Gain-of-Function Mutations of Receptor Tyrosine Kinases in Gastrointestinal Stromal Tumors

K. Isozaki[1,2] and S. Hirota[1,*]

[1]*Department of Surgical Pathology, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan and* [2]*Hyogo Medical Education Program, Hyogo Medical Association, Kobe, Hyogo 651-8555, Japan*

Abstract: Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors in human gastrointestinal tract. We first found that most GISTs expressed KIT, a receptor tyrosine kinase encoded by protooncogene c-*kit* and that approximately 90% of the sporadic GISTs had somatic gain-of-function mutations of the c-*kit* gene. Since both GISTs and interstitial cells of Cajal (ICCs) were double-positive for KIT and CD34, GISTs were considered to originate from ICCs or their precursor cells. We also found that germline gain-of-function mutations of the c-*kit* gene resulted in familial and multiple GISTs with diffuse hyperplasia of ICCs as the preexisting lesion. Moreover, we found that about half of the sporadic GISTs without c-*kit* gene mutations had gain-of-function mutations of platelet-derived growth factor receptor alpha (PDGFRA) gene that encodes another receptor tyrosine kinase. Imatinib which is known to inhibit constitutively activated BCR-ABL tyrosine kinase in chronic myelogenous leukemia also inhibits constitutive activation of mutated KIT and PDGFRA, and is now being used for metastatic or unresectable GISTs as a molecular target drug. Mutational analyses of c-*kit* and PDGFRA genes are considered to be significant for prediction of effectiveness of imatinib and newly developed/developing other agents on GISTs. Some mouse models of familial and multiple GISTs have been genetically created, and may be useful for further investigation of GIST biology.

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Key Words: Gastrointestinal stromal tumors, gain-of-function mutation, c-*kit* gene, PDGFRA gene, molecular target therapy.

INTRODUCTION

Most mesenchymal tumors arising from the gastrointestinal (GI) musculature in humans are gastrointestinal stromal tumors (GISTs) [1]. We first found that a large proportion of GISTs were positive for c-*kit* gene product, KIT receptor tyrosine kinase (TK) and had gain-of-function mutation of the c-*kit* gene [1]. These findings resulted in a general understanding of cellular origin and developmental mechanism of GIST, and then rapid progress concerning diagnosis and treatment of GISTs has been made. Immunohistochemistry of KIT is essential for pathological diagnosis of GISTs, and molecular target therapy using TK inhibitors such as imatinib is now being carried out for patients with metastatic or unrectable GISTs. Moreover, we found that the minority of GISTs had gain-of-function mutations of platelet-derived growth factor receptor alpha (PDGFRA) gene [2]. Here, we describe a compendium for developmental biology, diagnosis and therapy of GISTs.

HISTORY OF PATHOLOGICAL CLASSIFICATION OF GI MESENCHYMAL TUMORS

Previously, most of the GI mesenchymal tumors had been thought to originate from smooth muscle cells and they were pathologically diagnosed as leiomyomas, leiomyoblastomas or leiomyosarcomas. After electron microscopic and immunohistochemical analyses, however, many investigators have noticed that the tumor cells lacked differentiation features of smooth muscle cells or schwann cells [3-5]. They have also known that a small proportion of GI mesenchymal tumors were genuine smooth muscle tumors or neurogenic tumors [3-5]. Based on such recognition, the concept of GISTs was proposed for non-myogenic and non-neurogenic GI mesenchymal tumors [6].

In the first half of the 1990's, it was reported that approximately 70% of non-myogenic and non-neurogenic GI mesenchymal tumors express CD34, which is one of the adhesion molecules [7-10]. But it was unclear whether approximately 30% of them which do not express CD34 but have similar histological findings should be included in the same tumor type, i.e. GISTs. Moreover, the CD34 expression in GISTs did not make a suggestion on cellular origin of GISTs. Therefore, a category of GISTs remained obscure. In 1998, we found that almost all non-myogenic and non-neurogenic GI mesenchymal tumors were positive for KIT by immunohistochemistry [1]. The discovery of common KIT expression in non-myogenic and non-neurogenic GI mesenchymal tumors contributed to understanding cellular origin of GISTs and to establishing the concept of GISTs [1]. At present, it is generally accepted that KIT-positive GI mesenchymal tumors are nearly equal to GISTs. Detailed description concerning cellular origin of GISTs appears in the later section.

c-*KIT* GENE, KIT AND RELATIONSHIP BETWEEN KIT AND PDGFRA

The c-*kit* gene was cloned in 1988 as a normal cellular homologue of v-*kit* oncogene in the genome of the Hardy

*Address correspondence to this author at the Department of Surgical Pathology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan; Tel: +81 798 45 6666; Fax: +81 798 45 6671; E-mail: hiros@hyo-med.ac.jp

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Zuckermann 4 feline sarcoma virus [11-13]. It encodes KIT receptor TK, which molecular weight is approximately 145 Kd. KIT consists of extracellular (EC) domain, transmembrane (TM) domain, juxtamembrane (JM) domain and TK domain Fig. (**1**). The EC domain is composed of five immunoglobulin-like repeats and the TK domain is split into two domains (TK I and TK II domains) by a kinase insert Fig. (**1**). The JM domain is a portion between TM domain and TK domain, and is considered to play a role in regulation of KIT dimerization. KIT is classified into type III receptor TK subfamily as well as PDGFRs and colony-stimulating factor 1 receptor. Particularly, amino acid sequence of KIT shows high homology with that of PDGFRA, and c-*kit* gene and PDGFRA gene are located adjacently on human chromosome 4 and mouse chromosome 5 [14-16]. Gene duplication is considered to make these two similar genes. The ligand for KIT is stem cell factor (SCF) that was identified in 1990 [17-19]. Under unbound condition of KIT and SCF, the structure of JM domain is considered to inhibit dimerization of KIT. However, the conformational change of the JM domain by the binding of KIT and SCF appears to release the inhibitory effect. Thus, KIT forms dimer by binding of dimerized SCF. The KIT dimerization induces autophosphorylation of KIT on particular tyrosine residues, and the activated KIT receptor TK leads several signal transduction systems such as PI3K/Akt, Ras/MAPK and Stat pathways to activation Fig. (**1**) [20]. Two types of c-*kit* gene mutations are recognized. One is loss-of-function mutation in which the TK activity of KIT is lost. The other is gain-of-function mutation in which TK activity of KIT is constitutively increased without the binding of SCF. The c-*kit* gene resides in the mouse W locus and the rat Ws locus [21-24]. Since SCF-KIT signal pathways play a crucial role for the development of several cell types such as erythrocytes, mast cells, germ cells and melanocytes, these cells are deficient in mice and rats possessing two alleles of loss-of function mutant genes at W locus and Ws locus respectively [21-24]. On the other hand, the gain-of-function mutations of the c-*kit* gene were first reported in human mast cell leukemia cell line (HMC-1) by Furitsu *et al*. in 1993 [25]. They demonstrated that the cells had constitutive phosphorylation of KIT and two point mutations of c-*kit* gene resulting in amino acid substitution of Gly for Val at codon 560 (Val560Gly) in JM domain and Val for Asp at codon 816 (Asp816Val) in TK II domain [25]. The mutation corresponding to Asp816Val of the human HMC-1 cell line was found in the P-815 mouse mastocytoma cell line and the RBL-2H3 rat mast cell leukemia cell line [26,27]. In addition, the Asp816Val has been found in several clinical cases with mast cell neoplasms [28,29]. These results suggested that gain-of-function mutations of the c-*kit* gene were a cause of mast cell neoplasms.

CELLULAR ORIGIN OF GISTs

Interstitial cells of Cajal (ICCs) were proved to play an important role as pacemakers of GI movement through the electrophysiological examination [30]. ICCs are distributed in musculature throughout the GI tract, and abundantly localize around myenteric plexus [31]. Since ICCs previously could not be easily identified by commonly used methods, ICC research had not fully progressed. In 1992, Maeda *et al*.

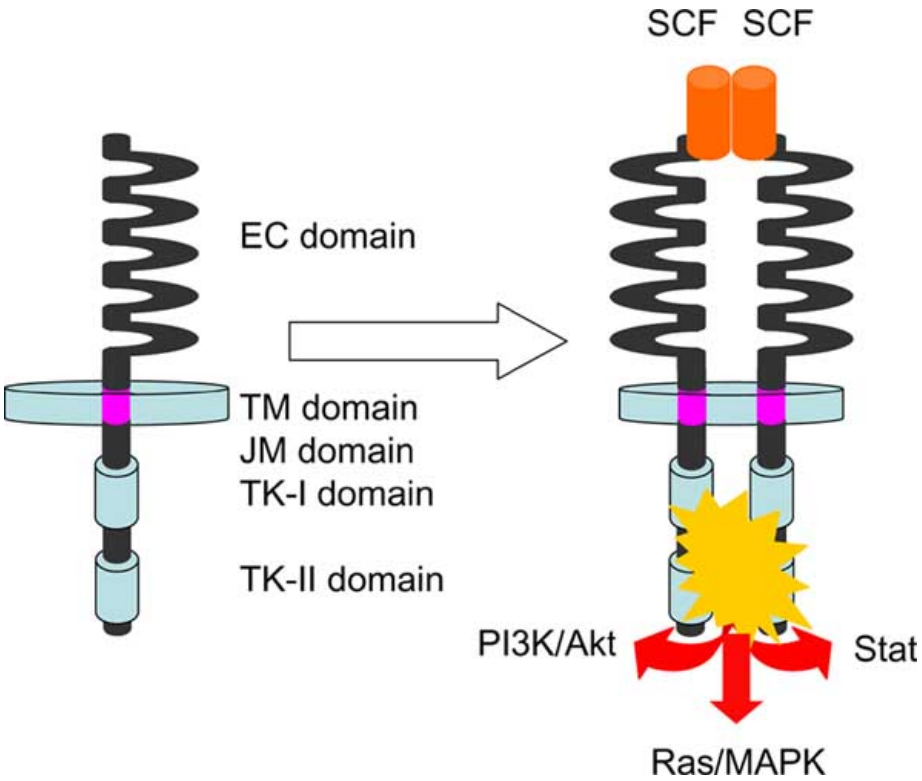

Fig. (1). Schemas of KIT structure and of KIT activation by SCF binding. EC, extracellular; TM, transmembrane; JM, juxtamembrane; and TK, tyrosine kinase.

[32] reported that there were KIT-positive cells in the GI musculature of mice and that the distribution of those cells was reminiscent of that of ICCs. They also showed that remarkable decrease of the KIT-positive cells was observed in the small intestine of W mutant mice [32]. We reported deficiency of c-*kit* mRNA-expressing cells in Ws mutant rats [33]. In 1995, Huizinga *et al*. [34] demonstrated that the KIT-positive cells were ICCs themselves. These results showed that ICCs could be easily and specifically identified by detection of KIT expression and that the SCF-KIT system was crucially required for the development of ICCs as well as erythrocytes, mast cells, germ cells and melanocytes [32-34]. During our ICC study, we hypothesized that 'ICC tumors' might be induced by gain-of-function mutation of c-*kit* gene, based on the facts that loss-of-function mutation of c-*kit* gene results in deficiency of ICCs and mast cells and that gain-of-function mutation of c-*kit* gene results in mast cell tumors as described above. Since the origin of GISTs had not been clarified at that time, we supposed that GISTs might originate from ICCs. KIT was used as a possible marker for identification of 'ICC tumors', and immunohistochemistry for KIT was performed on GI mesenchymal tumors. Although true smooth muscle tumors and true schwannomas were negative for KIT, almost all GISTs showed positive KIT staining Fig. (**2**) [1]. As described previously [10], approximately 70% of non-myogenic and non-neurogenic GI mesenchymal tumors, i.e, GISTs, expressed CD34 [1]. We also demonstrated that ICCs were positive for CD34 like GISTs [1]. We concluded that GISTs originate from ICCs because 1) both GISTs and ICCs are double-positive for KIT and CD34, 2) ICCs are the only cells that are double-positive for KIT and CD34 in the normal GI wall of humans [1]. The finding described by Mazur *et al*. in 1983 that the tumor cells of GISTs resembled the cells surrounding Auerbach's ganglion cells in ultrastructure might indicate the ICC origin of GISTs [6]. Our conclusion was supported by some additional data that both GISTs and ICCs expressed the embryonic isoform of smooth muscle myosin heavy chains, intermediate filament nestin and protein kinase C theta [35-37]. After our discovery of KIT expression in GISTs, KIT became the most reliable marker for pathological diagnosis of GISTs.

SOMATIC GAIN-OF-FUNCTION MUTATIONS OF c-*KIT* GENE IN SPORADIC GISTs

We examined whether GISTs also had the gain-of-function mutations of c-*kit* gene like mast cell tumors. When sequencing of the whole coding region of the c-*kit* gene was carried out, mutations were detected in five of six GISTs [1]. Each mutation was different but all were located at JM domain encoded by exon 11 [1]. We examined whether the c-*kit* gene mutations found in GISTs were of gain-of-function. The mutant c-*kit* cDNAs were transiently introduced into 293T human embryonic kidney cell line, and the KIT autophosphorylation was analyzed. The mutant KIT found in GISTs showed constitutive tyrosine phosphorylation without SCF stimulation [1]. When the equivalent mutations were introduced into the mouse c-*kit* cDNA and they were stably transfected into the interleukin-3 (IL-3)-dependent Ba/F3 murine pro-B cell line, the Ba/F3 cells became to grow autonomously without IL-3 [1]. Transplanted Ba/F3 cells with mutated c-*kit* cDNA formed tumors in back of nude mice [1]. These results suggested that the c-*kit* gene mutations in GISTs are of gain-of-function and these mutations

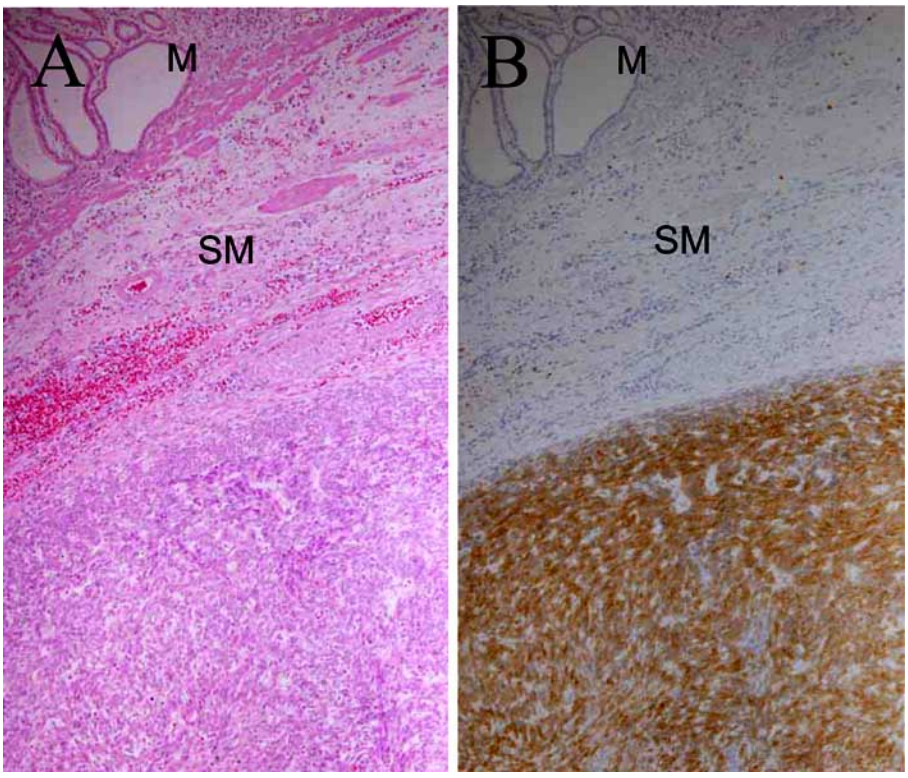

Fig. (2). Histology (A) and KIT immunohistochemistry (B) of a GIST. M, mucosal layer; SM, submucosal layer.

induce autonomous cell proliferation, i.e. development of GISTs.

Further analyses using many primary sporadic GIST cases revealed that 85 to 90% of all sporadic GISTs had c-*kit* gene mutations [38-41]. Seventy-five to eighty per cent of all sporadic GISTs have c-*kit* gene mutations at JM domain, 5 to 13% of them at EC domain encoded by exon 9, less than 4% at TK I domain encoded by exon13, and less than 4% at TK II domain encoded by exon17 Fig. (**3**) [39-45]. Various mutations are observed at JM domain, but the particular mutations are detected at EC, TK I, and TK II domains [38-45]. The EC domain mutation is usually duplication of codons 502 and 503, the JM I domain mutation Lys642Glu, and the TK II domain mutation Asp822Lys or Asp822His [42-45].

GERMLINE GAIN-OF-FUNCTION MUTATIONS OF c-*KIT* GENE IN FAMILIAL AND MULTIPLE GISTs

At least twelve families with multiple GISTs and germline c-*kit* gene mutations have been reported [46-58]. Patients of eight families have the mutation at JM domain [46-48,50,51,53-56], and those of one family at EC domain [57], those of one family at TK I domain [49], and those of two families at TK II domain [52,58]. The germline mutations are demonstrated to be of gain-of-function, and are considered to be the cause of multiple GIST development. Interestingly, remarkable diffuse proliferation of ICCs is observed as a preexisting lesion of multiple GISTs in small intestine of the patients [46,48,49,52]. Although mild symptom of achalasia-like dysphagia with manometrically abnormal simultaneous contraction of the esophagus has been reported in some patients [52], most of the patients do not appear to complain of any GI symptoms other than mild dysphagia. The c-*kit* gene mutation or ICC proliferation might not induce severely abnormal GI motility. Patients of some families show hyperpigmentation of perineal, perioral and digital area and/or mast cell neoplasms [46,50,51,53-55]. These findings might be attributable to the effect of germline gain-of-function mutation of c-*kit* gene on melanocytes and mast cells, but the reason why all of the patients with the mutation do not show the symptoms is not unclear.

GAIN-OF-FUNCTION MUTATIONS OF PDGFRA GENE IN GISTs

As described above, c-*kit* gene mutations are not detected in 10 to 15% of sporadic GISTs. In 2003, Heinrich *et al.* and we reported that somatic mutations of PDGFRA gene were observed in sporadic GISTs lacking c-*kit* gene mutations [2,59]. The frequency reported by Heinrich *et al.* was 35% (14 of 40 GISTs without c-*kit* gene mutations), and that in our result was 62.5% (5 of 8 GISTs without c-*kit* gene mutations) [2,59]. The mutations were localized at JM domain encoded by exon 12 or TK II domain encoded by exon 18 of PDGFRA gene [2,59]. Recently, PDGFRA gene mutation of TK I domain encoded by exon 14 has been reported as a rare mutation-type [60,61]. KIT and PDGFRA have extremely similar amino acid sequence as described above, and exon 12 and exon 18 of PDGFRA gene are comparable to exon 11 and exon 17 of the c-*kit* gene respectively. Most frequent PDGFRA mutation appears to be Asp842Val at exon 18, and the mutation is structurally comparable to Asp816Val at exon 17 of c-*kit* gene. Interestingly, the Asp816Val is the mutation that commonly found in human mast cell tumors but never in primary GISTs [2,59]. The PDGFRA gene mutations were proved to result in constitutive tyrosine phosphorylation of PDGFRA without PDGF-AA stimulation, and they were considered to be of gain-of-function [2,59]. These results suggested that the PDGFRA gene mutations are also another cause of GISTs. Sporadic GISTs with both c-*kit* and PDGFRA gene mutations have not been found, and these two types of mutations are considered to be mutually exclusive. Only one family with germline PDGFRA gene mutation of Asp846Tyr in the TK II domain has been reported [62].

MOLECULAR TARGET THERAPY FOR GISTs

Surgical resection is the basis of treatment for GISTs both in the past and now. However, the patients with unresectable or metastatic GISTs did not have practically remarkable effect of surgery and conventional chemotherapy. One of the TK inhibitors, imatinib, has completely revolutionized the status. Imatinib, a 2-phenylaminopyrimidine derivative, was initially developed as a specific inhibitor of

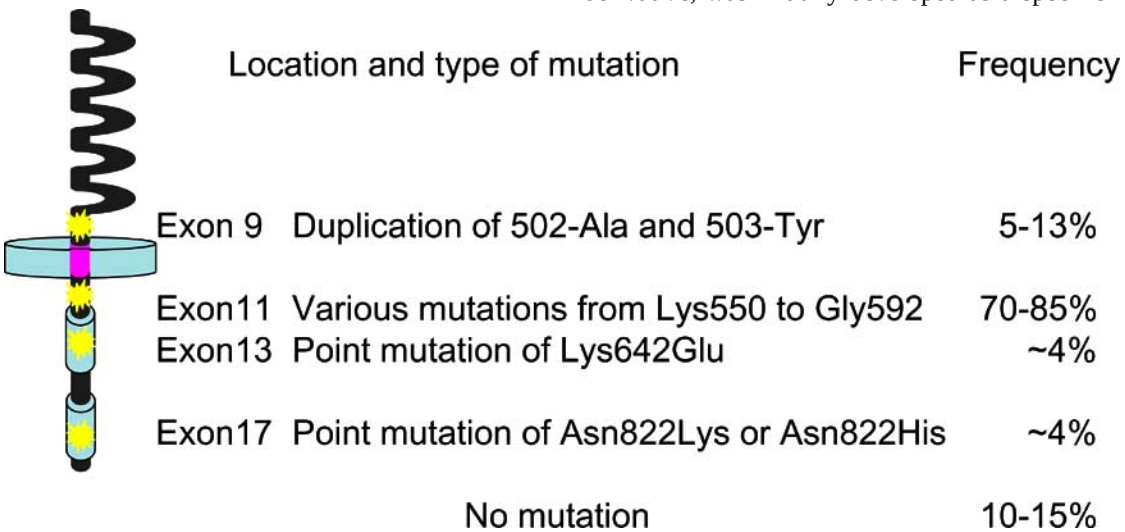

Fig. (3). Locations and types of c-*kit* gene mutations and their frequency in sporadic GISTs. EC, extracellular; TM, transmembrane; JM, juxtamembrane; and TK, tyrosine kinase.

PDGFRs and BCR-ABL [61]. BCR-ABL, constitutively activated non-receptor TK, is a product of gene fusion formed by translocation of chromosomes (Philadelphia chromosome) in patients with chronic myelogenous leukemia (CML) [63]. Its constitutive activation is the cause of CML, and imatinib is now successfully applied to patients with CML. In addition to the inhibitory effect of imatinib on PDGFR and BCR-ABL, Buchdunger *et al.* [64] found an inhibitory effect on wild-type KIT. Furthermore, it was confirmed by us [65] and others [66] that imatinib also inhibits various types of mutant KIT found in GISTs. These results suggested that imatinib might be effective for the treatment of patients with advanced GISTs, and in fact imatinib was first administered for a recurrent GIST patient with JM domain c-*kit* gene mutation in Finland [67]. Marvelous effect was observed in the patient, and multicenter trials of imatinib treatment for patients with unresectable and metastatic GISTs were performed thereafter. Successful clinical effect was confirmed in the majority of the patients, [68] and the agent has been approved as a therapeutic drug in many countries. During the trials, it became clear that the locations of c-*kit* and PDGFRA gene mutations were related to the effectiveness of imatinib on GIST patients [65,69-71]. The rate of partial response on GIST patients with JM domain mutations of the c-*kit* gene was 83.5%, whereas that with EC domain mutations was 47.8% [69]. GIST patients with PDGFRA gene mutation or with wild-type c-*kit* and wild-type PDGFRA gene appear to show lower rate of partial response. Mutational analyses in c-*kit* and PDGFRA genes are considered to be valuable for the prediction of imatinib effectiveness. As described above, imatinib is effective in the majority of GISTs, but there are some primarily resisitant GISTs for imatinib. Moreover, the secondarily resistant clones often develop after the initial successful treatment by imatinib. The main cause of the secondary resistance for imatinib is considered to be the secondary c-*kit* gene mutation on the identical gene [72-74]. The frequently observed secondary mutations are Val654Ala at exon 13, Thr670Ile at exon 14, Tyr823Asp and so on. The types of secondary mutations are basically not observed in primary GISTs. Interestingly, codon 820 and codon 822 that are the mutation sites observed in multiple and familial GISTs and sporadic GISTs respectively have been reported as the mutation sites in secondarily resistant GISTs. New agents against the imatinib-resistant tumors have been developed or are developing. Among these drugs, one of indolinone-derivative TK inhibitors, SUTENT/SU11248, has been clarified to show clinical effect for imatinib-resistant GISTs with exon 9 c-*kit* gene mutation, and in 2006 it has been approved by Food and Drug Administration in USA.

CONCLUSIONS

Although it is clear that gain-of-function mutations of c-*kit* and PDGFRA genes play an important role for the development of GISTs, unsolved subjects concerning the mechanism of GIST development still remain. Since multiple GISTs develop in later with ICC hyperplasia as a preexisting lesion [46,48,49,52], another event such as other gene mutations in addition to c-*kit* gene mutation appears to be required for neoplastic change from ICC hyperplasia. Such gene mutations have not been reported yet. In some NF1 patients, development of multiple GISTs has been reported [75,76], but we [77] and others [78,79] demonstrated that the rate of c-*kit* gene mutations was very low in NF1 GISTs. There may be an unknown mechanism for GIST development in NF1 patients. Further analyses for the development of GISTs without c-*kit* and PDGFRA gene mutations other than NF1 patients also have to be done.

ABBREVIATIONS

CML = Chronic myelogenous leukemia
EC = Extracellular
GI = Gastrointestinal
GIST = Gastrointestinal stromal tumor
ICCs = Interstitial cells of Cajal
IL-3 = Interleukin-3
JM = Juxtamembrane
PDGFRA = Platelet-derived growth factor receptor alpha
SCF = Stem cell factor
TM = Transmembrane
TK = Tyrosine kinase

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