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Review Article

Kit mutations in cancer and their treatment with protein kinase inhibitors*

Ugo Testa
Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. Correspondence: ugo.testa@iss.it

CONTENTS

Abstract

The Kit receptor, or the receptor for stem cell factor (SCF), is a member of the type III subclass of receptor tyrosine kinases (RTKs). Signaling by SCF and Kit plays an important role in hematopoiesis, gametogenesis, melanogenesis and mast cell development and function. Kit is frequently activated in neoplastic diseases; in fact, more than 30 gain-of-function mutations in Kit, either single amino acid changes or small deletions/insertions, have been identified in various tumors, including gastrointestinal stromal tumors (GISTs), mastocytosis, acute leukemias, melanomas and other cancers. Targeting of Kit by imatinib mesilate, a TK inhibitor, was found to be an effective anticancer strategy for the treatment of GISTs. However, the occurrence during treatment of secondary mutations of Kit renders the tumors resistant to imatinib. A growing generation of second-line TK inhibitors, such as nilotinib and dasatinib, are active against imatinib-resistant Kit mutant cancers and could thus offer a valuable therapeutic strategy. Non-TK inhibitors like rapamycin, 17-AAG and IMD-0354 have been added to the therapeutic armamentarium, with the hope that their use in combination therapy might have a synergistic antitumor effect or prevent/delay the development of drug resistance.

Kit structure and function

In multicellular organisms, a fundamental mechanism for regulating cell survival and proliferation and for allowing communication between cells is represented by the binding of protein ligands to cell-surface receptors possessing tyrosine kinase (TK) activity. These receptors play a key role in the regulation of cell survival, proliferation, differentiation, migration and metabolism through different signaling pathways.

Tyrosine phosphorylation has a key role in intracellular signaling. Inappropriate proliferation and survival cues in tumor cells often occur as a consequence of unregulated TK activity. Tyrosine kinase receptors are frequently activated and/or mutated in various cancers: FLT3 is frequently mutated in acute myeloid leukemia (AML), ret is often mutated in neuroendocrine tumors, CSF1R is dysregulated in metastatic breast cancer and Kit is frequently activated and/or mutated in GISTs, mastocytosis and AML. Kit is a receptor tyrosine kinase (RTK) that normally controls the function of hematopoietic stem cells/progenitors, erythroid cells, melanocytes, germ cells and mast cells.

During the last decade, it has become clear that uncontrolled activity of Kit contributes to the formation of an array of human tumors. Unregulated Kit activity may be related either to its overexpression or autocrine loop or activating mutations. The latter mechanisms seem to operate in various tumors.

The *KIT* proto-oncogene encodes for a class III transmembrane RTK, Kit, that is composed of an extracellular portion containing five immunoglobulin (Ig)-like domains and an intracellular portion consisting of a juxtamembrane and two TK domains separated by an interkinase domain (Fig. 1) (reviewed in Refs. 1 and 2). The human *KIT* gene, located on chromosome 4q12, spans approximately 89 kb and comprises 21 exons which are transcribed and translated, giving rise to a peptide of 976 amino acids with a molecular mass of 145 kDa. The five Ig-like subdomains (the first three form the binding site for stem cell factor [SCF]) of the peptide extracellular domain span from amino acid residues 23 to 520 and are encoded by exons 1-9. The transmembrane domain is formed by amino acid residues 521-543 and is encoded by exon 10. The juxtamembrane domain, endowed with an autoinhibitory function, spans from amino acid residues 544 to 581 and is encoded by exon 11. The TK domain, encoded by exons 12-21, spans from amino acid residues 582

* See also: Systemic mastocytosis, p 187 this issue.

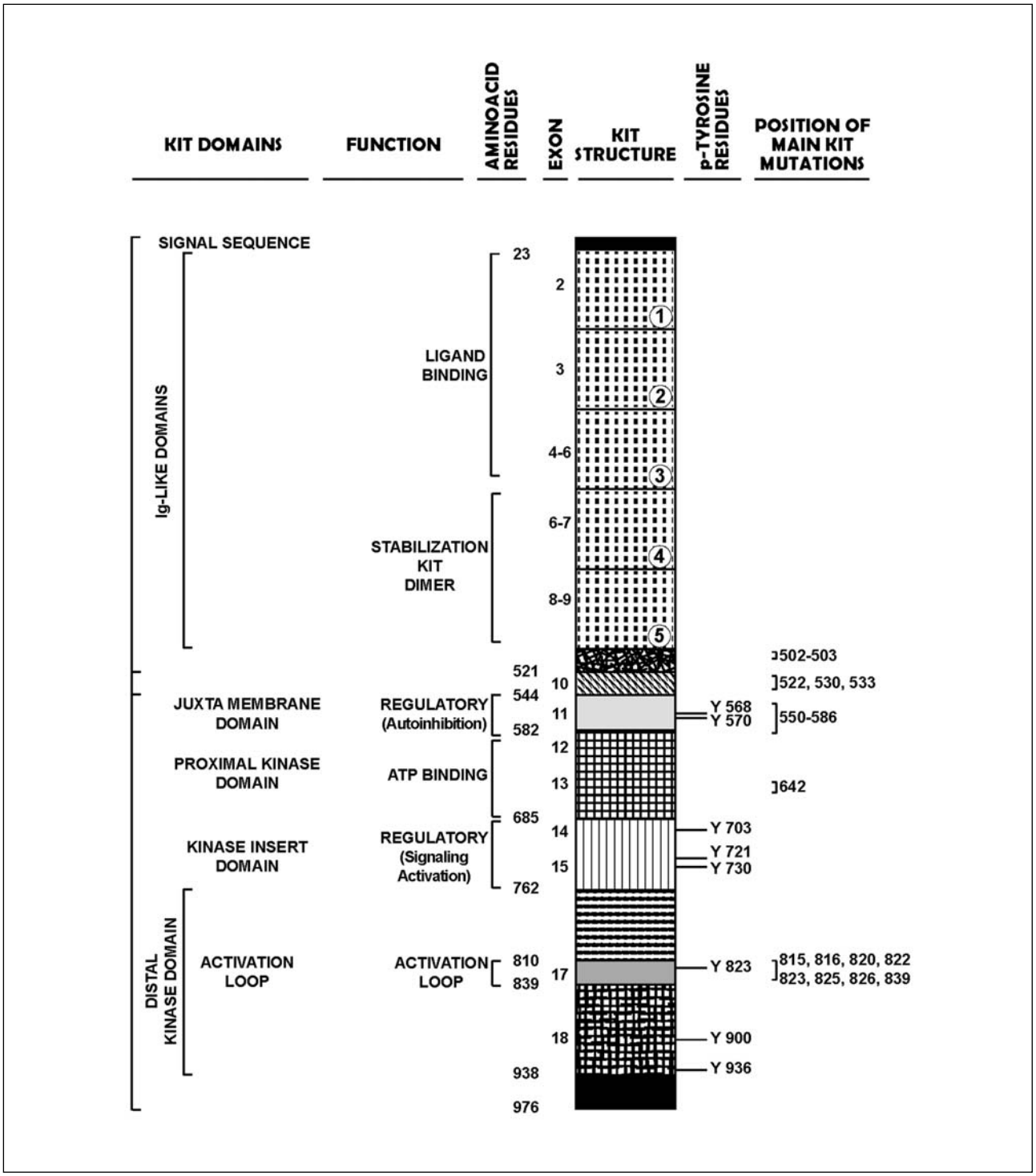

Fig. 1 Schematic representation of the structure of the Kit receptor. The receptor is comprised of an extracellular domain composed of five immunoglobulin (Ig)-like domains involved in ligand binding and receptor dimer formation, a transmembrane domain anchoring the receptor to the cell membrane and an intracellular domain involved in cell signaling and regulation of receptor kinase activity; at the level of the intracellular domain, several subdomains have been identified, including a juxtamembrane domain, a proximal kinase domain, a kinase insert domain and a distal kinase domain, containing an activation loop. The intracellular domain contains eight tyrosine residues (Y) that are phosphorylated following the activation of Kit through binding with its ligand. Kit-phosphorylated tyrosine residues interact with adapter proteins that mediate the activation of several cell signaling pathways: Ras/MAPK activation involved in the stimulation of cell proliferation (Y568, Y570, Y703 and Y936); PI3K and Akt activation involved in cell survival (Y721); PKC activation involved in cell proliferation (Y730); and JAK/STAT activation exerting a negative control on cell proliferation (Y570). On the right, the localization of the more frequently observed mutations in the *KIT* sequence is shown.

to 937 (1, 2). The TK domain of the Kit receptor is divided into two subdomains, separated by a kinase insert: 1) the proximal kinase domain spans from amino acids 582 to 684 and contains the ATP binding site; and 2) the distal kinase domain expands from amino acids 762 to 937 and contains the phosphotransferase and the activation loop (Fig. 1). The natural ligand of Kit is a cytokine known as SCF, or Kit ligand. The kinase insert domain is about 80 residues in length: this domain is phosphorylated and serves as a docking site for some important signal transduction proteins (reviewed in Ref. 3).

Kit is a typical example of a class III RTK in which receptor activation implies ligand-mediated receptor dimerization. In fact, recent crystallization studies of the ectodomain of the receptor have shown that the bivalent ligand (SCF) binds simultaneously to two receptor molecules and effectively crosslinks them to form a dimer (4, 5). In particular, each SCF molecule in the dimer binds to the first three Ig-like domains (D1, D2 and D3) of Kit; in the D1-D3 region there are no contacts between the two receptor molecules, although there are clear contacts between the two receptor molecules in the membrane proximal region of the dimer, involving domains D4 and D5.

Upon binding of SCF to the extracellular Ig-like domains, Kit undergoes homodimerization and autophosphorylation at the Y568 and Y570 tyrosine residues of the juxtamembrane domain. This leads to the phosphorylation and activation of multiple signaling pathways, such as Janus kinase/signal transducer and activator of transcription (JAK/STAT), Src kinases, mitogen-activated protein (MAP) kinases and phosphatidylinositol 3-kinase (PI3K). The activation of these complex cell signaling pathways depends on the phosphorylation of several tyrosine residues present on the cytoplasmic tail of the Kit receptor and on the binding of enzymes or adapter proteins to these Tyr residues. The adapter proteins APS, SHC and SHP2 (tyrosyl phosphatase) bind to Tyr568. SHP1 and the adapter protein SHC bind to Tyr570. The different Tyr residues present in the kinase insert domain are phosphorylated and bind some signaling components: Tyr703 binds the GRB2 adapter protein, Tyr721 binds PI3K and Tyr730 binds phospholipase C γ (PLCγ). Finally, two Tyr residues present in the distal TK domain are phosphorylated, and one of them (Tyr900) binds PI3K, while the other (Tyr936) binds the adapter proteins APS, GRB2 and GRB7. SCF induces the recruitment of Kit at the level of membrane lipid rafts, where molecules involved in cell signaling are concentrated. The integrity of lipid rafts is required for efficient activation of PI3K/Akt proteins and Kit-mediated proliferation (6).

In addition to the cell membrane, the Kit receptor is also present in intracellular compartments, such as the Golgi apparatus. The cellular localization of some mutant Kit proteins is different compared to the wild-type (wt) protein, with a predominant intracellular localization (7). In normal tissues, clear Kit expression was observed in bone marrow, secretory cells of mammary glands, basal cells of skin, thymic epithelial cells, mast cells, interstitial cells of Cajal and spermatogonia in testicular tubules (8).

The expression and mutational status of Kit have been explored in detail in the large majority of human tumors. Kit positivity was detected in AML (80-85%), systemic mastocytosis (100%), GISTs (100%), seminomas (> 80%), salivary gland adenoid cystic carcinoma (65%), malignant melanoma (35%), small cell lung cancer (38%) and large cell carcinomas of the lung (17%). The other tumors were Kit-negative or displayed < 10% positive cases (8). In parallel, abnormalities of the sequence or the copy number of the *KIT* gene have been explored in large panels of human neoplasias, showing that *KIT* mutations are observed in GISTs, systemic mastocytosis and AML, while *KIT* gene amplification was relatively common in other tumors with strong Kit protein expression (9).

Kit mutations in GISTs

The term gastrointestinal stromal tumors (GISTs) was introduced in 1983 by Mazur and Clark to identify a group of gastrointestinal tract tumors previously erroneously classified as leiomyomas, leiomyoblastomas and leiosarcomas (10). Immunohistochemical and electron microscopy studies provided evidence that these tumors are related not to myogenic cells, but to a population of spindle cells present in the gut wall and known as interstitial cells of Cajal (ICCs). ICCs play an important role as pacemakers of gut motility; in particular, their intercalation between enteric nerve varicosities and smooth muscle cells makes it very likely that ICCs have a role in neurotransmission (11). Interestingly, ICCs fail to develop in mice deficient in the expression of Kit (12). In line with this observation, immunohistochemical studies showed that Kit is strongly expressed in the large majority of GISTs (13). Kit and CD34 positivity is considered a common histochemical criterion for the identification of GISTs (14). However, although Kit expression is a reliable marker of GISTs and is an important criterion for differential diagnosis with respect to other gastrointestinal sarcomas, about 4-10% of GISTs fail to express Kit, while about 25% of leiomyosarcomas express Kit. Therefore, simple positivity for Kit is not sufficient to distinguish GISTs from leiomyosarcomas and this point is of great clinical relevance, since the former tumors are sensitive to imatinib treatment, while the latter do not respond to this protein kinase inhibitor. Given these limitations, recent studies have shown that whole-genome gene expression studies using microarray technology allowed to distinguish GISTs from leiomyosarcomas in about 98% of cases (15).

Following these initial findings on the basic biology of ICCs, subsequent studies (16) have shown that Kit is very frequently mutated in GISTs. These studies have demonstrated that 80-85% of GISTs harbor activating mutations of the *KIT* gene: in 66% of cases at the level of exon 11, in 13% of cases at the level of exon 9, and more rarely, in 1.2% and 0.6%, respectively, at the level of exon 13 and 17 (16). Various types of mutations occurring at the level of exon 11, which encodes the juxtamembrane domain, have been observed, including deletions and insertions

affecting the first part of exon 11, particularly codons 557 and 559, point mutations limited to four codons within the exon (557, 559, 560 and 576), and internal tandem duplications observed near the end of the exon. *KIT* oncogenic mutations abrogate juxtamembrane region autoinhibition of the Kit kinase (17). The mutations in exon 9, which encodes a part of the extracellular domain, correspond more frequently to AY 501-502 duplication/insertion and more rarely to FAF 506-508 duplication/insertion. Exon 9 mutant GISTs occur at the level of the small intestine. The mutation at the level of exon 13 (proximal kinase domain) consists of the point mutation K642E, while the mutation at the level of exon 17 (activation loop) is represented by N822K or N822H point mutations.

As mentioned above, about 15-20% of GIST patients do not harbor *KIT* mutations. However, about 40% of these wt *KIT* GISTs display mutations of the platelet-derived growth factor receptor alpha gene *PDGFRA* (18). The analysis of 1,105 GIST patients showed that 7.2% of them displayed a *PDGFRA* mutation, the large majority (82%) in exon 18 and the remaining in exons 12 and 14. Exon 12 and 14 *PDGFRA* mutants are imatinib-sensitive, while the large majority of exon 18 mutants and, particularly the D842V mutant (corresponding to 65% of *PDGFRA* mutant GISTs), are imatinib-resistant (19). The D842V mutant was potently inhibited by the staurosporine derivative PKC-412, thus supporting the clinical testing of this kinase inhibitor for the treatment of mutant *PDGFRA* (20).

The key role of *KIT* gain-of-function mutations in GIST pathogenesis is directly supported by the analysis of families harboring germline *KIT* mutations. These mutations are transmitted in an autosomal dominant manner. The clinical manifestations observed in these families are largely dependent on the *KIT* domain affected by the mutation: *KIT* mutations at the level of the juxtamembrane domain are usually associated with cutaneous hyperpigmentation, mastocytosis, hyperplasia of the GIST progenitor ICCs and, in some cases, progression to the development of GISTs; *KIT* mutations at the level of the split kinase domain lead to the development of GISTs, but are not associated with mastocytosis and hyperpigmentation (21-25). The analysis of a transgenic knock-in murine GIST model harboring a germline *KIT* K641E mutation confirmed these findings. In fact, homozygous and heterozygous *KIT* K641E mice are viable and develop GISTs and/or hyperplasia of ICCs (26). Together, these findings strongly support a pivotal role for *KIT* activating mutations in the pathogenesis of GISTs.

The presence or not of *KIT* or *PDGFRA* mutations implies important differences between the various groups of GISTs. In particular, the responsiveness of various GISTs to treatment with the kinase inibitor imatinib varies substantially depending on the exonic location of the *KIT* or *PDGFRA* mutation. The molecular elucidation of the pathogenesis of GIST has provided a rational basis for molecularly targeted therapy of this disease. The idea underlying this approach was to block the constitutive kinase activity of the mutated Kit receptor using small-molecule TK inhibitors.

Imatinib (Glivec, Gleevec; Novartis) is a small-molecule TK inhibitor with activity against various TKs, including ABL, BCR-ABL, Kit, PDGF-R-α and PDGF-R-β. The structure of this drug mimics ATP and it binds to the ATP binding sites of the target kinases. The rationale for using this compound came from two different observations: 1) it inhibits the kinase activity of both wt and mutant Kit; and 2) it inhibits the growth of a GIST cell line harboring a *KIT* gene mutation (27).

Clinical evidence supporting the efficacy of imatinib for GIST was obtained from phase I and II studies in which partial responses were obtained in the majority of patients (reviewed in Ref. 28). Responses in GIST patients depend on the presence and genomic location of *KIT* oncogenic mutations: 1) patients with exon 11 *KIT* mutations have a partial response rate of about 85-90%, compared to a 0% partial response rate among patients without *KIT* mutations; 2) patients with exon 9 mutations might benefit from the use of higher imatinib doses; and 3) patients with *PDGFRA* mutations do not have durable responses to imatinib treatment. The majority of exon 11 *KIT* mutant GIST patients are heterozygous for the mutant *KIT* allele. However, in a minority of GIST patients, homozygous exon 11 *KIT* mutations have been observed and are strongly associated with malignant clinical behavior and limited survival (29).

The translation of imatinib response to survival benefit has been shown in long-term follow-up studies. In this context, data from a phase III trial in which doses of either 400 or 800 mg/day imatinib were administered showed progression-free survival at 24 months of 48% and 56%, respectively, in a group of 946 patients with GISTs. In particular, complete responses, partial responses and stable disease, according to RECIST, were achieved in 5%, 45-50% and 25-30% of patients, respectively. This study showed that imatinib prolongs the survival of GIST patients when compared to historical controls, with a 2-year survival of 69% *versus* 17% (30). Follow-up of these patients showed that responses are durable, with a median overall survival time of 5 years (31). Response rates and overall survival are poorer in GIST patients with tumors harboring wt genes or an exon 9 mutation than in patients with tumors expressing an exon 11 mutation. However, it is of interest to note that GIST patients with exon 9 mutations have a longer progression-free survival during imatinib treatment at 800 mg than at the standard dose of 400 mg, while for other mutants there is no difference between the two doses (32). These studies, however, also showed that in some patients disease progression develops over time despite continued imatinib therapy.

As indicated above, imatinib administration to GIST patients very rarely induces complete disease remission, indicating that this drug can prevent disease progression but only rarely cure the disease. In line with this observation, imatinib interruption results in rapid progression in most patients with advanced GIST and cannot be recommended in routine practice unless the patient experiences severe toxicity to imatinib (33).

In some patients, disease progression develops over time despite continued imatinib therapy. Two types of resistance to imatinib have been described: 1) a primary resistance occurring early during treatment (< 6 months; this category is represented by tumors with no detectable kinase mutation or tumors with a *KIT* exon 9 mutation); and 2) a secondary resistance occurring later during treatment (> 6 months; this category is represented by tumors acquiring new kinase mutations that interfere with imatinib activity). The majority of these new *KIT* mutations occur at the level of the ATP binding pocket, thus inhibiting the capacity of the drug to bind the mutant Kit molecule. For yet unknown reasons, secondary mutations develop more frequently with a primary exon 11 mutation (about 60%) compared to exon 9 (about 20%). This finding provides evidence that exposure to imatinib drives the selection of double *KIT* mutant clones. It is likely that the heterogeneous secondary *KIT* mutant subclones exist at very low levels in most primary tumors, providing the point of departure for clonal selection and development of drug resistance during imatinib therapy. Interestingly, these secondary resistance mutations can vary between different progressing metastases in each patient.

Desai *et al*. recently explored the radiological pattern of tumor progression in 89 GIST patients undergoing imatinib therapy. Forty-eight of these patients developed progressive disease and 23 of the 48 patients displayed a peculiar radiological pattern of tumor progression (34). In fact, these patients displayed a “resistant clonal nodule”, corresponding to a new enhancing nodular focus enclosed within a pre-existing tumor mass. These nodules are thought to represent the emergence of clones resistant to imatinib, as suggested by two observations: 1) the median survival among patients whose first progression was nodular was 35 months, compared to 45 months for patients whose first progression was not nodular; and 2) comparative genotypic analysis in tumor biopsies at baseline and from progressive nodules showed new activating kinase mutations in Kit and PDGF-R-α receptors in 80% of these patients (34).

The approach for the treatment of imatinib-resistant GISTs consists of the use of other TK inhibitors. One of these alternative drugs is sunitinib (Sutent; Pfizer). This compound is an oral small-molecule TK inhibitor that is able to inhibit various TKs, including wt and mutant Kit, and wt and mutant FLT3 and VEGFR-2. This compound emerged from a drug discovery program designed to identify a more potent inhibitor of VEGFRs and PDGFRs (35). The *KIT* V654A and *KIT* T670I mutations, located in the proximal kinase domain of the gene, have been reported as common, recurrent secondary mutations in GIST patients treated with imatinib, and have been proven to confer resistance to imatinib; cell lines harboring these *KIT* mutants were potently inhibited *in vitro* by sunitinib, while they were resistant to imatinib (36). A phase I/II trial showed that patients with GIST tumors with exon 9 mutations or wt GISTs had better and more durable responses than those with *KIT* exon 11 mutations (37). In this study, 43% of patients taking sunitinib experienced clinical benefit. A double-blind phase III study of the use of sunitinib in the treatment of patients with advanced GIST who were resistant to or intolerant of previous treatment with imatinib showed a median time to progression of about 6.3 months compared to 1.5 months with placebo; partial responses were observed in 6.8% of sunitinib-treated patients (38). Patients more responsive are represented by those exhibiting exon 9 and *PDGFRA* mutations. Based on these findings, in January 2006 the FDA approved sunitinib for the treatment of patients with imatinib-refractory or -intolerant GIST (39). The drug was subsequently (January 2007) also approved in the E.U. for these indications. A GIST treatment case study is also being performed to provide sunitinib to patients who are ineligible to be included in sunitinib studies but who, in the opinion of the treating physician, are likely to benefit from this treatment; a median time to tumor progression of 23.7 weeks has been reported to date (40).

In a recent study, the safety and efficacy of PTK-787/ZK-222584 (vatalanib), an oral mulitargeted TK inhibitor that inhibits all VEGFRs, Kit and PDGFRs, was evaluated in a group of 15 GIST patients who had progressed after a few weeks of treatment with imatinib: 2 patients achieved partial responses, 8 had stable disease and 5 progressed (41).

In addition to these drugs, other TK inhibitors have been screened for their ability to inhibit imatinib-resistant *KIT* mutants. Thus, the T670I *KIT* mutant (a frequent secondary *KIT* mutation occurring in GISTs) resistant to imatinib was found to be sensitive to sorafenib (42) and PKC-412 (midostaurin) (43). Recently, the efficacy of second-line TK inhibitors such as sorafenib (Nexavar; Bayer), dasatinib (Sprycel; Bristol-Myers Squibb) and nilotinib (Tasigna; Novartis) against the commonly observed imatinib-resistant *KIT* mutations (*KIT* V654A, *KIT* T670I, *KIT* D820Y, *KIT* N822K) was compared. Sorafenib inhibited all the *KIT* mutants, nilotinib was more potent against *KIT* V654A and *KIT* D820Y mutants than dasatinib and sorafenib, and the *KIT* T670I mutant was inhibited by sorafenib, but not by nilotinib or dasatinib (44). Since these drugs have been introduced in therapy for the treatment of other cancer types, they represent promising agents for the treatment of imatinib-resistant GIST patients. Recently, INNO-406, also known as NS-187, a novel TK inhibitor that is 25-55-fold more potent that imatinib against BCR-ABL (45), was shown to be able to inhibit Kit kinase (46). In fact, INNO-406 was effective against cells expressing wild-type *KIT* (IC_{50} = 840 nM) and mutant *KIT* V560G (IC_{50} = 51 nM), but was unable, like imatinib, to inhibit cells expressing the mutant *KIT* D816V (IC_{50} >10 μM) (46). These findings suggest clinical potential for INNO-406 in *KIT* V560G-expressing malignancies.

The above-mentioned studies were carried out in GIST patients with metastatic and inoperable malignant disease. The role of adjuvant imatinib in the treatment of GIST is unclear and is under active investigation in several ongoing clinical trials. These studies (*e.g.,* ACOSOG Z9000 [NCT00025246], ACOSOG Z9001

[NCT00041197], EORTC 62024 [NCT00103168] and SSGXVIII [NCT00116935]) include patients with different malignant potential, but basically aim to evaluate the effect of imatinib in GIST patients, including those who have undergone radical surgery, at low, intermediate and high risk. Awaiting the results of these important multicenter trials, Nilsson *et al*. recently reported their experience with adjuvant imatinib therapy in 23 GIST patients, showing that 1 year of imatinib (400 mg/day) treatment after radical surgical resection of high-grade GIST dramatically reduces the risk of recurrent disease (47).

It is of interest to note that, before the introduction of imatinib in clinical practice, the large majority of GIST patients were treated by surgery; however, complete resection was not possible in > 50% of these patients. Since the introduction of imatinib, the role of surgery in GIST treatment must be carefully reassessed. In this context, two studies have evaluated the role of surgery in patients with advanced GISTs while receiving imatinib therapy. Basically, the two studies showed that in a high percentage of patients achieving stable disease with imatinib therapy, it is possible to obtain a complete resection of the tumor, and in a lower percentage of patients achieving limited progression or generalized progression after imatinib therapy, surgery removed all the tumor bulk (48, 49). Alternatively, imatinib can be administered after surgery. Thus, a randomized phase III study performed by the American College of Surgeons (ACOSOG Z9001, NCT00041197) was based on a comparison of imatinib treatment for 1 year following complete surgical resection of GIST compared to treatment with a placebo control. After 1 year of treatment, the recurrence rate was higher in the placebo group than in the imatinib-treated group, mostly for GISTs > 10 cm in size (33% *vs*. 4%).

An alternative strategy to inhibit mutated Kit consists of enhancing its cellular degradation via ubiquitination/proteasome-mediated processing. Thus, Fumo *et al*. (50) showed that Kit activation depends on protein stabilization through interaction with the molecular chaperone heat shock protein 90 (HSP90), HSP90 inhibition causing degradation of both wt Kit and the imatinib-resistant Kit D816V mutant. The interaction between HSP90 and its client proteins, including Kit, is inhibited by the geldanamycin 17-AAG. 17-AAG was shown to induce *in vitro* degradation of both wild-type and mutant Kit (both imatinib-sensitive and -resistant) (51). The dramatic inactivation of imatinib-resistant Kit oncoproteins induced by 17-AAG suggests that HSP90 inhibition provides a potentially important therapeutic perspective in the treatment of imatinib-resistant tumors. It is of interest to note that a recent study provided evidence that imatinib inhibits the interaction between HSP90 and wt Kit or imatinib-sensitive Kit mutants (52). Finally, a completely different therapeutic strategy to be used in GIST patients with imatinib-resistant disease could be based on the use of molecules such as flavopiridol, which act as repressors of *KIT* gene transcription (53). The advantage of this type of agents is due to the fact that, unlike Kit kinase inhibitors, their biological effect consisting of repression of *KIT* gene expression cannot be hampered by any type of mutation of the coding sequence of the *KIT* gene.

In contrast to adult GISTs, pediatric GISTs, although they express Kit at levels comparable to those observed in adult GISTs, display Kit mutations in only about 15% of cases. However, surprisingly, pediatric wt Kit GISTs exhibit Kit activation at levels comparable with mutant Kit pediatric and adult GISTs (54). These observations suggest that targeted therapies for pediatric GISTs should focus on inhibitors of Kit activation, particularly those with high activity against wt Kit.

Kit mutations in leukemias

Kit mutations occur in only 2.5% of unselected AML cases, but occur very frequently in certain AML subtypes. Gain-of-function point mutations in the *KIT* kinase domain or at the level of the juxtamembrane domain result in ligand-independent constitutive activation of Kit signaling, which leads to uncontrolled proliferation and resistance to apoptosis. Kit expression in acute leukemias is a hallmark of AMLs (about 70% of AMLs are Kit-positive), while the large majority of acute lymphoid leukemias (ALLs) are Kit-negative (about 3-4% of ALLs are Kit-positive). In particular, the small proportion of Kit-positive T-ALLs (about 7-9%) are represented by mixed-phenotype pro-T-ALLs, co-expressing both lymphoid and myeloid antigens. Among the AMLs, the highest Kit expression was observed at the level of FAB M0, M1 and M2 AMLs, *i.e.,* phenotypically the most immature AMLs (55-57).

Mutations of the *KIT* gene occurring in AMLs may affect two portions of the molecule: either the juxtamembrane domain involved in the regulation of the enzymatic site of the Kit receptor, such as an internal tandem duplication (ITD) of exon 11 or insertion/deletion at the level of exon 8 of the *KIT* gene, or the structure of the TK domain through mutations such as the substitution of a single amino acid at codon 816 (TDK816) (Fig. 1). Both of these types of *KIT* mutations have been identified predominantly in specific genetic subgroups of AML: exon 8 mutations in patients with inv(16) and D816 mutations in patients with t(8;21), respectively. In these AML subgroups (which represent about 15-20% of adult AMLs), the transcription factor CBF is targeted: *AML1* (CBF-α) in t(8;21) and *CBFB* (CBF-β) in inv(16), leading to the common nomenclature of “core-binding factor leukemia” for inv(16)/t(16;16)- and t(8;21)-positive AMLs. On the molecular level, the translocation (8;21) leads to the generation of the *AML1-ETO* fusion gene, while inv(16) results in the creation of the fusion gene *CBFB-MYH11*. Activating mutations of the *KIT* gene have been reported in 13-46% of adult CBF AMLs and in 12% of pediatric non-acute promyelocytic leukemia (APL) AMLs (58-61).

The most common activating alleles in the context of *AML1-ETO* leukemias are D816V, D816Y, D816H, D816I and N822K. *KIT* D816 mutations in *AML-ETO*-positive AMLs are associated with impaired event-free and overall survival (62-64). These mutations lead to spontaneous ligand-independent autophosphorylation of Kit protein

and confer to the cells a proliferative and survival advantage. Enforced expression of the *KIT* mutants induces factor-independent growth of hematopoietic cell lines and transforms normal hematopoietic cells (66). Induction of a constitutive PI3K activation is required for the oncogenic activity of the murine equivalent (D814V) of the human D816V mutant (65). In line with this observation, genetic disruption of p85α, the regulatory subunit of PI3K, or treatment with PI3K inhibitors suppressed the hyperproliferative ligand-independent stimulus induced by *KIT* D814V (63). Finally, the D816V mutant, but not wt *KIT,* elicited the activation of Fes, a cytoplasmic TK. Therefore, Fes activation is an essential effector for the proliferative signal activated by *KIT* D816V (67).

Schnittger *et al*. (68) explored the frequency of the *KIT* D816 mutation in 1,930 unselected cases of AML: 1.7% of these AMLs displayed *KIT* D816 mutations. The analysis of D816 mutations in AML subtypes showed that: 1) D816 mutations were most frequently observed in t(8;21) (11% of cases) and in trisomy 4 (14% of cases); 2) D816 mutations were more rarely observed in AMLs with a normal karyotype (about 1%) or with inv(16)/t(16;16); and 3) D816 mutations were never observed in t(15;17) M3 AMLs. From a morphological point of view, the large majority of these AMLs correspond to M2 FAB AMLs and less frequently to M1 and M4. In another survey, 2,136 *de novo* AMLs were explored and a frequency of 1.8% *KIT* D816 mutants was observed. The frequency of *KIT* D816 mutants was similar in primary, secondary and relapsed AMLs (69).

In addition to the D816 mutation, the N822K mutation is another TK domain mutation frequently observed in AMLs. In a group of Chinese t(8;21) AML patients, the N822K mutation was more frequent than the D816 mutation (70).

In line with the studies reported above, about 30-45% of t(8;21) AMLs possess a mutated *KIT* allele. Interestingly, the analysis of Kit expression at the mRNA and protein level provided evidence that in t(8;21) AMLs, including those not displaying a mutated Kit receptor, this receptor is overexpressed compared to other AMLs (70). According to these findings, it was suggested that increased Kit signaling is seemingly operating in virtually all t(8;21) AMLs (65).

D816 mutations have also been explored in pediatric AMLs. An initial study by Beghini *et al*. (68) provided an estimation of about 8% TK domain mutations in pediatric CBF leukemias. Subsequently, in a larger series of patients it was estimated that 5 of 16 t(8;21) pediatric AML patients displayed TK mutations (71). Shimada *et al*. (57) studied 46 pedatric t(8;21) AML patients and showed that 8 of them exhibited *KIT* mutations at the level of the TK domain: 3 N822K mutations and 5 D816 mutations. These AMLs had a poor prognosis (72).

The other most frequent mutation of the *KIT* gene occurring in AMLs is represented by mutations occurring in exon 8, which encodes an evolutionarily highly conserved region in the extracellular portion of the Kit receptor, believed to play a role in receptor dimerization (73). All the mutations occurring at the level of exon 8 correspond to in-frame deletion plus insertion mutations involving the loss or the replacement of the codon encoding for Asp419, which is highly conserved across species and is located in the receptor extracellular domain (74). Several studies have explored the occurrence of exon 8 mutations in inv(16) AMLs: 15 of 63 (24%) (57), 9 of 46 (20%) (74) and 7 of 61 (11.5%) (64). The analysis of the impact of *KIT* exon 8 mutations on the response to therapy suggested that these mutations did not significantly change overall survival; however, the cumulative incidence of relapse was higher in patients with *KIT* mutations than in patients without (58, 64, 74).

Internal tandem duplications (ITD) of exon 11, which encodes the juxtamembrane domain, are constitutively activating mutations found in 7% of GISTs, but are rarely observed in adult AML patients. Only three cases of ITD of the juxtamembrane domain of the Kit receptor have been reported among the cases of adult AMLs studied up to now (59, 70). However, a recent study provided evidence for a high frequency (7%) of an ITD mutation involving exon 11 and exon 12 in childhood AML; like the D816 mutation, this type of mutation leads to ligand-independent receptor activation and cell growth (75).

The discovery of prognostically relevant *KIT* mutations in CBF AMLs is important because it has potential therapeutic impact. Mutations in the *KIT* gene, such as those observed in GIST, have previously been shown to constitute potential targets for TK inhibitors. In particular, imatinib is effective in the majority of patients with GISTs harboring *KIT* mutations. *KIT* is most commonly activated in GISTs by small deletions in the juxtamembrane domain that are thought to constitutively activate the TK via loss of autoinhibitory function. These observations have suggested that imatinib might be of value in the treatment of AMLs that have activating *KIT* mutations. However, the most common activating alleles in the context of AMLs, represented by the D816 mutation in the activation loop, are resistant to imatinib both *in vivo* (76) and *in vitro* (77) (Table I). In fact, the D816V mutation involves the substitution of aspartate to valine in codon 816 in the activation loop lying at the entrance of the Kit enzymatic pocket, thus interfering with imatinib binding. In contrast, imatinib was active against exon 17 mutations involving N822 in the activation loop, or variants of mutations in exon 8 (70). On the other hand, exon 17 D816 mutations can be successfully targeted with other TK inhibitors, such as PKC-412 (78, 79), dasatinib (80) and EXEL-0862 (81) (Table I). Furthermore, as reported in Table I, other TK inhibitors have been screened for their ability to inhibit the autophosphorylation of various Kit mutants, including those present in AML.

A phase II pilot study of imatinib in patients with AML expressing *KIT*, but without mutations, resulted in complete or partial responses in 5 of 21 patients (82), whereas another study of 36 *KIT* expression-positive patients with AML (but with no information about *KIT* mutations) reported no significant responses (83). Significant responses to Kit inhibitors have been reported in individ-

Table I: List of Kit inhibitors and their IC_{50} values for inhibiting the autophosphorylation of wt and mutant Kit.

Compound	*KIT* WT	*KIT* V560G	*KIT* D816V	*KIT* D816F	*KIT* D816Y	*KIT* V560D	*KIT* N822K	*KIT* T670I	*KIT* V654A	*KIT* V559D	*KIT* L576P
Imatinib	25-50 nM	200 nM	10 μM	10 μM	1 μM	10 nM	140 nM	> 10 μM	3.9 μM	63 nM	253 nM
Sorafenib	50-100 nM	ND	ND	ND	ND	ND	3 nM	60-900 nM	1 μM	66 nM	ND
Dasatinib	6 nM	100 nM	200 nM	50 nM	5 nM	10 nM	868 nM	7.5 μM	585 nM	27 nM	54 nM
EXEL-0862	8.5 nM	100 nM	42 nM	ND	ND	ND	ND	100 nM	ND	ND	ND
PKC-412 (midostaurin)	80 nM	100 nM	50-100 nM	ND	33 nM	ND	ND	ND	ND	ND	ND
Nilotinib	100 nM	100 nM	1 nM	ND	ND	ND	ND	> 10 μM	192 nM	44 nM	185 nM
OSI-930	10 nM	10 nM	ND	ND	ND	ND	ND	ND	ND	ND	ND
MLN-518 (tandutinib)	170 nM	40 nM	160 nM	ND	ND	ND	5 nM	1 μM	ND	4 nM	ND
SU-5416 (semaxanib)	50 nM	ND	> 10 μM	ND	ND	ND	ND	ND	ND	ND	ND
SU-011248	1-10 nM	ND	ND	ND	ND	ND	5 nM	50-100 nM	50-100 nM	100 nM	ND
SU-5614	50 nM	ND	1 nM	ND	ND	ND	ND	ND	ND	ND	ND
SU-9529	30 nM	ND	10 nM	1 mM	1 mM	10 nM	ND	ND	ND	ND	ND
AP-23464	85 nM	ND	10 nM	11 nM	10 nM	ND	ND	ND	ND	ND	ND
VX-680	300 nM	ND	ND	ND	ND	ND	100 nM	600 nM	ND	300 nM	ND
PD-180970	50 nM	25 nM	1	1-5 μM	ND	ND	4 nM	3 μM	ND	1 nM	ND
INNO-406 (NS-187)	840 nM	50 nM	> 10 μM	ND	ND	ND	ND	> 10 μM	ND	ND	ND
PTK-787/ ZK-222584 (vatalanib)	700 nM	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND, not determined.

ual AML cases (84-86), including 2 patients with exon 8 mutations, 1 who had a complete remission with imatinib and mild chemotherapy at second relapse (85), and 1 who had disappearance of the *KIT* mutation but no hematological response (86). The efficacy of TK inhibitors as part of therapy administered to patients with CBF AML, associated with the exact definition of the *KIT* mutation in each case, should be investigated in future clinical trials.

According to the "two-hit" model of leukemogenesis, it is tempting to suggest that *KIT* mutations occurring in CBF AMLs are involved in the pathogenesis of this disease and contribute to the development of more aggressive neoplasia. Experimental evidence supporting this view comes from the analysis of the expression of *AML1-ETO* and *KIT* mutant transcripts in AML patients after chemotherapy (69). The analysis carried out in 3 of these patients achieving complete remission post-treatment showed that, at 1 year after the start of therapy, hematopoietic cells were still positive for *AML1-ETO* but not for *KIT* mutant transcripts. These observations suggest that mutant *KIT* AML cells may arise from a less aggressive "preleukemic" clone that harbors the fusion transcript and acts as a second, but crucial, hit to provide preleukemic cells with growth and survival advantages (70).

Kit mutations in mastocytosis

The *KIT* gene is very frequently mutated in mastocytosis, a heterogeneous disorder characterized by the proliferation and accumulation of mast cells in various organs and tissues. The clinical course of systemic mastocytosis can vary from indolent, as in cutaneous mastocytosis, to aggressive mast cell leukemia. In fact, the D816V *KIT* mutation was observed in 93% of patients with systemic mastocytosis (87). Some patients negative for the D816V mutation display other *KIT* mutations. Finally, patients with a rare form of aggressive systemic mastocytosis display *KIT* mutations of the juxtamembrane domain (Val559Ile) that are resistant to TK inhibitors (88). The management of patients with systemic mastocytosis involves attempting to control symptoms related to the release of mediators from mast cells and organ dysfunction caused by mast cell infiltration. Aggressive mastocytosis and mast cell leukemia are associated with a poor prognosis. The strong association between this disease and the presence of *KIT* mutations offers the opportunity to attempt treatment using Kit kinase inhibitors. As mentioned above for AMLs, the D816V mutation was resistant to imatinib and, therefore, it is not surprising that the majority of the 31 patients treat-

ed with this drug did not show any significant response. Furthermore, the only significant clinical responses to this treatment were observed in cases lacking the D816V mutation (reviewed in Ref. 89).

According to *in vitro* studies, other TK inhibitors have been clinically evaluated for the treatment of patients with mastocytosis. An initial case report study evaluated the effect of PKC-412 in a patient with mast cell leukemia, showing a partial response to the treatment (90). An ongoing phase II study is evaluating the safety and the efficacy of the TK inhibitor PKC-412 in the treatment of systemic mastocytosis. Preliminary results of this study showed 6 partial responses in a total of 9 patients (91). Finally, in a pilot phase II study for the treatment of systemic mastocytosis with dasatinib, an overall response rate of 37% was observed, with 2 of 24 complete responses (92).

Interestingly, *in vitro* studies carried out in cell lines displaying the D816V mutation and in fresh cells derived from patients with systemic mastocytosis showed high sensitivity to the mTOR inhibitor rapamycin (93). According to these observations, it was proposed that rapamycin could be used for the treatment of systemic mastocytosis. Finally, a constitutive NF-κB activation was observed in mastocytes carrying the D816V mutation and the NF-κB inhibitor IMD-0354 induced the death of these cells (94).

Kit mutations in other cancer types

Kit is clearly expressed in normal human melanocytes. Immunohistochemical studies of human melanomas have shown that Kit is highly expressed in the *in situ* and junctional components of invasive lesions, but expression is lost once the melanoma becomes invasive and metastatic (95-97). It was suggested that the loss of Kit expression allows the melanoma cells to escape from SCF-induced apoptosis (*i.e.*, the cells survive even in the absence of SCF) (98).

Kit is frequently mutated in melanomas. In fact, in a recent study, *KIT* aberrations (*i.e.*, mutations or copy number increases) were found in 39% of mucosal melanomas, 36% of acral melanomas, 28% of skin melanomas with chronic sun-induced damage and 0% of melanomas without chronic sun-induced damage (99); 69% of these mutations (K642E) were predicted to affect the juxtamembrane domain, presumably resulting in constitutive activation of Kit. A study carried out specifically in a subtype of mucosal melanoma, anal melanoma, showed a 13% frequency of *KIT* mutations (all consisting of the L576P mutation), associated with strong immunoreactivity of the tumor cells with anti-Kit antibodies (100). The same L576P *KIT* mutation was observed by Willmire-Payne *et al*. in 2 of 29 *KIT*-positive melanomas (101).

These findings suggested that imatinib might be a promising treatment option for melanoma. However, two phase II clinical trials testing this drug in melanoma failed to show objective responses and poor survival was seen in assessable patients (102, 103). However, a re-evaluation of the patients participating in these studies showed that only 2 of them displayed the characteristics of high *KIT* aberration (acral and mucosal melanomas). According to these observations, Becker *et al*. proposed a clinical trial investigating imatinib in metastatic melanoma originating from primary tumors of acral skin, mucosa or chemically sun-damaged skin, correlating the treatment outcome with the mutational status of *KIT* (104).

Kit is frequently overexpressed and mutated in testicular germ cell tumors (TGCTs). Kit is necessary for the migration and survival of primordial germ cells and is expressed in intratubular neoplastic germ cells and seminomas. Two types of tumors have been identified: seminoma, exhibiting features of primordial germ cells, and nonseminoma, exhibiting features of embryonal and extraembryonal structures. Overexpression of Kit is a typical feature of seminomas, with nonseminomas rarely showing expression of Kit (105). In particular, Kit protein was expressed in 88% of seminomas and 44% of nonseminomas (106). Kit is mutated in TGCTs, particularly in bilateral TGCTs.

In an initial study, the frequency of codon 816 mutations was found to be 1.3% in unilateral TGCTs and 97% in bilateral TGCTs (107). In a more recent study, the mutation frequency in *KIT* exon 17 was significantly higher in bilateral (64%) compared to unilateral TGCTs (6%) (108). The COSMIC database reports an overall somatic mutation rate of 8% for all TGCTs. The majority of *KIT* mutations occur in seminomas (about 20%), with only rare examples in nonseminomas (2%). Furthermore, 48% of extragonadal seminomas contain *KIT* mutations, while there are no examples of *KIT* mutations in extragonadal nonseminomas (109). Exon 17 mutations are much more frequent than exon 11 mutations in seminomas (108). *KIT* exon 17 mutations in seminomas mostly occur at the level of the 816 (D816V and D816H), 820, 822 (N822K) and 823 (Y823D and Y823C) amino acid residues, while *KIT* exon 11 mutations occur at the level of 555, 557 and 576 amino acid residues (110). Mutant *KIT* isoforms D816V, D816H, Y823D and N822K were constitutively phosphorylated in the absence of SCF; mutants N822K and Y823D, but not D816V and D816H, were not inhibited by imatinib (109). It is of interest to note that, in addition to point mutations, other types of *KIT* gene abnormalities have been observed in seminomas. In fact, an amplification of the *KIT* gene was observed in 21% of seminomas and only rarely in nonseminoma testicular tumors. *KIT* activating mutations and *KIT* gene amplification have never been observed in testicular carcinoma *in situ*, while they are frequent in seminomas, suggesting that *KIT* gene abnormalities play a role in the progression of carcinoma *in situ* to disseminated seminoma (111).

KIT mutations are very rare in epithelial ovarian cancers (112). However, ovarian dysgerminoma, a relatively rare variant of ovarian cancer of germ cell origin, frequently exhibits *KIT* mutations; 5 of 14 ovarian dysgerminomas displayed a codon 816 *KIT* mutation (113).

Furthermore, Kit protein was highly expressed in ovarian dysgerminomas.

Kit expression has been frequently (about 40% of cases) observed in small cell lung cancer (SCLC), and in 8% of cases the *KIT* gene was found to be mutated (at the level of either exon 9 (Asn495Ile) or exon 11 (Asn 567Lys), but Kit expression or mutational status had no impact on survival (114). Given these observations, therapy with imatinib for relapsing SCLC was attempted; however, in spite of patient selection for Kit-expressing SCLC, imatinib failed to demonstrate any clinical activity (115).

The *KIT* gene is frequently amplified in gliomas: 28% of anaplastic astrocytomas and 5% of astrocytomas (116). *KIT* amplification was associated with Kit protein expression. Furthermore, amplified *KIT* was more frequently present in recurrent gliomas than in newly diagnosed gliomas (117). However, no mutations of the *KIT* gene have been observed in gliomas (118). Given these observations, the therapy of glioma patients with imatinib was attempted. Single-agent studies of imatinib showed that this drug had only minimal activity in malignant gliomas (119, 120). In two other studies, imatinib was used in combination with hydroxyurea. The combination showed modest antitumor activity in patients with recurrent gliomas (121, 122). However, responding patients appeared to have durable antitumor acivity (121).

The lack of clinical activity of imatinib in gliomas and SCLC, where the *KIT* gene is amplified but not mutated, further supports the paradigm that the prediction of efficacy for novel therapeutic agents based on target expression rather than pathways of activation through activating mutations may not be a valid approach for drug development.

Sinonasal natural killer/T-cell lymphoma (NKTCL) is a condition of lethal midline granuloma that exhibits necrotic and granulomatous lesions in the upper respiratory tract, especially in the nasal cavity. The disease is more frequent in Asian countries than in Western countries. In a group of 23 Asian patients, it was observed that 10 of 14 Chinese patients (71.4%) had mutations in exon 11 or exon 17, whereas only 2 of 9 non-Chinese patients had mutations. Seven of the eight mutations in exon 17 occurred at codon 825 and three of four mutations in exon 11 occurred at codon 561. However, *in vitro* studies showed that *KIT* 825 is not a gain-of-function mutation (123).

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