one case with lymph node metastases did not have the $BRAF^{V600E}$ mutation while 5 of the 29 cases with no lymph node metastases were negative for the mutation. Distant metastases were present in 4 of the 46 cases and all were positive for the mutation. There was no statistically significant relationship between any of these three abovementioned parameters and the $BRAF^{V600E}$ mutation (p value for T, 0.895; p value for N, 0.390; and p value for M, 1.00).

The stage distribution was as follows: Stage 1 in 21, stage 2 in 8, stage 3 in 8, and stage 4 in 9 cases. Among the six $BRAF^{V600E}$ mutation negative cases, five were in stage 1, and one was in stage 2. All stage 3 and stage 4 cases were positive for the $BRAF^{V600E}$ mutation. However, this distribution was not statistically significant (p=0.195).

Discussion and Conclusion

We have evaluated 46 PTC cases for the $BRAF^{V600E}$ mutation in this study and compared the results with the well-known histopathological prognostic factors. Although we found no statistical significance, the six cases without $BRAF^{V600E}$ mutations seemed having favorable prognostic features. There are only a few studies in the literature concerning the relationship between $BRAF^{V600E}$ mutation and the prognosis [10, 11], nodal recurrence [12], biological behavior [13], and treatment [14] in PTC cases.

The $BRAF^{V600E}$ mutation is generally thought to be associated with an unfavorable prognosis. A meta-analysis by Kim et al. [15] has reviewed 5,655 PTC patients in a total of 26 studies. The mean mutation rate was 49.4 %. Of these 26 studies, 18 were about the relationship between the $BRAF^{V600E}$ mutation and lymph node metastasis, 22 on the relationship with stage, and 9 on the relationship with recurrence and therapy-resistant disease. This meta-analysis has shown that PTC patients with the $BRAF^{V600E}$ mutation have 1.5 to 2.1 times more risk regarding resistant disease, recurrence, extrathyroidal extension and lymph node metastasis compared to those without the mutation.

There are, however, some conflicting articles which report no meaningful relationship between the $BRAF^{V600E}$ mutation and established prognostic parameters. A study from Korea in 2005 [9] has reported no relationship between the $BRAF^{V600E}$ mutation and age, gender, extrathyroidal extension, multifocality, and lymph node metastasis. Another study from Japan has also reported no relationship between the prognosis and the $BRAF^{V600E}$ mutation.

All the PTC cases without the $BRAF^{V600E}$ mutation in our study had favorable prognostic features. Only one of the six cases which were negative for the mutation had invasion of thyroid capsule and extrathyroidal extension. We also found that all except one of the cases with lymph node metastasis and all cases with distant metastases were positive for the mutation. We think that the relatively limited number of our cases may either weaken or mask some potentially important relationship between $BRAF^{V600E}$ mutation and the established prognostic factors.

The $BRAF^{V600E}$ mutation was present in 86.9 % of our cases. This value is higher than other series studying this mutation in PTC cases [14-16]. However, there are also other studies reporting higher rates in PTC. Martur et al. [5] have reported in 2011 that the detected $BRAF^{V600E}$ mutation in thyroid cancers has increased over the years. They evaluated a total of 628 PTC cases seen between 1991 and 2005. The cases were grouped according to their year as 1991–1995 (group 1), 1996–2000 (group 2), and 2001–2005 (group 3). There was no difference between the groups for age, gender, ethnic origin, tumor size, extrathyroidal invasion, and stage. However, the $BRAF^{V600E}$ mutation was present at a rate of 51 % in group 1, 43 % in group 2, and 88 % in group 3. Most of our cases were seen after the year 2000. This was interpreted as the $BRAF^{V600E}$ mutation contributing more to carcinogenesis in PTC cases compared to the past.

The development of molecular methods has led to increasing research on targeted treatment for malignancies [17]. There are several studies on the association between the $BRAF^{V600E}$ mutation and targeted treatment. Solit et al. [18] have shown in 2006 that tumors containing the $BRAF^{V600E}$ mutation are dependent on the RAF-MEK-ERK growth pathway. Later studies [2] have revealed a cytostatic effect of MEK inhibitors on PTC cell lines. Salerno et al. [8] have studied targeted treatment in PTC cases with the $BRAF^{V600E}$ mutation in 2009. They tried to inhibit the mutant BRAF oncoprotein using the PLX4032 agent. This agent binds to the mutant BRAF ten times more specifically than the wild-type BRAF. It inhibits the MEK-ERK kinase pathways at increased concentrations. It has also been shown that it did not inhibit other kinases under in vivo conditions [13]. However, the contribution of RAF inhibition to treatment is currently a matter of debate.

Sensitivity of methods detecting point mutations differs. To our knowledge, there is no study comparing of sensitivity of methods for detection of $BRAF^{V600E}$ mutation in thyroid cancer. However, $BRAF^{V600E}$ is a point mutation, and Lanthaler et al. has compared methods including real-time PCR and RFLP for their sensitivity in detection of K-RAS mutations, which are also point mutations [19]. In this study, 14 of 31 colorectal cancer samples are positive for K-RAS mutations. Comparison has been made in two different scenarios. In the first one, tumor tissue was up to 30 % of the observed area of section, and in second one, tumor tissue was at least 70 % of sectioned tissues. In the first scenario, real-time PCR did better than RFLP in detecting any given point mutation, but in the second condition results were practically the same. Since we have used tissues which are composed of completely neoplastic in origin, we think that our results with RFLP are reliable enough.

Thyroid fine-needle aspiration is quite frequently used for the diagnosis of thyroid nodules. It has great diagnostic accuracy and contributes much to the therapeutic approach to the nodule. However, it is unable to differentiate between benign and malignant in 10–15 % of cases and these patients end up in surgery to avoid risk. Most of these cases are benign and surgery is unnecessary. Jo et al. [7] have performed fine-needle aspiration accompanied by US in 101 thyroid nodules and reported the results as 43 benign, 30 malignant, 24 suspect, and 4 nondiagnostic cases. The $BRAF^{V600E}$ mutation was then studied in all cases. They found the mutation in 22 malignant and also in 7 suspects and 1 nondiagnostic case. These 8 cases were resected in addition to the malignant ones and all cases were found to have PTC. Similarly, there are many studies showing greater diagnostic accuracy of fine-needle aspiration when used together with $BRAF^{V600E}$ mutation studies [20, 21].

The current guidelines state the treatment of choice for PTC lesions over 1 cm as total or almost total thyroidectomy [22]. We know that some non-neoplastic nodules are categorically evaluated as “suspected for malignancy” in fine-needle aspiration cytology (FNAC). Therefore, unnecessary thyroidectomies are still being performed in certain patients. Detection of $BRAF^{V600E}$ mutation in FNAC samples may reduce the need of surgery. Adrienne et al. [22] have investigated in 2001 whether the BRAF status could change the first surgical intervention. They evaluated 44 $BRAF^{V600E}$ positive PTC cases in the preoperative stage and found 31 to be positive and 13 to be negative. These results indicate that a positive $BRAF^{V600E}$ mutation status in the preoperative stage leads to cancer on the histopathological evaluation, i.e., a 100 % positive predictive value. The authors report that the preoperative BRAF ^{V600E} status influences their surgical approach and that they make use of the patient's mutation status in the management during their clinical work.

In conclusion, we found the $BRAF^{V600E}$ mutation rate as 86.9 % in patients with classical PTC. Although statistical analysis did not reveal any significance, it was noteworthy that $BRAF^{V600E}$ negative cases had favorable histopathological prognostic indicators. We think that the limited number of our cases may either weaken or mask some potentially important relationship between $BRAF^{V600E}$ mutation and the established prognostic factors. Studies in larger series are needed to make more reliable conclusions.

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