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The frequencies of c-kit proto-oncogene (*KIT*) mutations in gastrointestinal stromal tumours: a population based study from Aegean Region of Turkey

[Gastrointestinal stromal tümörlerde c-kit proto-onkogeni *(KIT)* mutasyon sıklıkları: Türkiye'nin Ege Bölgesi kaynaklı populasyon çalışması]

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ABSTRACT

Objectives: The c-kit proto-oncogene *(KIT)* exon 9, 11, 13 and 17 activation mutations are associated with clinical behaviour in gastrointestinal stromal tumors with highly variable biological behaviour. The purpose of this study is to determine the frequency of *KIT* gene exon 9, 11, 13 and 17 mutations and associate them with histopathologic features for the first time in the Turkish population.

Materials and Methods: DNAs were isolated from formalin fixed paraffin embedded tumour tissues of sixty patients diagnosed with gastrointestinal stromal tumor between 2006 and 2010, by spin column method. Isolated DNAs were sequenced by DNA sequencing system based on capillary electrophoresis for the presence of mutations in *KIT* gene exons 9, 11, 13 and 17.

Results: *KIT* mutations frequencies determined for exon 9, 11, 13 and 17 were 8.3%, 47.5%, 11.7% and 1.7%, respectively. Correlation analysis revealed the presence of overall mutations and exon 11 mutations of males to be higher than those of females (p=0.004 and p=0.003). Small intestine tumor mutation frequency was higher than gastric tumors (p=0.05).

Conclusion: This study represents the first mutational results from Turkish gastrointestinal stromal tumor patients. Our cases showed different mutational trends when compared to other reports, indicating the significance of personalized medicine, which could be intensified by the use of genotyping tools in cancer management. Redefining the molecular features associated with tumor behaviour is important for management of diagnostic and therapeutic processes in gastrointestinal stromal tumors.

Keywords: Gastrointestinal stromal tumors, *KIT* gene mutations, DNA sequencing, pharmacogenomics.

Conflict of Interest: Authors declare no conflict of interest.

ÖZET

Amaç: Biyolojik davranışın oldukça değişken olduğu gastrointestinal stromal tümörlerde c-kit proto-onkogeni *(KIT)* ekzon 9, 11, 13 ve 17 aktivasyon mutasyonları tümörün klinik davranışı ile ilişkilidir. Bu çalışmanın amacı Türk popülâsyonunda ilk kez gastrointestinal stromal tümörlerde *KIT* geni ekzon 9, 11, 13 ve 17 mutasyonlarının dağılımlarının belirlenmesi ve histopatolojik özellikler ile ilişkilendirilmesidir.

Gereç ve Yöntemler: 2006-2010 yılları arasında gastrointestinal stromal tümör tanısı almış altmış hastanın formalinle fikse edilmiş parafine gömülmüş tümör dokularından, spin kolon yöntemi kullanılark DNA izole edilmiştir. Elde edilen DNA'lar kapiller elektroforez tabanlı DNA dizileme sisteminde *KIT* geni ekzon 9, 11, 13 ve 17 mutasyonlarının varlığı açısından dizilendi.

Bulgular: Olguların *KIT* geni ekzon 9, 11, 13 ve 17 mutasyon sıklıkları sırasıyla % 8.3, % 47.5, % 11.7 ve % 1.7 olarak saptandı. Klinikopatolojik özellikler ile mutasyon durumu arasındaki ilişki analizine göre, genel mutasyon ve ekzon 11 mutasyon varlığı erkeklerde kadınlara göre anlamlı olarak fazlaydı (p=0.004 ve p=0.003). İnce bağırsak tümörlerinde mutasyon oranı da mide tümörlerine göre fazlaydı (p=0.05).

Sonuç: Sonuç olarak bu çalışma Türk gastrointestinal stromal tümör olgularının moleküler sonuçlarının sunulduğu ilk çalışmadır. Olgularımız diğer çalışmalarla karşılaştırıldığında farklı mutasyon eğilimlerine sahiptir ve bu durum kanser yönetiminde genotipleme araçlarının kullanılması ile yoğunlaşan bireyselleştirilmiş tıbbın önemini açığa çıkarmaktadır. Gastrointestinal stromal tümörlerde tümör davranışı ile ilişkili moleküler özelliklerin tanımlanması tanı ve sağaltım süreçlerinin yönetiminde önemlidir.

Anahtar Kelimeler: Gastrointestinal stromal tümörler, *KIT* gen mutasyonları, DNA dizileme, farmakogenomik

Çıkar Çatışması: Yazarlar çıkar çatışması olmadığını beyan ederler.

Introduction

Gastrointestinal stromal tumours (GISTs) are the most frequent mesenchymal tumours of gastrointestinal tract in humans [1, 2]. GISTs can arise at any location of the gastrointestinal tract from the oesophagus to the rectum [3, 4]; this tumour group is thought to arise from interstitial cells of Cajal (ICC) or their precursors in the gastrointestinal tract [5]. Immunohistochemically, c-kit proto-oncogene (KIT) over-expression is a phenotypic tool for differentiating GISTs from other soft tissue sarcomas, since 90% of GISTs are positive for KIT [6]. GISTs are molecularly characterized by mutations in *KIT* oncogene. *KIT* has located in the long arm of chromosome 4 (4q11–12) and expressed the 145-kD KIT receptor tyrosine kinase (transmembrane glycoprotein), which is a receptor for stem cell factor (SCF). When the SCF binds to KIT, this binding induces receptor dimerization and activation of the signal pathways responsible for cell survival, proliferation, differentiation, adhesion and cellular escape from apoptosis [7, 8]. KIT function is crucial for the development of the ICC, hematopoietic progenitor cells, mast cells and germ cells. Mutations in this oncogene, resulting in constitutive phosphorylation, activation of receptors and signal transduction for cell survival or proliferation, are responsible for 90% of GISTs. There is a wide range of *KIT* mutations in GISTs varying from 20% to 80% [9- 11]. Most of the mutations located in exon 11 and in exon 9, encoding the juxtamembrane domain and extracellular domain, respectively. The mutations in exons of *KIT* 13 and 17 which encode for tyrosine kinase domains 1 and 2 are rare [12].

The prognostic role of different types of mutations is still controversial. Several studies have discussed that some specific type of *KIT* mutation, rather than the presence or absence of any, predicts GIST outcome. The KIT protein extracellular domain mutations cluster in exon 9 region and these mutations seem to define a specific subset of GISTs with unfavourable course [13].

Exon 11 mutations have been shown to manage ligand-independent phosphorylation of the tyrosine kinase of KIT. These include a spectrum of deletion/deletion-insertions, single nucleotide substitutions and duplications. *KIT* gene exon 11 mutation status in GISTs has been studied and demonstrated a relationship between presence of mutations and malignant tumor behaviour [14- 16]. Especially for exon 11, deletions indicate worse prognosis than missense mutations and the detection of Tyr557_Lys558del in GISTs predicts unfavourable outcomes [17, 18].

KIT tyrosine kinase subunit mutations have been defined in exon 13, which encodes the "First tyrosine kinase (TK1)-ATP binding" domain of the KIT protein and in exon 17 encoding the "Second tyrosine kinase (TK2)-enzymatic loop" domain in GISTs. In untreated GISTs, *KIT* exon 13 and exon 17 mutations were only reported sporadically [19, 20]. However, at the same time, these domains can be affected by secondary mutations during imatinib mesylate therapy and acquired resistance to this therapy [21].

Imatinib mesylate (Glivec/Gleevec, Novartis, Basel, Switzerland), a selective inhibitor of RTKs, is used in the treatment of unresectable and metastatic GISTs. It targets KIT's ATP binding site and inhibits phosphorylation of downstream intracellular signalling molecules [22]. Some studies have demonstrated the significance of *KIT* molecular status in the imatinib therapy response. In particular, exon 11 *KIT* mutations are more likely to respond to imatinib therapy than those with either exon 9 *KIT* mutations or undetectable mutations [9, 11].

The incidence of *KIT* gene mutations in GISTs is unknown in Turkish population. The molecular analysis of *KIT* gene becomes important in determining which patient will positively respond to standard imatinib treatment when different genotypic variants give rise to different drug responses and prognosis. The aim of this study is to identify the frequency of *KIT* gene mutations in a series of Turkish GIST patients and to associate them with histopathologic features for the first time in the Turkish population.

Materials and Methods

Tissue samples

Sixty formalin-fixed paraffin-embedded (FFPE) tumor tissues which were diagnosed as GISTs were retrieved from archive (2006–2010) of the Pathology Department of Dokuz Eylül University Hospital, İzmir, Turkey. From each FFPE tissue sample containing at least 75% of tumor tissue, 5 micron sections were dissected into micro centrifuge tubes using a sterile needle. Tumor tissues were analysed by comparison of blood samples.

DNA isolation

Genomic DNA was extracted from 5 micron sections from each sample using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according the manufacturer's instructions. Tumor tissue sections were deparaffinised by a serial extraction with xylol, ethanol and allowed to dry in air, and were digested with proteinase-k treatment.

KIT mutation analysis

KIT gene exons 9, 11, 13, and 17 were screened for known and unknown mutations by using the polymerase chain reaction (PCR) amplification (Tag DNA Polymerase, dNTPack 4x250U, Roche Diagnostics GmbH Mannheim, Germany) with specifically designed primers. PCR amplicons were purified from excessive primers and nucleotides (Roche High pure PCR Product Purification Kit, Roche Diagnostics GmbH, Mannheim, Germany). Purified amplicons were sequenced in both directions by using DTCS-Quick Start Kit (Beckman Coulter, USA) with the same primer sets and Genome-

Lab™ GeXP Genetic Analysis System (Beckman Coulter, USA). The generated DNA sequences were analyzed by using GenomeLab Software, version 5.1 (Beckman Coulter, USA) and specific bioinformatics tools for DNA sequencing chromatograms analysis. Analytical validation was performed by comparing three laboratories' DNA sequencing results. The numbering of specific mutations and SNPs has been referenced according to human *KIT* gene sequences (ENST00000288135) from Ensemble Database http://www.ensembl.org.

Statistical analysis

SPSS (Version 19.0; SPSS, Inc., Chicago, IL, USA) program was used for statistical analysis. The following parameters were analysed: age at diagnosis, gender, tumour location, cell type and molecular status. The association of clinicopathologic variables with the presence of mutation was tested by chi-square test. P value <0.05 was considered to be significant.

Ethics statement

The study was based on pathological archive material approved by Clinical Research Ethics Committee of Dokuz Eylul University School of Medicine (Ethics committee approval date: 17.09.2009, protocol number: 207/2009).

Results

Patient demographics and tumour characteristics

In this study, we analysed sixty patients diagnosed with GISTs between 2006 and 2010. Among sixty patients enrolled in the study, twenty-eight patients were male and thirty- two were females. Median age at diagnosis was 61 years (range 20-86). The most prevalent tumour location was the stomach (45%, 27/60) followed by the small intestine (26.7%, 16/60), esophagus (5%, 3/60), omentum (5%, 3/60), large intestine (3.3%, 2/60) and other sites (15%, 9/60). The median tumour size was 5 cm (range 0.3-35 cm). The most common cell type was spindle (78.3%, 47/60) followed by mixed types (13.3%, 8/60) and epitheloid (8.3%, 5/60).

Molecular results

KIT mutation analysis showed that thirty-five of sixty GISTs (58.3%, 35/60) presented *KIT* activating mutations at any four exons (exon 9, 11, 13 and 17). All detected mutations were heterozygote. The number of mutations in exon 11 was remarkably higher than those of others.

Five cases showed mutation in exon 9 (8.3%, 5/60). The exon 9 nucleotide alterations consisted of Ala–Tyr insertions between codons 503 and 504 in two GIST cases (3.3%, 2/60) (Figure 1) and single nucleotide substitutions (Q460H and S451C) in three case (5%, 3/60). Q460H (c.1380G>C) is one of these two single nucleotide substitutions. It is a novel nucleotide alteration, which could not found in the scientific literature, and is identified in two cases.

Twenty- eight cases showed mutation in exon 11 (47.5%, 28/59). One patient's exon 11 PCR was unsuccessful; therefore, patient number was accepted as fifty-nine for exon 11 frequency calculations. Among the exon 11 mutations, we observed single nucleotide substitutions in twelve tumors (20.3%, 12/59), deletions in fourteen tumours (23.7%, 14/59) (Figure 2), both deletion and substitution in one (1.7%, 1/59) and in-frame insertion in one tumor (1.7%, 1/59). Most of the exon 11 mutations were located between codons 550 and 580, called as "hot spot region".

Seven cases showed mutation in exon 13 (11.7%, 7/60). All of them were single nucleotide substitutions. Five cases had K642E substitution and two of these were associated with another substitution N649D (c.1945A>G), which is a novel nucleotide alteration in the literature. Two cases had P627L substitution (Table 1).

Only one case showed mutation in exon 17 (1.7%, 1/60). The same case presented two mutations A795P and D820A. The A795P mutation was a novel nucleotide alteration in the scientific literature (Table 1).

The statistical analysis was performed for correlation of clinic pathological parameters (gender, tumor location, cell shape and age) with KIT gene mutation status.

KIT gene mutations and *KIT* gene exon 11 mutations were observed in 78.6% (22/28) and 67.9% (19/28) of all male patients, respectively. Overall *KIT* gene and exon 11 mutation frequencies were lower in females than in males. *KIT* gene and exon 11 mutations in females were 40.6% (13/31) and 29% (9/31) respectively. One female's exon 11 PCR was unsuccessful, therefore female patient number was accepted as thirty-one for exon 11 frequency calculations. Overall mutations and exon 11 mutations in males was higher than those of females (p=0.004 and p=0.003).

Study of the correlation between stomach-small intestine localized tumours and mutation status revealed frequency of small intestine tumors to be higher than stomach tumours (p=0.05). *KIT* gene mutations were identified in 48.1% (13/27) of all stomach localized tumours and 81.3% (13/16) of all small intestine localized tumours.

The morphology of the cell types and mutations in the exons were examined, mutation was detected in 57.4% (27/47) of all the spindle-cells, 80% (4/5) of all epitheloid cells and 50% (4/8) of all mixed type tumors. No significant correlation was found between cell shape and mutation status.

Discussion

In recent years, researches are focused in cancer therapy, emphasizing the inhibition of tyrosine kinases that play an important role in the pathogenesis of cancer. In this context, *KIT* gene in the pathogenesis of GISTs and

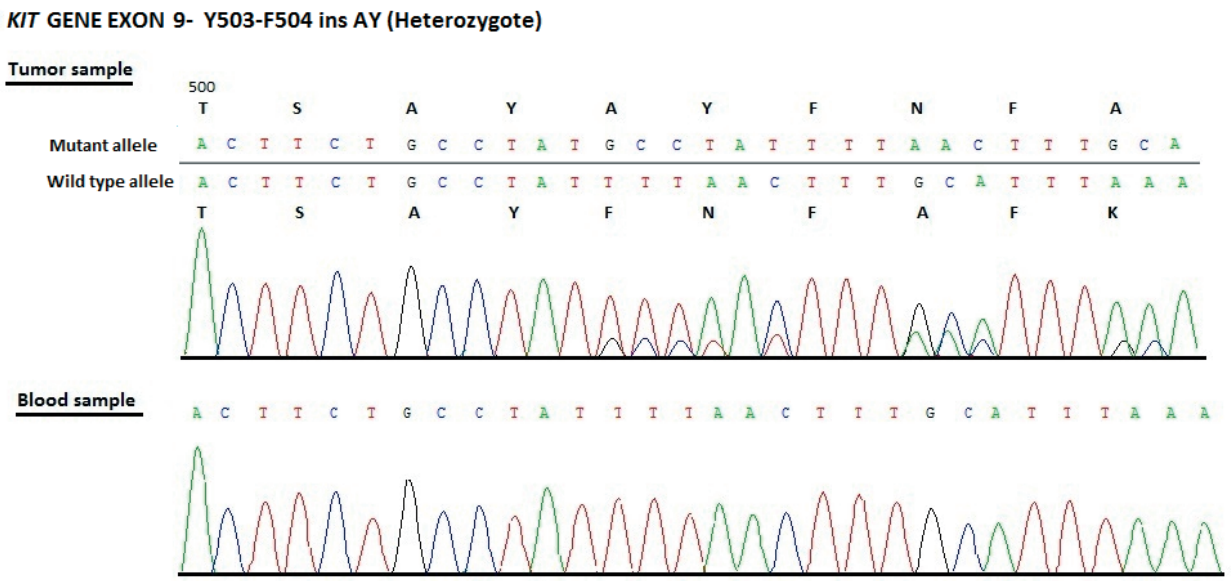

Figure 1. *KIT* gene exon 9- Y503-F504 ins AY (Heterozygote) mutation. The most common exon 9 nucleotide alterations consisted of Ala–Tyr amino acids insertions in GISTs. Six nucleotides were inserted between codons 503 and 504.

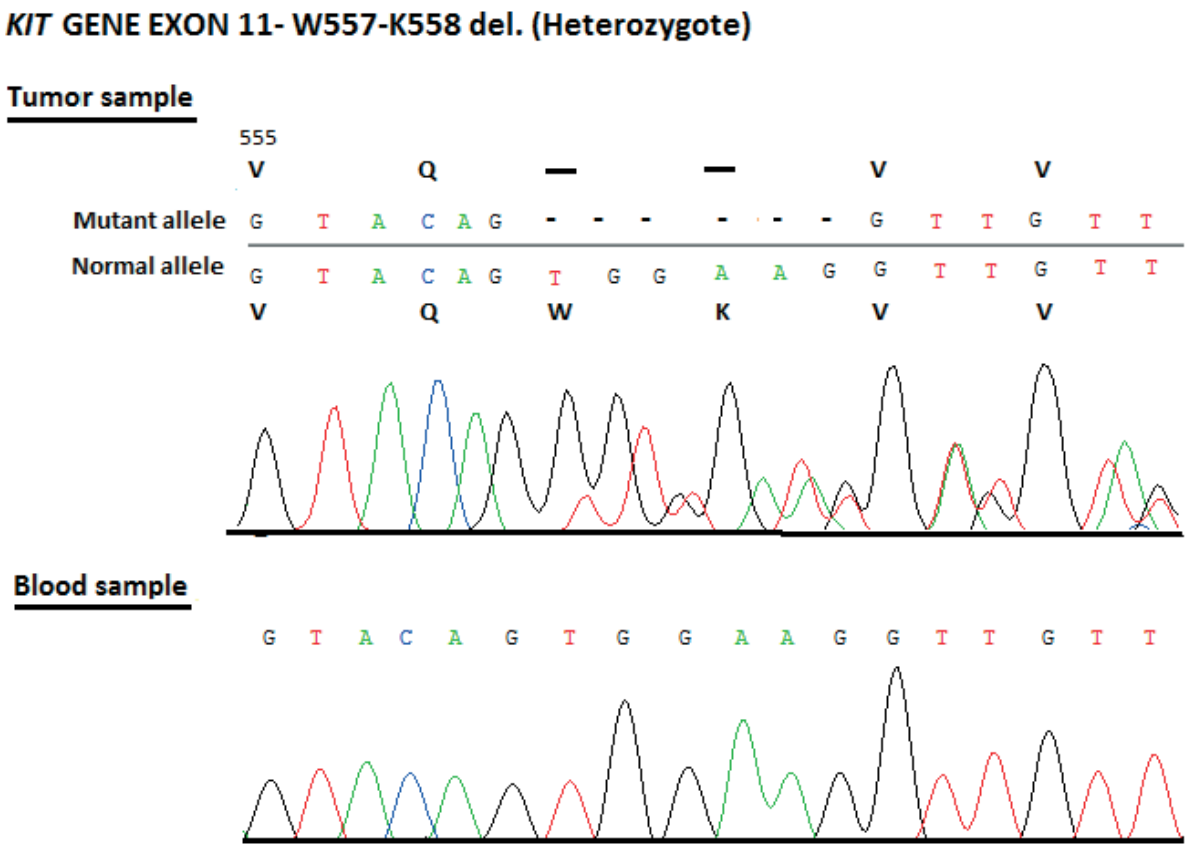

Figure 2. *KIT* gene exon 11, W557-K558 deletion (Heterozygote). The most common exon 11 nucleotide alterations consisted of Tyr- Lys deletions in GISTs. Six nucleotides were deleted at codons 557 and 558.

Table 1. Distribution of *KIT* mutations in gastrointestinal stromal tumours according to different populations.

***KIT* mutations**	**Mutation frequency %**	**Mutation frequency %**	**Mutation frequency %**	**Mutation frequency %**	**Mutation frequency %**	**Mutation frequency %,**
	Current study (mutant case / total case)	**Norwegian study[26]† (n:89)**	**Canadian study[24]† (n:54)**	**Italian study[6]† (n:104)**	**Portuguese study[5]† (n:78)**	**Korean study[27]† (n:86)**
Mutant- Total	58.3% (35/60)	75.3%	74%	79%	56%	74.4%
Exon 9	8.3% (5/60)	4.5%	19%	11%	9%	3.5%
Substitution	5% (3/60)					
Duplication	3.3% (2/60)					
Exon 11	47.5% (28/59*)	65.2%	52%	67%	91%	70.9%
Deletion	23.7% (14/59)					
Deletion + substitution	1.7% (1/59)					
Substitution	20.3% (12/59)					
Insertion	1.7% (1/59)					
Exon 13	11.7% (7/60)	3.4%	4%	No mutation	No mutation (1 silent mutation)	No mutation
Substitution	11.7% (7/60)					
Exon 17	1.7% (1/60)	1 case	No mutation	1%	No mutation (1 silent mutation)	No mutation
Substitution	1.7% (1/60)					

*One patient's exon 11 PCR was unsuccessful, therefore patient number was taken as fifty-nine for exon 11 frequency calculations. †Reference numbers.

imatinib, a tyrosine kinase inhibitor, has been one of the most evident targets in the management of cancer. However, observations and studies conducted in the years indicated changes in tyrosine kinase inhibitor response due to KIT protein mutations. Therefore, *KIT* gene activation mutations are further studied [5, 23].

In literature, the identified *KIT* gene mutations are variable. In our study, 58.3% (35/60) of our cases have any mutation in the *KIT* gene exon 9, 11, 13 or 17. Generally, this frequency was reported to be between 66.9% and 78% [13, 21, 23-25]. The reason of variable mutation frequencies might have been caused by methodological differences (pathological or molecular methods) or ethnicity. Since there is no multi-national study about mutations in the *KIT* gene and ethnicity, it is difficult to evaluate the effects of the ethnicity on the mutations. On the other hand, there are lots of articles about analysis of *KIT* mutation in a population based study design. In some population based studies, mutation frequencies were as follows: 75% in a Norwegian study, 79% in an Italian study, 56% Portuguese study and 74.4 % in a Korean study [5, 6, 26, 27]. Depending on these results, it might be said that there is no relationship between mutation frequencies and geographical variances. This study is important to be the first of its kind in Turkish patients.

We identified different mutation frequencies in the exons. Exon 9 of *KIT* gene encodes extracellular subunit of KIT protein, which takes part in receptor dimerization. In literature, the exon 9 mutation frequencies are reported to be between 5% and 18% [19, 25, 28]. We found exon 9 mutation frequency as 8.3% (5/60) and this result was lower than the other studies in literature [6, 24]. Approximately 95% of the exon 9 mutations are associated with small intestine localized tumors. Antonescu et al. identified the relationship between mutations in exon-9 and small intestine localized aggressive tumors [13]. Kontogianni-Katsarou et al. reported two Y503-F504insAY cases in small intestine with malign characters and they reported spindle shaped cells in their study [29]. We also observed this mutation in our two cases with spindle cell shapes. This mutation mechanism has not been yet clarified; however, some researchers have considered that it may impair the anti-dimerization motif in the extracellular subunit [21].

Exon 11 of *KIT* gene encodes the juxta-membrane subunit of the KIT protein and it provides auto-inhibition of the tyrosine kinases. Mutations in this region form constant auto-phosphorylation. These mutations represent great variation (20-92%) according to the literature. In this study, the mutation frequency in exon 11 was found as 48.3% (28/59). This value is close to the results of Canadian, Italian and Korean studies, their mutation frequencies are 52%, 67% and 70.9%, respectively [6, 24, 27]. However, for exon 11 mutation frequency, no ratio can be given according to ethnic origin. In our study, exon 11 mutations were in well-known "hot spot" region (550-580) as it has been reported in many researches. All of the identified mutations in our study were in this hot spot region. Exon 11 mutations were commonly due to substitutions and deletions. Exon 11 substitutions frequency was determined as 20.3% (12/59) and deletions frequency was 23.7% (14/59). The most common mutation residues in the literature, W557-K558, have great significance for receptor's tyrosine kinase activity or constant phosphorylation, respectively. If there is a mutation at these residues, it might be associated with the carcinogenesis, malignancy potential and poor prognosis [18, 29, 30]. At 557 and 558 residues, the identified mutation frequency was 43% (12/28) of all exon 11 mutant cases.

KIT gene exon 13 region encodes tyrosine kinase subunit-1 of KIT protein. Tyrosine kinase subunits of KIT protein have roles in phosphorylation and signal transduction pathways. Single nucleotide substitutions are mostly reported in *KIT* exon 13 [19]. Especially, 1945A>G mutation, leading to Lys642Glu variation in KIT exon 13 after imatinib treatment, and other exon 13 mutations cause conformational change in imatinib binding site in KIT protein and secondary imatinib resistance. In GISTs exon 13, mutation frequencies were reported as 1.5-4.5% [19, 25, 28]. In our study, the frequency of mutations determined in exon 13 of *KIT* was 11.7% (7/60) among all cases. Considering population based differences for exon 13 mutations; the frequency of exon 13 mutations in Norwegian and Canadian studies were 3.4% and 4%, respectively [24, 26]. There was no mutation in exon 13 from Italian and Korean based studies [6, 27].

According to our results, Turkish GIST population shows a low mutation ratio at exon 9 and high mutation ratio at exon 13 compared to the studies conducted on Northern European populations. In this context, it might be suggested that exon 9 mutation frequency is low, but exon 13 mutation frequency is high for GISTs in Aegean region of Turkey. According to this approach, Northern Europe population can have more aggressive GISTs (due to high exon 9 mutation frequency) and Aegean Turkish population can have possibility of secondary resistance to clinical therapy (due to high exon 13 mutation frequency). In this respect, it is an important issue on the cancer management for oncologists that *KIT* gene exon 17 region encodes tyrosine kinase subunit-2 of the KIT protein. In literature, the mutation frequencies for exon 17 were between 1.5 and 4.5% [19, 28]. Mostly, 2485A>C or 2487T>A substitutions, leading to Asn822Lys and Asn822His variations, are reported [19]. In our study, only one case (1.7%, 1/60) had mutation in exon 17. This case had two mutations, and both of them were single nucleotide substitutions. Only one mutation in exon 17 was reported in a Norwegian and an Italian based studies [6, 27]. There was no mutation in exon 17 in a Korean based study [27]. Our results support the other population based studies in literature.

Comparing stomach and small intestine localized tumours, the general *KIT* gene mutation frequency was significantly higher in patients with small intestine localized GISTs than stomach localized GISTs ($p<0.05$). There

was no significant correlation between tumour location and the mutation status in most studies [31, 32]. Mostly exon 9 mutated tumours were located in small intestine [19]. Similar is valid in patients with mutations at exon 17. In most of the studies, exon 11 mutations were more frequent in stomach localized tumours [13, 18, 27, 28, 33, 34]. The anatomical distribution of kinase mutations in GISTs suggests various ICC populations in different parts of the digestive system due to their biology and kinase dependence.

According to the gender and *KIT* gene mutation correlation analysis, the presence of overall mutations and exon 11 mutations of males were significantly higher than females (p=0.004 and p=0.003). In Turkey, smoking and alcohol consumption of females is relatively rare. This can be the main reason of lower *KIT* mutation frequency in females. The other reason can be the possibility of different ICC populations between females and males. If females and males have different ICC populations, male's tendency to the mutations may be more than females. These possible reasons may affect the carcinogenesis process. In this respect, an etiological study can be designed to find reasons behind the GIST carcinogenesis differences between females and males. There was no significant correlation between gender and the mutation status in Portuguese (as a Mediterranean country) and Korean (as an Asian country) based studies [5, 27]. In the literature, there have been studies about the association of *KIT* gene and imatinib response. According to these results, *KIT* exon 11 tumours gives the best response, exon 9 tumours show an intermediate response and wild-type GISTs (no mutations in *KIT* or *PDGFRA* gene) have a poor response [25]. Our result may suggest that therapeutic responses may be different in males and females, and reasons and explanation of this difference need studies with larger groups.

In summary, this study represents the first mutational results from Turkish GIST patients. In this study, different mutation frequencies were determined and compared to other population studies. *KIT* gene mutation frequency was 58.3%, indicating that the frequency of *KIT* gene mutations in Turkish GIST patients is lower than other population based studies. *KIT* gene molecular status has been the most important predictor of how GISTs will respond to imatinib therapy. This condition exhibits the significance of personalized medicine, which could be intensified by the use of genotyping tools in the cancer management. This mutation analysis has a prognostic value because of indicating the sensitivity of treatment. More studies are needed to understand GIST pathogenesis in our population.

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