

Presence of homozygous KIT exon 11 mutations is strongly associated with malignant clinical behavior in gastrointestinal stromal tumors

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of gastrointestinal tract. GISTs range from benign indolent neoplasms to highly malignant sarcomas. Gain-of-function mutations of tyrosine kinase receptors, *KIT* or *PDGFRA*, have been identified in most GISTs. In this study, we report 36 GIST patients whose tumors had homozygous *KIT* exon 11 mutations detected by direct sequencing of PCR products. Loss of heterozygosity in *KIT* locus and other chromosome 4 loci were documented in majority of these tumors. However, fluorescence *in situ* hybridization with *KIT* locus-specific probe and chromosome 4 centromeric enumeration probe showed no evidence of *KIT* hemizyosity in a majority of analyzed cases. These findings are consistent with duplication of chromosome 4 with *KIT* mutant allele. Homozygous *KIT* exon 11 mutations were found in 33 primary tumors and 7 metastatic lesions. In two cases, shift from heterozygosity to homozygosity was documented during tumor progression being present in metastases, but not in primary tumors. Among primary GISTs, there were 16 gastric, 18 intestinal and 2 from unknown locations. An average primary tumor size was 12 cm and average mitotic activity 32/50 HPFs. Out of 32 tumors 29 (90.6%) with complete clinicopathologic data were diagnosed as sarcomas with more than 50% risk of metastatic disease, and 26 of 29 patients with follow-up had metastases or died of disease. An average survival time among pre-imatinib patients, who died of the disease was 33.4 months. Based on these findings, we conclude that presence of homozygous *KIT* exon 11 mutations is associated with malignant course of disease and should be considered an adverse prognostic marker in GISTs.

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of gastrointestinal (GI) tract. GISTs can occur in any part of GI tract, but are most frequently found in the stomach and small intestine. GISTs show spindle or epithelioid cell morphology, and occasionally pleomorphic features. A great majority of GISTs express KIT.¹ KIT expression links GISTs to interstitial cells of Cajal, hypothetic GIST progenitor cells.^{2,3}

KIT or *PDGFRA* gain-of-function mutations have been identified in a great majority of GISTs and are considered to be one of the first molecular events in their pathogenesis; these mutations lead to pathological activation of KIT or PDGFRA signaling pathways.^{3,4} Both KIT and PDGFRA belong to the type III tyrosine kinase receptor family and play an important role in different cell functions including cell proliferation.⁵

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Tumor size and mitotic activity are the most important prognostic parameters in GISTs.¹ However, recent studies have shown that molecular genetic markers, such as the type of *KIT* mutation, might have prognostic value. One study reported gastric tumors with *KIT* exon 11 deletions to have more malignant clinical outcome than the ones with point mutations.⁶ Other studies have shown that GISTs with *KIT* 1690_1695del (Tyr557_Lys558del at the protein level) have a significantly worse prognosis than ones with other *KIT* exon 11 mutations.^{7,8}

Since 2000, the tyrosine kinase inhibitor, imatinib mesylate (Gleevec[®], Novartis, USA) has been successfully used in the treatment of clinically advanced and metastatic GISTs.⁹ The type of *KIT* or *PDGFRA* mutation indicates tumor responsiveness to imatinib treatment. Whereas *KIT* exon 11 (juxtamembrane domain) mutant GISTs achieve the highest level of response, tumors with Asp842Val, the most common *PDGFRA* exon 18 (tyrosine kinase domain) mutation, do not respond to imatinib mesylate treatment.¹⁰ A more recent study showed that GISTs with *KIT* exon 9 (extracellular domain) mutations required two times higher dose of imatinib mesylate to achieve a response similar to that observed in *KIT* exon 11 mutant tumors.¹¹

Although GISTs with homozygous *KIT* exon 11 mutations have been sporadically reported in the literature, the clinicopathologic profile of such tumors is not known.^{12–19} In this study, we report a series of 36 primary and metastatic GISTs with homozygous *KIT* exon 11 mutations and review the clinicopathologic profile and natural history of such tumors before the availability of tyrosine kinase inhibitors. Moreover, we report responsiveness of GISTs with homozygous *KIT* exon 11 mutations to imatinib treatment.

MATERIALS AND METHODS

Material

Formalin-fixed, paraffin embedded (FFPE) samples of tumor and corresponding normal tissue were retrieved from the files of the following institutions: Armed Forces Institute of Pathology (AFIP), Washington DC, USA; M. Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Warszawa, Poland; Haartman Institute of the University of Helsinki, Helsinki, Finland; University Hospital Northern Norway, Tromsø, Norway; Otto-von-Guericke University, Magdeburg, Germany; Collegium Medicum of the Jagiellonian University, Krakow Poland; Department of Pathology, Pomeranian Medical University, Szczecin, Poland; and University College Hospital, Ibadan, Nigeria. Demographic, clinical and follow-up data were obtained according to the Institutional Review Board approval.

Tumors were diagnosed as GISTs using previously established histological and immunohistochemical criteria.¹ Based on tumor size and mitotic activity, primary GISTs were classified into eight prognostic groups, indicating likelihood of malignant behavior (Table 1). Response to imatinib treatment was evaluated following the criteria provided

Table 1 Tumor size and mitotic criteria used to assess the malignant potential of GISTs, according to previously published studies

Group	Tumor parameters		Risk for metastasis (% patients with progressive disease)	
	Size (cm)	Mitosis (per 50 HPF)	Gastric ^a	Small Intestinal ^b
1	≤2	≤5	None	None
2	>2 ≤5	≤5	Low (1.9%)	Low (4.3%)
3a	>5 ≤10	≤5	Low (3.6%)	Moderate (24%)
3b	>10	≤5	Moderate (12%)	High (52%)
4	≤2	>5	Unknown	High (50%)
5	>2 ≤5	>5	Moderate (16%)	High (73%)
6a	>5 ≤10	>5	High (55%)	High (85%)
6b	>10	>5	High (86%)	High (90%)

^aBased on study on 1055 gastric GISTs.⁶

^bBased on studies 629 small intestinal.²⁰

by RECIST (response evaluation criteria in solid tumors) guidelines.²¹

KIT Mutation Status

GIST *KIT* mutation databases at the Department of Soft Tissue Pathology, AFIP and at the Department of Molecular Biology, M. Sklodowska-Curie Memorial Cancer Center were screened for tumors with homozygous *KIT* exon 11 mutations. These mutations were previously identified at the DNA level by PCR amplification and direct sequencing, following published procedures.²² In two cases, 'hot spots' in *KIT* exon 13, 14 and 17 were evaluated for secondary mutations acquired during imatinib mesylate-based treatment. The following primers were used for PCR amplification: CK13.5F (76271_76290) and CK13.2.1R (76369_76388) for exon 13; CK14.7F (77544_77563) and 14.6R (77670_77690) for exon 14; CK17.1F (81351_81370) and CK17.2R (81451_81570) for exon 17. PCR conditions were standard with annealing temperatures 55°C in all reactions.

Nomenclature of the mutations was based on the recommendations of Human Genome Mutation Society (www.hgvs.org). Mutations at the protein level were deduced with the assumption that all changes identified at the genomic level involved one allele. The following *KIT* (HSU63834, XO6182) and *PDGFRA* (ACO98587) reference sequences were obtained from National Center for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov.

Loss of Heterozygosity Studies

Loss of heterozygosity (LOH) was evaluated by PCR amplification of three chromosome 4q (D4S3045, D4S1619, D4S392) and one chromosome 4p (D4S2950) microsatellite

markers. Marker positions and primer sequences were obtained from human genome microsatellite marker databases linked to the NCBI webpage at www.ncbi.nlm.nih.gov. PCR amplifications were performed using the standard conditions recommended by Applied Biosystems (www.appliedbiosystems.com). PCR products were analyzed on ABI PRISM[®] 310 Genetic Analyzer, following the Applied Biosystems procedure. A ratio of the peak high values (fluorescence intensity) between the longer and shorter allele was calculated for normal and tumor tissues. To obtain the LOH value, an allele ratio from normal tissue was divided by an allele ratio from tumor tissue. The values ≤ 0.5 and ≥ 1.5 were considered to indicate LOH, as recommended by PE Biosystems and reported previously.²³ The borderline values $> 0.5 < 0.6$ and $> 1.5 < 1.6$ were considered to represent a partial LOH.

Twelve *KIT* and two *PDGFRA* single-nucleotide polymorphisms (SNPs) were evaluated by PCR amplification and direct sequencing. Locations of SNPs were obtained from databases available at NCBI. In addition, during the screening, two previously unreported SNPs in exon 10 and exon 17 were identified and evaluated as well. The frequencies of these two SNPs were 10 and 6.7%, respectively, and were based on 30 normal DNA samples from unrelated healthy individuals tested in this study. Primer positions and PCR conditions used to amplify *KIT* and *PDGFRA* SNPs are listed in Table 2. All *KIT* and *PDGFRA* SNPs evaluated in this study are listed in Table 3.

Also, six SNPs reported in Huntington's Disease gene (*HD*), located at 4p16.3, were evaluated in 18 cases. Primer sequences and PCR conditions used for *HD* SNPs studies are listed in Table 4.

All microsatellite marker- and SNP-based LOH studies were carried out independently in different laboratories.

FISH Studies

Interphase FISH studies were performed on standard 5- μ m sections of FFPE tissues prepared for hybridization using SPoT-Light Cell Pretreatment Kit, following manufacturer's protocol (Zymed, CA, USA). BAC clone RP11-959G16 containing the full length of *KIT* was used as the locus-specific probe (LSP), together with the chromosome 4 centromeric enumeration probe (CEP) (Vysis Inc., Downers Grove, IL, USA). The probes were assessed individually or simultaneously following previously reported procedures.¹¹ The images were analyzed with Zeiss Axioplan 2 (Zeiss, Germany) fluorescence microscope and captured by cooled black-and-white charged-couple device camera coupled with Isis FISH Imaging System version 5.1 software (Metasystems, Germany).

In each case, at least 100 intact, nonoverlapping nuclei were chosen for scoring fluorescent signals. The percentage of tumors nuclei containing 0, 1, 2, 3, 4, >4 signals were calculated for each probe. Based on a previously published study,²⁴ the following criteria for FISH anomalies were applied: (1) abnormal gain required 10% nuclei with three or

Table 2 PCR assays used in this study (previously unpublished) to evaluate *KIT* and *PDGFRA* SNPs

Gene	Amplification target	Primer	Location	Annealing temp (°C)
KIT	SNP in intron 1	CK1.6F	6555_6575	60
		CK1.7R	6685_6676	
	SNP in intron 1	CK1.8F	7922_7941	50
		CK1.9R	8021_8040	
	SNP in intron 4	CK4.4F	50235_50254	55
		CK4.5R	50349_50369	
	SNP in intron 8	CK8.6F	72661_72780	60
		CK8.7R	72780_72799	
	SNPs in exon 10	CK10.6F	75400_75419	55
		CK10.4R	75594_75613	
	SNPs in intron 16	CK17.4F	81141_81160	55
		CK17.3R	81320_81339	
SNP in exon 17	CK17.3F	81320_81339	55	
	CK17.8R	81425_81444		
SNP in intron 17	CK17.8F	81425_81444	55	
	CK17.9R	81533_81552		
SNP in exon 21	CK21.1F	86861_g.8680	55	
	CK21.3R	87001_87020		
PDGFRA	SNP in intron 17	P18.3F	24562_24581	55
		P18.4R	24672_24691	
	SNP in exon 18	P18.4F	24672_24691	55
		P18.1R	24819_24849 ^a	

^aPreviously reported.⁴

more signals; (2) abnormal loss required $\geq 65\%$ nuclei with 0 or 1 signal. All FISH analyses were carried out on coded slides without knowledge of other data.

Statistical Studies

Prognostic comparative data were analyzed using Kruskal-Wallis test. All statistical tests were two-sided and 5% level of significance was used.

RESULTS

KIT Mutation Studies

Screening of 700 *KIT* exon 11 mutant GISTs from the AFIP database and 32 *KIT* exon 11 mutant GISTs from the M. Sklodowska-Curie Memorial Cancer Center database revealed 27 (3.86%) and 9 (28.1%) homozygous mutations, respectively. There were 27 (75%) deletions (del) or deletion-insertions (delins), 7 (19.4%) single-nucleotide substitutions (point mutations (pm)) and 2 (5.6%) duplications (dup). Deletions ranged from 3 to 57 nucleotides and mostly involved 5' part of *KIT* exon 11. The most common deletion, 1690_1695del leading at the protein level to

Table 3 *KIT* and *PDGFRA* SNPs evaluated in this study

Gene	Location	Position ^a	Type	Codon	SNP NCBI ID	PCR assay
KIT	Intron 1	6619	C/G		rs999021	CK1.6F/CK1.7R
	Intron 1	8016	A/C		rs2865813	CK1.8F/CK1.9R
	Intron 4	50315	A/G		rs3111795	CK4.4F/CK4.5R
	Intron 8	72701	G/T		rs4864920	CK8.6F/CK8.7R
	Exon 10	75544	A/C/G	541	rs3822214	CK10.6F/CK10.4R
	Exon 10	75561	A/G	546	Unreported ^b	CK10.6F/CK10.4R
	Intron 16	81210	A/T		rs11935331	CK17.4F/CK17.3R
	Intron 16	81213	A/T		rs1573615	CK17.4F/CK17.3R
	Intron 16	81240	A/G		rs4864921	CK17.4F/CK17.3R
	Exon 17	81349	C/T	798	Unreported ^b	CK17.3F/CK17.8R
	Intron 17	81517	C/T		rs1008658	CK17.8F/CK17.9R
	Exon 21	86961	A/G	Untranslated	rs17084733	CK21.1F/CK21.3R
	PDGFRA	Intron 17	24624_24625	-/T		rs3830355
Exon 18		24706	A/C	824	rs2228230	P18.4F/P18.1R

^aPosition based on reference sequences HSU63834 for *KIT* and ACO98587 for *PDGFRA* from NCBI nucleotide database (www.ncbi.nlm.nih.gov).

^bUnreported by NCBI.

Table 4 PCR assays used in this study to evaluate Huntington's Disease gene (*HD*) SNPs

SNP NCBI ID amplification target	Primer	Sequence	Annealing temp. (°C)
SNP rs4690074 (exon 29)	Hu29.1F	5' TGGATCTTCAGAACAGCACG 3'	60
	Hu29.1R	5' ATCAAGTGTGCCAGCCACAA 3'	
SNP rs363125 (exon 39)	Hu39.1F	5' GGCATGGGTTAGTTATAGGC 3'	60
	Hu39.2R	5' AGCTCTCCTTCTCCGTAT 3'	
SNP rs362331 (exon 50)	Hu50.1F	5' CTCCTCCACAGAGTTTGTA 3'	56
	Hu50.2R	5' GGTGAAGCAGACAGGA ACTA 3'	
SNP rs362273 (exon 57)	Hu57.1F	5' CTGAGGACAGAAACGGACAG 3'	60
	Hu57.2R	5' TGTTACAGGTGCCCTCATC 3'	
SNP rs2276881 (exon 60)	Hu60.1F	5' CAGTTCTGTCAGCGTCACAT 3'	55
	Hu60.2R	5' GGATTCTAACAGCGGATTTC 3'	
SNP rs362272 (exon 61)	Hu61.1F	5' GTCGCACTCCAGCACATAGA 3'	55
	Hu61.2R	5' TGTCTGTGTTCCTAGGAC 3'	

Tyr557_Lys558del, was found in three cases. Deletions 1756_1758del (Asp579del) and 1682_1738del (Glu554_Pro573delinsAla) were identified in two cases each. Remaining deletions and deletion-insertions were unique. Substitutions were found at codons 557 ($n=2$), 560 ($n=4$) and 576 ($n=1$). Two duplications consist of in-frame repeat of 6 and 39 nucleotides in the 3' part of *KIT* exon 11. Genomic sequences of all homozygous mutations and deduced mutant *KIT* protein sequences are listed in Table 4. Representative examples of direct sequencing of *KIT* exon 11 PCR products are shown in Figure 1a.

Two cases with metastatic lesions resistant to imatinib treatment were screened for secondary *KIT* mutations in exon 13, 14 and 17. A 1982T>C substitution leading to heterozygous Val654Ala mutation was identified in one case.

LOH Studies

Normal tissue was available in 29 of 36 cases. These cases were evaluated, for LOH at chromosome 4q loci, using 3 microsatellite markers and 14 *KIT*, *PDGFRA* SNPs and for LOH at chromosome 4p loci, using 1 microsatellite marker and 6 *HD* SNPs. Successful amplification was obtained in 531

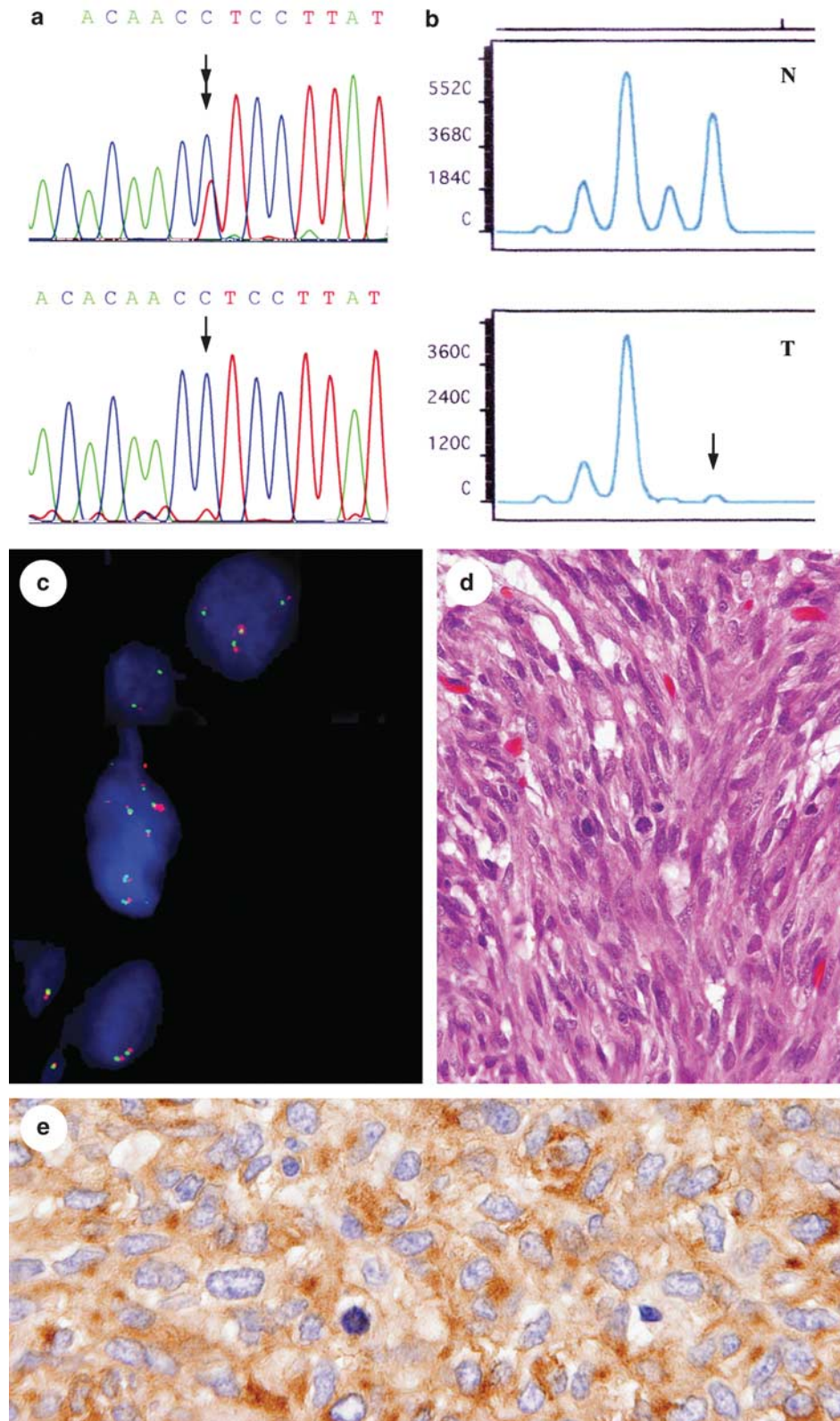


Figure 1 (a) Example of direct sequencing of *KIT* PCR amplification products in Case 35. Upper panel primary tumor, lower panel metastatic lesion; double and single arrows indicate heterozygous and homozygous point mutations, respectively. (b) Example of LOH detected by evaluation of microsatellite D4S1619 marker in Case 35; arrow indicates lost allele. (c) Example of FISH with *KIT* LSP (red signal) and CEP 4 (green signal) in Case 17. Note increased number of *KIT* and centromeric signals, indicating increased copy number of chromosome 4. (d, e) Representative histological and immunohistochemical images of GISTs analyzed in this study. (d) Malignant intestinal GIST with spindle cell morphology (Case 22). (e) Strong *KIT* immunoreactivity in a malignant gastric GISTs with spindle cell morphology (Case 1).

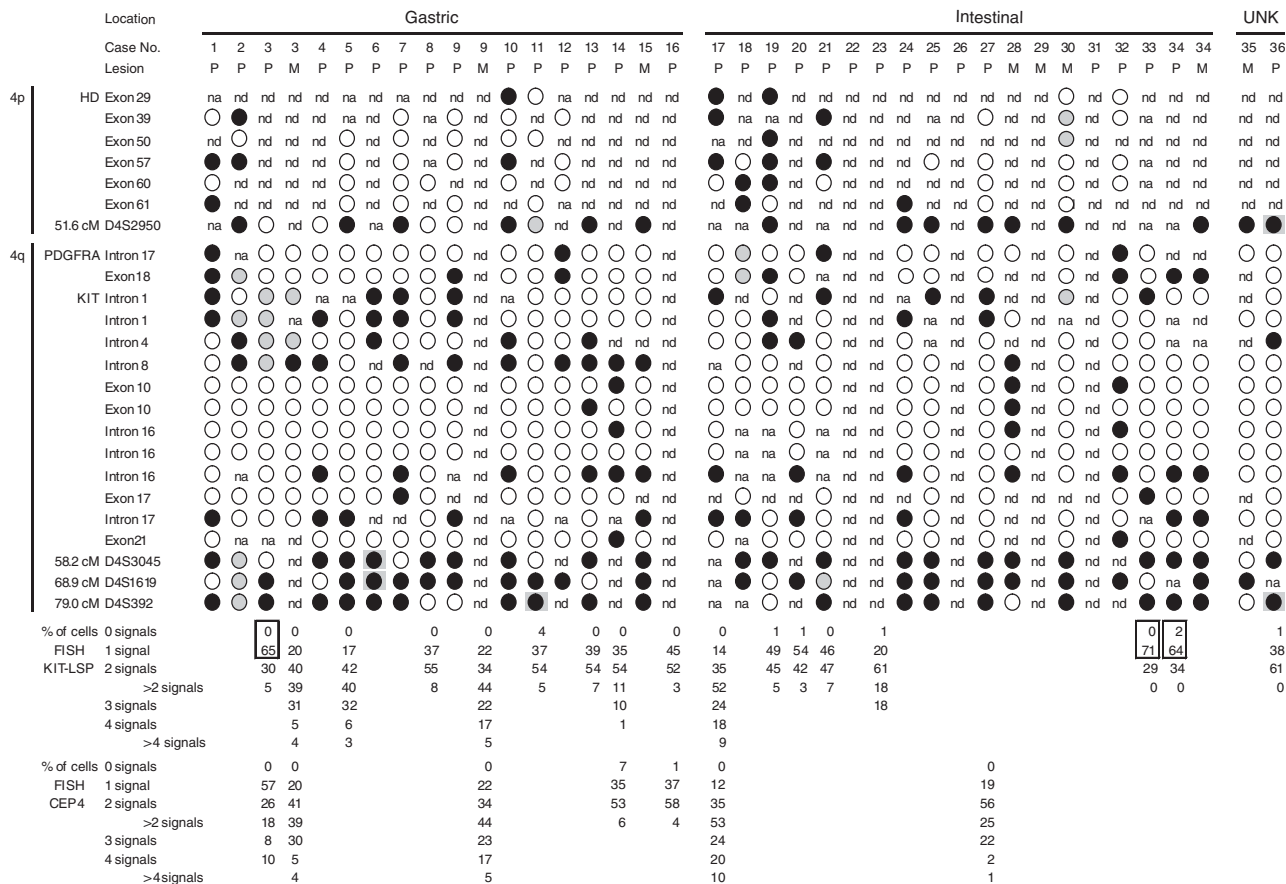


Figure 2 Summary of LOH and FISH studies. Single assay is represented by a circle. Gray and black colors indicate constitutional heterozygosity with retention of both allele and loss of one allele, respectively. White color indicates homozygosity (non-informative loci). Gray squares with black circles indicate borderline LOH values. Abbreviations: P: primary tumor; M: metastatic tumor; nd: not done; na: no PCR amplification products. Tumors with $\geq 65\%$ of nuclei with none or one FISH signal are indicated by rectangles.

(89.9%) of 591 analyses. LOH was identified in 174 (90.2%) of 193 informative microsatellite and SNP loci. A representative microsatellite-based LOH assay is shown in Figure 1b. Results of all LOH studies are summarized in Figure 2.

In 28 tumors, multiple informative SNPs and microsatellite markers were lost indicating possible loss of the entire copy of chromosome 4. In two tumors (Cases 3 and 30), one to four *KIT* SNPs clustered between introns 1 and 8 remained polymorphic. Also, the two *PDGFRA* SNPs clustered in intron 17 and exon 18 remained polymorphic in one case (Case 18). Moreover, in one tumor (Case 11), lack of LOH was documented at D4S2950, chromosome 4p microsatellite marker and in another tumor (Case 30), two *HD* SNPs were preserved.

In one tumor (Case 2), LOH was detected only in *KIT* intron 4 and 8 and at chromosome 4p loci. In this case, microsatellite markers telomeric to *KIT/PDGFR*A locus remained polymorphic, indicating retention of a large portion of chromosome 4q.

FISH Studies

FISH with *KIT* LSP was successful in 17 tumors. A representative FISH image is shown in Figure 1c. In three (17.7%) tumors, no or one signal was found in $\geq 65\%$ of nuclei, suggesting loss of *KIT* locus. In 14 (82.3%) tumors, the percentage of nuclei with ≥ 2 *KIT* LSP signals ranged from 45 to 99%, suggesting either a diploid karyotype or abnormal gain. Moreover, in five of these tumors, a ratio of *KIT* LSP to CEP4 FISH signals was 1 or close to 1. In Case 3, FISH analysis of the primary and subsequent metastatic lesion revealed a decrease of the percentage of nuclei with 1 signal from 65% in primary tumors to 24% in metastatic one, and an increase of the percentage of nuclei with > 2 signals from 5% in primary tumor to 34% in metastatic tumors.

Demographic, Clinical and Pathologic Features

All demographic, clinical and pathologic data are summarized in Table 5. The patient age varied from 37 to 80 years with median age of 62 years. The male to female ratio was

Table 5 *KIT* mutations identified in this study

Case	Tumor	Mutation type	Mutations identified at the DNA level	Mutations deduced at the protein level
1	Primary	Homozygous	1688_1693delinsCGC	Gln556_Lys558delinsProGlu
2	Primary	Homozygous	1672_1692del	Pro551_Tyr557del
3	Primary	Homozygous	1682_1738del	Glu554_Pro573delinsAla
3	Metastasis	Homozygous	1682_1738del	Glu554_Pro573delinsAla
4	Primary	Homozygous	1674_1691del	Met552_Tyr557del
5	Primary	Homozygous	1692_1693delinsTT	Tyr557_Lys558delinsCysGlu
6	Primary	Homozygous	1690_1695del	Tyr557_Lys558del
7	Primary	Homozygous	1691_1696del	Tyr557_Val559delinsPhe
8	Primary	Homozygous	1692_1697del	Trp557_Val559delinsCys
9	Primary	Homozygous	1756_1758del	Asp579del
10	Primary	Homozygous	1689_1700del	Gln556_Val560delinsHis
11	Primary	Homozygous	1690_1704del	Tyr557_Glu561del
12	Primary	Homozygous	1676_1695delinsCT	Met552_Lys558delinsThr
13	Primary	Homozygous	1690_1695del	Tyr557_Lys558del
14	Primary	Homozygous	1676_1681del	Tyr553_Glu554del
15	Primary		Not available for testing	
15	Metastasis	Homozygous	1749_1787dup	Pro577_Leu589dup
16	Primary	Homozygous	1689_1739del	Trp557_Pro573del
17	Primary	Homozygous	1699_1701del	Val560del
18	Primary	Homozygous	1696_1716del	Val559_Gly565del
19	Primary	Homozygous	1753_1758dup	Tyr578_Asp579dup
20	Primary	Homozygous	1674_1691del	Met552_Trp557del
21	Primary	Homozygous	1690T>A	Trp557Arg
22	Primary	Homozygous	1700T>A	Val560Asp
23	Primary	Homozygous	1756_1758del	Asp579del
24	Primary	Homozygous	1673_1684del	Pro551_Val555delinsLeu
25	Primary	Homozygous	1696_1704del	Val559_Glu561del
26	Primary	Homozygous	1681_1698del	Glu554_Val559del
27	Primary	Homozygous	1690T>A	Trp557Arg
28	Primary	Heterozygous	1700_1701delinsAG	Val560Glu
28	Metastasis	Homozygous	1700_1701delinsAG	Val560Glu
29	Primary		Not available for testing	
29	Metastasis	Homozygous	1690_1695del	Tyr557_Lys558del
30	Primary		Not available for testing	
30	Metastasis	Homozygous	1681_1734del	Glu554_Ile571del
31	Primary	Homozygous	1690_1695del	Tyr557_Lys558del
32	Primary	Homozygous	1700T>A	Val560Asp
33	Primary	Homozygous	1700T>A	Val560Asp
34	Primary	Homozygous	1674_1686delinsT	Met552_Val555del
34	Metastasis	Homozygous	1674_1686delinsT	Met552_Val555del
35	Primary	Heterozygous	1748T>C	Leu576Pro
35	Metastasis	Homozygous	1748T>C	Leu576Pro
36	Primary	Homozygous	1700T>A	Val560Asp

17:19. There were 16 gastric, 15 small intestinal, 2 colonic and 1 rectal GIST. The primary location could not be established for two tumors.

Thirty (83.3%) tumors had spindle cell morphology. There were two and three epithelioid gastric and intestinal tumors, respectively. One gastric GIST revealed mixed histology with both spindle and epithelioid components. KIT (CD117) expression was documented immunohistochemically in all analyzed cases. Representative histological and immunohistochemical images are shown in Figure 1d and e.

The size of primary tumors ranged from 2 to 30 cm (median 12.3 cm). Twenty-nine of 34 (85.3%) primary GISTs with known size of the primary tumor were >5 cm in diameter. Mitotic activity in the primary tumors varied from 1 to >100 per 50/HPF with an average of 32 mitoses per 50 HPF.

Complete or partial clinical and follow-up data were available in 33 cases (Table 6). Twenty-eight (84.5%) patients developed metastasis or died of disease. The average survival time for the 14 pre-imatinib patients who died of disease was 33.4 months. Based on previously published criteria (Table 1), three GISTs without follow-up data represented malignant tumors with 55 to 85% chance of developing metastatic diseases. Also, two patients that died of unknown causes had malignant tumors with higher than 55% risk of metastasis. Only 3 patients diagnosed with GIST with low to moderate risk of metastases were alive with follow-up ranging from 7 to 15 months (average 11.6 months).

Eight patients with advanced, disseminated GISTs were treated with imatinib mesylate. Initially, a partial response and stable disease were seen in six and one cases, respectively. There was insufficient clinical follow-up in one case. Subsequently, a patient with stable disease and three patients with partial response developed progressive disease (Table 7).

The metastatic tumors resistant to imatinib mesylate treatment were available for molecular studies (Case 9 and 13). Both lesions were screened for secondary mutations affecting *KIT* exons 13, 14 and 17. A heterozygous 1982T>C substitution in *KIT* exon 13, leading to Val654Ala mutation at the protein level was identified in Case 13.

The average survival time for the three patients treated with imatinib mesylate, who died of disease was 27 months. Three other patients remained in partial response at the time of this study.

Statistical Studies

Risk of progressive disease in gastric and intestinal homozygous *KIT* exon 11 mutant GISTs was compared with risk of progressive disease in the cohorts of gastric and intestinal tumors with heterozygous *KIT* exon 11 mutations or without determined mutational status. Previously published data on gastric and small intestinal GISTs were included in statistical studies.^{6,20} All results are summarized in Tables 8 and 9.

The risk of progressive disease was significantly higher in GISTs with homozygous *KIT* exon 11 deletion/deletion-in-

sertions than in GISTs with heterozygous *KIT* exon 11 deletions/deletion-insertions or without determined mutational status. No difference in risk of progressive disease was detected when small intestinal GISTs with homozygous single nucleotide substitutions (point mutations) were compared with heterozygous mutants. However, the cohort of tumors with homozygous single-nucleotide substitutions was relative small and included only four cases.

DISCUSSION

GISTs encompass a spectrum of mesenchymal tumors from benign, indolent lesions to highly malignant sarcomas.¹ GISTs are believed to originate from interstitial cells of Cajal or their precursor cells being driven by gain-of-function *KIT* and PDGFRA mutations.²⁻⁴

Most *KIT* mutations are heterozygous. However, in some cases, only the mutant allele can be identified by direct sequencing of PCR amplification products. There are several possible explanations for such findings. These include the presence of the same mutation in both alleles (truly homozygous mutation), presence of *KIT*-mutant (MT) and absence of *KIT*-(wild type) WT allele (hemizygous mutation), and selective amplification of mutant *KIT* locus or polysomy of *KIT*-MT chromosome 4.

In this study, we have examined the nature of homozygous *KIT* exon 11 mutations, as detected by direct sequencing of PCR products. Lack of polymorphism at multiple polymorphic sites indicated loss of *KIT*-WT chromosome 4. However, dual-color FISH using *KIT* LSP and chromosome 4 CEP showed no evidence of *KIT* deletion or loss of one copy of chromosome 4 in majority of analyzed tumors. Also, shift from chromosome 4 monosomy in primary tumor to disomy in metastatic lesion was documented by FISH in one case. Together, these data suggested that a loss of *KIT*-WT chromosome 4 is followed by a duplication of *KIT*-MT chromosome 4. A similar molecular mechanism was previously reported in two *KIT* exon 13 mutant GISTs.²⁵

In one tumor (Case 2), multiple polymorphic markers retained polymorphism and only those located in the vicinity of *KIT* exon 11 showed homozygosity. The presence of genetic changes other than loss of entire chromosome 4 should be considered in this case. For example, coexistence of a large *KIT* deletion in another *KIT* allele or complex genomic rearrangements could explain this finding. Also, in a few cases, selected SNPs and microsatellite markers remained polymorphic, indicating retention of genetic material from *KIT*-WT chromosome 4, most likely involved in complex genetic rearrangements. Identification of such changes was beyond the scope of our investigation based on archival FFPE material.

Numeric changes of chromosome 4 copies identified by classical karyotyping or FISH are relatively rare among GISTs.^{24,26} Also, comparative genomic hybridization studies have failed to identify losses or gains of genetic material from chromosome 4 in GISTs.²⁷ The current study showed that

Table 6 Summary of demographic, clinical and pathologic data of 36 cases analyzed in this study

Case	Age	Sex	Eth	Loc	His	Size (cm)	Mitosis/50HPs	Group	Metastasis	Imatinib treatment	Follow-up
1	60	M	C	St	Sp	30	85	6b		NO	DOD 20m
2	65	F	B	St	Sp	27	50	6b		NO	UNK
3	55	M	C	St	Sp	19	16	6b	Liver 21m	YES	DOD 71m
4	62	F	C	St	Sp	15	> 100	6b	IAB 24m	NO	DOD 43m
5	50	M	C	St	Sp	14	59	6b		NO	DUNK 11m
6	70	M	C	St	Sp	13	13	6b		NO	DOD 39m
7	37	M	C	St	Ep	12	100	6b	IAB	NO	DOD 29m
8	58	F	C	St	Sp	11	> 100	6b		NO	UNK
9	63	M	C	St	Sp	11	> 50	6b	Liver, IAB	YES	DOD 21m
10	41	F	C	St	Sp/Ep	10	> 100	6a	IAB	NO	AWD 18m
11	68	M	C	St	Sp	10	34	6a		NO	DOD 16m
12	67	M	C	St	Ep	9.5	8	6a	IAB	NO	UNK
13	43	M	C	St	Sp	8	6	6a	IAB	YES	DOD 24m
14	73	F	C	St	Sp	3.5	2	2		NO	ANED 7m
15	64	F	C	St	Ep ^a	UNK	UNK	UNK	IAB 40m	YES	AWD 84m
16	54	F	C	St	Sp	21	13	6b	Liver 60m	YES	AWD 86m
17	60	M	UNK	SB	Sp	26	35	6b	IAB	NO	UNK
18	70	F	C	SB	Sp	18	0	3b	IAB	NO	UNK
19	72	F	UNK	SB	Ep	12	24	6b	IAB	NO	DOD 12m
20	54	F	C	SB	Sp	11	12	6b	Liver 36m	NO	DOD 46m
21	62	F	UNK	SB	Sp	10.5	3	3b		NO	DOD 54m
22	UNK	F	UNK	SB	Sp	10	100	6a		NO	DOD 10m
23	60	M	UNK	SB	Sp	9	9	6a		NO	DOD 35m
24	72	F	C	SB	Ep	9	1	3a		NO	ANED 15m
25	62	M	UNK	SB	Ep	8.5	8	6a		NO	DOD 6m
26	47	M	C	SB	Sp	7	<5	3b	Liver, IAB 29m	YES	AWD 51m
27	46	F	C	SB	Sp	4	3	2		NO	ANED 13m
28	53	M	C	SB	Sp	2	10	4	IAB 9m	YES	AWD 25m
29	80	F	C	SB	Sp	UNK	UNK	UNK	IAB 72m	NO	UNK
30	40	M	C	SB/M	Sp ^a	> 20 kg	UNK	UNK	IAB	NO	DOD 84m
31	52	M	C	SB/M	Sp	14	6	6b	IAB	YES	AWD 3m
32	64	M	C	Col	Sp	16.5	1	3b	IAB	NO	DOD 8m
33	74	M	UNK	Col	Sp	5	13	5		NO	DUNK 84m
34	68	F	C	Rec	Sp	3	38	5	IAB 24m	NO	DOD 66m
35	63	F	C	UNK	Sp	19	1	3b	IAB 24m, 65m	NO	AWD 69m
36	53	F	C	UNK	Sp	7	23	6a		NO	UNK

Abbreviations: C, Caucasian; B, black; UNK, unknown; St, stomach; SB, small bowel; Col, colon; Rec, rectum; IAB, intra-abdominal; DOD, died of disease; DUNK, died of unknown causes; AWD, alive with disease; ANED, alive no evidence of disease.

^aBased on evaluation of metastatic lesions.

loss of *KIT*-WT chromosome 4 could be masked by duplication of *KIT*-MT chromosome 4, a molecular event that can not be identified by classical karyotyping or comparative genomic hybridization.

In the AFIP *KIT* mutation database, homozygous *KIT* exon 11 mutations represented only a small fraction, approximately 4%, of all *KIT* exon 11 mutations. Also, a recent study based on population of Northern Norway reported

Table 7 Type and duration of response to imatinib treatment among eight GIST patients with homozygous *KIT* exon 11 mutations

Case	Response to imatinib mesylate treatment		Follow-up	Secondary <i>KIT</i> mutation
3	Primary response (39 months)	Progressive disease (5 months)	Died of disease (44 months)	ND
9	Primary response (18 months)	Progressive disease (5 months)	Died of disease (23 months)	WT
13	Stable disease (17 months)	Progressive disease (5 months)	Died of disease (22 months)	1982T > C (Val654Ala)
15	Primary response		Alive with disease (37 months)	ND
16	Primary response		Alive with disease (18 months)	ND
27	Primary response (16 months)	Progressive disease (2 months)	Alive with disease ^a (18 months)	ND
28	Primary response		Alive with disease (17 months)	ND
31	To be determined		Alive with disease (2 months)	ND

^aModified treatment.

Table 8 Comparison of risk of progressive disease among gastric GISTs with homozygous *KIT* exon 11 del/delins vs GISTs with heterozygous del/delins, GISTs with heterozygous point mutations and all gastric GISTs

Prognostic group	Gastric GISTs <i>KIT</i> exon 11 mutants			
	Del/delins homozygous	Del/delins heterozygous ^a	Point mutations heterozygous ^a	All ^a
1	0	4 (5.8%)	1 (2.9%)	116 (7.5%)
2	1 (0.7%)	15 (21.7%)	12 (35.3%)	456 (29.4%)
3a	0	10 (14.5%)	15 (44.1%)	301 (19.4%)
3b	0	7 (10.1%)	2 (5.9%)	193 (12.4%)
4	0	0	0	8 (0.5%)
5	0	7 (10.1%)	1 (2.9%)	137 (8.8%)
6a	4 (26.7%)	9 (13%)	3 (8.8%)	153 (9.9%)
6b	10 (66.7%)	17 (24.6%)	0	188 (12.1%)
Total:	15	69	34	1552
Risk of progressive disease was higher in gastric GISTs with homozygous del/delins				
Significance:		$P = 0.0005$	$P < 0.0001$	$P < 0.0001$

^aBased on previous study on gastric GISTs.⁶

homozygous *KIT* mutations in 4.5% (4 of 89) analyzed GISTs.²⁸ However, one study based on 322 GISTs, including 127 malignant tumors from a clinical imatinib mesylate trial, identified homozygous *KIT* mutations in 17.8% of analyzed cases.¹⁰ Also, a substantially higher frequency (28%) was seen among cases contributed to our study by Sklodowska-Curie Memorial Cancer Center, which specializes in the treatment of advanced GISTs. The higher frequency of homozygous *KIT* exon 11 mutations in the materials from clinical trials and large cancer centers clearly reflects selection bias and further support the idea that homozygous mutations are enriched among patients with malignant, highly advanced tumors.

A combination of clinicopathologic features such as tumor size and mitotic activity is considered to be the most important prognostic parameter in GISTs.¹ However, differences in clinical outcome between GISTs from different locations

have been reported.¹ In general, small intestinal tumors tend to follow a more malignant course of disease than gastric ones.²⁰ In this series, almost entirely based on malignant GISTs, small intestinal tumors were overrepresented, if compared with the data reported by population-based studies.^{29,30} This confirms previous observations that tumors from small intestinal locations are enriched in cohorts of malignant GISTs.¹

Recent studies have also shown that the type of *KIT* mutation might correlate with the clinical outcome. Gastric GISTs with *KIT* exon 11 deletions have more malignant clinical outcomes than the ones with point mutations.⁶ In the present study, none of the 16 gastric GISTs had *KIT* exon 11 point mutations. In contrast, point mutations were found in 31.6% of intestinal GISTs. Previous studies have shown that *KIT* codons 557_558 deletion indicates unfavorable

Table 9 Comparison of risk of progressive disease among small intestinal GISTs with homozygous *KIT* exon 11 del/delins vs GISTs with heterozygous del/delins, GISTs with heterozygous point mutations and all small intestinal GISTs

Prognostic group	Small intestinal GIST <i>KIT</i> exon 11 mutants					
	All homozygous	All ^a	Del/delins homozygous	Del/delins heterozygous ^a	Point mutations homozygous	Point mutations heterozygous ^a
1	0	69 (8.7%)	0	6	0	1 (3.6%)
2	1 (7.7%)	174 (22%)	0	6	1 (25%)	7 (25%)
3a	1 (7.7%)	177 (22.4%)	1 (12.5%)	18	0	7 (25%)
3b	3 (23.1%)	99 (12.5%)	2 (25%)	11	1 (25%)	4 (14.3%)
4	1 (7.7%)	2 (0.3%)	0	0	1 (25%)	1 (3.6%)
5	0	37 (4.7%)	0	3	0	0
6a	3 (23.1%)	108 (13.7%)	2 (25%)	7	1 (25%)	3 (10.7%)
6b	4 (30.8%)	125 (15.8%)	3 (37.5%)	9	0	5 (17.9%)
Total	13	791	8	60	4	28
Risk of progressive disease was higher in GISTs with homozygous del/delins						No difference in risk was detected
Significance		<i>P</i> = 0.01		<i>P</i> = 0.04		<i>P</i> = 0.42

^aBased on previous study on small intestinal GISTs.²⁰

prognosis in GISTs.^{7,8} This deletion was identified in two malignant gastric and one small intestinal/mesenteric GIST, in this study. Duplications in the 3' end of *KIT* exon 11 have been linked to gastric GISTs with rather benign clinical outcome.^{31,32} Contrary to previous observations, two malignant tumors with such mutations including one of intestinal origin were reported in this study.

The current series of 36 GISTs showed a strong association between the presence of homozygous *KIT* exon 11 mutations and malignant clinical outcome. Tumors with such mutations generally had histologic features of sarcomas and developed intra-abdominal and liver metastases in the majority of cases. Therefore, the presence of homozygous *KIT* mutations was coupled with overall malignant features. However, GISTs with homozygous deletion/deletion-insertions showed a significantly higher risk of developing metastases than heterozygous mutants from similar locations.

In most cases, homozygous *KIT* mutations were already found in primary tumors. However, a great majority of these GISTs were at an advanced stage of disease, often with metastases. In two cases, a shift from heterozygosity to homozygosity was seen in metastatic lesions. This might indicate that *KIT*-MT(+)/*KIT*-WT(-) clones have a higher metastatic potential than *KIT*-MT(+)/*KIT*-WT(+) clones and supports the hypothesis that the presence of *KIT*-WT allele can moderate the effect of a *KIT*-MT allele. Also, two separate studies have reported two and three GISTs with a shift from heterozygosity to homozygosity seen only in metastatic lesions.^{11,19} Thus, a shift from heterozygosity to homozygosity might be acquired during disease progression and accumulation of secondary genetic changes. However, one

study reported homozygous *KIT* exon 11 mutations in 2 (15.4%) of 13 incidental, <1 cm GISTs.¹⁴ These findings could suggest that a shift from heterozygosity to homozygosity can also occur at an early stage of tumor development. However, two recent, separate studies failed to find homozygous *KIT* exon 11 mutations among 16 minimal GISTs (Agaimy personal communication).^{29,33} In our series, no minimal GISTs with homozygous *KIT* exon 11 mutations were identified; however, two relatively smaller tumors (Cases 14 and 27) with low risk of developing progressive disease were reported. It is possible that homozygosity detected in small, benign GISTs differs in nature from that found in malignant tumors. Further studies employing different molecular techniques and based on a large number of cases are necessary to clarify this issue.

Familial GIST syndrome is associated with germline *KIT* mutation and development of multiple GISTs. Two studies have reported homozygous *KIT* mutations in large, malignant tumors diagnosed in familial GIST patients, whereas smaller, benign lesions have remained heterozygous.^{30,34} Recently, we have identified a homozygous 1756_1758del leading to the loss of *KIT* codon 579 in a tumor from a patient with familial GIST syndrome. However, this tumor behaved indolently over 16 years.³⁵ These findings suggest that the behavior of familial GISTs with homozygous *KIT* mutations may vary.

An amplification of *KIT* and *PDGFRA* has been reported in gliomas and shown to be more frequent in anaplastic and recurrent tumors than in low-grade lesions.^{36,37} Also, selective *KIT* amplification leading to *KIT* overexpression has been reported in the seminoma subtype of testicular germ cell

tumors and is linked to the progression of carcinoma *in situ* lesion to seminoma.³⁸ In GISTs, amplification of the *KIT*/*PDGFRA* locus or selective amplification of *KIT* appears to be an extremely rare molecular event and has been reported only in a few cases.^{11,39} Also, in our series, the *KIT* locus was not amplified. However, in the majority of analyzed GISTs, at least two copies of mutated *KIT* were present in a substantial number of tumor cells due to the duplication of *KIT*-MT chromosome 4.

In one study, a shift from heterozygosity to homozygosity was observed in two tumors at the time of resistance to imatinib; however, the resistance was also associated with secondary *KIT* mutation and it was unclear whether homozygosity or secondary mutation contributed to insensitivity to imatinib mesylate in these cases.¹⁹ In another study, *KIT* LOH was detected in highly cellular areas in the primary lesion and in liver metastasis resistant to imatinib.¹¹

In our series, four of seven cases treated with imatinib mesylate developed progressive disease after relatively short time of 16–18 months. Moreover, the average survival time for the patients treated with imatinib mesylate, who died of the disease, was not different from the one calculated for the pre-imatinib patients. A second heterozygous *KIT* mutation, 1982T>C (Val654Ala) identified in one case, indicated that mutation related to resistance to imatinib mesylate treatment arose after chromosome 4 duplication. Although, secondary *KIT* mutation could account for tumor resistance, loss of *KIT*-WT allele, followed by duplications of *KIT*-MT allele and possibly increased of *KIT*-MT expression might also contribute to a more malignant clinical behavior and lower sensitivity to imatinib mesylate treatment. However, further clinicopathologic studies based on larger number of cases are necessary to verify this hypothesis.

In summary, this study documents the loss of *KIT*-WT allele and duplication of *KIT*-MT allele as molecular mechanisms leading to a shift from heterozygosity to homozygosity in a subset of GISTs. The risk of progressive disease was significantly higher among gastric and small intestinal GISTs with homozygous *KIT* exon 11 deletion/deletion-insertions than in tumors with heterozygous *KIT* exon 11 mutations. Also, follow-up data showed that the presence of such *KIT* exon 11 mutation represents a sign of disease progression and is associated with malignant course of disease. Thus, detection of homozygous *KIT* exon 11 mutations should be considered an additional adverse prognostic marker in GISTs.

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