C-kit Gene Abnormalities in Gastrointestinal Stromal Tumors (Tumors of Interstitial Cells of Cajal)

Shinji Sakurai, Tomoki Fukasawa, Ja-Mun Chong, Akira Tanaka and Masashi Fukayama

Department of Pathology, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachigun, Tochigi 329-0498

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the GI tract, and expresses KIT and CD34 in most cases. Gain-of-function mutation of the c-kit proto-oncogene has been described, but its significance in GIST has not yet been fully evaluated. Mutation in exon 11 of the c-kit gene was determined by both polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing in primary and metastatic GISTs and esophageal leiomyomas in Japanese subjects. C-kit gene mutation was identified in 15 of 48 primary GISTs (31%), four of seven metastatic GISTs, but none of the leiomyomas. Three mutations were mis-sense point mutations, and 16 were in-frame deletions of 3–48 bp. C-kit gene mutation was observed equally in low- and high-risk groups, and was not related to any clinical and pathologic factors, phenotypes or Ki-67 labeling index (LI) of tumor cells. In five of 15 deletion mutations (four in primary tumors and one in a metastatic tumor), the mutations were present at the distal location of exon 11 of the c-kit gene, which was a minor mutation in previous reports from Finland and the USA. C-kit gene mutations in GIST are not always related to a poor prognosis, but further comparative studies are necessary in Western and Japanese populations.

Key words: C-kit — Mutation — GIST

Gastrointestinal stromal tumor (GIST), once assumed to be of smooth muscle cell origin, consists of mesenchymal cells with or without partial differentiation to smooth muscle, neuronal cell, or both.1–5) The normal cellular counterpart of GIST in the gastrointestinal (GI) tract was not clarified until CD34, KIT and the embryonic form of smooth muscle myosin heavy chain were commonly demonstrated in GIST and interstitial cells of Cajal (ICC).6–8) Based on these findings, it is now considered that most GISTs are tumors of ICC (TICC), or at least show differentiation to ICC. With regard to genetic alterations in GIST, Hirota et al. recently demonstrated gain-of-function mutations of the c-kit gene in five of six tumors, in the region between the transmembrane domain and the tyrosine kinase domain of KIT (codons 550–560).6) They also reported a familial GIST with germline mutation of the c-kit gene, which is occasionally accompanied by hyperpigmentation and mast cell hyperplasia.9) Both findings indicate that gain-of-function mutations of the c-kit gene may be essential for the development of GIST. On the other hand, three subsequent reports demonstrated a correlation between c-kit gene mutation and a poor prognosis, suggesting that this mutation plays a role in progression of the tumor.10–12) Thus, the significance of c-kit gene mutation in the development and progression of this particular tumor of GI tract has not yet been fully clarified. Since we previously demonstrated that telomerase reactivation also plays a role in progression of the tumor,13) in the present study we evaluated c-kit gene mutation in a large number of cases including the previous series, which enabled us to compare multiple pathologic parameters of GIST with c-kit gene mutation. Furthermore, we also compared the mutation in six pairs of primary and metastatic tumors.

MATERIALS AND METHODS

GIST In this study, we defined ‘GIST’ as an inclusive category, i.e., primary non-epithelial tumors of the stomach and intestine, regardless of immunohistochemical phenotypes. Forty-eight GISTs were surgically resected from 47 patients (age: 33–80 years, average 60 years, 20 males and 27 females). Twenty-seven of these tumors were examined in our previous studies and the clinicopathological details and telomerase activities of the tumors have been reported. The sites of the primary tumors were the stomach (n=27), small bowel (n=14), and large bowel (n=7). Of these cases, seven metastatic tumors of six patients (four from the liver and three from the peritoneum) were obtained and also examined. Formalin-fixed and paraffin-embedded specimens were used for histopathological and immunohistochemical studies. In addition to GISTs, eight leiomyomas of the esophagus were similarly examined. All of the GISTs were histologically classified as high- or low-risk according to the criteria of Franquemont with some modifications, as previously reported13): high-risk GIST = a) size >5 cm and mitotic count >2/10 high-power fields (HPF) or b) size >5 cm or mitotic rate >2/10 HPF, and Ki-
Analysis of c-kit: gene mutation  All 48 primary and seven metastatic GISTs and eight leiomyomas of the esophagus were examined for mutation in c-kit gene exon 11 by both polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing of PCR products. DNA was extracted from tumor tissues which were either frozen (n=35) or formalin-fixed and paraffin-embedded (n=20). In one case, DNA was extracted from both materials. The DNA was amplified by PCR with upstream primers (5′-TAGCTG-GCATGATGTGCATT-3′) and downstream primers (5′-TGGAAAGCCCCTGTTTACA-3′) to amplify exon 11 of the c-kit gene.

PCR-SSCP analysis was carried out as described previously. The amplified DNA was electrophoresed on 12.5% acrylamide gel (150×100×1 mm) for 4 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 with an ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction KIT (Applied Biosystem, Chiba) on an ABI PRISM TM 377 DNA Sequencer using the same primers as were used for amplification. PCR products, in which abnormally shifted bands were detected as abnormally shifted bands by PCR-SSCP analysis, and three were only detected by direct sequencing. The mutant bands were accompanied by normal bands in the samples derived from frozen tumor tissues (11/35) or formalin-fixed and paraffin-embedded tumor tissues (31%), four of seven metastatic GISTs and none of eight esophageal leiomyomas showed mutations in exon 11 of the c-kit gene (Fig. 1). Sixteen of 19 mutations were detected as abnormally shifted bands by PCR-SSCP analysis, and three were only detected by direct sequencing. The mutant bands were accompanied by normal bands in most cases, but only abnormally shifted bands were amplified in the SSCP gel in cases 14 and 42. With regard to the source of DNA, the mutation detection rates were similar in the samples derived from frozen tumor tissues (11/35) and formalin-fixed and paraffin-embedded tumor tissues (8/20). In case 48, the same mutations were found in DNA extracted from both paraffin-embedded sections at the primary site and frozen tissue at a metastatic site.

Mutant bands were observed in three metastatic GISTs, while no mutation was observed in the corresponding primary tumors (cases 7, 13 and 16). In case 7, the mutation was identified in the hepatic metastasis, but not in the peritoneal metastasis. In case 14, the same shifted bands were observed in both primary and metastatic tumors.

Sequence of the mutation: The sites of the mutations and the predicted amino acid sequences of the mutant c-kit genes are presented in Fig. 2. The predominant type of mutation was an in-frame deletion and others were point mutations that changed the amino acid base sequences. Sixteen GISTs, including four metastatic tumors, showed in-frame deletions of 3–48 bp, including two combined
### Table I. Clinicopathologic Data, Immunohistochemistry and C-kit Mutations of GISTs at Primary Sites

| Case No. | Age  | Gender | Primary site | Size of the primary site (cm) | Ki-67-LI (%) | Telomerase activity | Immunohistochemistry | C-kit mutation | Follow-up |
|----------|------|--------|--------------|-------------------------------|-------------|---------------------|----------------------|-----------------|-----------|
|          |      |        |              |                               |             |                     | C-kit CD34 α-SMA S-100 | p/f      |           |           |
| High risk|      |        |              |                               |             |                     |                      |                 |           |
| 1        | 56   | M      | stomach      | 10                            | 16.3        | ND                  | + + − − − − f         | 21 bp deletion | alive, 7 mos |
| 2        | 57   | M      | stomach      | 17                            | 4.2         | + − − + + − − f     | wild                | DOD, 59 mos    |
| 3        | 58   | M      | stomach      | 15                            | 7.8         | − + + + − − − − f   | wild                | DOD, 27 mos    |
| 4        | 74   | F      | stomach      | 19.5                          | 5.6         | + + + + − − − − f   | wild                | DOD, 47 mos    |
| 5        | 68   | F      | stomach      | 9.5                           | 4.9         | − + + + − − − − f   | 21 bp deletion      | alive, 97 mos  |
| 6        | 59   | F      | stomach      | 12.5                          | 14.8        | + + + + − − − − f   | 6 bp deletion       | DOD, 3 mos     |
| 7        | 66   | F      | stomach      | 11                            | 11.8        | ND                  | + + − − − − p         | wild            | DOD, 60 mos  |
| 8        | 70   | F      | stomach      | 10                            | 14.9        | ND                  | + − − − − − − p       | wild            | alive, 5 mos  |
| 9        | 66   | F      | stomach      | 4                             | 8.6         | ND                  | + + − − − − p         | wild            | alive, 18 mos |
| 10       | 52   | M      | stomach      | 7                             | 4           | ND                  | + + − − − − p         | wild            | alive, 20 mos |
| 11       | 41   | M      | stomach      | 12                            | 16.3        | ND                  | + + − − − − p         | wild            | alive, 17 mos |
|          |      |        |              |                               |             |                     |                      |                 |           |
| Low risk |      |        |              |                               |             |                     |                      |                 |           |
| 12       | 66   | M      | stomach      | 8                             | 9.9         | ND                  | + + − − − − p         | point mutation    | DOD, 104 mos |
| 13       | 77   | M      | small int.   | 13                            | 11.1        | + + + − − − − f     | wild                | alive, 53 mos   |
| 14       | 46   | M      | small int.   | 10.5                          | 10.5        | ND                  | + − − − − − − p       | 24 bp deletion   | DOD, 22 mos  |
| 15       | 40   | M      | small int.   | 25                            | 8.1         | ND                  | + + − − − − − − f     | DOD, 38 mos     |
| 16       | 70   | F      | small int.   | 3                             | 17.8        | ND                  | + − − − − − − p       | wild            | alive, 162 mos|
| 17       | 49   | M      | small int.   | 4.5                           | 10.0        | ND                  | + − − − − − − p       | point mutation    | alive, 33 mos |
| 18       | 60   | M      | small int.   | 7                             | 15.1        | + + + + − − − − f   | wild                | alive, 27 mos   |
| 19       | 47   | F      | small int.   | 4                             | 9.0         | ND                  | + + − − − − − − p     | wild            | alive, 14 mos |
| 20       | 33   | M      | colon        | 12.5                          | 21.1        | + − − − − − − p     | wild                | DOD, 15 mos     |
| 21       | 71   | M      | colon        | 20                            | 18.9        | ND                  | + − − − − − − p       | DOD, 24 mos     |
| 22       | 56   | F      | colon        | 9                             | 16.6        | + − − − − − − p     | wild                | alive, 5 mos    |
| 23       | 80   | M      | colon        | 4.5                           | 17.1        | ND                  | + − − − − − − p       | wild            | alive, 14 mos |
| 24       | 53   | F      | colon        | 2.8                           | 14.4        | ND                  | + − − − − − − p       | 6 bp deletion    | DOD, 22 mos  |
| 25       | 48   | F      | stomach      | 14                            | 6.1         | + + + + + + f       | wild                | alive, 26 mos   |
| 26       | 47   | F      | stomach      | 11                            | 3.9         | ND                  | + − − − − − − f       | 9 bp deletion    | alive, 17 mos |
| 27*      | 58   | M      | stomach      | 1.1                           | 0           | − + + + + + f       | wild                | alive, 16 mos   |
| 28*      | 58   | M      | stomach      | 5                             | 6.7         | − + + + − − − f     | wild                | alive, 16 mos   |
| 29       | 67   | F      | stomach      | 25                            | 1.7         | + + + − − − − f     | wild                | liver metastasis, 54 mos later, alive, 57 mos |
|          |      |        |              |                               |             |                     |                      |                 |           |
| 30       | 75   | F      | stomach      | 5.2                           | 1           | − + + + + + f       | wild                | alive, 82 mos   |
| 31       | 65   | M      | stomach      | 8                             | 3.4         | − − − − − − − − f   | wild                | alive, 80 mos   |
| 32       | 55   | F      | stomach      | 4.5                           | 2.9         | ND                  | + + + + + + f        | wild            | alive, 15 mos |
| 33       | 64   | F      | stomach      | 5                             | 2.1         | − + + + + + f       | point mutation      | alive, 98 mos   |
| 34       | 62   | F      | stomach      | 6                             | 1.8         | + + + + + + f       | 28 bp deletion+1 bp insertion | alive, 5 mos |
| 35       | 49   | F      | stomach      | 3.5                           | 3.6         | − + + + + + f       | 6 bp deletion       | alive, 6 mos    |
| 36       | 61   | F      | stomach      | 4.5                           | 1           | − + + + + + f       | wild                | alive, 62 mos   |
| 37       | 45   | M      | stomach      | 2                             | 4           | − + + + + + f       | wild                | alive, 31 mos   |
| 38       | 45   | F      | stomach      | 6                             | 6.4         | − + + + + + f       | wild                | alive, 15 mos   |
| 39       | 58   | M      | stomach      | 3.5                           | 1.5         | − + + + + + f       | wild                | alive, 11 mos   |
| 40       | 61   | F      | small int.   | 3                             | 5.7         | ND                  | + − − − − − − p       | 48 bp deletion   | alive, 86 mos |
| 41       | 74   | F      | small int.   | 3.4                           | 2.1         | − + + − − − − f     | wild                | alive, 51 mos   |
| 42       | 64   | F      | small int.   | 3                             | 5.3         | − + + + + + f       | 24 bp deletion+3 bp insertion | alive, 121 mos |
|          |      |        |              |                               |             |                     |                      |                 |           |
| 43       | 68   | F      | small int.   | 2.4                           | <1          | ND                  | + − − + − − − − f     | wild            | alive, 18 mos |
| 44       | 71   | F      | small int.   | 1.1                           | 2.3         | ND                  | + + + + + + p         | wild            | unknown       |
| 45       | 63   | F      | small int.   | 6.5                           | 3.7         | ND                  | + − − − − − − p       | 21 bp deletion   | alive, 40 mos |
| 46       | 51   | M      | small int.   | 4.5                           | 7.0         | ND                  | + + + + + + p         | wild            | alive, 22 mos |
| 47       | 65   | M      | colon        | 6.5                           | 3.1         | ND                  | + + + + + + p         | 6 bp deletion    | alive, 56 mos |
| 48       | 55   | F      | colon        | 10                            | 2           | ND                  | + + + + + + p         | liver metastasis, 84 mos later, alive, 129 mos |

*LI, labeling index; α-SMA, α-smooth muscle actin; p/f, paraffin sections/frozen tissue; M, male; ND, not done; +, positive; −, negative; bp, base pair; mos, months; F, female; small int., small intestine. * Two gastrointestinal tumors originated in the same patient.
mutations: one with a 28 bp deletion and a 1 bp insertion (case 34) and the other with a 24 bp deletion and a 3 bp insertion (case 42). In one case, the mutation consisted of the same in-frame deletion in the primary and metastatic GISTs (case 14).

There seemed to be two types of deletion mutations with regard to the location in exon 11; proximal location involving codons 550–565 and distal location involving codons 566–580 (Fig. 2). In our study, deletion mutations were present at the distal location in five of 15 cases (cases 5, 34, 42, 45 at primary site and case 16 at metastatic site; 33%) and nine at the proximal location. In one case, the site of deletion extended from the proximal to the distal location (case 40).

**Clinicopathologic correlations with c-kit gene mutation**

Clinicopathological and immunohistochemical data were compared between cases of primary GISTs with and without c-kit gene mutation (Table III). Cases 7, 13, and 16, in which mutations of the c-kit gene were found only in metastatic sites and not in primary sites, were classified as mutation-negative in Table III. The proportion of high-risk tumors was nearly equal in GISTs with or without this mutation. No difference was observed between GISTs with and without c-kit gene mutation with regard to age, gender, primary site, tumor size, proliferating activity (Ki-67-LI), or telomerase activity. All of the GISTs with c-kit gene mutations were immunohistochemically positive for KIT, except for one case (case 26), which was only positive for CD34. In the present study, five tumors negative for KIT and CD34 showed no mutation. With regard to the prognosis of the patients, 11 patients died of the disease from 3 to 104 months after the initial surgery, all of whom had high-risk tumors (Table I). As expected from the equal proportion of high-risk tumors, the presence or absence of c-kit gene mutation in the primary GIST did not affect the survival rates of patients with GISTs (Fig. 3).

As for the correlation between location of the deletion mutation and clinicopathological factors (Table IV), all of the patients with deletion at the distal location were 62- to 70-year-old females, and were alive, though one of the five cases showed hepatic metastasis (case 16).

**C-kit gene mutation** was observed only in metastatic tumors in cases 7, 13 and 16. When Ki-67-LI was compared in the primary and metastatic GISTs in these three cases, LI was similar in both tumors (Tables I and II).

**DISCUSSION**

C-kit gene mutation was identified in 15 of 48 primary GISTs (31%) and 3 metastatic GISTs, but in none of the leiomyomas of the esophagus. This finding is consistent with two previous reports, although the rates of the
mutations were higher than those reported by Moskaluk et al. The absence of c-kit gene mutation in esophageal leiomyomas further supports the notion that GISTs differ from smooth muscle tumors. Interestingly, no c-kit gene mutation was observed in the KIT(−)CD34(−) GISTs. These tumors may be a distinct group of GISTs, since four of five tumors were high-risk tumors derived from the colon.

In three cases, c-kit gene mutations were found only in metastatic tumors. Such cases have not been reported in previous studies. In these cases, mutations might occur only in a small proportion of tumor cells at primary sites or after tumor metastasis, indicating that c-kit gene mutations might participate in a late stage in the tumor development.

The predominant gene abnormality was a simple in-frame deletion in 13 cases, and this deletion and an additional insertion resulted in an in-frame deletion in two cases (cases 34 and 42). There was no frameshift mutation in the present study, as in other studies. Since KIT is critical for the maintenance of ICC in the GI tract, it is possible that clones harboring a loss-of-function mutation of the c-kit gene can not proliferate in GIST. In two GISTs with a c-kit gene mutation, normal bands were not amplified by PCR-SSCP analysis. Ernst et al. also reported a homozygous deletion in two of 13 GISTs. Although no abnormalities have been reported on chromosome 4q11-12, where the c-kit gene is present, by analyses using comparative genomic hybridization or microsatellite markers (Fukasawa et al., in preparation), it is possible that an additional deletion occurs in the limited region of the paired allele of the c-kit gene.

In our study, deletion mutations were present at the distal location in five of 15 cases (case 5, 34, 42, 45 at primary site and case 16 at metastatic site, 33%), though this seems to be a minor type in previous reports from Finland and the USA. Only one of 10 reported cases of deletion mutation by Ernst et al., one of 13 cases by Lasota et
Table III. Comparison of Clinicopathologic Data and Immunohistochemical Data between GISTs with and without C-kit Gene Mutations

|                | C-kit Mutation (+) n=15 | C-kit Mutation (-) n=33 | NS |
|----------------|-------------------------|-------------------------|----|
| Age at initial operation | 46–68 (average 58) | 33–80 (average 60) | NS |
| Gender (male:female) | 5:10 (n=32) | 16:16 (n=32) | NS |
| Primary site (stomach:small intestine:colon) | 8:5:2 | 19:9:5 | NS |
| Maximum size of the primary site | 6.8 +/- 0.9 | 9.2 +/- 1.1 | NS |
| Pathologic diagnosis (high risk:low risk) | 7:8 | 17:16 | NS |
| Alive without disease | 11/15 | 19/32 | NS |
| Ki-67-LI | 7.3 +/- 1.3 | 8.0 +/- 1.1 | NS |
| Telomerase activity | 1/6 | 6/12 | NS |
| Immunohistochemistry | | | |
| KIT | 14/15 | 27/30 | NS |
| CD34 | 11/15 | 24/30 | NS |
| α-SMA | 6/15 | 14/30 | NS |
| S-100 | 1/15 | 0/30 | NS |
| KIT (-)/CD34 (-) | 0/15 | 5/33 | NS |

NS, not significant; LI, labeling index; α-SMA, α-smooth muscle actin.

Fig. 3. The survival rates of patients with primary gastrointestinal stromal tumors with or without c-kit gene exon 11 mutations. ○ mutation (+) (n=15), □ mutation (-) (n=29).

Table IV. Age, Gender and Ki-67-LI in GISTs with Deletion Mutation at Proximal and Distal Locations in C-kit Gene Exon 11

|                | Deletion at proximal site (n=9) (Cases 1, 6, 7, 13, 14, 24, 26, 35, 47) | Deletion at distal site (n=5) (Cases 5, 16, 34, 42, 45) | NS |
|----------------|------------------------------------------------------------------------|---------------------------------------------------------|----|
| Age at initial operation | 46–77 (average 58) | 62–70 (average 65) | NS |
| Gender (male:female) | 4:5 | 0:5 | NS |
| Ki-67-LI | 9.3 +/- 1.7 | 7.9 +/- 4.0 | NS |

NS, not significant; LI, labeling index.
al. and one of seven cases by Moskaluk et al. showed deletions at the distal location in exon 11 of the c-kit gene. Nakahara et al. reported a single Japanese case with a deletion mutation at codon 579, and showed that it was a gain-of-function mutation.19) Interestingly, all of the patients with deletion at the distal location were 62- to 70-year-old females in the present study. Thus, certain specific factors, such as race and sex, may favor deletion at the distal location of exon 11 in GISTs.

C-kit gene mutations were identified equally in high-and low-risk GISTS in the present study. While the mean size of the primary tumor, Ki-67-LI and telomerase activity were significant determinants of prognosis in our previous study, no correlation was found between these factors and c-kit mutation. The finding in the present study is in sharp contrast to those of Lasota et al. and Ernst et al.,27),28) who found that mutation was associated with poor prognosis. The discrepancy does not seem to be due to the different criteria for benign and malignant GISTS, though the mitotic index used by Lasota et al. to define benign GISTS was twice that in our criteria. In the present study, c-kit gene mutation was observed in the metastatic, but not in the primary GISTS in three cases. Despite the presence of the mutation in metastatic tumors, the Ki-67-LI was not different between primary and metastatic tumors. Although we examined only exon 11 of c-kit gene and could not exclude the possibility that mutations occurred in other exons and contributed to the malignant potential of GISTs, mutations in other regions have not been reported in previous studies. One possible explanation for this discrepancy is that c-kit gene mutation has a different effect according to the site of deletion: all of the patients with a deletion at the distal location were alive, even though one of the five cases showed hepatic metastasis (case 16). However, the number of cases was too small and the follow-up periods were too short to clarify different effects of mutations at different locations in exon 11 of the c-kit gene. Further comparative studies in Western and Japanese populations are necessary to clarify the significance of c-kit mutation in GIST.

Based on the findings in the present study, c-kit gene mutation occurs either at an early or a late stage in the development and progression of GIST. A high frequency of activated c-kit gene mutation in GISTs may be due to selection of tumor cells during their development. However, in our study and others, two-thirds of the GISTS did not show c-kit gene mutation, which indicates that additional genetic or epigenetic alterations may be necessary for the development and progression of GIST.

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