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Are BRAF V600E and K-Ras Mutations Associated With Tumor Aggressiveness in Well-Differentiated Thyroid Cancer?

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Aim: Many clinical studies have shown an association between B-type rapidly growing fibrosarcoma kinase [BRAF(V600E)] mutation and aggressive clinicopathologic features, although some results from others are controversial. Besides, Kirsten rat sarcoma (K-Ras) mutations are more common in endemic iodine deficiency regions, as our country is. However, use of the biologic markers are questioned in clinical practice; they are beginning to be used for the management of patients with thyroid nodules and cancers. The aim of the present study was to evaluate the prevalence of the BRAF(V600E) mutation in tumor samples and its relationship to high-risk clinicopathologic features.

Methods: From 2000 to 2007, 82 patients with well-differentiated thyroid cancer (WDTC) who underwent surgery in Ege University were enrolled retrospectively in the study. Univariate and multivariate analyses were performed to analyze associations between BRAF(V600E) and K-Ras mutations and clinicopathologic features. We identified 82 patients with WDTC (male:female = 1:3.2).

Results: The median follow-up was 96 months. The mean age was 46.4 (16–80). None of the all analyzed prognostic factors—age; sex; lymph node metastasis; multifocality; multicentricity; invasion; tumor diameter; and tumor, node, metastasis staging—were correlated with BRAF(V600E) mutation status in the univariate analysis. Meanwhile, none of the analyzed prognostic factors were correlated with K-Ras mutation status.

Discussion: Although many studies suggest BRAF(V600E) and K-Ras mutations as prognostic factors in WDTC, our results are controversial. BRAF(V600E) and K-Ras mutations have no significant effects on tumor aggressiveness in Turkish patients with WDTC. Our results underline that it is too early to reach a conclusion that BRAF(V600E) and K-Ras mutations are involved with poor clinical outcomes.

Key words: Mutation – BRAF(V600E) – K-Ras – Thyroid cancer

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Thyroid cancer is the most common type of endocrine malignancy, and the incidence of thyroid cancer is increasing in some areas of the world, presumably because of increased detection of small papillary carcinomas. By contrast, evaluation and management of thyroid nodules have changed dramatically over the past years.[1] Imaging nodules (especially with [insert definition of USG here]), fine needle aspiration (FNA), and cytopathologic practices were the most important enhancements for the management of thyroid nodules. The use of molecular markers for thyroid cancer diagnosis, prognosis, and surveillance has been an exciting area of study that has seen changes in the last decade. These developments in surgery promise to allow expanded surgical treatment options and potentially make thyroid cancer surgery safer and better accepted by patients.[2] Most thyroid operations are performed for known or potential thyroid cancers. Investigations in the past decade have led to a better understanding of carcinogenesis of various types of thyroid cancers. Understanding the molecular genetic alterations of thyroid cancers can potentially help in their diagnosis and treatment. The aim of this special topic is to provide an update on recent advances in understanding of thyroid tumorigenesis and their implications in clinical practice.

The majority (95%) of all thyroid cancers are originated from thyroid follicular cells. These cancers are divided into well-differentiated (follicular and papillary), poorly differentiated, and undifferentiated (anaplastic) subtypes. Papillary (PTC) and follicular (FTC) thyroid carcinoma and their subtypes represent the majority of well-differentiated thyroid cancers (WDTC). Genetic alterations, most commonly associated with WDTC, are point mutations of the B-type rapidly growing fibrosarcoma kinase (BRAF) and rat sarcoma (RAS) genes as well as the gene rearrangements, rearranged during transfection/papillary thyroid cancer (RET/PTC) and paired box gene 8/peroxisome proliferator-activated receptor gamma (PAX8/PPARγ).

The BRAF mutation is a common somatic mutation in thyroid cancer, occurring exclusively in about 45% of PTC and 25% of anaplastic cancer.[3] There are three isoforms of the serine–threonine kinase Raf in mammalian cells: ARaf, BRaf, and CRaf or Raf1. CRaf is expressed ubiquitously, whereas BRaf is expressed at higher levels in hematopoietic cells, neurons, and testis.[4] BRaf is also the predominant isoform in thyroid follicular cells. Although all Raf isoforms activate MEK, they are differentially activated by oncogenic Ras. In addition, BRaf has higher affinity for MEK1 and 2 and is more efficient in phosphorylating MEKs than other Raf isoforms.[5] More than 90% of BRAF mutations in PTC are characterized by a change of valine to glutamic acid at codon 600, designated BRAF(V600E). In PTC, BRAF(V600E) is associated in most retrospective studies with histopathologic findings of aggressive disease such as extrathyroidal extension and lymph node metastasis.[6,7] Besides, there are studies suggesting that patients with BRAF(V600E)-positive PTC are more likely to recur and associated with decreased disease-specific survival.[8,9] Reoperation was also more likely for BRAF(V600E)-positive PTC.[10] Conversely, several studies have not shown any correlations, including two studies that analyzed a relatively large number of patients. The clinical and pathologic implications of BRAF mutations in PTC are, in part, still controversial.

HRAS, *KRAS*, and *NRAS* genes are members of the *RAS* family coding for a G-protein. When activated, RAS protein starts the intracellular signal transduction through the release of GTP and the activation of MAPK and PI3K/AKT pathways. Therefore, an increase of the affinity for GTP and inactivation of the GTPase function are explained by the presence of point mutations in the *RAS* domains. Point mutations of *RAS* are found in 10% to 20% of PTCs.[11–13] *RAS* mutations have also been found in 40% to 50% of FTCs and in 20% to 40% of follicular adenomas. The distinguishing features of K-Ras mutation from other RAS mutations are also attractive. K-Ras mutations are more common in PTC than FTC. Meanwhile, K-Ras mutations are more common in endemic iodine deficiency regions.

The aim of the study was to evaluate the effects of K-Ras and BRAF(V600E) mutations on tumor behavior.

Patients and methods

From January 2000 to December 2007, 82 WDTC patients with fully archived data, who underwent surgery in Ege University Medical School, were enrolled retrospectively in the study. Data of the patients were obtained from a recorded database. The median follow-up was 96 months. All patients underwent total thyroidectomy. Central compartment or lateral neck dissections were not routinely performed. Central compartment or lateral neck dissections were only performed in cases due to abnormality reported by preoperative imaging,

preoperative proven metastasis, or pathologic intervention during intraoperative examination.

Only patients with WDTC were included in the study. These patients had papillary thyroid carcinomas or papillary microcarcinomas. Histologic subtypes were not investigated because of the small number of patients in the study. We reviewed the recorded database for clinicopathologic features. The clinicopathologic features expected to effect tumor aggressiveness included gender; sex; lymph node metastasis; multifocality; invasion; tumor diameter; multicentricity; and the tumor, node, metastasis (TNM) staging system.

DNA was isolated from paraffin-embedded blocks by using a high pure PCR template preparation kit (Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions. DNA quality was measured with a Nanodrop ND1000 spectrophotometer (Nanodrop Technologies, Waltham, Massachusetts). Primers used to amplify BRAF(V600E) exon 15 and K-Ras exon 2 were synthesized by TıbMolBiol (Berlin, Germany).

The PCR and high-resolution melting (HRM) were performed with a LightCycler 480 Instrument (Roche Diagnostics, Penzberg, Germany) in a reaction mix containing 25 ng of genomic DNA, 400 nM (exon 15 and exon 2) of each primer and 3 mM $MgCl_2$ in the LightCycler 480 HRM Master containing ResoLight dye (Roche Diagnostics) with PCR grade water adjusted to a total volume of 20 μL. The reaction condition included an activation step at 95°C for 10 minutes followed by 55 cycles of 95°C for 10 seconds, a touch down of 65°C to 55°C for 10 seconds (1°C/cycle), and 72°C for 30 seconds. Before the high-resolution melting step, the products were heated to 95°C for 1 minute. The HRM was carried out over the range from 72°C to 95°C rising at 1°C per second with 30 acquisitions per degree. All reactions were performed in duplicate.

Upon completion of the run (approximately 2 hours), HRM curve analysis was performed using the LightCycler 480 Software version 1.3 supplied with the LightCycler 480 Instrument. The melting curves were normalized, to allow for direct comparison of samples, and the temperature was shifted. Difference plots were generated by selecting a negative control, converting the melting profile to a horizontal line and normalizing the melting profiles of the other samples against this sample. Significant differences in fluorescence from the horizontal baseline were indicative of mutations. Differences were judged as significant if the replicates fell outside the range of variation seen in the wild-type samples.

Statistical analysis

Data analysis was performed with SPSS for Windows, version 11.5 (SPSS Inc., Chicago, Illinois). Data were shown as mean ± standard deviation (SD) or number of cases and percentage, where applicable. While, the mean age differences between groups were compared by Student's *t*-test; otherwise, Mann-Whitney *U* test was applied for comparisons of the pathologic tumor size and TNM levels. Categoric data were analyzed by continuity corrected χ^2 or Fisher's exact test, where applicable. A value of $P < 0.05$ was considered statistically significant.

Results

The average age of all patients reviewed in the study was 46.4. A total of 33 (40.2%) patients were under the age of 45 and 49 (59.8%) were older than 45 years. The ratio of female patients was higher, as expected (76.8%). K-Ras mutation was found to be present in 16 (19.5%) patients. BRAF(V600E) mutation was positive in 15 (18.3%) patients. Papillary carcinoma was seen in 72 (87.8%) patients and papillary microcarcinoma in 10 (12.2%). Tumor size was larger than 4 cm in 8 (9.8%) patients, while in 74 (90.2%) it was smaller than 4 cm. Multifocality was found in 19 (23.2%) patients and 20 (24.4%) patients had multicentric tumors. Lymph node metastasis was found to be present in 13 (15.9%) patients. In 26 (31.7%) patients, tumoral invasion was seen. Vascular invasion was found in only 2 (2.4%) patients. According to TNM classification, 61 (74.4%) patients were in stage I, while 12 (14.6%) were in stage II, 7 (8.5%) in stage III, and 2 (2.4%) in stage IV (Table 1).

K-Ras mutation status

Out of 33 patients, 8 (24.2%) were younger than 45 years and had K-Ras mutation positivity, while 8 (16.3%) out of 49 patients older than 45 years, were positive according to K-Ras mutation analysis. In the comparison of K-Ras mutation status with age, there was no statistically significance determined. K-Ras mutation was found to be present in 2 (10.5%) male and 14 (22.2%) female patients. The relationship between sex and K-Ras mutation was statistically insignificant. Histopathologically, 14 (87.5%) cases with K-Ras mutation had papillary carcinoma,

Table 1. Patient demographic and clinical characteristics

Variables	n = 82
Age, y	46.4 ± 13.3 16–80
Age groups, n (%)	
<45	33 (40.2)
≥45	49 (59.8)
Sex, n (%)	
Male	19 (23.2)
Female	63 (76.8)
Pathology, n (%)	
Papillary carcinoma	72 (87.8)
Papillary microcarcinoma	10 (12.2)
Tumor diameter, n (%)	
≤1.0 cm	13 (15.9)
1.1–2.0 cm	40 (48.8)
2.1–3.0 cm	15 (18.3)
3.1–4.0 cm	7 (8.5)
4.1–5.0 cm	2 (2.4)
>5 cm	5 (6.1)
Tumor diameter, n (%)	
≤4 cm	75 (91.5)
>4 cm	7 (8.5)
K-RAS+, n (%)	16 (19.5)
BRAF+, n (%)	15 (18.3)
Multicentricity, n (%)	20 (24.4)
Multifocality, n (%)	19 (23.2)
Lymph node metastasis, n (%)	13 (15.9)
Invasion, n (%)	
(−)	56 (68.3)
(+)	26 (31.7)
Surrounding tissue	9 (11.0)
Vascular	2 (2.4)
Capsule	15 (18.3)
TNM, n (%)	
I	61 (74.5)
II	12 (14.6)
III	7 (8.5)
IV	2 (2.4)

while 2 (12.5%) had papillary microcarcinoma. There was no significant relationship with K-Ras mutation status. According to tumor diameter, K-Ras mutation was present in 14 (17.1%) patients with tumor size <4 cm. Only 2 (2.4%) patients with a tumor size ≥4 cm had K-Ras mutation. The relationship between tumor diameter and K-Ras mutation was statistically insignificant (Table 2).

Multicentricity was absent in 14 patients with mutation positivity, while 2 of the K-Ras positive cases had multicentric tumors. Meanwhile, multifocality was not found 13 (81.9%) patients with K-Ras mutation. Lymph node metastasis was found to be present in only 4 (25%) patients with K-Ras mutation, while 7 (8.5%) patients had tumoral invasion. Lymph node metastasis, tumor size, tumoral invasion, multicentricity, and multifocality had no statistically significant relation with K-Ras mutation status (Table 2). The stage distribution of patients with K-Ras mutation positivity was as follows: 10 (62.5%) patients had stage I, 3 (18.8%) patients had stage II, and 3 (18.8%) patients had stage III, according the TNM classification.

BRAF(V600E) mutation status

BRAF (V600E) mutation was positive in 15 (18.3%) cases, as mentioned above. A total of 5 (15.2%) were <45 years old, while 10 (20.2%) were ≥45 years. There was no statistically significant difference. Of those in the study, 2 out of 15 patients were male, while 13 were female. The relationship between sex and BRAF(V600E) mutation showed no significance. Histopathologically, 11 (73.3%) patients had papillary carcinoma and 4 (26.7%) had papillary microcarcinoma. Statistically, the effects of BRAF(V600E) mutation on histologic subtypes was insignificant.

According tumor size, all patients with BRAF(V600E) mutation positivity had a tumor <4 cm; 8 (11.9%) patients had no BRAF(V600E) mutation and had tumors ≥ 4 cm. Tumor diameter was significantly smaller in patients with BRAF V600E mutation ($P = 0.017$; Table 3). Multifocality was found in 3 (20%) patients, lymph node metastasis was found in 1 (7.7%) patient and multicentricity was found in 2 (13.3%) patients with a BRAF(V600E) positive result. A total of 13 (86.7%) patients had no tumoral invasion, and 2 (13.3%) had tumoral invasion in the presence of BRAF(V600E) mutation (Table 3). According to the TNM staging system, 12 (80%) patients were in stage I, 1 (6.7%) in stage II, and 2 (13.3%) in stage III. Lymph node metastasis, tumor diameter, tumoral invasion, multicentricity, multifocality, and TNM staging had no statistically significant relation with BRAF(V600E) mutation status.

Both BRAF(V600E) and K-Ras mutations were found in only 3 (3.7%) patients. Presence of a dual mutation showed no significant effect on tumor aggressiveness.

Discussion

The BRAF(V600E) mutation is thought to be an important factor of the oncogenic transformation in thyroid cancer.[14] Numerous studies from various groups have reported that BRAF(V600E) mutations were found to be in a range between 26% to 84% of patients.[15,16] Interestingly, in our study we found that the BRAF(V600E) mutation rate was 18.3% in

Table 2. Demographic and clinical characteristics of patients with K-Ras mutation status.

Variables	K-RAS− (n = 66)	K-RAS+ (n = 16)	*P* value
Age, y	47.1 ± 13.4	43.6 ± 13.0	0.349
Age groups, n (%)			0.547
<45	25 (37.9)	8 (50.0)	
≥45	41 (62.1)	8 (50.0)	
Sex, n (%)			0.338
Male	17 (25.8)	2 (12.5)	
Female	49 (74.2)	14 (87.5)	
Pathology, n (%)			1.000
Papillary carcinoma	58 (87.9)	14 (87.5)	
Micropapillary carcinoma	8 (12.1)	2 (12.5)	
Tumor diameter, n (%)			0.449
≤1.0 cm	11 (16.7)	2 (12.5)	
1.1–2.0 cm	33 (50.0)	7 (43.8)	
2.1–3.0 cm	11 (16.7)	4 (25.0)	
3.1–4.0 cm	6 (9.1)	1 (6.3)	
4.1–5.0 cm	2 (3.0)	–	
>5 cm	3 (4.5)	2 (12.5)	
Tumor diameter, n (%)			0.618
≤4 cm	61 (92.4)	14 (87.5)	
>4 cm	5 (7.6)	2 (12.5)	
Multicentricity, n (%)	18 (27.3)	2 (12.5)	0.334
Multifocality, n (%)	16 (24.2)	3 (18.8)	0.752
Lymph node metastasis, n (%)	9 (13.6)	4 (25.0)	0.270
Invasion, n (%)			0.393
(−)	47 (71.2)	9 (56.3)	
(+)	19 (28.8)	7 (43.8)	
Surrounding tissue	7 (10.6)	2 (12.5)	1.000
Vascular	2 (3.0)	–	1.000
Capsule	10 (15.2)	5 (31.3)	0.157
TNM, n (%)			0.224
I	51 (77.3)	10 (62.4)	
II	9 (13.6)	3 (18.8)	
III	4 (6.1)	3 (18.8)	
IV	2 (3.0)	–	

WDTC patients, which was lower than what was reported in the literature. This may be explained by the low number of cases or less aggressive tumoral behavior in WDTC.

Numerous studies have reported that BRAF(V600E) mutations have no relationship with sex and age.[17,18] Correspondingly, in our study, we did not find any correlation with BRAF(V600E) mutation status on age and sex. In controversy, some studies have reported that BRAF(V600E) mutations were more frequent in older patients.[8,9]

Previous studies have reported the association of BRAF(V600E) mutations with aggressive clinicopathologic findings of papillary thyroid cancers, such as tumoral invasion, lymph node metastasis, tumor diameter, and advanced tumor stage.[8,9,14] Nevertheless, several studies suggested that BRAF(V600E) mutation is a risk factor for disease persistence and recurrence in WDTC.[19,20] However, we could not verify these findings with our controversial results. In our observation, it was noted that BRAF(V600E) mutation did not cause aggressive tumor behavior. On the other hand, there are several studies supporting our results in the literature.[17,18,21–26] Moses *et al*[27] reported that BRAF(V600E) mutation did not cause aggressive tumoral behavior, but they mentioned that this mutation might be a reason for thyroid cancer in younger patients.

Similarly, the effect of K-Ras mutation on tumor behavior is still controversial. K-Ras mutation is more frequent in iodine deficiency regions, like the Aegean (Western) part of Turkey, from where all patients from this study were habitants.[27] Besides, K-Ras mutation is associated with the classical type of papillary thyroid cancer. Goutas *et al*[18] studied the effects of K-Ras and BRAF(V600E) mutations on MTC and PTC. They reported that there were no correlations between aggressive clinicopathological findings and these mutations.[18] Similarly, some

Table 3. Demographical and clinical characteristics of patients with BRAF V600E mutation status

Variables	BRAF− (n = 67)	BRAF+ (n = 15)	*P* value
Age, y	47.3 ± 13.5	42.7 ± 11.9	0.235
Age groups			0.755
<45	28 (41.8)	5 (33.3)	
≥45	39 (58.2)	10 (66.7)	
Sex, n (%)			0.501
Male	17 (25.4)	2 (13.3)	
Female	50 (74.6)	13 (86.7)	
Pathology, n (%)			0.079
Papillary carcinoma	61 (91.0)	11 (73.3)	
Papillary microcarcinoma	6 (9.0)	4 (26.7)	
Tumor diameter, n (%)			**0.017**
≤1.0 cm	7 (10.4)	6 (40.0)	
1.1–2.0 cm	34 (50.7)	6 (40.0)	
2.1–3.0 cm	13 (19.4)	2 (13.3)	
3.1–4.0 cm	6 (9.0)	1 (6.7)	
4.1–5.0 cm	2 (3.0)	–	
>5 cm	5 (7.5)	–	
Tumor diameter, n (%)			0.340
≤4 cm	60 (89.6)	15 (100.0)	
>4 cm	7 (10.4)	–	
Multicentricity, n (%)	18 (26.9)	2 (13.3)	0.339
Multifocality, n (%)	16 (23.9)	3 (20.0)	1.000
Lymph node metastasis, n (%)	12 (17.9)	1 (6.7)	0.445
Invasion, n (%)			0.127
(−)	43 (64.2)	13 (86.7)	
(+)	24 (35.8)	2 (13.3)	
Surrounding tissue	9 (13.4)	–	0.200
Vascular	1 (1.5)	1 (6.7)	0.334
Capsule	14 (20.9)	1 (6.7)	0.283
TNM, n (%)			0.649
I	49 (73.1)	12 (80.0)	
II	11 (16.4)	1 (6.7)	
III	5 (7.5)	2 (13.3)	
IV	2 (3.0)	–	

other investigators suggested that K-Ras mutation did not affect the clinicopathologic features such as, age, lymph node metastasis, tumor size, multicentricity, and TNM stage.[28,29] On the contrary, some other studies suggested that K-Ras mutation was associated with aggressive clinicopathologic features.[30,31]

In this study, we only used the TNM staging system for the determination of WDTC prognosis, since it is widely accepted that the TNM staging system can determine the prognosis in a manner consistent with clinical findings.[32] Both studied mutations had no effect on the TNM stage of the patients.

Compared to other studies, we found that the incidence of BRAF(V600E) mutation was lower. The low ratio of this mutation can be caused by the low patient sample or due to geographic reasons. Other studies carried out in Turkey, one from Ankara and the other from Istanbul—which are not Aegean cities—reported higher BRAF(V600E) mutation rates (86% versus 40%).[21,33] Another possible discussion for this low rate of mutation might be the method used for DNA isolation, where sequencing, which is another method for DNA isolation, may decrease the sensitivity of the test. Since we did not use sequencing for DNA analysis, it's not worth to question this. On the other hand, it has been shown that radiation-induced thyroid tumors demonstrated a low prevalence BRAF mutations and a high prevalence of RET/PTC rearrangements.[34] BRAF(V600E) mutation has been less frequently (4%–24%) observed in postradiation.[35] Inhabitants of the current study were stable inhabitants of the region that was heavily affected after the Chernobyl disaster in 1986. This could be an important factor for the low incidence of BRAF(V600E) mutation. Dietary iodine may modulate the mutations in thyroid carcinogenesis.[27,36] In iodine deficiency areas, RAS oncogene activation may play a more

important role in thyroid carcinogenesis.[27] These factors can explain the high ratio of K-Ras mutation and the low ratio of BRAF(V600E) mutation. It is certain that BRAF(V600E) mutation is the most common genetic alteration in thyroid cancers. Besides, there is a tendency of BRAF(V600E) mutation to be a poor prognostic factor for thyroid cancers. On the other hand, there are a lot of dissident studies from all over the world, that we cannot undervalue.[19,20,23,25,37–39]

In conclusion, this study showed that BRAF(V600E) and K-Ras mutations did not show any correlation concerning tumor aggressiveness. Mutations in thyroid cancer has become a very attractive and important topic in the last decay. Molecular investigations help and influence the clinicians in decision-making. But what impact it has and whether these will lead to a paradigm shift in management, recently we do not know. These molecular studies may solve the major problem of the undetermined FNA in the very near future. The effects of K-Ras and BRAF(V600E) mutations on tumorigenesis are certain, but we still need much more clinical studies concerning tumor behavior.

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