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Short Communication

Mutations in Exons 9 and 13 of *KIT* Gene Are Rare Events in Gastrointestinal Stromal Tumors

A Study of 200 Cases

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Gastrointestinal stromal tumors (GISTs), the most common mesenchymal tumors of the gastrointestinal tract, typically express the KIT protein. Activating mutations in the juxtamembrane domain (exon 11) of the c-*kit* gene have been shown in a subset of GISTs. These mutations lead into ligand-independent activation of the tyrosine kinase of c-*kit*, and have a transforming effect *in vitro*. Several groups have studied the clinical implication of the c-*kit* mutation status of exon 11 in GISTs and a possible relationship between c-*kit* mutations and malignant behavior has been established. Recently, a 1530ins6 mutation in exon 9 and missense mutations, 1945A>G in exon 13 of the c-*kit* gene were reported. The frequency and clinical importance of these findings are unknown. In this study we evaluated 200 GISTs for the presence of mutations in exons 9 and 13 of c-*kit*. Six cases revealed 1530ins6 mutation in exon 9 and two cases 1945A>G mutation in exon 13. All tumors with mutations in exon 9 and 13 lacked mutations in exon 11 of c-*kit*. None of the analyzed tumors had more than one type of c-*kit* mutation. All but one of the eight tumors with mutations in exon 9 or 13 of the c-*kit* gene were histologically and clinically malignant. All four of six cases with exon 9 mutation of which location of primary tumor was known, were small intestinal, suggesting that this type of mutation could preferentially occur in small intestinal tumors. Exon 9 and 13 mutations seem to be rare, and they cover only a small portion (8%) of the balance of GISTs that do not have mutations in exon 11 of c-*kit*. This finding indicates that other genetic alterations may activate c-*kit* in GISTs, or that KIT is not activated by mutations in all cases. ***(Am J Pathol 2000, 157:1091–1095)***

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract. GISTs differ from other gastrointestinal mesenchymal tumors histologically, immunohistochemically, and genetically. Immunohistochemically they are typically positive for CD117 (KIT protein) and CD34 but negative for S100-protein and desmin and express smooth muscle actin in 20 to 40% of cases.[1]

The c-*kit* gene encodes for a receptor for a growth factor termed stem cell factor. The KIT protein (stem-cell factor receptor) contains an internal tyrosine kinase component and regulates cell growth and survival.[2–5] Constitutional KIT expression has been shown in germ cells,

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Table 1. Oligonucleotide Primers Used in this Study

Primer	Sequence	PCR products
CK9.1F	5′-TCC TAG AGT AAG CCA GGG CTT-3′	Exon 9
CK9.3R	5′-TGG TAG ACA GAG CCT AAA CAT CC-3′	283 base pair
CK10.4F	5′-CCA GAG TGC TCT AAT GAC TG-3′	Exon 11
CK11.3R	5′-AGC CCC TGT TTC ATA CTG AC-3′	284 base pair
CK13.1F	5′-GCT TGA CAT CAG TTT GCC AG-3′	Exon 13
CK13.2R	5′-AAA GGC AGC TTG GAC ACG GCT TTA-3′	193 base pair

melanocytes, hematopoietic stem cells, mast cells, and the interstitial cells of Cajal.[6–8]

Specific mutations in the c-*kit* gene have been found in myeloproliferative disorders,[9] mast cell neoplasms,[10] seminoma,[11] and in acute myeloid leukemia.[12] Mutations in the juxtamembrane domain (exon 11) of the c-*kit* gene have been shown in GISTs,[13,14] and in a mast-cell leukemia cell line.[15] The juxtamembrane domain mutations have been shown to lead into ligand-independent activation (phosphorylation) of the tyrosine kinase of c-*kit* and have a transforming effect *in vitro*.[13] The c-*kit* mutation status of exon 11 has been studied in GISTs by several groups[16–20] and the relationship between presence of mutations and malignant behavior has been demonstrated.[16,17,19]

Recently, 1530ins6 mutation in exon 9 (extracellular domain) and 1945A>G mutation in exon 13 (kinase domain) of the c-*kit* gene were reported in a small group of GISTs, that lacked mutations in exon 11 (juxtamembrane domain) of c-*kit*.[21] In this study, we evaluated the frequency and clinicopathological importance of these mutations in a large series of benign and malignant GISTs.

Materials and Methods

Tissue Material

Two hundred GISTs were obtained from the files of the Armed Forces Institute of Pathology, Washington, DC; the Haartman Institute of the University of Helsinki, Helsinki, Finland; the Maria Sklodowska-Curie Memorial Institute, Krakow, Poland; and the Medical University, Lodz, Poland.

Immunohistochemistry

The tumors were immunohistochemically analyzed for CD34, CD117 (the c-*kit* proto-oncogene protein product), α-smooth muscle actin, desmin, and S100-protein. Immunohistochemistry was performed by using the avidin-biotin peroxidase complex system and diaminobenizidine as the chromogen, as previously described.[22] Negative and positive controls were included in each run.

Molecular Studies

Exons 9, 11, and 13 of the c-*kit* gene was evaluated for the mutations by polymerase chain reaction amplification and direct sequencing of the amplification products. Formalin-fixed tumor and normal tissue were microdissected from paraffin blocks. DNA was extracted as previously described.[17] Primer sequences are shown in Table 1. Annealing temperatures for all sets of primers was 56°C. Cycling condition and the reaction mix were standard as recommended by Perkin-Elmer-Cetus (Foster City, Ca). Previously described precautions[23] were followed to avoid and monitor possible cross-contamination. The polymerase chain reaction assays amplified fragments containing the entire sequences of the exons 9, 11, and 13 of the c-*kit* gene. The amplification products were size-fractionated on 2% agarose gels, purified from the gel, and sequenced directly using forward and reverse primers. The sequences were analyzed using the Lasergene software (DNASTAR, Madison, WI) in connection with the data of the GenBank 110/EMBL55 database (January 99 edition).

Results

Clinicopathological Features

The study consisted of 188 primary and 12 recurrent or metastatic GISTs. Primary tumors represented 10 esophageal, 79 gastric, 42 small intestinal, 19 colonic, 25 rectal, three mesenteric, and three omental GISTs. In seven cases, with the large abdominal mass at presentation, the primary localization could not be established. Nine intra-abdominal recurrences and three liver metastasis represented two gastric, four small intestinal, one colonic, and five GISTs of unknown primary location. Of the 200 patients, 116 were male and 84 were female. The age of the patients ranged from 17 to 90 years (median, 60 years). GISTs were diagnosed based on previously published criteria.[1] All analyzed tumors showed GIST-specific immunophenotype; KIT protein expression was documented in all cases. Seventy-four percent of cases showed co-expression of CD34. Forty-one percent and 20% of cases expressed α-smooth muscle actin and S100 protein, respectively. All but four GISTs were desmin-negative. Examples of the histological and immunohistochemical features are shown in Figure 1, A–E.

Evaluation of c-kit Mutation

Two hundred GISTs were evaluated for the presence of c-*kit* mutations. One hundred three (52%) GISTs had mutation in exon 11 of the c-*kit* gene. Mutation in exon 9, representing insertion of GCC TAT and resulting in duplication of amino acid residues Ala[502] and Tyr[503], was seen in six GISTs. The exon 13 1945A>G mutation re-

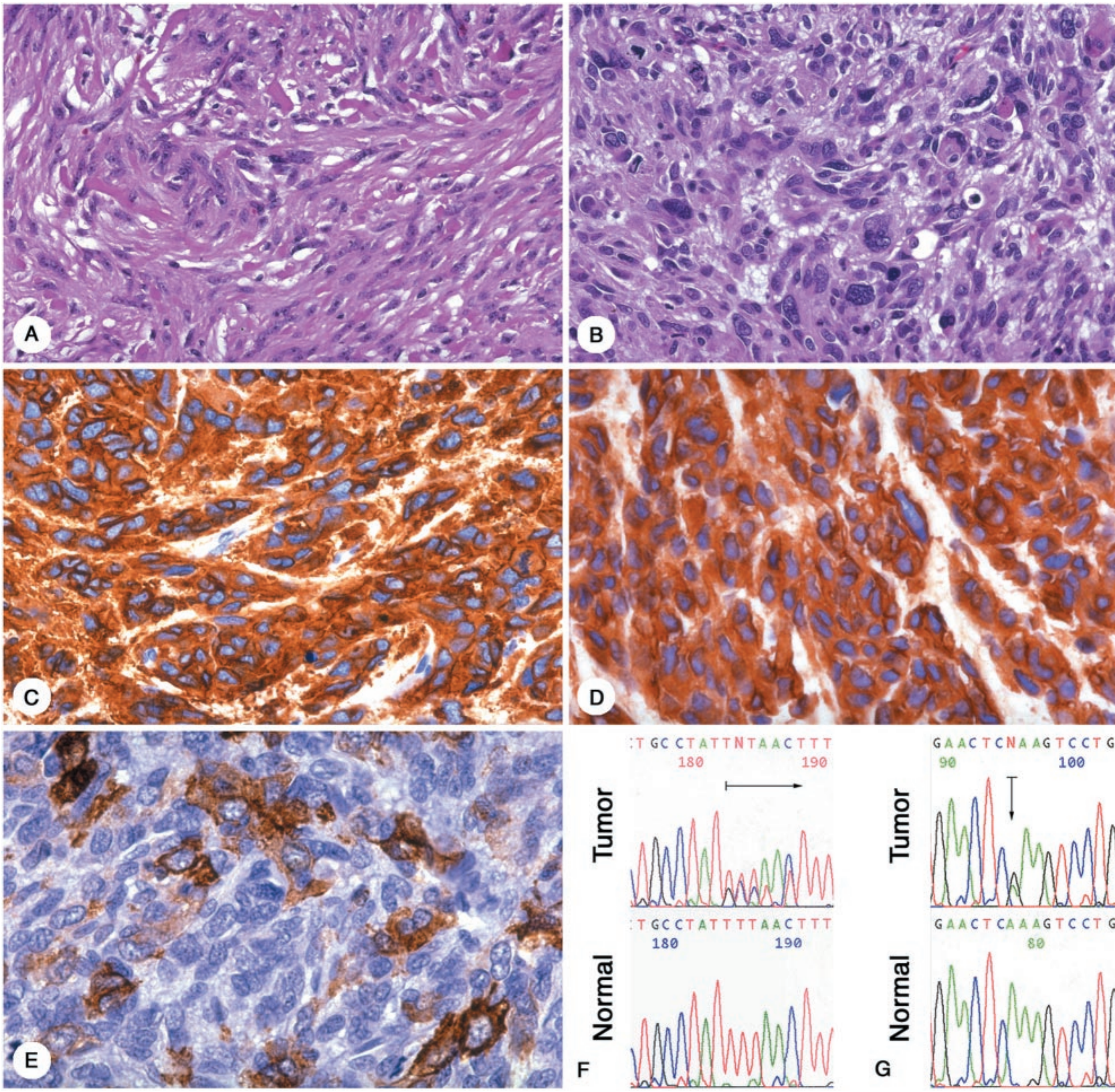

Figure 1. A: Case 1, spindle-cell GIST from small intestine with so-called skeinoid fibers. **B:** Case 8, pleomorphic GIST from stomach. **C:** Case 8, positive for CD117. **D:** Case 8 positive for CD34. **E:** Case 8, focally positive for α-smooth muscle actin. **F:** Case 1, sequence analysis of exon 9 from the tumor and corresponding normal tissue. **Arrow** indicates heterozygous 1530ins6 mutation (GCC TAT). **G:** Case 7, sequence analysis of exon 13 from tumor and corresponding normal tissue. **Arrow** indicates heterozygous 1945A>G mutation.

sulting in substitution of a Glu to Lys^{642} was seen in two GISTs. All mutations found in exon 9 and 13 were heterozygous. Examples of mutated sequences are shown in Figure 1, F and G. None of the analyzed tumors revealed the presence of more than one type of c-*kit* mutation. No mutations were found in polymerase chain reaction products amplified from microdissected normal tissue. Exon 9 1530ins6 mutation was found in 6% and exon 13 1945A>G mutation was found in 2% of GISTs that lacked mutation in exon 11. Together mutations in exons 9, 11, or 13 were found in 56% of all analyzed GISTs.

Correlation between Pathological and Genetic Features

Four of six tumors with mutation in exon 9 were localized in the small intestine. In two GISTs (cases 5 and 6), the primary localization could not be clearly established because of the large size and infiltration of different organs. However, in neither case was the small intestine ruled out as a primary location. Six of eight tumors with duplication in exon 9 or point mutation in exon 13 had an aggressive clinical behavior. Clinical follow-up was not available in

Table 2. Clinicopathological Data of Five GISTs Containing 1530ins6 Mutation in Exon 9

Case	Age/Sex	Ethnicity	Localization	Tumor size	Cell type	Mitoses/ 10 HPF	Immunophenotype**					Status and follow-up in months
							CD117	CD34	SMA	DES	S100	
1	35/F	Hispanic	Small intestine	5 cm	Spindle	0	100%	5%	0%	0%	0%	NA
2	68/M	Caucasian	Small intestine	7 × 6 cm	Spindle	17	100%	0%	0%	0%	0%	DOD (36)
3	54/M	Caucasian	IAR, primary tumor in small intestine	5 cm	Spindle	2	100%	0%	0%	0%	0%	Abdominal wall metastasis (48)
4	48/M	Caucasian	Small intestine	6–7 cm	Epithelioid	3	100%	0%	0%	0%	60%	DOD (15)
5*	67/F	Caucasian	Retroperitoneal mass	15 cm	Epithelioid	5	100%	50%	0%	0%	0%	Liver metastasis at operation
6*	68/F	Caucasian	Large abdominal mass	16 × 14 × 12	Spindle	4	100%	40%	0%	0%	0%	DOD (45)

*Localization of primary tumor could not be clearly established; **, percentage of cells showing positive staining. NA, not available; DOD, died of disease; IAR, intra-abdominal recurrence.

case 1 who had a 5-cm-large small-intestinal GIST with low mitotic index. The clinicopathological data of GISTs carrying mutation in exons 9 and 13 are summarized in Tables 2 and 3.

Discussion

Activating mutations in the KIT gene have been found in the spectrum of different tumors. The kinase domain of the KIT gene is mutated in mast cell neoplasms and seminomas,[10,11] whereas extracellular and transmembrane domains are mutated in acute myeloid leukemia and in myeloproliferative disorders.[9] In the first study on GISTs mutations were mapped exclusively to exon 11, the KIT juxtamembrane domain.[13]

Approximately 40 to 50% of GISTs, mostly the malignant variants, have mutations in the juxtamembrane domain of the c-*kit* gene,[16,17,19] although some studies found mutations in only 15%[20] or others in as many as 80%[21] of analyzed cases. Lack of mutations in exon 11 in a significant portion of GISTs may suggest that mutations may occur in other domains of the KIT gene. However, no mutations were found in exon 17 (kinase domain) in a large group of GISTs studied;[19] this is the area where KIT mutations occur in mastocytoma[10] and seminoma.[11]

Recently, new mutational hotspots were identified as a result of comprehensive sequencing of KIT cDNA obtained from 13 GISTs, that were negative for exon 11 (juxtamembrane domain) mutations. Mutations in exon 9 and exon 13 were detected in six (46%) and two (15%) of 13 cases suggesting that such mutations may be relatively common in GISTs.[21]

In this study, we analyzed 200 GISTs, including 97 cases that did not have mutations in exon 11 of c-*kit*. Mutations in exon 9 were found in six cases. These mutations were all two codon duplications (Ala^{502} and Tyr^{503}), similar to those described by Lux et al.[21] Exon 13 mutation (missense mutation leading to substitution of Glu to Lys^{642}) was seen in only two cases. Our results confirm that some GISTs may have mutations in the previously described hotspot areas in exon 9 and exon 13 of c-*kit*, but indicate that such mutations are rare. Therefore, these mutations do not offer a KIT mutation-based pathogenetic explanation for GIST oncogenesis for any substantial portions of those GISTs that are negative for exon 11 mutations.

Malignant clinical behavior of the tumor was documented in seven of eight cases that had mutation in exons 9 and 13, suggesting that similar to mutations in exon 11 of c-*kit*, those in exons 9 and 13 predominantly occur in malignant *versus* benign GISTs.[16,17,19]

1530ins6 mutation in exon 9 seems to occur preferentially in malignant small intestinal tumors and may be the marker of malignant behavior for the GISTs of this location. However, a larger group of small intestinal GISTs with clinical follow-up should be evaluated to establish the statistical significance of this observation.

The apparent difference in the frequency of exon 9 and exon 13 mutation (combined 8% in this study *versus* 61% a previous study[21]) has several potential explanations. They most importantly include case selection bias, as larger malignant tumors may have been included in the frozen-tumor bank collection available for cDNA analysis in the earlier study. Another study showed that larger

Table 3. Clinicopathological Data of Two GISTs Containing 1945A>G Mutation in Exon 13

Case	Age/Sex	Ethnicity	Localization	Tumor size	Cell type	Mitoses/ 10 HPF	Immunophenotype**					Status and follow-up in months
							CD117	CD34	SMA	DES	S100	
7	70/F	Caucasian	Esophagus	25 cm	Spindle	5	100%	100%	80%	0%	0%	DOD (18)
8	53/M	unknown	Stomach	3.5 cm	Spindle/epithelioid/ pleomorphic	29	100%	100%	15%	0%	0%	IAR (6)

*Localization of primary tumor could not be clearly established; **, percentage of cells showing positive staining. NA, not available; DOD, died of disease; IAR, intra-abdominal recurrence.

tumors more frequently revealed mutations in exon 11 of the c-*kit* gene than the smaller ones.[19]

Geographic genetic differences cannot be completely ruled out, as our material was predominantly composed of individuals of Caucasian ethnicity (80%) from northern and central Europe. An extensive study on GISTs from ethnically different populations has to be performed to exclude such a possibility.

Technical factors, including normal tissue contamination, could impair the detection of mutation. In this study, tumor tissue was carefully microdissected to maximize the tumor content in the samples to be analyzed for mutation. Also, frequent detection of mutation in exon 11, using the same strategy, provided an internal positive control in our study.

In summary, the present study showed that mutations in exons 9 and 13 of c-*kit* may occur in GISTs, predominantly in malignant tumors. However, such mutations seem to be rare. Other structural or functional genetic alterations may activate KIT in GISTs, or other genetic mechanism may be operational.

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