Extracellular domain *c-kit* mutation with duplication of Ser501Ala502 found in gastrointestinal stromal tumors is more imatinib- and nilotinib-sensitive than that with duplication of Ala502Tyr503

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The great majority of gastrointestinal stromal tumors (GISTs) have gain-of-function mutations of the *c-kit* gene, which encodes KIT receptor tyrosine kinase. Most of the mutations are located at exon 11, but some are at exon 9 or at other exons. Mutation types at exon 11 vary, while most mutations at exon 9 are a particular duplication of Ala502Tyr503 (KIT-Dup-Ala502Tyr503). Recently a duplication of Ser501Ala502 (KIT-Dup-Ser501Ala502) at exon 9 has been reported in two cases of pediatric mastocytosis and one case of adult mast cell leukemia. Although KIT-Dup-Ser501Ala502 had not been reported in GISTs, we found two GIST cases possessing the mutation in 45 GIST cases with exon 9 *c-kit* gene mutations, among a total of approximately 500 GIST cases examined. In this report, we briefly summarize clinicopathological findings of the two cases, and characterize the biology of the mutation. When autophosphorylation of KIT-Dup-Ser501Ala502 was examined by transient transfection of *c-kit* cDNA with Dup-Ser501Ala502 into CHO-K1 cells, KIT-Dup-Ser501Ala502 was ligand-independently activating. The inhibitory effect of selective tyrosine kinase inhibitors, imatinib and nilotinib, on KIT-Dup-Ser501Ala502 was examined and compared with that of KIT-Dup-Ala502Tyr503. Imatinib efficiently inhibited constitutive activation of KIT-Dup-Ser501Ala502 at a concentration of 0.1 μ M, whereas it inhibited that of KIT-Dup-Ala502Tyr503 at a concentration of 10 μ M. Constitutive activation of KIT-Dup-Ser502Ala503 was not inhibited by nilotinib even at a concentration of 10 μ M but that of KIT-Dup-Ala501Tyr502 was almost completely inhibited at a concentration of 1 μ M. The results suggest that imatinib and nilotinib could be more effective on GISTs with KIT-Dup-Ser501Ala502 than those with KIT-Dup-Ala502Tyr503. In fact, a patient with KIT-Dup-Ser501Ala502 showed long-term stable disease with administration of the usual dose of 400 mg imatinib. Although mutation sites of KIT-Dup-Ser501Ala502 and KIT-Dup-Ala502Tyr503 are closely located, imatinib- and nilotinib-sensitive KIT-Dup-Ser501Ala502 are distinguishable from KIT-Dup-Ala502Tyr503.

Laboratory Investigation (2013) 93, 502–507; doi:10.1038/labinvest.2013.43; published online 4 March 2013

KEYWORDS: *c-kit* gene; exon 9; gain-of-function mutation; gastrointestinal stromal tumor; imatinib; nilotinib

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors arising in the gastrointestinal wall.¹ Most GISTs express a type III receptor tyrosine kinase, KIT, which is encoded by the *c-kit* protooncogene.^{1,2} Structurally, KIT contains an extracellular domain with five immunoglobulin-like repeats, a transmembrane domain, a juxtamembrane domain, tyrosine kinase domain (TKD) I and TKD II.^{3–5} Physiologically, KIT is phosphorylated by the binding of its ligand, stem cell factor (SCF),⁶ and some signal

transduction pathways are activated to induce proliferation and differentiation of several types of cells, which essentially require the function of SCF-KIT system.^{7–10}

Approximately 90% of sporadic GISTs have gain-of-function mutations of the *c-kit* gene, and considerable numbers of the remaining GISTs without the *c-kit* gene mutations have gain-of-function mutations of the platelet-derived growth factor receptor alpha (PDGFRA) gene encoding another type III receptor tyrosine kinase.^{11,12} Thus, the *c-kit* and PDGFRA

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Received 25 December 2012; revised 1 February 2013; accepted 3 February 2013

gene mutations are considered to be the main causes of most GISTs.^{1,11,12} The majority of the *c-kit* gene mutations (~90%) are present at exon 11 encoding the juxta-membrane domain,^{1,13,14} fewer (~10%) at exon 9 encoding the extracellular domain,^{13–15} and rare (~1%) at exon 13 encoding the TKD I^{14,16} or at exon 17 encoding the TKD II.^{14,16} Various types of the mutations have been reported at exon 11, while most exon 9 mutations are a particular internal tandem duplication of Ala502Tyr503 (KIT-Dup-Ala502Tyr503).^{13–15} Only a few GIST cases have been reported to possess duplication of Phe506Ala507Phe508,^{17,18} deletion of Lys484His485Asn486Gly487,¹⁸ complex deletion/insertion of 14 amino acid (490–503) to AspHisIle ValValSerLeuThr¹⁹ and substitution of Pro456Ser.²⁰

Imatinib is a selective tyrosine kinase inhibitor for BCR-ABL, KIT and PDGFRs.^{21,22} It is clinically used for treatment of both chronic myelogenous leukemia²³ and metastatic or unresectable GISTs.^{24,25} In GIST treatment, however, effectiveness of imatinib is dependent on the types of the *c-kit* and PDGFR gene mutations.^{17,18,26,27} Resistance to imatinib may occur either from the beginning of the administration or after the initial control of the disease. The former is called primary resistance and the latter secondary resistance. GISTs with PDGFR-Val842Asp at exon 18 of the PDGFR gene are well known as one of the major causes of the primary resistance,¹² and some GISTs with KIT-Dup-Ala502Tyr503 at exon 9 of the *c-kit* gene might show primary resistance.²⁸ On the other hand, a major mechanism of the secondary resistance for imatinib is the addition of various types of point mutation at exon 13, 14, 17 or 18 of the *c-kit* gene in GISTs with primary *c-kit* gene mutation.^{29–32} Nilotinib is another selective tyrosine kinase inhibitor for BCR-ABL, KIT and PDGFRs.³³ It is now used for the patients with chronic myelogenous leukemia as either a first-line or a second-line drug,^{34,35} but its effectiveness for GIST patients has not been established.

We discovered two cases of primary sporadic GISTs of the small intestine with an internal tandem duplication of Ser501Ala502 (KIT-Dup-Ser501Ala502) at exon 9 of the *c-kit* gene, which is different from the more common exon 9 mutation of KIT-Dup-Ala502Tyr503. Recently, two cases of pediatric mastocytosis and one case of adult mast cell leukemia with KIT-Dup-Ser501Ala502 have been reported.^{36,37} In the present study, we describe the brief clinicopathological features of the two GIST cases with KIT-Dup-Ser501Ala502, and show whether the mutation is of gain-of-function and whether the mutation is sensitive to both imatinib and nilotinib by using CHO-K1 cells transiently transfected with the mutated *c-kit* cDNA.

MATERIALS AND METHODS

Methods for Analyses of *c-kit* and PDGFR Gene Mutations

We have analyzed *c-kit* and PDGFR gene mutations in approximately 500 authentic GIST cases. When fresh-frozen

samples were available, total RNA was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Almost all coding regions of the *c-kit* and/or PDGFR genes were amplified by PCR after reverse transcription of the extracted RNA as described previously.^{1,14} When fresh-frozen samples were not available, genomic DNA was extracted from paraffin sections using QIAamp DNA Mini Kit (QIAGEN). Exons 9, 11, 13 and 17 of the *c-kit* gene and exons 12, 14 and 18 of the PDGFR gene were amplified by PCR. Primers used for PCR were as described previously.^{1,12} Direct sequencing of the amplified products was carried out with ABI BigDye terminator ver.3.1 (Applied Biosystems, Foster City, CA, USA) and ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). The informed consent for the present study was obtained, and the study was approved by the institutional review boards.

Histology and Immunohistochemistry of GISTs

Resected GIST tissues were fixed with 10% formalin and embedded in paraffin. Sections (3 µm thick) were cut and used for hematoxylin and eosin staining and immunohistochemistry. Immunohistochemistry was performed using ENVISION + KIT HRP (DAB) system (DAKO, Glostrup, Denmark). Rabbit polyclonal antibody against human KIT (A4502; DAKO) and mouse monoclonal antibody against human CD34 (Novocastra Laboratories, Newcastle upon Tyne, UK) were used as the primary antibodies.

Generation and Transfection of *c-kit* cDNA with Dup-Ser501Ala502

Human-type whole *c-kit* cDNA with Dup-Ser501Ala502 was generated by ligation of a doubly digested SnaBI-MroI fragment (nucleotides 1131–2683) of the PCR product amplified using complementary DNA of case 2 in Table 1 and a large fragment of expression vector pEF-BOS containing the whole human wild-type *c-kit* cDNA doubly digested by SnaBI-MroI. The expression vector with KIT-Dup-Ser501Ala502 was transfected into CHO-K1 cells using Nucleofector (Lonza Cologne GmbH, Köln, Germany) and Amaxa Cell Line Nucleofector Kit T (Lonza Cologne GmbH). The expression vector pEF-BOS containing the whole wild-type *c-kit* cDNA or whole *c-kit* cDNA with Dup-Ala502Tyr503 was generated

Table 1 Clinicopathological characteristics of GISTs with KIT-Dup-Ser501Ala502

Case No.	Age	Sex	Size (cm)	Site	KIT	CD34	Mitosis/50 HPFs	Metastasis
1	56	M	11.0	Jejunum	+	+	7	Metachronous (2 years later)
2	75	M	4.2	Jejunum	+	+	2	Synchronous

M, male; HPF, high power field.

previously,^{1,15} and was similarly transfected into CHO-K1 as comparisons.

Autophosphorylation of KIT-Dup-Ser501Ala502

CHO-K1 cells transfected with pEF-BOS containing whole *c-kit* cDNA with Dup-Ser501Ala502 or Dup-Ala502Tyr503 were used for the experiment. After 24 h of transfection, cells were collected and incubated with or without recombinant human SCF (10 μ g/ml, PeproTech Inc., Rocky Hill, NJ, USA) at 37 °C for 15 min. Cell lysis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis were carried out as described previously.¹ Immunoblotting was performed with a rabbit polyclonal antibody specific for phosphorylated forms of KIT (OPA-1-03163, Thermo Scientific, Rockford, IL, USA). Antibody binding was detected by an enhanced chemiluminescence western blotting detection system (GE Healthcare UK, Little Chalfont, UK). The membrane was stripped and reprobed with a rabbit anti-human KIT polyclonal antibody (A4502, DAKO).

Effect of KIT Inhibitors on Autophosphorylation of KIT-Dup-Ser501Ala502

Similarly, CHO-K1 cells transfected with pEF-BOS containing whole *c-kit* cDNA with Dup-Ser501Ala502 or Dup-Ala502Tyr503 were used for the experiment. After 24 h of transfection, cells were cultured at various concentrations of imatinib (0.001, 0.01, 0.1, 1 or 10 μ M) or nilotinib (0.001, 0.01, 0.1, 1 or 10 μ M) at 37 °C for 90 min. Imatinib and nilotinib were generous gifts from Novartis (Basel, Switzerland). Cell lysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting were done as described above.

RESULTS

Detection of GIST with KIT-Dup-Ser501Ala502

Exon 9 *c-kit* gene mutation was detected in 45 out of approximately 500 GISTs examined. Among them, 43 GISTs had KIT-Dup-Ala502Tyr503 (data not shown) and 2 had KIT-Dup-Ser501Ala502 (Figure 1).

Clinicopathological Features of GISTs with KIT-Dup-Ser501Ala502

Clinicopathological features of two GIST cases with KIT-Dup-Ser501Ala502 are shown in Table 1. Characteristically, both GISTs occurred at the jejunum and showed metastasis. The tumors of both cases consisted of spindle-shaped cells (Figures 2a and d). Immunohistochemistry revealed that the tumor cells of both cases were diffusely positive for KIT (Figures 2b and e). CD34 is also positive in both the cases, but case 1 showed diffuse positivity and, case 2, partial positivity (Figures 2c and f).

Brief Clinical History of Patients

After complete resection of GIST, case 1 patient had undergone 1 year of imatinib adjuvant therapy (400 mg/day)

Figure 1 Sequencing of *c-kit* cDNA in a GIST with KIT-Dup-Ser501Ala502. A heterozygous mutation is shown.

because the tumor was considered to be in high risk for recurrence. One year after completion of imatinib adjuvant therapy, multiple liver metastases were found by abdominal CT. Then, imatinib (400 mg/day) was administered again, and the metastatic foci had been controlled for 40 months. Because of progression of liver metastasis, the patient received sunitinib therapy, but he had severe adverse events. Sunitinib therapy had been stopped and the patient died 3 months after the progression of liver metastasis. Case 2 patient had a jejunal tumor and a focus of liver metastasis at S3. The jejunal tumor had been excised, and it was diagnosed as GIST. The metastatic focus in the liver had not been resected. Imatinib was administered for metastatic disease, but the grade 3 adverse event occurred. Half a year after stopping imatinib, the focus of liver metastasis enlarged, and the metastatic tumor was resected. A 7-year follow-up period after the resection of liver metastasis has revealed no recurrence.

Constitutive Activation of KIT-Dup-Ser501Ala502

To clarify whether the KIT-Dup-Ser501Ala502 is constitutively activated, *c-kit* cDNA with Dup-Ser501Ala502 was transiently transfected into CHO-K1 cells and phosphorylation of mutant KIT was examined in the presence or absence of SCF. Wild-type KIT was apparently phosphorylated only when SCF was added (Figure 3). On the other hand, autophosphorylation of KIT-Dup-Ala502Tyr503 was observed without SCF stimulation as reported (Figure 3).¹⁶ Similarly, autophosphorylation of KIT-Dup-Ser501Ala502 was detected even without SCF stimulation, demonstrating that the mutation is also of gain-of-function (Figure 3).

Effect of Imatinib on Autophosphorylation of KIT-Dup-Ser501Ala502

To clarify whether KIT-Dup-Ser501Ala502 is imatinib-sensitive or not, the inhibitory effect of imatinib on autophosphorylation of KIT was examined in CHO-K1 cells transfected with *c-kit* cDNA with Dup-Ser501Ala502. Imatinib completely inhibited autophosphorylation of KIT-Dup-Ala502Tyr503 at a concentration of 10 μ M (Figure 4). On the other hand, autophosphorylation of KIT-Dup-Ser501Ala502 was inhibited at a concentration of 0.1 μ M (Figure 4), suggesting that activation of KIT-Dup-Ser501-

Figure 2 Histology and immunohistochemistry of two GISTs with KIT-Dup-Ser501Ala502. (a–c), case 1; (d–f), case 2. (a, d), hematoxylin and eosin staining; (b, e), immunohistochemistry for KIT; (c, f), immunohistochemistry for CD34. Tumor cells of case 1 are spindle-shaped and diffusely positive for KIT and CD34. Those of case 2 are also spindle-shaped and diffusely KIT-positive, but they are partially CD34-positive.

Figure 3 Constitutive activation of KIT-Dup-Ser501Ala502. Wild-type KIT is phosphorylated only when SCF is added. Conversely, KIT-Dup-Ser501Ala502 is phosphorylated without SCF stimulation as observed in KIT-Dup-Ala502Tyr503.

Figure 4 Inhibitory effect of imatinib on phosphorylation of KIT-Dup-Ser501Ala502. Autophosphorylation of KIT-Dup-Ala502Tyr503 is inhibited by imatinib at a concentration of 10 μ M while that of KIT-Dup-Ser501Ala502 is almost completely inhibited by imatinib at a concentration of 0.1 μ M.

Ala502 was more effectively inhibited by imatinib than that of KIT-Dup-Ala502Tyr503.

Effect of Nilotinib on Autophosphorylation of KIT-Dup-Ser501Ala502

To clarify whether the KIT-Dup-Ser501Ala502 is nilotinib-sensitive or not, inhibitory effect of nilotinib on autophosphorylation of KIT was examined in CHO-K1 cells transfected with *c-kit* cDNA with Dup-Ser501Ala502. Nilotinib could not completely inhibit autophosphorylation of KIT-Dup-Ala502Tyr503 even at a concentration of 10 μ M

(Figure 5). On the other hand, autophosphorylation of KIT-Dup-Ser501Ala502 was inhibited at a concentration of 1 μ M (Figure 4), suggesting that activation of KIT-Dup-Ser501Ala502 was more effectively inhibited by nilotinib than that of KIT-Dup-Ala502Tyr503.

DISCUSSION

In this report, we described clinicopathological features of two GIST cases with KIT-Dup-Ser501Ala502 and characterized the mutation. We examined whether the mutation is of gain-of-function and whether the mutation is sensitive to

Figure 5 Inhibitory effect of nilotinib on phosphorylation of KIT-Dup-Ser501Ala502. Autophosphorylation of KIT-Dup-Ala502Tyr503 is not inhibited by nilotinib even at a concentration of 10 μ M. On the other hand, that of KIT-Dup-Ser501Ala502 is almost completely inhibited by nilotinib at a concentration of 1 μ M.

both imatinib and nilotinib by using CHO-K1 cells transiently transfected with human *c-kit* cDNA with the mutation. The mutation was found to be of gain-of-function, and both imatinib and nilotinib could be more effective on GISTs with KIT-Dup-Ser501Ala502 than those with KIT-Dup-Ala502Tyr503. In fact, a GIST patient with KIT-Dup-Ser501Ala502 demonstrated long-term stability with administration of the usual dose of imatinib, 400 mg.

Recently, two cases of pediatric mastocytosis and one case of adult mast cell leukemia with KIT-Dup-Ser501Ala502 were reported.^{36,37} Most of the *c-kit* gene mutations observed in mast cell neoplasms are Asp816Val at exon 17, and exon 9 *c-kit* gene mutation is rare in them. As various types of exon 9 *c-kit* gene mutations are detected in mast cell neoplasms,³⁶ those with KIT-Dup-Ser501Ala502 are very rare. On the other hand, GISTs with exon 9 mutations comprise approximately 10% of all GISTs. However, most of exon 9 *c-kit* gene mutations are a type of KIT-Dup-Ala502Tyr503 in GISTs. In fact, we have records of 45 GIST cases with exon 9 mutations, and most of them had KIT-Dup-Ala502Tyr503. Only two GIST cases (<5% among GISTs with exon 9 mutations) had KIT-Dup-Ser501Ala502. Therefore, GISTs with KIT-Dup-Ser501Ala502 are also considered to be very rare (<0.5% of all GIST cases).

GISTs with KIT-Dup-Ala502Tyr503 preferentially occur in the small intestine, and are considered to have poorer prognosis than those with other types of *c-kit* gene mutations because of high rate of recurrence and/or metastasis. Two GIST cases with KIT-Dup-Ser501Ala502 detected primarily developed at the small intestine and showed metastasis. There is a possibility that GISTs with KIT-Dup-Ser501Ala502 might have similar clinicopathological features to those with KIT-Dup-Ala502Tyr503. However, the number of GIST cases with KIT-Dup-Ser501Ala502 is too small to draw conclusions about features, and further effort to collect those GIST cases is needed.

In a case of adult mast cell leukemia with KIT-Dup-Ser501Ala502, the mutant KIT was demonstrated to be

constitutively activated using transiently transfected Cos cells.³⁷ The authors also showed that masitinib, a new inhibitor of tyrosine kinase, including KIT,³⁸ effectively inhibited KIT autophosphorylation. In the present report, we showed that autophosphorylation of KIT-Dup-Ser501Ala502 was detected even without SCF stimulation as well as that of KIT-Dup-Ala502Tyr503 by using transiently transfected CHO-K1 cells. We also demonstrated that imatinib completely inhibited the autophosphorylation of KIT-Dup-Ser501Ala502 at a concentration of 0.1 μ M, whereas KIT-Dup-Ala502Tyr503 was inhibited at a concentration of 10 μ M. These results suggest that the activation of KIT-Dup-Ser501Ala502 was more effectively inhibited by imatinib than that of KIT-Dup-Ala502Tyr503. Although KIT-Dup-Ala502Tyr503 is considered to be a cause of primary resistance to imatinib in GIST treatment and usually needs 800 mg/day imatinib to improve survival of the patients,³⁹ GISTs with KIT-Dup-Ser501Ala502 might show good response to ordinary dose of 400 mg/day imatinib. In fact, the patient of GIST with KIT-Dup-Ser501Ala502 showed long-term stability with the usual 400 mg dose of imatinib. The finding should be verified in more cases harboring the mutation.

We also examined the effect of nilotinib on autophosphorylation of KIT-Dup-Ser501Ala502. Nilotinib completely inhibited the autophosphorylation of KIT-Dup-Ser501Ala502 at a concentration of 1 μ M, whereas KIT-Dup-Ala502Tyr503 was not inhibited at a concentration of 10 μ M. These results suggested that the activation of KIT-Dup-Ser501Ala502 was more effectively inhibited by nilotinib than that of KIT-Dup-Ala502Tyr503.

In summary, GISTs with KIT-Dup-Ser501Ala502 might share some clinicopathological features, including preferential site of development and high frequency of metastasis with those with KIT-Dup-Ala502Tyr503, but the results of imatinib and nilotinib treatment of GISTs with KIT-Dup-Ser501Ala502 are better than those with KIT-Dup-Ala502Tyr503. As mutations of KIT-Dup-Ser501Ala502 and KIT-Dup-Ala502Tyr503 are very closely situated, there is a possibility that KIT-Dup-Ser501Ala502 is confused with KIT-Dup-Ala502Tyr503. Precise separation of KIT-Dup-Ser501Ala502 and KIT-Dup-Ala502Tyr503 might be important for determining proper treatment with target drugs.

ACKNOWLEDGEMENTS

This work was partially supported by Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (Grant Number 23390094).

DISCLOSURE/CONFLICT OF INTEREST

Dr Nishida and Dr Hirota have received donations for research support from Novartis. The other authors declare no conflict of interest.

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