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ORIGINAL ARTICLE

$BRAF^{V600E}$ mutation in Turkish patients with papillary thyroid cancer: strong correlation with indicators of tumor aggressiveness

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Abstract Papillary thyroid cancer (PTC) constitutes more than 90 % of the thyroid cancers. MAP kinase/ERK pathway plays an important role in the development of several cancers. BRAF which is a member of Raf-kinase family activates this way. BRAF gene activating mutations lead to neoplastic transformation in thyroid follicle cells. In PTC, this mutation itself is a poor prognostic sign independent of other clinicopathological characteristics. We evaluated $BRAF^{V600E}$ mutation and clinical–pathological characteristics in Turkish population with PTC. We assessed 109 patients with PTC (88 female, 21 male). The average age was 38.7 ± 9.9 (17–71). $BRAF^{V600E}$ mutation was detected using polymerase chain reaction and fluorescent melting curve analysis. The results show that $BRAF^{V600E}$ mutation rate was found in 39.45 % of our patients. We observed that $BRAF^{V600E}$ mutation was significantly higher in men, in tumors larger than 1 cm in size, and in patients with classical PTC. Moreover, statistically significant correlations of $BRAF^{V600E}$ with indicators of tumor aggressiveness such as thyroid capsular invasion, multifocality, lymph node metastasis, and extrathyroidal spread were found. Patient groups below and over the age of 45 did not differ in mutation frequency. Patients with micro-PTC were evaluated separately, it was found that $BRAF^{V600E}$ mutation was more frequent in the classic type and that lymph node metastasis rate significantly increased when the mutation was present. We concluded that $BRAF^{V600E}$ was correlated with indicators of tumor aggressiveness in our study population. This fact is taken into consideration in treatment and follow-up of our patients with PTC and positive $BRAF^{V600E}$ mutation.

Keywords $BRAF^{V600E}$ mutation · Thyroid papillary cancer · Thyroid papillary carcinoma

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Introduction

Thyroid cancers, which arise from follicular epithelial cells, are the most frequent endocrine cancers [1]. Papillary thyroid cancer (PTC) constitutes more than 90 % of the thyroid cancers. A rapid rise in frequency is observed, particularly in micropapillary thyroid cancer (MPTC). Environmental and genetical factors are responsible in etiology and pathogenesis of PTC [1–3]. PTC usually responds to treatment well and has a good prognosis. The relapse rate at follow-up is 20 and 30 % by the 10th and 30th years, respectively [4]. The mortality rates are low in patients receiving standard treatment (surgery and/or radioactive iodine, RAI), but high in patients with inoperable tumors or in tumors lacking iodine

retention capability [5]. In these tumors, different treatment modalities can be used [5, 6]. In addition, long-term follow-up studies revealed a 10-year cause-specific survival rate of 31 % in poorly differentiated thyroid carcinoma patients [7, 8]. The parameters used in assessment of progression, relapse, and morbidity–mortality are age, gender, tumor diameter, extrathyroidal invasion, lymph node metastasis, distant metastasis, stage of the disease, and treatment method administered initially. MAP kinase/ERK pathway plays an important role in the development of several cancers [9]. Primary mutations that cause PTC by creating aberrant activation of this pathway are BRAF (23–83 %), RET/PTC (10–50 %), RAS (1–10 %) [10, 11].

BRAF is a serine-threonine kinase which is a member of Raf-kinase family. It is expressed abundantly in thyroid follicular cells. It activates mitogen extracellular kinase (MEK)1 and MEK2 [12]. Among A, B, C—Raf kinases, the one with the most powerful activation potential is BRAF [13]. BRAF gene activating mutations lead to neoplastic transformation in thyroid follicle cells. After the role of BRAF mutation in the occurrence of cancer was established, more than 40 mutations have been reported in the gene [14]. The most common mutation is "BRAF (V600E)" (as a result of thymine → adenosine transformation at position 1799 at exon 15 → valine–glutamate substitution at residue 600) [15]. This is not a germline mutation, it is a somatic genetic alteration, which is rare in children. $BRAF^{V600E}$ mutation is observed in PTC and anaplastic cancer, not in follicular thyroid cancer [16]. It not only triggers tumorigenesis but also leads to tumor progression [17]. It incites an aggressive character in PTC by suppressing many tumor-suppressor genes, increasing pro-tumor and pro-angiogenetic molecules, reducing differentiation of the tumor and RAI retention capability (by reducing TPO, NIS, thyroglobulin, and pendrine expression) [18–21].

$BRAF^{V600E}$ mutation is associated with extrathyroidal invasion, lymph node metastasis, and advanced tumor stage; all of which are poor prognostic indicators. Besides, this mutation itself is a poor prognostic sign independent of other clinicopathological characteristics [17, 22, 23].

In light of these informations, several investigations focusing on $BRAF^{V600E}$ mutation in PTC were reported in populations of different countries. In this study, we present the initial data on $BRAF^{V600E}$ mutation presence and clinical–pathological characteristics in Turkish population with PTC.

Patients and methods

We assessed 109 patients with PTC diagnosed in our thyroid diseases clinic, between 2009 and 2011. Eighty-eight of them were females and 21 were males. The average age was 38.7 ± 9.9 (17–71). All patients were operated by the same endocrine surgeon. All patients had undergone total thyroidectomy. The patients that pathological lymph node was detected by preoperative ultrasonography and/or it was confirmed as metastasis by fine needle aspiration biopsy underwent to dissection. Tissue samples were studied by the same group of pathologists. We used the UICC/AJCC TNM staging system that it is most widely adopted for tumor staging [24]. $BRAF^{V600E}$ mutation analysis was performed in Genetic Department. A total of 109 formalin-fixed, paraffin-embedded (FFPE) papillary thyroid carcinoma specimens were evaluated in this study. Two 10-μm sections were deparaffinized by immersion in three changes of xylene for 5 min each. The tissue BRAF mutations were detected using sections were then hydrated in a graded series of ethanol, followed by immersion in dH_2O for 1 min. The slides were allowed to air-dry completely. Sample was treated with proteinase K (3 mg/ml) in digestion solution (50 mM Tris, 1 mM EDTA, pH 8.0, 1 % Tween 20) overnight. For genomic DNA preparation, the QIAamp DNA Mini Kit was used following the manufacturer's instructions. BRAF V600E mutation was detected using polymerase chain reaction and fluorescent melting curve analysis as described by Rowe et al. [25]. In brief, an amplicon of 250 bp in length was generated using a PCR forward primer, 5′-CTCTTCATAATGCTTGCTCTGATAGG-3′, and a reverse primer, 5′-TAGTAACTCAGCAGCATCTCAGG-3′. A sensor probe, 5′-AGCTACAGTGAAATCTCGATGGAG-Fluoroscein-3′, and an anchor probe, 5′-LCRed640-GGTCCCATCAGTTTGAACAGTTGTCTGGA-Phosphate-3′, were used to perform melting curve analysis. PCR was carried out in glass capillaries, in a total volume of 10 μl, containing 10 ng of genomic DNA, 1 μl of 10× LightCycler DNA Master Hybridization Probes (Roche Molecular Biochemicals, Mannheim), 0.8 μl of 25 mM $MgCl_2$, 1 μl (5 μM) forward and reverse primer, and 1 μl (2 μM) anchor and sensor hybridization probes. The reaction mixture underwent 45 cycles of rapid PCR. Post-amplification fluorescent melting curve analysis was performed by gradual heating of the samples at a rate of 0.1 °C/s from 45 to 95 °C. All PCR products that showed deviation from the WT genomic DNA melting peak and benign control samples were confirmed by direct sequencing of exon 15 as described elsewhere [25]. The informed consent was obtained from the patients for the genetic analyses.

Statistical analysis

Data were presented in terms of frequency percentage rate and arithmetic mean. Chi-square and Fisher's exact Chi-square tests were used in order to analyze the categorical

Table 1 $BRAF^{V600E}$ mutation and clinicopathological features in our patients with papillary thyroid cancer

Clinical–pathological features	$BRAF^{V600E}$ *n*	(+) %	$BRAF^{V600E}$ *n*	(−) %	*p* value
Mean tumor size (mm) (min–max)	13.30 ± 6.7	(4–40)	11 ± 9.8	(2–55)	**0.002**
Gender					**0.01**
Female ($n = 88$)	30	34.09	58	65.91	
Male ($n = 21$)	13	61.90	8	38.10	
Age at diagnosis (years)					0.96
<45	33	39.80	50	60.2	
≥45	10	38.50	16	61.5	
Tumor size (cm)					**0.01**
≤1	19	29.70	45	70.3	
>1	24	53.3	21	46.7	
Histological type					**0.02**
Classical	38	45.2	46	54.8	
Sub-type	5	20.0	20	80.0	
Thyroid capsule invasion					**0.04**
Yes	17	53.1	15	46.9	
No	26	33.8	51	66.2	
Lymph node metastasis					**0.02**
Yes	12	63.2	7	36.8	
No	31	34.4	59	65.6	
Multifocality					**0.02**
Yes	23	52.3	21	47.7	
No	20	30.8	45	69.2	
Extrathyroidal invasion					**0.002**
Yes	13	72.2	5	27.8	
No	30	33.0	61	67.0	
Lympocytic thyroiditis					0.26
Yes	18	35.3	33	64.7	
No	25	43.1	33	56.9	

Bold values are statistically significant at $p < 0.05$

variables. Kolmogorov–Smirnov and Shapiro–Wilks tests were used to control the suitability of continuous variables to normal distribution. The variables that were not suitable for normal distribution were checked with Mann–Whitney U test. Logistic regression was performed for variables which were significant in univariate analysis. A *p* value of <0.05 was regarded as being significant.

Results

While 84 patients (77.1 %) had the classical variant of papillary cancer, 25 patients (22.9 %) had been diagnosed with the less frequent histological sub-types [follicular ($n = 20$), tall cell ($n = 2$), oncocytic variant ($n = 3$)]. $BRAF^{V600E}$ mutation rate was found in 39.45 % of patients. The relationship between clinicopathological characteristics and $BRAF^{V600E}$ mutation is shown in Table 1. According to TNM classification, most of our patients were in stage 1 (97 %). In patients with sub-types PTC, 22 were female and 3 were male. Mean age was 38.2 years (range 17–61 years) and mean tumor size was 15 mm (range 10–20 mm). There was no lymph node metastasis in these patients. Besides, other indicators of tumor aggressiveness were not frequent in patiens with sub-types. $BRAF^{V600E}$ mutation was positive as 25 % ($n = 5$) in patients with follicular variant papillary carcinoma. In other patients with sub-types PTC, mutation was negative. We observed that $BRAF^{V600E}$ mutation presence was significantly higher in men, in tumors larger than 1 cm in size, and in patients with classical PTC. Moreover, statistically significant correlations of $BRAF^{V600E}$ with indicators of tumor aggressiveness such as thyroid capsular invasion, multifocality, lymph node metastasis, and extrathyroidal spread were found. There was no relation between $BRAF^{V600E}$ mutation presence and lymphocytic thyroiditis. Patient groups below and over the age of 45 did not differ in mutation frequency. Since increased tumor diameter is associated with extrathyroidal invasion, logistic regression analysis was performed and it was observed that

$BRAF^{V600E}$ mutation was an independent predictor of tumor aggressiveness ($p = 0.009$, OR = 4.6, 95 % CI 1.5–14.4). Likewise, by logistic regression analysis, it was evident that $BRAF^{V600E}$ is associated with higher percentage of lymph node metastasis and multicentricity. In multivariate analysis, strong correlations of $BRAF^{V600E}$ with indicators of tumor aggressiveness were also independent from histological sub-types. When patients with MPTC were evaluated separately, it was found that $BRAF^{V600E}$ mutation was more frequent in the classic type and that lymph node metastasis rate significantly increased when the mutation was present (Table 2). Extrathyroidal invasion and multifocality did not significantly differ between mutated and non-mutated groups. Follow-up duration of our patients was 15.8 ± 7.1 months (median 17 months).

Discussion

High prevalence in PTC suggested that $BRAF^{V600E}$ mutation could play an essential pathogenic role. The frequency of $BRAF^{V600E}$ mutation in different studies ranges from 18 to 87 % [26–30]. Some of them were summarized in Table 3. In our series, the $BRAF^{V600E}$ mutation frequency was found as 39.4 % ($n = 43/109$). While the frequency was high in the classic variant of PTC, no statistically significance with histological sub-type was observed ($p = 0.02$). It is hard to explain the wide frequency range, but one may consider that such difference could not arise from technical conditions, but rather from biological characteristics which may vary depending on the geographic region. In a study that observed the $BRAF^{V600E}$ mutation in patients with PTC ($n = 228$) in three different time zones between the years 1991–1995, 1996–2000, and 2001–2005, mutation rates were determined as 51, 43, and 88 %, respectively. Researchers stated that the high mutation rate in the third time zone could not be ascribed to any clinical variable, and that it could directly be associated with the increasing rate of papillary cancer within the recent years [31]. On the other hand, the study results of Ahn et al. [32] published recently are interesting in terms of reflecting the potential differences among the studied populations. They declared that $BRAF^{V600E}$ mutation rate was high in Korean patients with PTC (79.4 %) and therefore the presence of this mutation would not be of predictive value in PTC. Studies in different patient populations are of utmost importance, since definitive conclusions may only be cumulated from meta-analytic studies. We think that our results can provide guidance to Turkish patients with PTC, because the presence of $BRAF^{V600E}$ mutation in PTCs was significantly correlated with indicators of tumor aggressiveness in our study. In our patients with positive $BRAF^{V600E}$ mutation, thyroid capsular invasion, lymph node metastasis, multifocality, and extrathyroidal invasion of the tumor ($p = 0.04$, 0.02, 0.02, 0.002, respectively) were increased. This relationship was independent of tumor size, although $BRAF^{V600E}$ mutation was more frequent in tumors >1 cm ($p = 0.01$). Univariate and logistic regression analyses clearly revealed that $BRAF^{V600E}$ mutation presence directly correlated with thyroid capsular invasion, lymph node metastasis, multifocality, and extrathyroidal invasion regardless of the size of the tumor. In addition, $BRAF^{V600E}$ positivity in MPTCs was significantly related to lymph node metastasis ($p = 0.04$). The increase in patients can found a strong relationship between MPTCs with other indicators of tumor aggressiveness. Recently, Kim et al. reporting data of a meta-analysis of 27 studies, including 5,655 PTC patients, about the association of the $BRAF^{V600E}$ mutation with prognostic factors and poor clinical outcome in papillary thyroid cancer. This meta-analysis demonstrates that the $BRAF^{V600E}$ mutation is closely related to the high-risk clinicopathological factors and poorer outcome of PTC [33].

Table 2 $BRAF^{V600E}$ mutation and clinicopathological features in our patients with MPTC

Clinicopathological features	$BRAF^{V600E}$ *n*	(+) %	$BRAF^{V600E}$ *n*	(−) %	*p* value
Histological type					**0.01**
Classical	19	36.0	34	64.0	
Sub-type	0	0.0	11	100.0	
Lymph node metastasis					**0.04**
Yes	5	62.5	3	37.5	
No	14	25.0	42	75.0	
Multifocality					0.06
Yes	11	42.3	15	57.7	
No	8	21.1	30	78.9	
Extrathyroidal invasion					0.10
Yes	4	57.1	3	42.9	
No	15	26.3	42	73.7	

Bold values are statistically significant at $p < 0.05$

Table 3 Prevalence of $BRAF^{V600E}$ in some studies

Studies	Country	Number of patients with PTCs	Prevalence of $BRAF^{V600E}$ (%)	Correlation of $BRAF^{V600E}$ with increased PTC aggressiveness
Nikiforova et al. [16]	USA, Italy	320	38	Positive correlation
Xing [17]	USA, Ukraine, Italy	219	49	Positive correlation
Fugazzola et al. [38]	Italy	260	38	No correlation
Lupi et al. [29]	Italy	500	43.8	Positive correlation
Elisei et al. [23]	Italy	102	37.3	Positive correlation
Frasca et al. [34]	Italy	323	38.6	Positive correlation
Ito et al. [30]	Japan	631	38.4	No correlation
Czarniecka et al. [36]	Poland	88	43	No correlation
Ahn et al. [32]	Korea	107	79.4	No correlation
Stanojevic et al. [26]	Serbia	266	31.6	Positive correlation

Similar results were observed by Frasca et al. [34], who demonstrated significant correlations between $BRAF^{V600E}$ positiveness and indicators of tumor aggressiveness. In Kebebew's study [35], advanced age, lymph node metastasis, distant metastasis, advanced stage, recurrent and persistent disease were associated with the presence of mutation. Although most of our patients were women ($n = 88$), $BRAF^{V600E}$ mutation was statistically significant in men ($n = 13/21$, $p = 0.01$). This result was different than some other studies [36–38]. Most of the previous studies stated that mutation frequency increased in parallel with the age [16, 23, 35–38]. But, no correlation was found with age in our patients ($p = 0.96$). In our study, average age was younger than those of other groups. And, the mutation frequency did not increase with advancing age. Moreover, most of our patients had stage 1 disease and similarity of $BRAF^{V600E}$ mutation rate to those studies comprising advanced stage patients supports that this mutation could be responsible for early stages of carcinogenesis. Fugazzola et al. [38] reported a mutation rate of 38 % in 260 patients and significant positive correlation with classic variant and advanced age. In this study, patients were selected from different geographical parts of Italy in order to exclude geographical bias and it was emphasized that the frequency of mutation did not depend on geographical difference, except for a small region in the mid-country. However, a study from the Sicily region of Italy published 2 years later implicated a strong geographical influence on the rate of mutation [34]. In a different study, tumor aggressiveness criteria increased independently of age for the mutated patients, but recurrence risk increased when both advanced age and mutation were present [39]. Since both PTC and $BRAF^{V600E}$ mutation rates increased in parallel in recent years, we believe that further studies from different countries are warranted. Although MPTC is usually considered to have a good prognosis, some recent studies included $BRAF^{V600E}$ mutation as a criterion for tumor aggressiveness [34, 40, 41]. We detected a strong significant relationship with $BRAF^{V600E}$ mutation (29.68 %) and lymph node metastasis in MPTCs. Although there was a higher risk of multicentricity in patients with positive $BRAF^{V600E}$ mutation, the difference was not significant. $BRAF^{V600E}$ mutation probably plays role in microcancer progression and increases the risk of invasive and aggressive behavior. We believe that in the future, approach to MPTC may be modified by follow-up studies in different patient populations. While a successful surgery is deemed adequate for such patients currently, treatment and follow-up modifications may be established for patients with positive $BRAF^{V600E}$ mutation and MPTC. In our study, we had also planned to find out clinical–pathologic characteristics of our patient population and observed no recurrence for a period of median 17 months. However, follow-up is continuing since we care about long-term outcomes and plan to share them in the meantime.

In conclusion, a $BRAF^{V600E}$ mutation rate in our patients (39.45 %) is similar to previously studies. In most of these studies and in our study, patients with $BRAF^{V600E}$ mutation show a major aggressiveness at clinical presentation. This fact is taken into consideration in follow-up of our patients with PTC and positive $BRAF^{V600E}$ mutation. No adequately data about the prognostic value of $BRAF^{V600E}$ mutation are provided yet. However, long-term follow-up will clarify if the mutation implies also a bad prognosis.

Conflict of interest The authors declare that they have no conflict of interest.

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