

Association between BRAF^{V600E} mutation and the clinicopathological features in incidental papillary thyroid microcarcinoma: A single-center study in Turkish patients

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ABSTRACT

OBJECTIVE: In this study, we evaluated the influences of BRAF^{V600E} mutation on clinicopathological features in incidentally found papillary thyroid microcarcinomas (PTMCs).

METHODS: This retrospective cohort study included 72 patients with PTMC who underwent surgery from 2008 to 2012. The mean follow-up of the whole cohort was three years. DNA was isolated using QIAamp DNA formalin-fixed, paraffin-embedded (FFPE) tissue kit. BRAF gene was amplified by the polymerase chain reaction-restriction fragment length (PCR-RFL) polymorphism method with the following primers. The clinicopathologic features (age, gender, histologic subtype, tumor size, presence of tumor capsule, bilaterality, multifocality, extrathyroidal extension (ETE), thyroid capsular invasion, presence of Hashimoto's thyroiditis, lymph node metastasis (LNM) and distant metastasis) were compared between the BRAF (+) and BRAF (-) patient groups.

RESULTS: BRAF^{V600E} mutation was detected in 30 of the 72 patients (41.6%). The presence of the mutation was statistically significantly associated with classic variant ($p=0.046$), invasion of thyroid capsule ($p=0.002$) and absence of tumor capsule ($p=0.003$).

CONCLUSION: Although incidental PTMCs positive for the BRAF^{V600E} mutation had more invasive behavior, the presence of the mutation was not associated with recurrences within three years of follow-up.

Keywords: BRAF^{V600E}; clinicopathological features; incidental; papillary thyroid microcarcinoma.

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Differentiated thyroid carcinomas are the most common endocrine malignancy with good prognosis and a long-term survival [1]. Over the past decades, the incidence of papillary thyroid carcinoma (PTC) has increased worldwide [2]. The recent advances in ultrasonographic imaging and ultrasound-guided fine-needle aspiration biopsy (FNAB) have allowed more PTMCs to be discovered [3]. Approximately 50% of the new PTCs

are PTMCs [4]. PTMC is a type of PTC that is defined by the World Health Organization as having a maximum diameter of 1.0 cm or less [5]. Many studies support that PTMCs are mostly incidental findings in surgeries for other pathologies nonrelated to thyroid malignancy [6, 7]. Incidentally discovered that thyroid cancers are less aggressive compared to clinically apparent thyroid cancers [6, 7]. The clinical significance of PTMC is still unclear.



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PTMC generally has an excellent prognosis and is rarely associated with local or distant metastasis [8–10]. On the other hand, PTMC may progress with a significant recurrence rate and even mortality in some patients [11–13]. A study reported that cancer-related mortality rate was 1.0%, the distant metastasis rate was 2.5%, and the lymph node recurrence rate was 5% in PTMCs [14]. The presence of high-risk clinicopathological factors, including ETE, LNM, and high TNM stages, are classically known to be associated with a poor prognosis [15–18]. There is no consensus to support either a more conservative approach or a more aggressive treatment for this patient group. In recent studies, patients with cytologically-confirmed PTMC were monitored by regular ultrasound and were performed surgery only when the PTMC exhibited progression or spread [19–22]. Their results have shown that >90% of PTMCs did not have progress with time under the non-surgical approach; none of the patients developed distant metastasis or died [19–22].

Many genetic alterations were associated with the development of follicular cell-derived thyroid cancer [1]. The activating mutations in some genes play main role in the pathogenesis of thyroid cancer and cause a malignant transformation in thyroid follicular cells. The B-type Raf kinase (BRAF) is one of the members of the RAF gene family and plays a role in the pathogenesis of the PTC. BRAF gene encodes a protein that is in charge of the mitogen-activated protein kinase (MAPK) signaling pathway and it has a serine/threonine kinase activity [23]. The MAPK signaling pathway is a regulator of numerous cellular processes, including division, proliferation, differentiation, adhesion, migration and apoptosis [24]. The BRAF mutation induces aberrant activation of the MAPK pathway. The T1799A somatic point mutation results in a valine-to-glutamic acid switch at codon 600 (BRAF^{V600E}), which is located on chromosome 7q24 in exon 15. The BRAF activated point mutation is the most common genetic alteration, which has been determined in different series at a rate of 20–52% in PTMCs [25]. In the many recent studies, BRAF mutation is associated with recurrence, poor prognosis, LNM, ETE and aggressiveness in patients with PTC [26–30]. Its prognostic value is unclear in PTMCs. The BRAF mutation-induced molecular changes [(suppressing many tumor-suppressor genes, increasing pro-tumor and pro-angiogenic molecules, reducing differentiation of the tumor, and radioactive iodine (RAI) retention capability)] are likely to be responsible for PTMC aggressiveness [4, 11, 25, 26]. However, another study showed

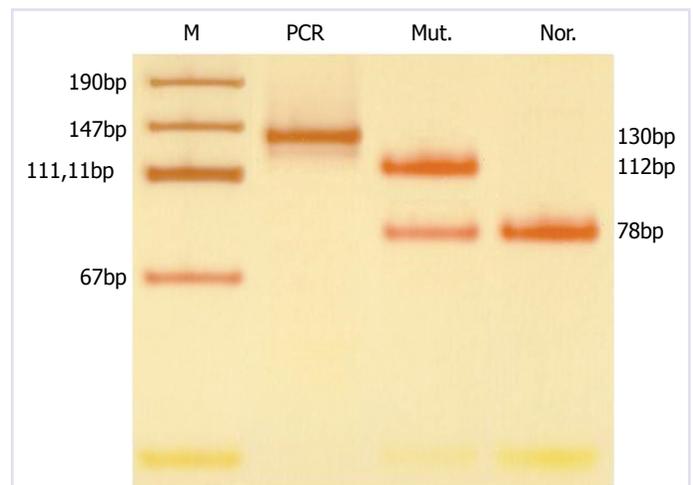


FIGURE 1. Electrodeposition imaging of a BRAF positive patient (Line 3).

M: Marker; Mut.: Mutant; Nor.: Normal; bp: Base pair.

no significant correlation between BRAF mutation and aggressive behavior [31]. American Thyroid Association (ATA) 2015 Guideline included BRAF^{V600E} mutation into the risk group of the recurrence in PTMCs.

Turkey is in a region that is with an increased risk of thyroid cancer. Iodine deficiency and being in close proximity to the Chernobyl accident have increased the incidence of thyroid cancer in Turkey [32]. There are data determining the aggressiveness of PTMCs with BRAF mutation in Turkish patients [28]. One study found a significant association between LNM and BRAF mutation [28], although conflicting findings were reported in another study [33]. Our study aimed to evaluate the influences of BRAF mutation on clinicopathological features in incidental microcarcinomas in a Turkish population.

MATERIALS AND METHODS

All the patients included in this study were diagnosed with PTMC between 2008 and 2012. This study was a retrospective study, and all procedures in this study were approved by our hospital's ethics committee. Follow-up data information was obtained from hospital records. The patients were followed up for at least one year. Seventy-two tumor samples from 72 patients were studied. If more than one PTMC was present, the largest tumor was considered for this study. The tumors were clinically asymptomatic. Thyroidectomy was performed on 26 of 72 patients (36.1%) due to benign thyroid disease and PTMC was diagnosed after thyroidectomy. In the rest of the group, in 46 patients (63.8%), FNAB was performed

according to the suspicious features of the nodules in thyroid ultrasonography and PTMC was diagnosed before surgery. Demographic factors (age and gender), clinicopathological factors, including tumor size, multifocality of the tumor, bilaterality, presence of thyroid capsular invasion, presence of tumor capsule, ETE, presence of Hashimoto's thyroiditis, LNM, distant metastasis and presence of BRAF mutation, were included into a risk analysis of these patients. We reviewed the preoperative thyroid-stimulating hormone (TSH). Tumor pathology in the surgical material was identified with an experienced pathologist. The genomic DNA was extracted from the paraffin-embedded tumor blocks (5 µm thick sections with a confirmed tumor). As for the DNA isolation, QIAamp (Qiagen) DNA FFPE tissue kit was used.

DNA Isolation

The paraffin was removed, 1.25 ml of xylol was added and centrifuged. Xylol was discharged, 1 ml of 100% ethanol was added and centrifuged. The ethanol was discharged, and the tubes were put into the heat block for evaporation of the remaining ethanol. One hundred and eighty µl tissue lysis buffer and 20 µl proteinase K enzyme were added. The tubes were kept at 56 °C and at 90 °C. Two hundred µl lysis buffer and 200 µl of 100% ethanol were added and centrifuged. The samples were kept at +4 °C and centrifuged. Five hundred µl washing buffer 1 was added and centrifuged. Five hundred µl washing buffer 2 was added and centrifuged. The upper filtered part was put into another tube and centrifuged. The upper filter was put into the DNA tubes, 100 µl DNA-protecting buffer was added and centrifuged. The DNA isolation was completed for PCR analysis.

Genotyping

Exon 15 of the BRAF gene was amplified by the polymerase chain reaction restriction fragment length (PCR-RFL) polymorphism method with the following primers. For PCR-RFLP, a 50 µL PCR mixture was composed of extracted DNA. The amplicon size was 130 bp.

Primers for BRAF mutation for the first PCR as follows: sense: 5'-TAAAAATAGGTGATTTTGGTCTAGCTGC-3',

antisense: 5'CCAAAAATTTAATCAGTGGAAAATA-3'. The PCR cycling conditions were as follows; initial denaturation step at 95 °C for 11 min, 30 cycles of: 95 °C 30 s, 58 °C 30 s, 72 °C 30 s and the final elongation for 5 min at 72 °C. The amplified 130 bp long

PCR products were digested at 37 °C for 3 h with BtsI restriction enzyme and separated by 10% PAGE for 20 min. The BRAF^{V600E} mutation had 112 and 18 bp fragments for the AA genotype and 78, 34 and 18 bp for the WT genotype.

Primers for BRAF mutation for the second PCR as follows: sense: F: 5'-TAAAAATAGGTGATTTTGGTCTAGCTGC-3',

antisense: R: 5'AAAAATTTAAGCAGTGGAAAATAAC-3'. The PCR cycling conditions were as follows; initial denaturation step at 95 °C for 11 min, 30 cycles of: 95 °C 30 s, 58 °C 30 s, 72 °C 30 s and the final elongation for 5 min at 72 °C. The amplified 224 bp long PCR products were digested at 65°C overnight with a TspRI restriction enzyme and separated by 8% PAGE for 20 min. Finally, samples were stained with silver nitrate and photographed.

Statistical Evaluation

The statistical analyses were performed using NCSS (Number Cruncher Statistical System) 2007 Statistical Software (Utah, USA) package program. In the evaluation of the data, apart from the descriptive statistical methods (mean, standard deviation), the independent samples T-test was used in comparing pairwise groups, while Chi-Square and Fisher's Exact Test and Odds Ratio were used in evaluating the qualitative data. The results were evaluated at a p<0.05 significance level with a 95% confidence interval.

RESULTS

The study population included 72 patients (Female/Male: 60/12). The cases of this study received the diagnosis of PTMC incidentally. Thyroidectomy was performed on 26 of 72 patients (36.1%) due to multinodular goiter and PTMC was diagnosed after thyroidectomy. In the rest of the group with 46 patients (63.8%), FNAB was performed according to the suspicious features of the nodules in thyroid ultrasonography and PTMC was diagnosed before surgery. The mean follow-up duration of the whole cohort was three years (min: 1 year, max. 5 years). The mean age was 46.3±11.7 years (range, 21–68). The mean size of the tumors was 6.7±2.3 mm (range, 1–10).

The BRAF^{V600E} mutation was present in 41.6% (30 of 72) of the patients. The presence of classical variant type (p=0.046), thyroid capsule invasion (p=0.002) and

TABLE 1. The relationship between BRAF mutation and clinicopathological features

	BRAF (+) group		BRAF (-) group		p
	n	%	n	%	
	Male	3	10.00	9	
Female	27	90.00	33	78.57	
Diagnostic age ≤45	14	46.67	12	28.57	0.11
Diagnostic age >45	16	53.33	30	71.43	
Follicular variant	8	26.66	21	50.00	0.046
Classical type	22	73.33	21	50.00	
Tumor diameter ≤5 mm	8	26.67	17	40.48	0.22
Tumor diameter >5 mm	22	73.33	25	59.52	
Thyroid capsule invasion	20	66.67	13	30.95	0.002
Extrathyroidal invasion	12	40.00	11	26.19	0.21
Lymph node metastasis	0	0	3	7.14	0.13
Multifocality	9	30.00	21	50.00	0.87
Bilaterality	9	30.00	9	21.43	0.40
Absence of tumor capsule	24	80.00	19	45.24	0.003
Hashimoto's thyroiditis	17	56.67	16	38.10	0.11
Found before surgery	22	73.33	24	51.14	0.15
Found after surgery	8	26.66	18	42.85	

BRAF: B-type Raf.

absence of tumor capsule ($p=0.003$) were statistically significantly higher in BRAF (+) group than in BRAF (-) group. The presence of the BRAF mutation in our patients strongly predicted the classical variant type of PTC, thyroid capsule invasion and the absence of tumor capsule. The mean size of the tumor was 7.1 ± 1.9 mm in the mutated patient group and 6.4 ± 2.5 mm in wild type patient group. There was no significant correlation between the BRAF mutation and tumor diameter ($p=0.087$). The mean age at diagnosis was 43.8 ± 12.1 years in BRAF (+) group and 48.2 ± 11.3 years in BRAF (-) group. The female sex was dominant in the study population (83.3%); however, there was no statistically significant difference in gender with BRAF^{V600E} mutation positivity ($p=0.20$). LNM was present in three cases; all of them were negative for the mutation and were tumors >5 mm in diameter. There was only one case with distant metastasis (to lung) that had BRAF wild type mutation and was a tumor >5 mm in size. There was no relationship between the BRAF mutation and LNM or distant metastasis in our study population. Total thyroidectomy was performed on 71 patients, while one patient had a

TABLE 2. Comparison of the clinicopathological features of the cases found before surgery versus found after surgery

	Found before surgery		Found after surgery		p
	n	%	n	%	
	Male	7	15.00	5	
Female	39	85.00	21	81.00	
BRAF mutation (+)	22	47.82	8	30.77	0.15
BRAF mutation (-)	24	52.17	18	69.23	
Thyroid capsule invasion	25	54.34	8	30.77	0.045
Bilaterality	11	23.90	7	26.90	0.77
Multifocality	17	36.90	13	50.00	0.28
Absence of tumor capsule	30	65.20	13	50.00	0.20
Extrathyroidal invasion	17	36.90	6	23.10	0.22
Hashimoto's thyroiditis	24	52.10	9	34.60	0.15
Tumor diameter >5 mm	35	76.10	12	46.10	0.010

lobectomy. One patient who had received radiation therapy in the neck region due to nasopharyngeal cancer, and one patient who was working as a radiology technician, were both negative for the mutation. Seven patients (9.7%) had thyroid cancer history in their family (3 patients with mutated, four patients with wild type BRAF). Forty-nine patients received RAI ablation therapy due to thyroid capsule invasion, ETE, lymph node involvement and distant metastasis (21 patients with mutated, 28 patients with wild type BRAF). The stage distribution was as follows: all cases positive for the BRAF mutation were in stage 1. Four cases negative for the mutation: two of them were in stage 2; two of them were in stage 3. There was no relationship between BRAF mutation presence and advanced stage. The mean TSH value (mIU/L) was 1.4 ± 1.2 in BRAF (-) patient group and 1.6 ± 0.98 in BRAF (+) patient group. In summary, there was no significant correlation with age, gender, multifocality, bilaterality, ETE, presence of Hashimoto's thyroiditis, and TSH value between mutated and wild-type patient groups. All of the patients had adequate follow-up information. No disease-specific mortality or regional or distant recurrences were demonstrated during the follow-up period.

The patients were divided into two groups as follows: the first group who were diagnosed after surgery included 26 patients, the second group who were diagnosed before surgery, included 46 patients. When the

TABLE 3. Comparison of the clinicopathological features of BRAF positive cases, found before surgery versus found after surgery

	Found before surgery		Found after surgery		p
	BRAF (+) group		BRAF (+) group		
	n	%	n	%	
Male	2	9.90	1	12.50	0.78
Female	20	90.10	7	87.50	
Thyroid capsule invasion	15	68.10	5	62.50	0.77
Bilaterality	7	31.80	2	25.00	0.71
Multifocality	7	31.80	2	25.00	0.71
Absence of tumor capsule	18	81.90	6	75.00	0.67
Extrathyroidal invasion	8	36.40	4	50.00	0.50
Hashimoto's thyroiditis	13	59.10	4	50.00	0.65
Diagnostic age ≤45	13	59.10	1	12.50	0.023
Tumor diameter >5 mm	16	72.00	6	75.00	0.90

BRAF: B-type Raf.

two groups were compared, the second group was more prone to have thyroid capsule invasion ($p=0.045$) and larger tumor size ($p=0.010$).

Patients had similar tumor characteristics; however, diagnostic age was statistically significantly lower in patients discovered before surgery ($p=0.023$).

DISCUSSION

In recent years, the incidence of thyroid cancer has rapidly increased worldwide [2]. This increase is mostly a result of the identification of smaller thyroid tumors due to increased use of high-resolution thyroid ultrasonography and imaging for non-thyroid related conditions [34]. PTCs equal to or less than 1 cm are classified as PTMCs. A small percentage of patients with PTMC has reported presenting with high-risk features (ETE, LNM), even clinically significant distant metastasis [8–10]. They may require more aggressive treatment. The activating somatic point mutation in the BRAF gene is specific to PTCs and it plays the main role in tumorigenesis and its progression [35]. The BRAF mutation may identify aggressiveness in patients with PTMC. Therefore, over the past decade, BRAF mutation has been widely studied in PTMCs.

Advances in ultrasonographic screening and USG-guided FNAB have allowed more PTMCs to be detected in their early stages [3, 35]. PTMC may be found nonincidentally or incidentally. Incidental microcarcinoma is detected following surgery or neck ultrasonography for benign thyroid diseases or nonrelated thyroid pathologies [36]. Nonincidental microcarcinoma is found on clinical presentation with local or distant metastasis [36]. PTMC determined incidentally has a better prognosis and lower recurrence risk than that determined nonincidentally [37]. We found that the presence of the BRAF mutation strongly predicted the thyroid capsule invasion and the absence of tumor capsule. The presence of a capsule within the tumor is a major factor for determining the prognosis, and the encapsulated tumors do not tend to have LNM [38, 39]. A meta-analysis showed that the BRAF mutation was associated with ETE and the absence of tumor capsule [40]. PTMC with thyroid capsule invasion may be more likely to have an increased tendency of having ETE [41]. Many studies found a significant association between BRAF mutation and ETE [10, 14, 27, 28, 31, 40, 42–50]. LNM and ETE were predictors of persistent/recurrent disease [49]. BRAF^{V600E} mutation might be a molecular marker of tumor invasiveness and this relationship was independent of tumor size [10, 40]. A recent multicenter study that included 743 patients reported that BRAF^{V600E} mutation differentiated the recurrence risk of PTMC, particularly low-risk PTMC [51]. In contrast, other studies reported that this mutation was not correlated with LNM [30, 31, 52, 53]. We reported no recurrence during the follow-up period in our study population; this might be associated with that all of the patients with BRAF mutation underwent total or near-total thyroidectomy. In addition, the whole cohort was discovered incidentally and 70% of the patients with BRAF mutation received RAI ablation therapy. The follow-up period might be too short a time to determine the recurrence rate and prognosis.

Many studies reported that a significant association existed between BRAF mutation and multifocality [14, 28, 31, 43, 45, 46]. Lin et al. found that multifocality can be associated with LNM in PTMCs [54]. In our study, multifocality did not significantly differ between the mutated and non-mutated groups. Kebebew et al. found that mutation frequency increased in parallel with age [55]. Six studies were analyzed for the association between BRAF mutation and age [10, 31, 41, 44–46], and found no significant association. In our

study, there was no significant association between age and the BRAF mutation. There was no significant association between BRAF mutation and male gender in many studies [10, 14, 28, 31, 41, 43–47, 50], except for two [29, 56]. We found no relation between the BRAF mutation and the male gender. This may be related to the low number of male cases in our study. Most of the current staging systems use 5 mm as the cut-off value to differentiate between low and high-risk PTMC [41]. Tumor size larger than 5 mm was found to be associated with recurrent PTMC [57]. In two studies, there was a significant association between BRAF mutation and tumor size being higher than 5 mm [43, 44], whereas another study showed no difference like our results [58]. Surprisingly, we found that tumors negative for the mutation, but were >5 mm in size had lymph node or distant metastasis. The relationship between the BRAF mutation and tumor size remains controversial. This study found the BRAF^{V600E} mutation prevalence of 41.6% was similar to earlier studies [25, 28, 50]. However, the mutation frequency was relatively high in Korean patients [31]. This may be related to geographical differences.

BRAF^{V600E} mutation-positive and negative PTCs have distinctive histology. Compared with the follicular variant, classical and tall cell variants of PTCs have a higher BRAF^{V600E} mutation rate [30, 59]. Three studies, including 243 patients, analyzed the association of BRAF mutation and tall cell variant (TCV) of PTMC (TCVPTMC). They found a significant association between BRAF mutation and TCVPTMC [10, 44, 47]. There was no patient with TCV in our study population. We found a significant association between the BRAF mutation and the classical variant.

Multiple studies have reported that PTC, along with Hashimoto's thyroiditis (HT), had more favorable clinicopathological features and a better prognosis than those without HT [60]. It has been reported that HT is a potential protective factor for PTC, regardless of the BRAF^{V600E} mutation [61]. However, the underlying molecular mechanism of the association between HT and PTC is still unknown. The theory of 'tumor defense-induced autoimmunity' may explain the co-existence of HT and PTC [62]. The immune cell infiltration into the thyroid is believed to be an ongoing antitumor immune response, which leads to a better prognosis among PTC patients [62]. Three studies reported the association between BRAF mutation and concomitant HT [10, 41, 46]. However, other studies

identified contradictory results [63]. In this study, no significant association was found between BRAF mutation and concomitant HT. This outcome may be due to the small study population. The relationship between BRAF mutation and chronic thyroiditis in PTMC requires further assessment.

A study from Turkey found that PTMCs harboring BRAF mutation had a more aggressive behavior [28], although conflicting findings were reported in another study [33]. The difference of our study was that we evaluated the BRAF mutation in incidental PTMCs. Incidental microcarcinomas have lower recurrence rate than nonincidental microcarcinomas [37]. Nevertheless, tumors positive for the mutation were more prone to spread the thyroid capsule in our study population.

We recognize some of the limitations of this study. First, it is a retrospective single-center study. Second, the sample size is small. The limited number of cases may mask the relationship between BRAF mutation and prognostic factors. Third, we have not long term follow-up time.

Conclusion

This report confirmed that BRAF mutation status was not associated with recurrences and poorer outcomes in a 3-year follow-up period in our study population. However, tumors positive for the mutation had more invasive behavior. There have been conflicting data on the management and treatment of PTMCs among the physicians. Currently, many studies discussed how BRAF mutation influences surgical type for PTMC patients. We believe that BRAF mutation screening of FNAB specimens preoperatively may give a guidance to determine the initial surgical approach. For better understanding of this issue, future prospective studies are needed with a long follow up period and larger patient groups.

Ethics Committee Approval: All procedures in this study were approved by our hospital's ethics committee.

Conflict of Interest: No conflict of interest was declared by the authors.

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Authorship Contributions: Concept – HS; Design – NU; Supervision – DY; Materials – NU; Data collection and/or processing – HS; Analysis and/or interpretation – NU; Literature review – DY; Writing – HS; Critical review – DY.

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