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Analysis of BRAF^{V600E}, KRAS Codon 12/13 and Novel Mutations in Patients with Thyroid Cancer in Blacksea Region of Turkey

Karadeniz Bölgesinde Tiroid Kanserli Hastalarda BRAF^{V600E}, KRAS Kodon 12/13 ve Yeni Mutasyonların Analizi

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ABSTRACT Objective: The incidence of thyroid cancer in many regions of the world has been steadily increasing over the past decades. Point mutations in the B-Raf (BRAF) and RAS proto-oncogenes are crucial in the molecular pathogenesis of thyroid cancers. In this study, it was aimed to investigate the BRAF^{V600E} and KRAS codon 12/13 mutations in thyroid cancer patients of the Turkish population. **Material and Methods**: In this study, totally 32 cases with thyroid cancer were investigated. Ten of the paraffin-embedded thyroid cancer tissues and 22 of peripheral blood samples from patients were included in the study. Genomic DNA was extracted from the paraffin-embedded sections and peripheral blood samples, and then polymerase chain reaction and DNA sequencing were performed for mutation analysis of BRAF and KRAS genes. **Results:** BRAF^{V600E} mutations were found in 13 (40.6%) patients, and 19 patients (59.4%) had wild-type profiles. G12N, G12V, and G13V mutations that are frequently seen in KRAS were not observed, but four new mutations (K16E, L19G, E31D, and M1I) were found in the current cohort. **Conclusion:** BRAF^{V600E} mutation was detected in patients with thyroid cancer, and four new mutations, which are in the GTP binding regions of the protein, were detected in the KRAS gene. These new mutations might be related to papillary thyroid cancer.

Keywords: Proto-oncogene proteins B-Raf; genes, Ras; thyroid cancer, papillary; mutation

ÖZET Amaç: Dünyanın pek çok bölgesinde tiroid kanseri görülme sıklığı, son yıllarda sürekli olarak artmaktadır. B-Raf (BRAF) ve RAS proto-onkogenlerindeki nokta mutasyonları, tiroid kanserlerinin moleküler patogenezinde çok önemlidir. Bu çalışmada, Türk toplumundaki tiroid kanseri hastalarında BRAF^{V600E} ve KRAS kodon 12/13 mutasyonlarının araştırılması amaçlandı. **Gereç ve Yöntemler:** Çalışmada, toplam 32 tiroid kanseri vakası incelendi. Parafine gömülü tiroid kanser dokularından 10'u ve hastalardan alınan periferik kan örneklerinden 22'si çalışmaya dâhil edildi. Parafine gömülü kesitlerden ve periferik kan örneklerinden genomik DNA ekstrakte edildikten sonra BRAF ve KRAS genlerinin mutasyon analizi için polimeraz zincir reaksiyonu ve DNA dizi analizi yapıldı. **Bulgular:** BRAF^{V600E} mutasyonları 13 (%40,6) hastada bulundu ve 19 (%59,4) hasta ise yabanıl tip profile sahipti. KRAS'ta sıklıkla görülen G12N, G12V ve G13V mutasyonları bu kohortta gözlenmedi, ancak 4 yeni mutasyon (K16E, L19G, E31D ve M1I) bulundu. **Sonuç:** Tiroid kanserli hastalarda BRAF^{V600E} mutasyonu ve KRAS geninde proteinin GTP bağlanma bölgelerinde meydana gelen 4 yeni mutasyon tespit edildi. Bu yeni mutasyonlar papiller tiroid kanseriyle ilişkili olabilir.

Anahtar Kelimeler: Proto-onkogen proteinleri B-Raf; genler, Ras; tiroid kanser, papiller; mutasyon

Among endocrine organs thyroid cancer is the most common type of malignancy. While most of the thyroid cancers are originated from thyroid follicular epithelial cells, the rest are from parafollicular cells or C cells. Thyroid cancers originated from follicular cells are subdivided into well-differentiated papillary, follicular, poorly-differentiated, and anaplastic carcinoma.[1]

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The frequency of thyroid cancer in many regions of the world has been steadily increasing over the past 40 years. Most of the increased cases are papillary thyroid cancer (PTC).[2] However, exposure to ionizing radiation, especially at an early age, is one of the best-known causes of thyroid cancer.[3] Other associated risk factors include body mass index, smoking, and exposure to various chemicals.[4] Gradual accumulations of genetic and epigenetic changes play a crucial role in the pathogenesis of thyroid cancer. These changes include activation and inactivation of somatic mutations, changes in gene expression pattern, deregulation of microRNAs and abnormal gene methylation.[1] Somatic mutations with the most data of these mechanisms occur in the early differentiation stage of cancer and play a crucial role in cancer progression.[5] It has been shown that important genes are frequently mutated in thyroid cancer due to molecular mechanisms such as point mutations and chromosomal rearrangements. These two different molecular mechanisms have been suggested to be related to certain etiological factors associated with thyroid cancer.[6]

Chromosomal rearrangements in the *RET* gene and point mutations in the *RAS* and *BRAF* genes are crucial in the molecular pathogenesis of PTC. These genetic changes give rise to activation in the mitogen activated protein kinase (MAPK) signal pathway.[7] In particular, *BRAF* mutations are related to clinically more invasive and more aggressive PTCs.[8]

The *BRAF* gene is a proto-oncogene consisting of 21 exons located in the chromosome 7q34 region, and encodes a protein from the *RAF* serine/threonine-protein kinase family. This protein has an important role in regulating the MAPK/ERK pathway, which controls important events such as cell division and differentiation. When the BRAF protein is activated, it causes phosphorylation and consequently activation of MAPK and other related pathways. *BRAF* gene mutations, particularly the most common mutation V600E, are among the causes of various cancers such as thyroid cancer and melanoma.[9] The most common mutation that lead to thymine>adenine substitution cause amino acid change of Val>Glu Val600Glu (V600E).[10] Val600Glu amino acid exchange in *BRAF* is the most common genetic change in PTC and is existed in approximately 45% of the tumors.[11] *Kirsten ras oncogene (KRAS)* is a proto-oncogene consisting of 6 exons located in the chromosome 12p12.1 region and is a homologous of the mammalian ras gene family. The *KRAS* gene encodes a protein from the GTPase superfamily. An amino acid substitution that occurs in this gene causes an activating mutation, and the protein that results from this mutation causes various malignancies.[12] In the majority of human tumors, point mutations in the *KRAS* gene occur in codons 12, 13, and 61.[13] Mutant *KRAS* encodes an oncoprotein, and this protein binds to GTP constantly then causes the formation of active RAS-GTP complex. While wild-type RAS proteins are inactivated shortly after, mutant RAS proteins cannot be inactivated, resulting in continuous activation of the RAS pathways.[14]

Several studies were reported in different countries and populations on the relationship between *BRAF* and *KRAS* mutations with thyroid cancer and the clinic-pathological characteristics of the disease.[2] Within the context of all this information, in this study, we aimed to investigate the *BRAF* V600E and *KRAS* codon 12-13 mutations in tumor tissue specimens and peripheral blood samples from thyroid cancer patients of the Turkish population.

MATERIAL AND METHODS

PATIENTS AND TUMOR SAMPLES

In the study, totally 32 cases with clinic and histologic diagnosis of thyroid cancer were investigated. This study was performed in line with the principles of the 1964 Helsinki Declaration and approval was given by the Ethics Committee of Ondokuz Mayıs University (approval number: KAEK 2011/426, date: 29.12.2011). All donors have given their written informed consent. Clinical and demographic information, including gender, age, tumor size, tumor stage and invasion were abstracted from clinical reports. All specimens from patients with thyroid cancer were obtained during routine surgery. Ten of paraffin-embedded thyroid cancer tissues and 22 of peripheral blood samples from patients with thyroid cancer were included. The tumor staging was performed according to The International Union against Cancer/Amer-

ican Joint Committee on Cancer tumor node metastasis (TNM) classification system.[15] Twenty-two of them were females and 10 of were males. The average age at diagnosis was 49±11. Patient and tumor characteristics are presented Table 1.

DNA ISOLATION

Genomic DNA was isolated from 5-µm paraffin-embedded sections and 200 µl peripheral blood samples using Nucleospin Genomic DNA Isolation Kit (Nucleospin, Germany), according to the manufacturer's protocol. The quantity and purity of DNA were determined with a NanoDrop spectrophotometer (Jenway Genova Nano, England).

MUTATION ANALYSIS OF BRAF AND KRAS GENES

Mutation analysis for *BRAF* codon 600 (*BRAF*^{V600E}) and *KRAS* codons 12 and 13 was performed using DNA sequencing system. Polymerase chain reaction (PCR) was utilized to amplify *BRAF* exon 15 before DNA sequencing using Taq DNA Polymerase (Thermo Fisher Scientific, Lithuania) and specific primers. The primer sequences were 5'-TCATAATGCTTGCTCTGATAGGA-3' for *BRAF* exon 15 Forward primer, and 5'-GGCCAAAAATTTAATCAGTGGA-3' for *BRAF* exon 15 Reverse primer. Mutational analyses for *KRAS* were performed by PCR and direct sequencing. The primer sequences were 5′-GTGTGACATGTTCTAATATAGTCA-3′ for KRAS Forward primer, and 5′-GAATGGTCCTGCACCAGTAA-3′ for KRAS Reverse primer. The PCR reaction was performed of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, and extension at 72 °C. In brief, amplicon of 215 bp in length for *BRAF* and 213 bp for *KRAS* codon 12-13 were generated by PCR. The Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) were used according to the manufacturer's instructions (G.M.L. SeqFinder Sequencing System) for DNA sequencing. Post-amplification analysis was performed with the Sequence Scanner Software (V2.x.x).

TABLE 1: Clinical and pathological characteristics of patients with thyroid cancer.

Characteristics	n (%)	BRAF^{V600E} Positive p value
Gender		
Female	22 (68.75%)	0.554
Male	10 (31.25%)	
Location		
Right	8 (25%)	0.874
Left	9 (28.1%)	
Bilateral	15 (46.9%)	
Tumor Histology		
Papillary	24 (75%)	0.528
Medullary	3 (9.4%)	
Folicullary	1 (3.1%)	
Papillary/Follicullary	4 (12.5%)	
Tumor Stage		
I	15 (46.9%)	0.125
II	7 (21.8%)	
III	4 (12.5%)	
IV	6 (18.8%)	
Lymph Node Metastasis		
Positive	1 (3.1%)	***0.002**
Negative	9 (28.1%)	
Unknown	22 (68.8%)	

*p<0.01

STATISTICAL ANALYSIS

SPSS V.22 software was used for analyzing the data from experiments. Data were presented as frequency percentage rate and mean±SD (standard deviation). The frequencies of mutations were determined with chi-square ($\chi 2$) test. Correlation analysis was performed using Spearman's test. p value <0.05 was considered as statistically significant.

RESULTS

CLINICAL AND PATHOLOGICAL DATA

The number of patients was 24 papillary (75%), 1 follicular (3.1%), 3 medullar (9.4%), and 4 papillary/follicular (12.5%) according to tumor histology. The number of patients was 15 grade T1 (46.9%), 7 grade T2 (21.8%), 4 grade T3 (12.5%), and 6 grade T4 (18.8%) according to tumor stage. In the current study, the clinical examination revealed that 1 patient had lymph node metastasis, 9 patients had no metastasis, and the other patients were unknown.

MUTATION ANALYSIS OF THE BRAF AND KRAS

In this study, common point mutations in *BRAF*^{V600E} and *KRAS* codon 12-13 were characterized in 32 pa-

TABLE 2: The new mutations in KRAS gene of thyroid cancer patients.

Sample no	Mutation	Histology	Tumor stage
TB-22	K16E (AAG>GAG) L19G (TTG>GGG) E31D (GAA>GAT)	Papillary	T-I
TB-38	M1I (ATG>ATT)	Papillary	T-III

tients with thyroid cancer. *BRAF*^{V600E} mutations were found in 13 (40.6%) patients and 19 patients (59.4%) had wild-type profiles for *BRAF*^{V600E} in the current cohort. In terms of the *BRAF*^{V600E} mutation presence, there was no statistical difference between men and women ($p>0.05$). Moreover, there were no relations between location, histology and stage of tumor and *BRAF*^{V600E} mutation presence ($p>0.05$). In addition, a statistically significant correlation of *BRAF*^{V600E} with lymph node metastasis was found ($p<0.01$). G12N, G12V and G13V mutations of the *KRAS* codon 12-13 were wild-type in all the studied samples.

There are four new mutations in *KRAS* gene of patients with thyroid cancer (Table 2). In a patient with T-I stage PTC, Lys16Glu (K16E) (missense), Lys19Gly (L19G) (missense) and Glu31Asp (E31D) (missense) mutations were detected as new mutations in the *KRAS* gene in blood sample. Additionally, in a patient with T-III stage PTC, Met1Ile (M1I) (missense) mutation was observed in blood sample. Representative images of DNA sequencing analysis were given in Figure 1.

The "uniprot.org/uniprot/P01116" database was used to determine in which functional region of the protein the mutations found and how they could affect the function of the protein. Amino acids in codons where Lys16Glu and Glu31Asp mutations found are located in the nucleotide binding sites of the protein. This region is GTP, GDP and GMP binding regions and is crucial for GTPase activity of KRAS protein.

DISCUSSION

Carcinogenesis involves a large number of genetic and epigenetic modifications.[16] Molecular markers including mutations have an important role in improving the preoperative diagnosis of cancer, including patients with thyroid cancer. Molecular mutation scans can be particularly useful for thyroid nodules with indeterminate cytology. Thyroid nodules that are positive for associated mutations indicate a high risk of thyroid cancer; therefore, more appropriate treatment methods can be used for the patient. In meta-analysis studies, *BRAF* mutations were found to be a highly related marker for thyroid cancer.[1] Although highly specific for cancer, it is not sufficient alone for patients who are negative in terms of *BRAF* mutation. Wherefore, determining other common thyroid cancer specific mutations and including them in the mutation analysis could improve the diagnostic efficiency.

In this study, common mutations of *BRAF* and *KRAS* proto-oncogenes were investigated in a Turkish population of thyroid cancer. Basically, DNA sequencing was carried out to identify mutations. The target proto-oncogenes *BRAF* and *KRAS* were genotyped in the current study. *BRAF*^{V600E} mutation was found in 13 (40.6%) patients of current samples and *KRAS* genes have wild-type genotype for codon 12/13. However, four new mutations which are K16E, L19G, E31D and M1I of KRAS gene were found in two patients with PTC. When we search

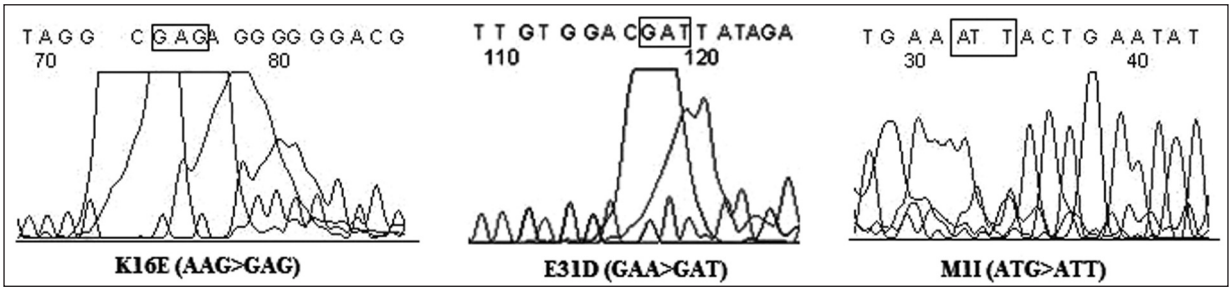

FIGURE 1: Representative images of DNA sequencing analysis for new mutations.

these new mutations in the uniprot database, we have found that K16E and E31D missense mutations occur in the GTP binding sites of KRAS protein. Therefore, we suggest that it could affect the protein's GTPase activity and the function of the protein. The mutant KRAS protein can lead to continuous activation of the RAS signaling pathways and consequently malignancies.[14] Since Met1Ile (M1I) (missense) mutation occurs in the start codon of protein synthesis, it could affect the amount of protein to be synthesized and cause deregulation in signaling pathways. Therefore, it could cause various malignancies including thyroid cancer.

In studies conducted in different populations, the frequency of *BRAF*^{V600E} mutation has been reported in the range of 18-87%.[17-21] In a study from Turkey, *BRAF*^{V600E} mutation and clinical-pathologic characteristics were examined in patients with PTC. In this study, the *BRAF*^{V600E} mutation rate was found as 39.45%. This rate is similar to the findings of our study (40.6%).[22] Previous studies have stated that the frequency of *BRAF*^{V600E} mutation increases with age.[23-25] However, in our study, no correlation was found between age and frequency of the mutation ($p>0.05$).

During the past decades, the frequency of *RAS* gene mutations has increased. Jung et al. analyzed the clinical, demographic and molecular characteristics of PTC patients between the years of 1974-2009.[26] In this study, it was determined that the rate of tumors with *RAS* mutation increased from 3% to 44%. In addition, it was determined that those of the follicular type increased from 18% to 44%. Although *BRAF* mutation rates are mostly stable, an increase in the mutation rate has been observed in papillary type thyroid cancer.[26] However, in our study, mutations frequently observed in the *KRAS* gene were not observed. These findings show that the increase in the incidence of thyroid cancer may be related to etiological factors.

CONCLUSION

BRAF^{V600E} mutation was detected in patients with thyroid cancer. G12N, G12V, and G13V mutations that are frequently seen in *KRAS* codon 12/13 were not found, but four new mutations (K16E, L19G, E31D and M1I) which are in the GTP binding regions of the protein were detected in the *KRAS* gene. These new mutations have been seen in PTC patients and might be related to PTC. However, larger population studies are needed to assess the clinical and biological effects of these mutations in *BRAF* and *KRAS* genes. Thus, better diagnostic criteria and treatment options can be developed for patients.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Ayfer Kamalı Polat, Zülfinaz Betül Çelik, Yurdanur Süllü;* ***Design:*** *Ayfer Kamalı Polat, Nurten Kara, Şengül Tural, Yurdanur Süllü;* ***Control/Supervision:*** *Ayfer Kamalı Polat, Nurten Kara;* ***Data Collection and/or Processing:*** *Ayfer Kamalı Polat, Yurdanur Süllü;* ***Analysis and/or Interpretation:*** *Ayfer Kamalı Polat, Zülfinaz Betül Çelik, Şengül Tural;* ***Literature Review:*** *Ayfer Kamalı Polat, Zülfinaz Betül Çelik;* ***Writing the Article:*** *Zülfinaz Betül Çelik, Ayfer Kamalı Polat, Nurten Kara;* ***Critical Review:*** *Ayfer Kamalı Polat, Nurten Kara, Şengül Tural;* ***References and Fundings:*** *Ayfer Kamalı Polat, Nurten Kara.*

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