Establishment and Characterization of a Novel Xenograft Model of Human Gastrointestinal Stromal Tumor in Mice

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Abstract. *Background: The clinical outcome of gastrointestinal stromal tumor (GIST) has been improved by the introduction of molecular-targeting drugs. However, resistance to these drugs appears during the course of treatment. The aim of this study was to establish and characterize a human xenograft model of GIST. Materials and Methods: GIST tissue from a patient with esophageal GIST was implanted under the skin of a NOD-SCID mouse. The tumor became successfully engrafted and we investigated the effects of imatinib and sunitinib on this model. KIT mutation was investigated by complementary DNA analysis, and c-KIT (CD117) expression was evaluated by immunohistological staining. Results: cDNA analysis of the tumor revealed a KIT mutation in exon 11. c-KIT expression was observed in each passaged tumor. Both imatinib and sunitinib significantly reduced the size of the xenograft tumor. Conclusion: We established a novel xenograft model of human GIST in mice. This xenograft model may be useful for studying GIST.*

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract and originates from the interstitial cells of Cajal (1, 2). The majority of GISTs have mutations in their thymidine kinase (TK) receptor (*KIT*) genes, usually in the regions that encode the auto-regulatory domains of TK. These mutations predominantly occur in exons 11 (66%) and 9 (10-18%) of the *KIT* gene (3). GISTs with mutations in exons 13 or 17 of the *KIT* gene are less common (3). In addition, a few GISTs harbor mutations in the gene for platelet-derived growth factor receptor-alpha (*PDGFRA*), which is related to the TK gene (3,4). Activating *KIT* or *PDGFRA* mutations have been defined as the underlying pathogenic events in GIST development.

Surgery is currently the first-line treatment for patients with primary resectable GIST. However, increases in our understanding of the molecular pathophysiology of GIST have led to the development of agents that target and selectively inhibit TK activity (5). For example, the suppression of signal transduction pathways in GIST cells with the TK inhibitors imatinib resulted in reduced cell proliferation and the induction of apoptosis (6). The introduction of these new targeted drugs has significantly improved the prospects of patients with locally advanced or metastatic GIST. Imatinib is currently the standard, first-line treatment for these patients and shows improved overall survival by four years, compared with conventional chemotherapeutic treatment (7).

However, the majority of patients for whom imatinib is initially effective, eventually develop resistance to the drug. Furthermore, about 19% of patients with GIST do not respond to imatinib, and another 5% develop unacceptable adverse effects (8). Secondary or late resistance develops after a median of almost two years' treatment (9). During the past few years, other signal transduction inhibitors such as sunitinib, nilotinib, and sorafenib have been developed (8, 10, 11). Out of these inhibitors, only sunitinib has been used in clinical practice as a second-line treatment for patients with imatinib-resistant GIST (8).

The toxicity and safety of imatinib and sunitinib have been confirmed, but they have not been confirmed to have direct antitumor effects *in vivo*. In a previous study, a human GIST cell line was subcutaneously implanted into mice to establish a human GIST xenograft mouse model, but the effects of TK inhibitors on this model were not examined (12).

The present study describes the establishment of a novel xenograft mouse model of human GIST, as well as its histology and immunohistology, and the effects of TK inhibitors on this model.

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Key Words: Gastrointestinal stromal tumor, xenograft, mouse, imatinib, sunitinib.

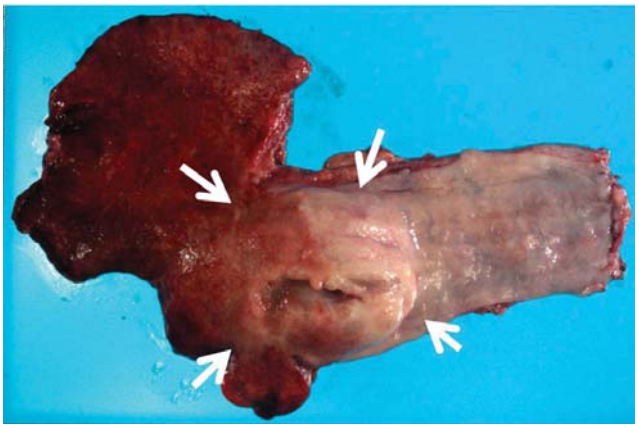

Figure 1. *Gross findings of the resected specimen. A solid, firm, elastic, ulcerated tumor measuring 9.0×6.8×3.5 cm in size was completely excised with the esophagus. The white arrow indicates the border of the Gastrointestinal stromal tumor.*

Figure 2. *Gastrointestinal stromal tumor tissue in the surgical specimen was cut into small pieces and subcutaneously inserted into the right and left flanks of a NOD-SCID mouse. It had become successfully engrafted by eight months after its transplantation.*

Materials and Methods

Patient history. A 72-year-old man was diagnosed with esophageal GIST by gastrointestinal endoscopy and biopsy. The GIST was located in the lower thoracic esophagus and had invaded into the stomach. In order to resect the tumor safely and prevent micrometastasis, the patient was treated with imatinib at a daily dose of 400 mg. One month after the chemotherapy, a marked reduction in the size of the tumor was observed; however, the continuous administration of imatinib for a further two months did not result in further reduction in tumor size. Thus, we performed subtotal esophagectomy through a right thoracotomy and reconstructed the esophagus with a gastric tube (Figure 1). Adjuvant imatinib treatment was planned, which the patient received for approximately three weeks. However, the patient stopped taking imatinib as he developed severe appetite loss. Ten months after the operation, a recurrent liver tumor was observed.

Establishment of the GIST xenograft. We aimed to develop a novel GIST xenograft model by transplanting human GIST xenograft tissue into a female non-obese diabetic (NOD) CB17-Prkdc(SCID)/J (NOD-SCID) mouse (Charles River Laboratories, Yokohama, Japan). Written informed consent for the use of resected specimens for research was obtained from the patient described above before surgery. The study was approved from the Institutional Review Board of the University of Toyama (approval number #22-11 & #Med-57). The GIST tissue in the surgical specimen was cut into small pieces and subcutaneously inserted into the right and left flanks of the NOD-SCID mouse. The xenograft tumor tissue had become successfully engrafted approximately four months after its transplantation (Figure 2). After eight months of transplantation, the xenografted tissue was subsequently transferred into 4-6 week-old female CAnN.Cg-Foxn1^{nu}/CrlCrlj (BALB/c) nude mice (Charles River Laboratories) (n=3).

Tumor growth curve. Tumor volume was measured weekly in xenografted mice with external calipers. Individual tumor volumes were calculated using the modified ellipsoid formula: tumor volume=1/2(a×b^2), where a is the longest longitudinal diameter (length) and b is the longest transverse diameter (width) of the xenograft.

Cell culture. The xenograft tissue was surgically removed from growth tumor of the xenograft mouse was mechanically minced with sharp scissors. Then, the minced tumor were digested by collagenase (Invitrogen Co, Grand Island, NY, USA) and dispase (Invitrogen Co, Grand Island, NY, USA) solution (1:1) for 90 min at 37˚C on a shaker. Cells were initially cultured in Dulbecco's Modified Eagle's medium + HAM's F12 medium (Wako, Osaka Japan), supplemented with 10% fetal bovine serum containing antibiotics (GIBCO, Grand Island, NY, USA) at 4˚C. The cells were maintained in humidified incubators at 37˚C in an atmosphere of 5% CO_2 and 95% air.

Agents. Imatinib mesylate (S1026) was purchased from Selleck Chemicals LLC (Houston, TX, USA), and sunitinib malate (PZ0012) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). These agents were dissolved in DMSO, and diluted in PBS. Control agent was adjusted by DMSO and PBS. For each treatment, a dose of imatinib at 100 mg/kg twice daily, a dose of sunitinib at 40 mg/kg once daily and PBS as control into 4 to 5 nude mice by oral gavage.

Mutation screening. To analyze the mutations in the *KIT* and *PDGFRA* genes, DNA was extracted from culture cells using the QIAamp DNA mini kit (Qiagen Inc, Valencia, CA, USA). DNA sample was amplified with polymerase chain reaction (PCR) for 40 cycles. After purification of the PCR products, mutation screening of exons 9, 11, 13, and 17 of *KIT* and exon 12, 14, and 18 of *PDGFRA* was performed by DNA direct sequencing analysis. The primers were obtained from FALCO biosystems Ltd (#7530, #7533, #7646, Kyoto, Japan).

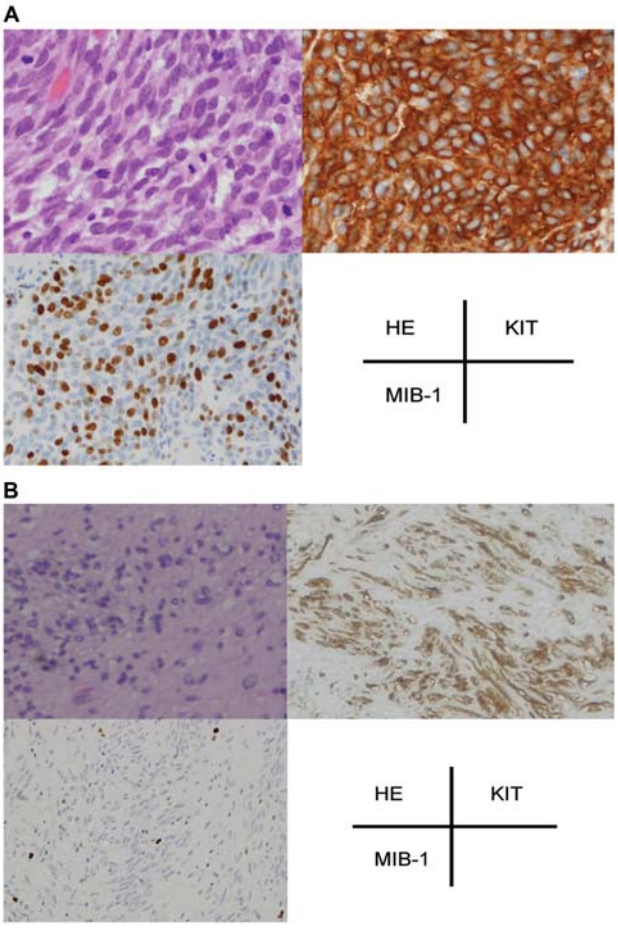

Figure 3. *A: Histopathological findings of an endoscopic biopsy sample obtained before imatinib treatment. Histologically, mitotic spindle cells were observed. Immunohistochemical analysis detected c-KIT- and MIB-1-positive cells. B: Histopathological findings of the resected specimen. Histologically, spindle cells were observed, a few of which were mitotic. Immunohistochemical analysis revealed c-KIT- and MIB-1-positive cells. However, there were fewer c-KIT- and MIB-1-positive cells in the resected specimen than in the biopsy sample.*

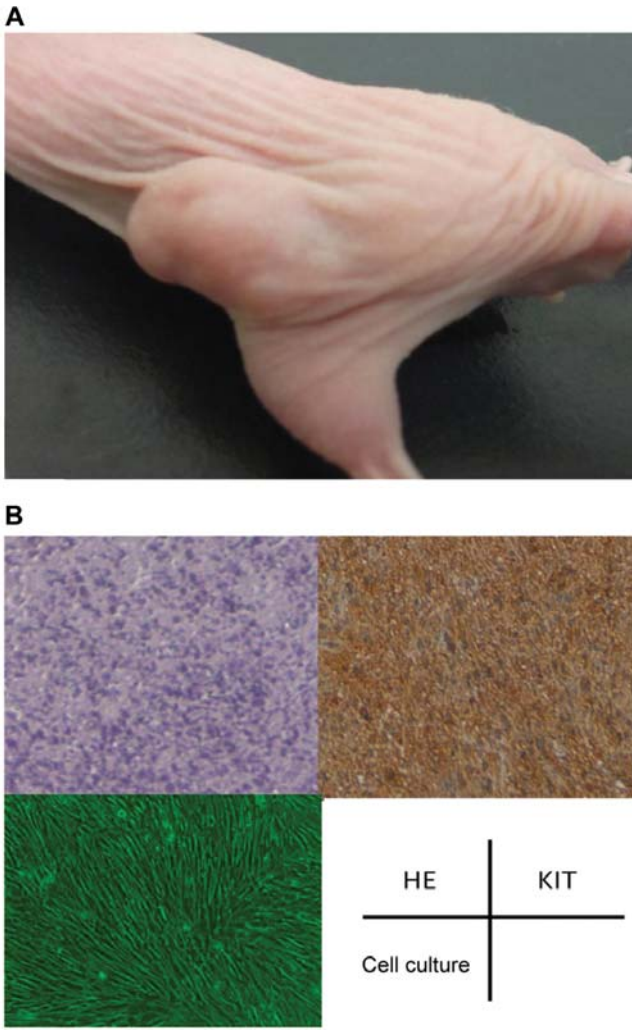

Figure 4. *A: The findings of a fifth-passage xenograft mouse model of human gastrointestinal stromal tumor. B: The tumor xenograft displayed spindle cells, and immunohistochemical staining detected c-KIT positivity. It was possible to culture the xenograft tissue.*

Immunohistochemistry. Immunohistochemical analysis of c-KIT expression was carried out using paraffin-embedded sections of the GIST xenograft tumors from fifth-passage BALB/c nude mice that were sacrificed by isoflurane. We used a rabbit polyclonal c-KIT antibody (A4502; Dako, Glostrup, Denmark) at a dilution of 1:100, Envision+dual link system-HRP (K4061, DAKO, Glostrup, Denmark) method.

Statistical analysis. The results are expressed as mean±standard error of the mean. Statistical significance was calculated by repeated measurements one-way analysis of variance (ANOVA) using JMP9 (SAS Institute Inc, Cary, NC, USA). Statistical significance is a comparison of the each entire graphs between zero week and four or five weeks. Any *p*-value is comparison of control group versus treatment group.

Results

Evaluation of clinical samples. An endoscopic biopsy sample obtained before the patient was treated with imatinib revealed the presence of spindle cells, and immunohistochemical staining detected c-KIT- and Ki-67 (MIB-1)-positive cells (Figure 3A). After imatinib treatment, the surgical specimen displayed reduced c-KIT expression compared with the biopsy sample (Figure 3B).

Model establishment and tumor characteristics. The tumor doubling time for the seventh passage was found to be approximately 3.4 weeks. We subcultured the xenografted tissue

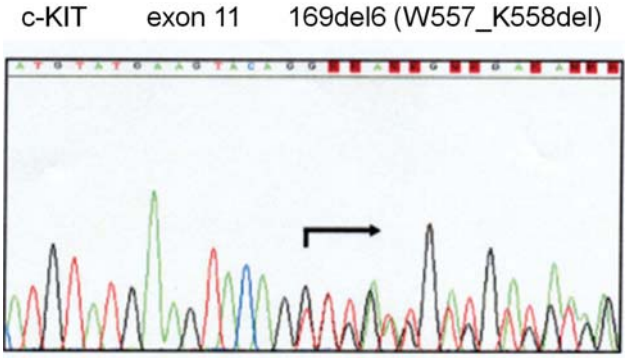

Figure 5. *Complementary DNA analysis of the tumor revealed a 1690del6 mutation in exon 11 (W557_K558del) of the c-KIT gene.*

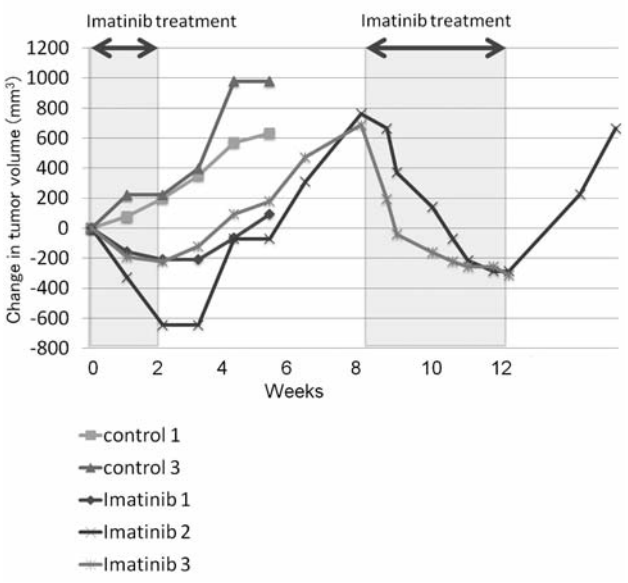

Figure 6. *In fourth-passage xenograft mice, imatinib treatment produced a significantly greater reduction in xenograft tumor size than did treatment with phosphate-buffered saline (control). However, the xenograft tumors grew again after the imatinib treatment was stopped. We subsequently treated the re-growing tumors with imatinib, which resulted in a further reduction in their size.*

into nude mice every four to six months (Figure 4A). The xenograft tissue contained spindle cells, and immunohistochemical staining detected c-KIT. It was possible to culture the xenograft tissue (Figure 4B); however, it was only possible to subculture it for a short period, *i.e.* until approximately the fifth passage. We also found that the c-KIT expression of the tumor tissue was reduced with each passage. Complementary DNA analysis of the cultured cells revealed a 1690del6 mutation in exon 11 (W557_K558del) of the *c-KIT* gene (Figure 5).

Tumor growth inhibition after imatinib treatment. We treated the mice xenografted with fourth passage GIST for two weeks with imatinib (100 mg/kg twice daily) or PBS (control) (imatinib: n=3, control: n=2) and observed the mice for a further six weeks. Imatinib treatment produced a greater reduction in tumor size than did PBS treatment (p=0.134). However, the tumors grew again after the imatinib treatment was stopped. After two weeks of treatment and six weeks of observation, we treated the regrowing tumors with imatinib, which produced further re-ductions in their size (Figure 6).

Next, we treated the mice xenografted with sixth passage GIST xenografted mice with imatinib for two weeks (imatinib: n=5, control: n=5) and examined them for a further three weeks. Imatinib treatment produced a significantly greater reduction in tumor size than did PBS treatment (p=0.0384) (Figure 7).

Tumor growth inhibition after sunitinib treatment. We treated the mice xenografted with sixth passage GIST with sunitinib (40 mg/kg once daily) or PBS (control) for two weeks (sunitinib: n=4, control: n=4) and then examined the mice for a further two weeks. Sunitinib treatment produced a significantly greater reduction in tumor size than did PBS treatment (p=0.0265) (Figure 8). However, the tumors began to grow again after the sunitinib treatment was stopped, as was found during the imatinib treatment.

Immunohistochemical analysis. Strong c-KIT expression was detected in the xenografted tumor tissue prior to imatinib treatment. However, c-KIT expression was reduced by imatinib treatment. These results were very similar to those observed in the patient with GIST (Figure 9).

Discussion

Immunodeficient mice have been used for various *in vivo* studies of human tissues, often as *in vivo* tumor models that have been implanted with *in vitro* cultured tumor cell lines (13-16). However, many of these models do not display the same proliferation patterns and structures as the original tissue and tend to be poorly-differentiated; thus, the engraftment of surgically excised human tumor tissue into immunodeficient mice is an alternative model that better preserves the characteristics of the original tumor (13-16). In addition, established tissue lines derived from such tissues could be used on demand and would provide a powerful tool for studying tumor biology. The most commonly cited benefit of patient-derived xenograft models is that they retain the intratumoral heterogeneity and histological characteristics seen in primary tumors (17, 18). Here, we established a novel xenograft mouse model of human GIST that did not involve the use of *in vitro*-cultured cells.

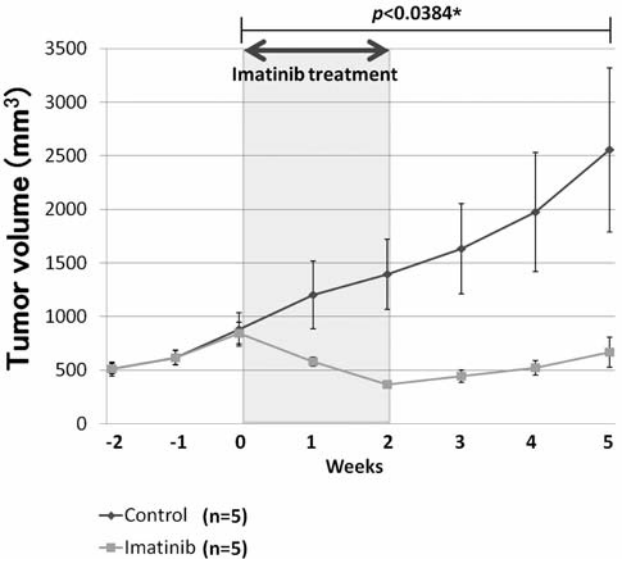

Figure 7. *In sixth-passsage xenograft mice, imatinib treatment produced a significantly greater reduction in xenograft tumor size than did treatment with phosphate-buffered saline (control) (p=0.0384*). Statistical significance was a comparison of the each-entire graphs between zero of the week and five weeks, and was calculated by repeated measurement analysis of variance (ANOVA).*

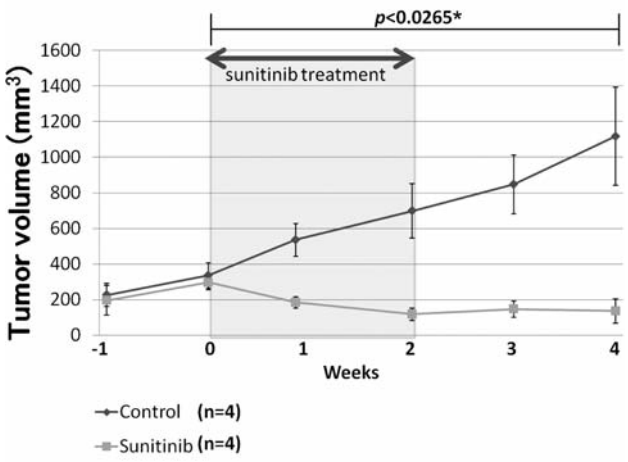

Figure 8. *In sixth passage xenograft mice, sunitinib treatment produced a significantly greater reduction in xenograft tumor size than did treatment with phosphate-buffered saline (control) (p=0.0265*). Statistical significance was a comparison of the each entire graphs between zero week and four weeks, and was calculated by repeated measures analysis of variance (ANOVA).*

There are a few reports about xenograft models of GIST that were established without *in vitro* culturing (12, 19, 20). Our model mimicked the clinical condition of the patient from whom the original GIST was obtained. Both the original GIST and the GIST xenograft were sensitive to imatinib. However, the GIST xenograft was grown in the absence of imatinib. In addition, in immunohistochemical analysis, the c-KIT expression of the xenograft tissue was found to be similar to that of the clinical specimen after imatinib treatment. Furthermore, the c-KIT expression of both the clinical specimen and the xenograft tissue were reduced by imatinib treatment. To our knowledge, there are no reports about GIST xenograft mouse models that mimicked clinical cases. Therefore, our xenograft model is a very valuable tool for studying GIST.

The surgical specimen displayed lower c-KIT expression than the biopsy sample obtained prior to imatinib treatment, and a previous report found that GIST stem cells displayed low c-KIT expression and suggested that low c-KIT expression aids the successful engraftment of GIST into NOD-SCID mice (21). Thus, we consider that implanting the surgical specimen into the NOD-SCID mouse after the original tumor had been treated with imatinib aided in the successful establishment of our GIST model, as it might have increased the xenograft's growth potential. Furthermore, we think that it is important to trim the specimen to an appropriate size, carefully observe it and patiently wait.

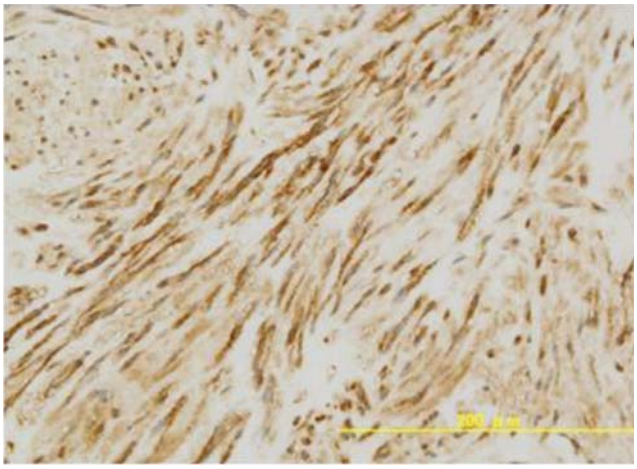

Figure 9. *Immunohistochemical analysis revealed that the c-KIT expression of the xenograft tumor was reduced by imatinib treatment, which was very similar to the findings obtained in the clinical case.*

Regarding the culturing of the xenograft tumor tissue, we found that it was impossible to subculture it for a long period. However, it was possible to subculture it for a short period, *i.e.* until approximately the fifth passage. We also found that the c-KIT expression of the tumor tissue was reduced with each passage. On the other hands, c-KIT expression-negative cells retained *KIT* mutation in exon 11. Thus, a few passage cultured cells may be useful for GIST study. Mutations in exon 11 of the *c-KIT* gene are also the

most common mutations in GIST; thus, our model, which displayed exon 11 mutation, might become a commonly used tool for studying GIST.

Although imatinib has been shown to be effective as a first-line treatment for GIST, most patients will eventually develop imatinib resistance (4, 8, 22). Such resistance can develop through various mechanisms, the most common being secondary *KIT* mutations such as in exon 17 in clonally expanded cancer cells (23, 24). Although the xenograft established in the present study only harbored exon 11 mutation, the continuous administration of imatinib might induce secondary *KIT* mutations.

The established GIST xenograft model could be used to study the resistance mechanisms of GIST, as well as combination and targeted therapies for GIST. It could also be used to develop new more specific biomarkers of GIST. However, the development of patient-derived xenograft models introduces logistical challenges, for example, it will often be necessary to freeze and revitalize tumor tissues after months on years of storage. Close coordination with surgeons and the implantation of specimens as rapidly as possible after their devascularization improves engraftment rates and might be critical for tumors with low engraftment rates.

In conclusion, a xenograft mouse model of human GIST was established from a human GIST. The xenograft model can be used to test new drugs such as TK inhibitors and other agents being developed to circumvent treatment resistance.

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