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Oncogene mutational analysis in imatinib naive population of gastrointestinal stromal tumor patients

Abdul Wahab Ali Abuderman[1*], Fahad M. Aldakheel[2]

[1] Department of Basic Medical Science, College of Medicine, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia
[2] Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

[*]**Correspondence to:** addulwahab.research1@gmail.com; aaabuderman@rediffmail.com

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Abstract: There are very scanty reports on gastro-intestinal stromal tumors (GISTs), a very common tumor of mesenchymal cells in GIT track primary resistance to imatinib. This comprehensive study identifies the prevalence, clinical presentation and GIST genotype association in the imatinib naïve population. Prospectively a record of anthropometric, baseline demographic data and clinical details for the patients diagnosed with GIST were scrutinized. Pathological information included the presence or absence of necrosis, tumor size, mitotic counts, immune-histochemical staining for CD 34, CD 117 and DOG1 was performed using biopsy sample. Selected exon genes of KIT, *PDGFRA* and *BRAF* were amplified and subjected to mutation analysis by direct sequencing. Appropriate statistical analyses were performed. The male/female ratio was 1.8:1 among 54 patients with GIST. The mean GIST size was comparatively bigger in females (2.49±0.855) than males (2.26±1.13). The stomach was the most common site for GIST followed by the Small bowel and rectum. The majority of the tumours were spindle cell. This study reports 12 different types of mutation among 39 KIT, 8 PDGFRA and 7 BRAF mutations. In KIT, the most prevalent was exon 11 mutation with the KITdelinc557/558 (14/30) being the major exon 11 type mutation. In PDGRFA, five exons 18 with p.D842V substitution and three exons 12 deletion mutation was reported. Seven patients had strong or diffuse BRAF staining having V600E type mutation as major BRAF type mutation. Drug-resistant GIST due to acquired mutations remains a serious issue, therefore genetic information of such mutational related to drug-resistant may provide the imperative clue for diagnosis and clinical treatment. These mutations are pivotal for prognosis and associated with imatinib as not all of them but only a few are reported resistant to the imatinib.

Key words: Gastrointestinal stromal tumors (GISTs); Imatinib; Mutation KIT; PDGRFA; BRAF.

Introduction

Gastrointestinal stromal tumors (GISTs) are very common tumors presented by mesenchymal cells of the GI tract (1). The usual and standard treatment of GIST is localized surgical resection and adjuvant imatinib is used to diagnose the high-risk GISTs (2). Imatinib works selectively to inhibit certain tyrosine kinases, particularly KIT (receptor tyrosine kinase gene), PDGFRA (platelet-derived growth factor receptor alpha), and ABL kinases (3). According to studies, 10-14% GISTs are primarily resistant to imatinib which likely to be dependent on the GIST genotype derived from mutation (4, 5). Most of the GISTs mutation are in *KIT* or (*PDGFRA*) which causes the activation or deactivation of downstream genes, including PI3K/AKT/mTOR and RAS/RAF/MAPK and therefore playing an essential role in the progression of GISTs (6, 7). The mutant KIT activates multiple downstream signals, therefore, these mutations not only control the biological behavior and clinical outcome of GISTs, but also characterize its risk category, and response to the drug (8, 9). Earlier studies indicated that *KIT* and *PDGFRA* mutations are initial events in GIST development, and malignant progression is then caused due to chromosomal aberrations accumulation. GISTs can be termed as true wild-type ones if they do not possess any major modifications in SDH family genes or *KIT, PDGFRA*, RAS signaling genes. In line with KIT, PDGFRA is also a member of the receptor tyrosine kinase family member. This PDGFRA contributes to cell viability by ERK-based stabilization among *KIT*-mutant GISTs (7, 8, 9). The studies conducted recently exposed the gene-level modifications of other tumor-related genes in GISTs, in addition to mutations found among well-known key driver genes inclusive of PDGFRA and KIT. For example, primary GISTs record about 0.93% (3/323) level of *EGFR* mutations and there is no overlapping found with that of the mutations in *KIT*, *PDGFRA*, *KRAS* or *BRAF.* There is an association exists between EGFR mutations and few scenarios such as stomach location, low recurrence rate and female gender. GIST case was reported with *PIK-3CA* mutation (p.H1047L), when there was a deletion of *KIT* exon 11 (10, 11) observed. Leaving behind PDGFRA and KIT, the mutations that occur in neurofibromin 1 (*NF1;* tumor suppressor gene) and succinate dehydrogenase (SDH) heterotetramer pose heavy threats in the progression of GISTs. RAS family gene mutations, as well as BRAF mutations, seem to play a similar role yet their frequency of occurrence in GISTs is less (9, 12, 13).

In case of low risk, surgical resection method is used

to treat the localized GIST while in case of heavy-risks, imatinib is utilized since it is a selective inhibitor of KIT, PDGFRA, and other kinases. Imatinib is mainly dependent on the GIST genotype yet it is unfortunate to know that >14% of GISTs exhibit primary resistance against imatinib (14). Imatinib faced secondary resistance within two years of treatment among 40% of GIST patients (15). There is an association exists between the tyrosine kinase mutations and the response of GIST to imatinib, and it is inclusive of mutations that occur in PDGFRA exons 12, 14, and 18 as well as KIT exon 9, 11, 13, and 17 (5, 6, 7, 14). But, these studies could not be generalized and remain inconclusive due to the low number of samples considered for the study. Further, these studies were reported only from developed countries while negligible or no such data has represented Saudi Arabia in terms of clinical and oncological perspectives of GISTs. The current comprehensive study portrays both these aspects of GISTs to expose the GIST genotype association in imatinib naïve population suffering from gastrointestinal stromal tumor patients and its clinical association.

Materials and Methods

This present work was designed and approved by the institutional review board before the study was actually executed. The study was performed prospectively on enrolled patients diagnosed with GIST, between January 2015 to December 2018. Only patients who were first time diagnosed with GIST were included in this study and patients with earlier exposure to the treatment of GIST were excluded. So, the study population was naïve to imatinib treatment. Patients were taken into the study only after they were ready to be enrolled for the study and provided their consent in writing. GIST was diagnosed based on endoscopy findings, clinical presentation, anatomical site and biopsy results. At the admission time history of all the patients was taken which include anthropometric details such as age, sex, clinical presentation, adjuvant therapy, medical history and type of surgical resection. A thorough clinical examination was done and an expert pathologist reviewed tumours for verification of diagnosis. The authors recorded the pathological data which was inclusive of mitotic counts, necrosis whether present or absent and size of the tumor. The authors conducted the standard hematoxylin and eosin staining with precise immune-histochemical staining for CD 34, CD 117 and DOG1. These staining procedures were conducted on a 4 mm section (Ventana Benchmark Ultra automated immunostainer; Ventana Medical Systems Tucson, AZ) taken from paraffin-embedded tissue that was fixed onto the formalin freshly (16, 17). The complete set of procedures, prior to staining, was automated into the system. The slides were kept under incubation with corresponding primary antibody i.e., mouse monoclonal VE1; Spring Bioscience, Pleasanton, CA. at a dilution of 1:150 for about 15 minutes at 37°C. The researchers used two kits such as VMS Opt iView DAB detection kit and VMS OptiView Amplification kit to localize the antigen-antibody complex. Standard grades of staining were set such as moderate/strong (stained in intermediate to dark brown), weak (stained in pale brown stronger than the background staining in smooth muscle) and negative (no staining at all). Slide grading was performed by two experts while a third expert gave the final solution in case of discrepancies in the former.

The DNA extraction was based on formalin-fixed tumor samples using the QIAamp DNA mini kit (a tissue DNA extraction kit) and quantified using a hybrid reader (Nanodrop). Selected exon genes of KIT, *PDGFRA* and *BRAF* were amplified using PCR and using primer sequences and PCR conditions as detailed in Data Table 1, as described by Patil et al. 2015 (18).

The PCR reaction contained 50 ng genomic DNA in

Table 1. Primer sequences details used in this study.

Gene	Exon	Primer	Sequence
KIT	8	forward	GACATATGGCCATTTCTGTTT
KIT	8	reverse	GAATCCTGCTGCCACACATT
KIT	9	forward	GCACAATGGCACGGTTGAAT
KIT	9	reverse	GAGCCTAAACATCCCCTTAAATTGG
KIT	11	forward	CCAGAGTGCTCTAATGACTG
KIT	11	reverse	CTCAGCCTGTTTCTGGGAAA
KIT	13	forward	GGAAGCCCTCATGTCTGAAC
KIT	13	reverse	ACACGGCTTTACCTCCAATG
KIT	17	forward	TCGGATCACAAAGATTTGTG
KIT	17	reverse	GCAGGACTGTCAAGCAGAGA
KIT	18	forward	TGTTCAATTTTGTTGAGCTTCT
KIT	18	reverse	CCAGACGTCACTTTCAAACG
PDGFRA	12	forward	GAAACCGAGGTATGAAATTCG
PDGFRA	12	reverse	TCTTGGAAACTCCCATCTTGA
PDGFRA	14	forward	GGCCAGATCCAGTGAAAAAC
PDGFRA	14	reverse	TCAGTGAGCCCACCTGACTT
PDGFRA	18	forward	CTCCTGGCACAAGGAAAAATT
PDGFRA	18	reverse	GTGAGGGAAGTGAGGACGTA
BRAF-F		forward	CTTCATGAAGACCTCACAGTAAAAATAGG
BRAF-R		reverse	TAGCCTCAATTCTTACCATCCACAAA

sterile distilled water, 0.2 µM of each primer, and PCR *Taq* master mix (Amplicon). The authors used Big Dye Terminator V.3.1. manufactured by Applied Biosystems, Foster City, California, to directly sequence all the exons in ABI Prism™ 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) so as to perform mutation analysis for the amplified DNA products. The sequences were then assessed with the help of mutation taster and Seq scape analysis software V2.5.

Statistical calculations were performed in SPSS Statistics version 19 (Chicago, IL, USA). Quantitative (mean ± standard deviation), and qualitative variables (frequencies; %) were presented. Significant variations were analysed using Pearson chi-square and Student's t-test was used to assess qualitative variables. Probability values (P values) ≤0.05 were taken as statistically significant.

Results

The present study was for a period of three years, 2015 to 2018 and all the patients diagnosed with GIST histologically and immunohistochemically were included in the study. During the study period, a total number of 54 proven cases of GIST were analyzed for the objective. The mean age of the study group was 56±7.9 years. Male were 65% of the study population while the male to female ratio was 1.8:1. The mean age of males (57±7.9 years) and females (55±7.6 years) were almost identical. The mean GIST size was 2.34±1.04 cm, comparatively bigger in females (2.49±0.855) than males (2.26±1.13) though not statistically significant (Table S1). The stomach (38.9%) was the most common site for GIST followed by the Small bowel (33.3%) and rectum (12.9%). The other two sites of GIST were colon (9.25%) and omentum/pelvis (5.5%). The most common symptoms were abdominal pain (n=33) followed by a gastrointestinal bleed (n=19) (Table 2). The other minor symptoms were weight loss, vomiting, abdominal distension, change in bowel habit, dyspepsia and nine cases were asymptomatic. Five of these cases were multifocal being four in female alone and three cases in the stomach. Two died with high risk and mutation at exon 11 region, were just initiated with imatinib therapy.

Fletcher criteria are generally used to classify the tumors based on size and mitotic count and the classifications are epithelioid type, spindle cell, and mixed type. In the current study, most cases i.e., 39 cases (72%) were spindle cell tumors while 9 cases were epithelioid and 6 cases had mixed type morphology. After segregating the patients under high and intermediate-risk categories, they were made to undergo adjuvant chemotherapy using imatinib mesylate. Surgical resection was performed for 78% (n=42) of the patients while imatinib therapy was rendered to 12 patients. According to the description given by Fletcher et al, the high-risk group GISTs were 22.2% while intermediate-risk, low and very-low risk groups were 24.0%, 40.7% and 9.3% respectively. A total of 3.7% GISTs was categorized under the no-risk category. When there is a strong and/or diffuse cytoplasmic positivity, one can infer that immunohistochemistry staining is positive. All the cases showed positivity in the case of immunohistochemistry staining for CD117. In the case of CD34 and DIG1, 46 (85.2%) cases showed positive. As shown in Tables 2 and 3, 7 cases (13%) were strong positive while 4 cases (4%) were weakly positive in the case of BRAF immunohistochemistry.

This study witnessed 12 different types of mutation among 39 KIT, 8 PDGFRA and 7 BRAF mutations. All 54 patients had one or another type of mutation. In KIT, the most prevalent was exon 11 (30/39) mutation with the KITdelinc557/558 (14/30) being the major exon 11 type mutation. Among the study population 15 were KIT wild type. There was no significant difference in mean age among different mutations of GIST patients however, exon 9 (A502-Y503 codon duplications) mutation which was the second common KIT type mutation was present in only male patients aged above 60 years (Table 2). In PDGRFA, five exons 18 with p.D842V substitution and three exons 12 deletion mutation was reported. All the PDGRFA mutations were seen to have spindle-shaped morphology and the risk factor was either low or very low. All exon 12 were seen in male patients. Seven patients had strong or diffuse BRAF immunohistochemistry staining and all were BRAF mutated having V600E type mutation as major BRAF type mutation. All BRAF V600E was seen in male patients. The other two BRAF mutations were L597S and G464E seen in female patients. The remaining week or negative BRAF expression harbored mutations in the KIT exon or PDGFRA exon. Risk factors varied significantly among various mutations and results indicated that KITdelinc557/558, junction deletion (Exon 11) and BRAF were only represented by high-risk GIST patients. The maximum number (10/12) of high-risk patients were exposed to KITdelinc557/558 mutation. Exon 9 and PDGFRA were mostly in the low and very low-risk group (<0.05). The majority of KITdelinc557/558 and BRAF mutation were having spindle-shaped morphology (Figure 1) (=0.09), but this was not significant as spindle-shaped morphology was seen in the majority of patients. The stomach was the primary site of GIST but PDGFRA and junction deletion (KIT, exon 11) were mainly seen in the small bowel site. Gender wise GIST

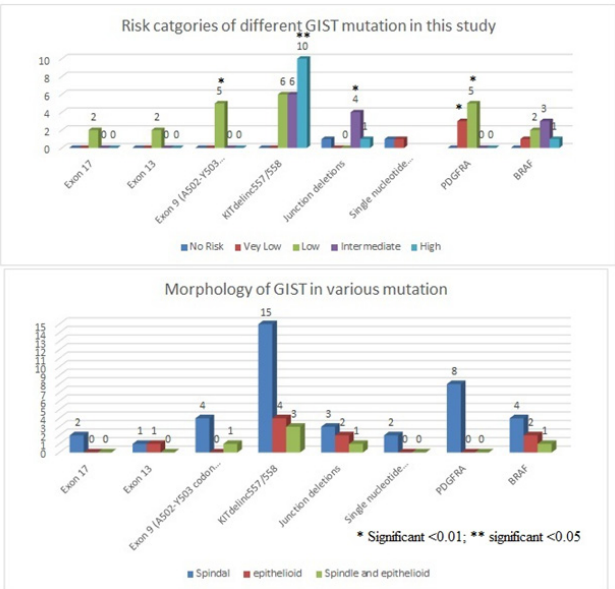

Figure 1. Risk categories of different mutation and their morphology.

Table 2. Clinical characterization of GIST patients.

Age (Mean)		Mean	SD	Significant and p-value
	Male	57.09	7.98	
	Female	54.63	7.61	
Age (Yrs)				
	Mean	56.2	7.6	
	<25	0		
	25-50	11		
	>50	43		
Imatinib therapy				
	Yes	12		
	No	42		
Survival				
	Yes	52		
	No	2		
Risk Classification				
	High	12		
	Intermediate	13		
	Low	22		
	Very Low	5		
	No	2		
Morphology				
	Spindle	39		<0.001
	Epithelioid	9		
	Mixed	6		
Mutation		Mutated	WT	
	KIT	39	15	
	PDGFRA	8	46	
	BFRA	7	47	
	WT			
Location		Unifocal	Multifocal	
	Stomach	18	3	<0.001 Significant
	Small bowel*	18	0	
	Colon	4	1	
	Rectum	7	0	
	Omentum/pelvis	2	1	
	*Duodenum (n=12), Jejunum (n=6)			
Treatment				
	Surgical resection	42		
	Imatinib therapy	0		
	Both	12		
Nature				
	Unifocal	49		
	Multifocal	5		

distribution was uniform among the various site and no differences were seen in morphology based on GIST location (Figure 2). High-risk patients were equally distributed among males (n=6/35) and females (n=6/19) although male patients dominated the study population. High-risk GIST had spindle-shaped morphology in the majority (<0.05), and KITdelinc557/558 as a major mutation type (<0.05) (Figure 3; Table 3).

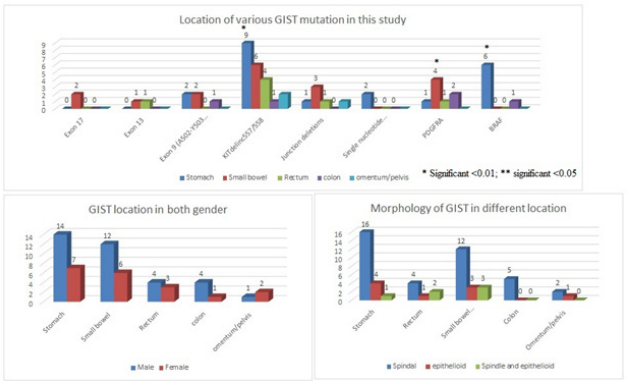

Figure 2. Location of GIST depending on the mutation, gender and morphology at a different location.

Discussion

With the advancement in molecular biological and computer-based bioinformatics analysis of pathogenesis, there is an increased understanding of GISTs and their novel alterations that are potentially related to GIST progress. Drug-resistant GIST due to acquired mutations remains a serious issue, therefore genetic information of such mutational related to drug-resistant may provide the clue for clinical diagnosis and treatment. In this present research work, we prospectively analyzed the mutational status of imatinib related kinases like KIT and factors such as PDGFRA. During the study period, 12 different mutations in all 54 samples, with an overall mutation frequency of 100%. No patients were having two different types of mutation at the same time. This Study is quite very similar

Table 3. Clinical characterization of a different mutation in GIST.

			Age (Mean+SD)	Male	Female	GIST Size (Cm)
KIT	Exon 11	Exon 17	52±0	1	1	2.45±0.5*
		Exon 13	52.5±4.95	1	1	1.85±0.5
		Exon 9 (*A502-Y503 codon duplications*)	61.4±7.2**	5	0	1.98±0.7
		KITdelinc557/558	55.3±8.0	12	10	2.98±3.68**
		Junction deletions	54.5±9.5	4	2	1.78±0.68
		Single nucleotide substitutions	54±12.7	2	0	0.55±0.49**
		PDGFRA	56.1±7.27	5	3	1.8±0.78
		BRAF	59.8±8.1	5	2	2.32±1.23*

* Significant <0.01; ** significant <0.05.

to report of 275 GIST cases, among which mutations were identified in 93.8% of the cases (19). Among these mutations, 72.2% were KIT type, 14.8% PDGFRA and 12.96% were BRAF mutations. Earlier studies have reported *KIT* mutations are prevalent in more than 60% of GISTs, while *PDGFRA* mutations are found in 5%–10%, therefore consistent with our findings (20, 21). As expected, the stomach was the most common site for GIST, however, the second most common site was the small bowel almost equally present in the population which was quite infrequent in earlier studies. *KIT* mutations are seen present in various gene regions, including exons 8, 9, 11, 13, 14, 15, and 17 (9, 22). All these domains are at a different place in exon and demonstrate various end coding functions as for example, exon 11 encodes the juxtamembrane domain, exons 8 and 9 encode the extracellular domain, and exons 13 and 17 encode the tyrosine kinase domain (23, 24, 25). Exon 11 mutation was the most prevalent type of KIT mutation in this study with the KITdelinc557/558 the major type of KIT mutation. According to the Polish registry study, KITdelinc557/558 were more frequent (88%) in larger (>5 cm) GISTs stratified as high-risk tumors. (22) The current study saw an overall 40.7% (n=14) KITdelinc557/558 mutation among the study population accounts for 73.3% of exon 11 type mutation. GISTs are generally positive for CD117 (c-kit) and considered to be a sensitive and specific marker, being positive in 95% of GIST. (26) Interestingly all exon 11, type KITdelinc557/558 mutation were positive for CD117, and 95.5% were positive for CD34 and DOG1. The majority of mutation of KIT is reported in exon 11 (>70%), which disrupt auto-inhibition and codon region 557-558 seems to be a hot spot site for mutations likely to promote constitutive activation and disrupt auto-inhibition of KIT leading to a metastatic phenotype (27, 28). Although earlier studies showed that 557/558, deletion-including codon mutations are associated with the high-risk grade of larger tumor size, which is not consistent with this study which showed 72.7% (n=16) were a high and intermediate-risk group with the average size of 2.98 cm (29). The other major exon 11 mutations was junction deletions found in six cases with a GIST size of 1.9 and 80% were in the intermediate-risk group. Single nucleotide substitutions in GISTs at the exon 11 region show lower mitotic activity, indolent phenotype, smaller tumor size, and favorable disease-free survival (9, 22, 30). The present study found only two single nucleotide substitutions mutation with a GIST size of 0.55 cm.

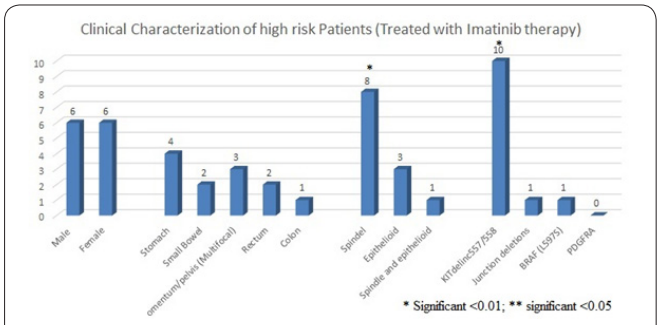

Figure 3. Clinical Characteristic of high-risk patients.

Exon 9 mutation which causes constitutive activation of KIT, is comparatively less frequent (7%). Exon 9 mutation was the second common (5/39) KIT type mutation only identified in male patients.

The overall mutations observed in exon 9 were p.A502_Y503dup type and were characterized by tandem duplication of six nucleotides at 502-503 codon site. These mutations have a close relationship with older age (>60 years), small bowel location, female gender, large tumor size and spindle cell morphology (31)

Similar to this study, we too found that all exon 9 mutation were spindle-shaped, but the tumor size was less than 2.0 cm and found in the stomach as well as in a small bowl. Very less approximately 1% to 2% of *KIT* mutations are found in exons 13 and 17 (24, 37, 40). Similarly, this study found only four cases had a mutation in region 13 (n=2) and 17 (n=2). As per the study conducted earlier, when imatinib is administered, the response rate of exon 11 mutant GIST becomes twice in comparison with exon 9 mutant or WT GISTs. When there is a high dosage of imatinib (800 mg) is administered, the exon 9 mutant GISTs tend to respond. The *PDFRA* mutations are generally exhibited by a mere 10-15% of GISTs and these mutations are found in the exon 12, exon 14 and exon 18 present in the juxtamembrane domain, ATP binding domain and activation loop. These exons result in the constitutive downstream activation of signaling pathways which are inclusive of MAPK, AKT, STAT1 and STAT3 (9, 33).

PDGFRA mutation was seen in exon 18 (5/8) and exon 12 (3/8) region, while all were spindle-shaped they all were in the low or very low-risk case and mostly found in small bowel site. *PDGFRA* GISTs site is prognostically more favorable gastric origin and p.D842V substitution is the most prevalent type of PDGFRA which was also prevalent exon 18 type in the present study.

PDGFRA D842V substitution mutation is said to occur in the exon 18 while the latter encodes the second kinase domain. This domain is touted to have an association with a disease-free survival condition. Other types of mutations, if and when occur, are predicted to trigger primary imatinib resistance (9, 34).

The other noted mutation such as T674I (exon 14) and D1071N (exon 22) which are an imatinib-resistant type of *PDGFRA*, were not identified (35, 36). A genome-wide association study to identify candidate genes is needed in this regard (37).

The exon 12 mutations are observed only on rare occasions in less than 1% of overall GISTs, yet they are inclusive of minor deletions, substitutions and insertions (9, 38). Among 54 cases, only three of them were having an exon 12 type of PDGFA mutation which was a deletion type of mutation found in the colon and rectum. Apart from mutation reported in this study, various others have been reported in connection with treatment and prognosis of GIST, including KIT and PDGFRA mutations in their exon's regions (9). We did not see KIT mutation in the exon region of 8, 14, and 15.

Oncogenes, or tumorigenic genes, are altered genes that normally express proteins that are involved in controlling cell growth and proliferation (39-42). These genes are normally called proto-oncogenes. But if mutations occur in proto-oncogenes, they turn into oncogenes (43-47). Oncogenes cause cancer. Mutations that convert proto-oncogenes to oncogenes often cause overexpression of control factors, increasing the number of genes encoding them, or altering control factors so that factor activity increases or their half-life in the cell increases. Oncogenes were first discovered in viruses called viral oncogenes. By mutation in the promoter of proto-oncogenes, they are converted to active oncogenes and their expression is increased, cell proliferation is increased and a tumor is formed (48-52).

Mutations play an important role in prognosis while the impact on imatinib from few mutations, though not all the mutations are reported to show resistance to it. The studies conducted earlier established that different mutations that occur in KIT and PDGFRA have an association with GIST response to sunitinib, including the mutations in KIT exons 9, 11, 13, and 17 as well as PDGFRA exons 12, 14, and 18 (5, 6, 7, 9, 25). The most frequent cause behind the resistance to imatinib in GIST is due to the secondary mutations that occur in *KIT* or *PDGFRA.* In spite of the availability of established adjuvant therapy practices for 'high-risk' WT GIST, there is still no optimal and systematic treatment or cutting-edge clinical guidelines available for metastatic WT GIST.

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