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Mutations in c-*kit* Gene Exons 9 and 13 in Gastrointestinal Stromal Tumors among Japanese

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Gain-of-function mutation in c-*kit* proto-oncogene exon 11 has been described in about 20–50% of gastrointestinal stroma tumor (GIST). Recently, additional mutational hot-spots in exon 9 and exon 13 of the c-*kit* gene have been reported in GISTs without mutations of exon 11, but a subsequent report in a Western population indicated that only a small portion of GISTs (eight of 200 GISTs, 4%) showed mutations in these regions. In this study, we evaluated mutations in exon 9 and exon 13 of the c-*kit* gene by both polymerase chain reaction-single strand conformation polymorphism analysis and direct sequencing in 48 GISTs in a Japanese population, for which the clinicopathological and immunohistochemical features and mutations in exon 11 had previously been reported. C-*kit* gene mutation in exon 9, representing insertion of GCC TAT, was identified in only 4 of 48 GISTs (8%), and none of the GISTs had mutations in exon 13. All four GISTs with mutation in exon 9 were high-risk, and the patients died of multiple tumor metastasis. Mutations in exon 9 and exon 13 of the c-*kit* gene were also rare events in Japanese GISTs and were related to a poor prognosis. These results in Japanese are consistent with those in Western populations, although a preferential occurrence of GISTs with exon 9 mutation in the small intestine, which was suggested in a previous report, was not observed.

Key words: C-*kit* — Mutation — Exons 9 and 13 — GIST

In the past, gastrointestinal stroma tumor (GIST) was believed to originate from smooth muscle, based on histological and immunohistochemical findings.[1–3] However, the expression of specific molecules such as KIT, CD34 and an embryonic form of smooth muscle myosin heavy chain isoform (SMemb) have been reported in most GISTs, and interstitial cells of Cajal (ICC) are the only cells in the gastrointestinal (GI) tract that express all of these molecules.[4–7] These findings lead us to consider that GIST is a tumor of ICC (TICC). Gain-of-function mutations in c-*kit* gene exon 11 were demonstrated in GISTs by Hirota and colleagues,[4] and subsequent reports have indicated a close correlation between c-*kit* gene mutations and a poor prognosis.[8–10]

We also analyzed mutations in c-*kit* gene exon 11 in GISTs and reported that 31% of primary GISTs, four of seven metastatic GISTs and none of eight esophageal leiomyomas, showed mutations.[11] However, in contrast to previous studies in Western populations, in our study, the presence or absence of c-*kit* gene mutations in the primary GISTs did not affect the survival rates of Japanese patients. The sites of mutations in c-*kit* gene exon 11 in our cases tended to be different from those in Western populations. In our study, mutations in some GISTs were located at the distal portion (codon 566–580) in c-*kit* gene exon 11, and all of the patients with mutations at this location were elderly females who showed long-term survival. Mutations at this location seem to be quite rare in previous studies in Western populations.[8–10] It has been suggested that mutations have different effects based on their location in exon 11 of the c-*kit* gene, and certain factors, such as race and sex, might induce this difference, although Taniguchi *et al.* reported different results in Japanese cases.[12]

Recently, Lux *et al.* reported mutations in the extracellular and kinase domain of the c-*kit* gene in all of eight GISTs without mutations in exon 11.[13] However, the frequency and clinical importance of these mutations in Japanese cases are still unknown. In this study, we analyzed mutations in c-*kit* gene exon 9 and exon 13 in GISTs, and compared multiple pathologic parameters of GISTs with these mutations. We also compared the results in Western and Japanese populations.

MATERIALS AND METHODS

GIST Forty-eight GISTs were previously examined, and clinicopathological data, telomerase activity, immunohistochemical phenotypes and mutations of c-*kit* gene exon

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11 have been reported.[11] As to the prognosis of the patients, recent follow-up data were obtained in some cases.

Analysis of c-*kit* gene mutation in exons 9, 11 and 13 All 48 GISTs were examined for mutation in c-*kit* gene exons 9 and 13 by both polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing of PCR products as described previously. Briefly, DNA was extracted from tumor tissues which were either frozen (n=30) or formalin-fixed and paraffin-embedded (n=18). The DNA was amplified by PCR using the primers listed in Table I to amplify exon 9 and exons 12–13 of the c-*kit* gene. Nested PCR was performed for amplification of exons 12–13. The amplified DNA was electrophoresed on 12.5% acrylamide gel (150×100×1 mm) for 3 h at 20°C. A single mutated band was then excised from the dried gel. Direct sequencing of the DNA extracted from the gel was carried out using an ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Chiba) and an "ABI PRISM" 377 DNA Sequencer using the same primers as were used for amplification. PCR products, in which abnormally shifted bands were not found in PCR-SSCP analysis, were also sequenced to avoid false-negative results.

Table I. Primers Used for Amplification of C-*kit* Gene Exons 9, 11 and 12–13

C-*kit* gene	
Exon 9	5′-ATTTATTTTCCTAGAGTAAGCCAGGG-3′
	5′-ATCATGACTGATATGGTAGACAGAGC-3′
Exon 11	5′-TAGCTGGCATGATGTGCATT-3′
	5′-TGGAAAGCCCCTGTTTCATA-3′
Exon 12, 13	
(outer)	5′-ATTTTGAAACTGCACAAATGGTCCTT-3′
	5′-GCAAGAGAGAACAACAGTCTGGGTAA-3′
(inner)	5′-CACCATCACCACTTACTTGTTGTCT-3′
	5′-GACAGACAATAAAAGGCAGCTTGGAC-3′

RESULTS

Of the 48 primary GISTs, only four (cases 3, 11, 12 and 18) (8.3%) showed mutations in exon 9 of the c-*kit* gene (Fig. 1), representing a 6-bp insertion of GCC TAT, as reported by Lux *et al*.[13] None of the GISTs had mutations in exon 13. Clinicopathological and immunohistochemical features of GISTs with mutations in c-*kit* gene exon 9 are summarized in Table II. In case 12, shifted bands showed a different mobility compared to those in other cases. Subsequent sequencing revealed an additional silent point mutation (GTG to GTA) in codon 497. In cases 3, 11 and 18, tumors lacked a mutation in exon 11, but a missense point mutation in exon 11 (TTT→TCT at codon 584: Phe→Ser) was also present in case 12. In this case, bands corresponding to normal sequences were not observed in either exon 9 or exon 11 by PCR-SSCP analysis.

All four GISTs with mutations in c-*kit* gene exon 9 were spindle cell-type, and were immunohistochemically positive for KIT and CD34 (Fig. 2) and negative for S-100 protein. Of these four cases, three were also weakly positive for α-smooth muscle actin (α-SMA). According to the

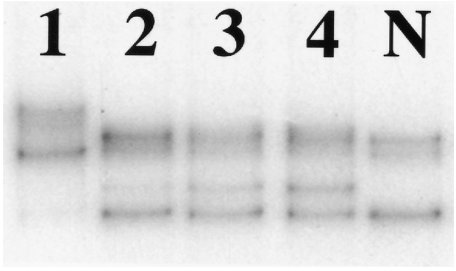

Fig. 1. SSCP analysis of exon 9 of the c-*kit* gene. Abnormally shifted bands were seen in GISTs. In case 12, shifted bands showed different mobility compared to those in other cases and bands corresponding to normal sequences were not observed (lane 1, case 12; lane 2, case 3; lane 3, case 11; lane 4, case 18; N, negative control).

Table II. Clinicopathological and Immunohistochemical Features of GISTs with Mutations in C-*kit* Gene Exon 9

Case No.	Gender	Age	Primary site	Maximum size (cm)	Ki-67 LI (%)	Telomerase activity	Immunohistochemistry				C-*kit* mutation		Follow-up
							C-*kit*	CD34	α-SMA	S100	Exon 11	Exon 13	
3	M	58	stomach	15	7.8	–	+	+	+	–	wild	wild	DOD, 27 mos
11	M	41	stomach	12	16.3	ND	+	+	+	–	wild	wild	DOD, 21 mos
12	M	66	stomach	8	9.9	ND	+	+	–	–	point mutation	wild	DOD, 104 mos
18	M	60	duodenum	7	15.1	+	+	+	+	–	wild	wild	DOD, 27 mos

LI, labeling index; α-SMA, α-smooth muscle actin; M, male; ND, not done; +, positive; –, negative; DOD, died of disease; mos, months.

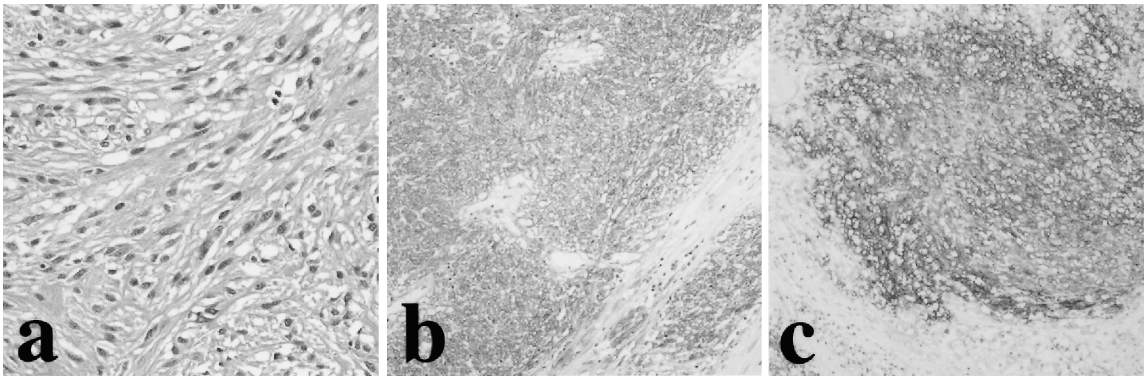

Fig. 2. GISTs with exon 9 mutation of the c-*kit* gene. All GISTs show a spindle cell morphology (a) and are immunoreactive for KIT (b) and CD34 (c).

criteria of Franquemont with some modifications, all four GISTs with mutations in exon 9 were classified as high-risk and all four patients died of disease from 21 to 104 months after the initial surgery due to multiple tumor metastasis.

Telomerase activity was found in case 18, but not in case 3. The two other cases were not examined.

DISCUSSION

Previously, c-*kit* gene mutations in exon 11 have been reported in approximately 20–50% of GISTs,[8–12] and a correlation between these mutations and a poor prognosis has been pointed out in some reports in Western populations.[8–10] However, in our study, we did not find any correlation between c-*kit* gene mutations in exon 11 and malignant potential of GIST in Japanese cases.[11] The location of a deletion mutation in exon 11 in our cases tended to be different from those in Western populations, and we speculated that a different effect of mutations based on the different location in exon 11 may be responsible for the different behavior of GISTs between Western and Japanese populations. However, more than half of GISTs seem to lack any mutation in c-*kit* gene exon 11 in both Western and Japanese populations, and there may be additional genetic alterations in these cases.

Recently, Losata *et al.* analyzed mutations in exons 9 and 13 of the c-*kit* gene in a large number of GISTs in a Western population.[14] However, in contrast to the first report by Lux *et al.*,[13] mutations in exons 9 and 13 were observed in only six (3%) and two (1%) of 200 GISTs in a Western population. Of these eight patients, six were Caucasian and one was Hispanic. The ethnicity of the other case is unknown. All four of the six cases with exon 9 mutations for which the location of the primary tumor was known were located in the small intestine. All but one of the eight tumors with exon 9 mutation or point mutation in exon 13 had an aggressive clinical behavior.[14]

Very recently, Isozaki *et al.* also reported a germline-activating mutation in exon 13, which was the same mutation as in previous reports,[13, 14] in a French family with multiple gastrointestinal stromal tumors in the small intestine and diffuse hyperplasia of the myenteric plexus layer.[15] All tumors were of low malignancy grade and there was no metastasis.

On the other hand, in this study in a Japanese population, mutation in c-*kit* gene exon 9 and exon 13 was observed in four (8.3%) and none of the 48 primary GISTs, respectively. All four of the GISTs with exon 9 mutation were high-risk and all of the patients died of multiple tumor metastasis. These results indicate a correlation between this type of mutation and a poor prognosis, and seem to be consistent with the results of Losata *et al.*,[14] but the rates of mutation were significantly lower than those of Lux *et al.*[13] However, three of the four GISTs with exon 9 mutation in our study were localized in the stomach, and a preferential occurrence of GISTs with this type of mutation in the small intestine, as suggested by Losata *et al.*, was not observed.

In case 12, a missense point mutation in exon 11 was found in addition to the mutation in exon 9. Cases with mutation in both exon 11 and other exons of the c-*kit* gene have not been reported in previous studies. In this case, bands corresponding to normal sequences were not observed by PCR-SSCP analysis in either exon 9 or exon 11. In our previous studies regarding mutation in c-*kit* gene exon 11 by PCR-SSCP analysis, lack of a normal allele was seen in some GISTs in the tubular GI tract and the omentum.[11, 16] Two cases of GISTs with exon 13 mutation reported by Lux *et al.* also lacked wild-type KIT sequences.[13] Loss of 4q11–12, where the c-*kit* gene is located, has not been reported using comparative genomic

hybridization,[17] microsatellite markers[18] and fluorescence *in situ* hybridization.[14] Although we hypothesized that additional deletions or mutations might occur at the sites of primers for the PCR amplification of exon 11, another possibility, that the loss of wild-type allele and duplication of the mutant occurred in the tumorigenesis of GIST, might explain the widespread lack of normal sequences from exon 9 to exon 13 in case 12.

In summary, mutations in c-*kit* gene exon 9 were infrequent in GISTs in a Japanese population. All of the GISTs with this type of mutation were high-risk and the patients died of multiple tumor metastasis, as seen in Western populations. However, alteration in the c-*kit* gene is still unknown in about half of GISTs in both Western and Japanese populations. Mutation of the c-*kit* gene may not be essential for tumorigenesis in these tumors. KIT-SCF autocrine growth regulation has been suggested in other tumors, such as small cell lung cancers, ovarian cancers, breast cancers and testicular tumors.[19–22] Further studies will be needed to understand the mechanism of KIT activation in the development of GIST.

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