Pediatric KIT–Wild-Type and Platelet-Derived Growth Factor Receptor α–Wild-Type Gastrointestinal Stromal Tumors Share KIT Activation but not Mechanisms of Genetic Progression with Adult Gastrointestinal Stromal Tumors

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Abstract

Fewer than 15% of gastrointestinal stromal tumors (GIST) in pediatric patients harbor KIT or platelet-derived growth factor receptor α (PDGFRα) mutations in contrast to a mutation rate of 80% in adult GISTs. However, some therapeutic inhibitors of KIT have efficacy in pediatric GIST, suggesting that KIT may, nevertheless, play an important role in oncogenesis. In adult GIST, characteristic cytogenetic changes occur during progression to malignancy. A better understanding of mechanisms of genetic progression and KIT and PDGFRα transforming roles in pediatric GIST might facilitate treatment advances. KIT and PDGFRα mutation analysis was done in 27 pediatric GISTs. The activation status of KIT, PDGFRα, and downstream signaling intermediates was defined, and chromosomal aberrations were determined by single nucleotide polymorphism assays. Mutations in KIT or PDGFRα were identified in 11% of pediatric GISTs. KIT and the signaling intermediates AKT and mitogen-activated protein kinase were activated in pediatric GISTs. In particular, most pediatric KIT–wild-type GISTs displayed levels of KIT activation similar to levels in adult KIT–mutant GISTs. Pediatric KIT–wild-type GISTs lacked the typical cytogenetic deletions seen in adult KIT–mutant GISTs. Notably, most pediatric KIT–wild-type GISTs progress to malignancy without acquiring large-scale chromosomal aberrations, which is a phenomenon not reported previously in malignant solid tumors. KIT activation levels in pediatric KIT–wild-type GISTs are comparable to those in KIT–mutant GISTs. Therapies that inhibit KIT activation, or crucial KIT signaling intermediates, should be explored in pediatric KIT–wild-type GIST. [Cancer Res 2007;67(19):9084–8]

Introduction

Gastrointestinal stromal tumor (GIST) is a neoplasm of mesenchymal origin in the gastrointestinal tract of adults and children. A key, early transforming event in 80% of adult GISTs is mutation, leading to ligand-independent activation of KIT or, less commonly, platelet-derived growth factor receptor α (PDGFRα; ref. 1). In adult GIST, oncogenic KIT or PDGFRα mutations are likely sufficient for transformation, but progression from benign to malignant GIST is characterized by sequential acquisition of chromosomal deletions at 14q, 22q, 1p, and 9p (1). Whereas pediatric GIST expresses KIT at levels comparable with adult GIST, only 15% of the pediatric tumors harbor activating mutations in KIT or PDGFRα (2).

GIST in both pediatric and adult patients is resistant to standard chemotherapeutic agents. However, therapeutic KIT and PDGFRα inhibitors, such as imatinib and sunitinib, have prolonged survival in adult patients with metastatic GIST (3–5). Although case reports suggest only limited therapeutic benefit of imatinib in metastatic pediatric GIST (6–8), there is recent evidence that sunitinib, a more potent inhibitor of wild-type KIT, can slow progression in pediatric GIST (9). A rational approach to investigation and use of drugs that inhibit receptor tyrosine kinases (RTK) in pediatric GIST requires a better understanding of the KIT and PDGFRα transforming roles in these tumors. To this end, we report herein the KIT and PDGFRα genotype in 27 pediatric GISTs and correlate genotype results with the activation status of KIT, PDGFRα, and downstream signaling intermediates.

Cytogenetic aberrations in adult GIST are similar, irrespective of whether the tumor contains a KIT or PDGFRα oncogenic mutation (10). Therefore, the mechanisms of genetic progression seem to be the same in adult GISTs with various activating RTK mutations. However, it is unknown whether similar chromosomal aberrations predominate in pediatric GIST. Evaluations of pediatric GIST have shown only normal (diploid) karyotypes (11), but such findings, in malignant solid tumors generally, are apt to result from overgrowth of the neoplastic population by reactive cells, such as normal myofibroblasts, during the cytogenetic analysis (12). To determine whether chromosomal aberrations in pediatric GIST are different from those in adult GIST, we investigated genomic alterations in 15 pediatric GISTs using 10K single nucleotide polymorphism (SNP) arrays.

Materials and Methods

Patient materials. Twenty-seven GISTs from patients <25 years at diagnosis were analyzed after Institutional Review Board (IRB) review and approval. All 27 cases were histologically reviewed and confirmed to be GIST by one of the authors (C.C.) and were tested for KIT and PDGFRα mutations. Fifteen cryopreserved tumor samples were available, and this subset was analyzed for KIT activation and KIT signaling by Western
blotting and genomic changes by SNP assays. Normal tissue or peripheral blood from three of the patients and from three healthy control subjects was also analyzed by SNP assays after IRB approval.  

**Reagents.** Polyclonal rabbit antibody to KIT was from DAKO. Polyclonal rabbit antibody to mitogen-activated protein kinase (MAPK) p42/p44 was from Zymed Laboratories. Polyclonal rabbit antibodies to phosphorylated KIT Y721, phosphorylated Akt S473, total Akt, and phosphorylated MAPK T202/Y204 were from Cell Signaling. Polyclonal goat antibody to PKCα was from Santa Cruz Biotechnology. Mouse monoclonal antibody to actin was from Sigma.

**Western blotting.** Whole cell lysates of cryopreserved tumors were prepared as previously described (13). The lysates were separated by gel electrophoresis using NuPAGE 4% to 12% Bis-Tris gels (Invitrogen) and blotted to nitrocellulose membranes. Immunostains were detected using enhanced chemiluminescence (ECL Amersham Pharmacia Biotech) and captured and quantified using a Fuji LAS1000-plus imaging system. A standard lane (GIST882 cell line lysate; ref. 14) was used to equalize the staining between gels.  

**KIT and PDGFRA mutational analyses.** Genomic DNA was isolated from paraffin-embedded or cryopreserved tumor. KIT exons 9, 11, 13, and 17 and PDGFRA exons 12 and 18 were amplified by PCR and screened for mutations by denaturing high-performance liquid chromatography. Primers, PCR conditions, and sequencing methods have been described previously (1). cDNA sequencing of the entire KIT coding sequence was also done in four pediatric GISTs lacking KIT or PDGFRA genomic mutations. The SuperScript one-step reverse transcription-PCR system with Platinum Taq (Invitrogen) was used for cDNA synthesis and PCR. KIT cDNA was synthesized in eight overlapping segments. Primer sequences used for PCR and sequencing are given in Supplementary Table S1. Sequencing was done with an ABI 3730 xl sequencer after gel purification of the PCR products.

**SNP assay.** DNAs were isolated from cryopreserved tumor using a standard phenol-chloroform method. The DNAs were analyzed using an Affymetrix 10K SNP array at the Dana-Farber Cancer Institute Microarray Core Facility. DNA (250 ng) was digested with XbaI, and linkers were ligated to the restriction fragments to permit PCR amplification. The PCR products were then purified and fragmented by treatment with DNase I. The fragmented PCR products were labeled and hybridized to microarray chips. The positions and intensities of the fluorescent emissions were analyzed using dCHIP/SPN software. Array intensity was normalized to the array with median intensity. Median smoothing was used to infer copy number. Loss of heterozygosity (LOH) analysis was done using a Hilden Markov Model (15).

**Results and Discussion**

**Patients.** Patients were 6 to 22 years at diagnosis with a mean age of 14 years. Twenty-three (85%) patients were female. Two patients had Carney Triad (cases 8 and 13). The age and gender distribution of the patients in this report are representative of previously published series of pediatric GIST (7, 16).

**KIT and PDGFRA mutation analyses.** Three of twenty-seven GISTs (11%) had KIT or PDGFRA mutations that included a homozygous KIT exon 11 mutation, resulting in deletion V559-S60 (case 1), a heterozygous KIT exon 9 mutation resulting in A1502-503 insertion (case 2), and a heterozygous PDGFRA exon 18 mutation resulting in D842V substitution. Patients with KIT-mutant GISTs were 17 and 22 years at diagnosis, and the patient with PDGFRA-mutant KIT was 14 years at diagnosis. To investigate the possibility that novel KIT mutations are present in pediatric GIST, cDNA sequencing of the entire KIT coding sequence was done. cDNA sequencing did not reveal mutations in four cases (cases 5, 6, 8, and 10) wherein the KIT and PDGFRA genomic mutation screening had been wild-type. The PDGFRA-mutant GIST did not have sufficient tumor material for inclusion in the subset analyzed by Western blotting and SNP assays. However, it is known that, in Chinese hamster ovary cells, the D842V PDGFRA variant leads to constitutive activation of PDGFRA in the absence of ligand stimulation. The D842V PDGFRA mutation is present in over half of adult GISTs with PDGFRA mutations (10).

KIT and PDGFRA genotyping in these 27 cases, together with data published previously for 31 pediatric GISTs, provides strong evidence that oncogenic KIT and PDGFRA mutations are found in only 15% of pediatric GISTs (2, 8). By contrast, >80% of adult GISTs feature KIT or PDGFRA mutations, with KIT exon 11, KIT exon 9, and PDGFRA mutations found in 66%, 10%, and 7%, respectively (1). These findings also suggest that the distribution of KIT and PDGFRA mutations differ between pediatric and adult GISTs, with KIT exon 11, KIT exon 9, and PDGFRA mutations found in 5%, 9%, and 3% of pediatric GISTs, respectively.

**KIT and KIT signaling.** KIT and PKCα expression are highly specific characteristics of GIST with the exception of PDGFRA mutant tumors, in which KIT expression is often nearly undetectable (13). All 15 cryopreserved pediatric GISTs variably expressed KIT, and 14 coexpressed PKCα, confirming the diagnosis of GIST (Fig. 1).

Twelve of thirteen KIT–wild-type pediatric GISTs expressed tyrosine phosphorylated KIT, consistent with a nonmutational mechanism of KIT activation (Fig. 1). In general, the level of KIT activation in pediatric KIT–wild-type GISTs was similar to that in adult and pediatric KIT-mutant GISTs. The AKT and MAPK downstream signaling intermediates were expressed and activated in all pediatric GISTs, except for case 8 which did not have MAPK activation. The level of total KIT expression was similar to adult KIT-mutant GISTs in all pediatric GISTs with the exception of slightly lower levels in one pediatric KIT–wild-type and one pediatric KIT-mutant case. None of the pediatric KIT–wild-type GISTs expressed PDGFRA (data not shown). The specificity of the phosphorylated KIT antibody used is underscored by the lack of phosphorylated KIT staining in case 12, which does express total KIT. The antibodies detecting phosphorylated downstream signaling intermediates have been validated in GIST cell lines treated with Imatinib (17).

KIT activation can serve as an initiating event in GIST oncogenesis. Early GISTs are often diagnosed incidentally and are characterized by small size and a low mitotic index. In adults, these early GISTs have activating KIT mutations. Furthermore, germline KIT mutations are associated with familial GIST syndromes. In adult KIT-mutant GIST, imatinib therapeutic responses correlate with KIT inactivation, indicating that KIT activation is essential in GIST progression (18). Likewise, recent clinical observations suggest that KIT activation might be important in pediatric GIST, even though oncogenic KIT mutations are uncommon. Namely, four of six pediatric patients with metastatic KIT–wild-type GIST had either disease stabilization or partial response during therapy with sunitinib, a potent inhibitor of wild-type KIT10 (9). In the present study, we find that most pediatric KIT–wild-type GISTs have KIT activation at levels comparable with KIT-mutant GISTs. Together with the therapeutic responses to sunitinib, these findings suggest that KIT, although lacking mutations, nonetheless might provide an important transforming mechanism in pediatric GIST. Thus, therapies that inhibit KIT activation or crucial KIT signaling intermediates should be explored in pediatric KIT–wild-type GIST.
and potent inhibitors of wild-type KIT might be particularly effective in this disease.

There are several mechanisms by which KIT might be activated in KIT–wild-type pediatric GISTs. One possibility is an autocrine/paracrine loop involving overexpression of the KIT ligand stem cell factor. There is precedent for such a mechanism in dermatofibrosarcoma protuberans, wherein PDGFRB activation results from a genomic mutation, causing overexpression of PDGFB (19). Alternatively, KIT activation in KIT–wild-type pediatric GIST could be due to impaired degradation, as can result from mutation of a Cbl ubiquitin-protein ligase or the adaptor containing PH and SH2 domains protein (20). Or, unchecked KIT activation could result from a phosphatase mutation, such as has been reported in juvenile myelomonocytic leukemia (21). Finally, tyrosine kinase heterooligomerization could result in KIT cross-phosphorylation by another RTK family member. This type of heterooligomerization is well-described in the epidermal growth factor receptor family. In addition, forced interaction between KIT and FLT-3 has been shown to result in KIT activation and cell proliferation (22).

In vitro and in vivo data confirm that activation of MAPK and AKT is dependent upon KIT activation in KIT-mutant GIST. In GIST cell lines, treatment with the selective KIT inhibitor imatinib abolishes KIT, AKT, and MAPK activation. Furthermore, analysis of GIST biopsies show complete inhibition of KIT, AKT, and MAPK shortly after initiation of imatinib (18). The KIT-dependent nature of AKT and MAPK activation in most adult GISTS suggests that AKT and MAPK activation in pediatric wild-type GISTS might also be regulated by KIT. Alternatively, AKT and MAPK activation in some pediatric GISTS might not be entirely regulated by KIT or PDGFRα signaling. Such possibilities are underscored by our immunoblotting data, which suggest that signaling relationships vary in pediatric GISTS. Case 8, with a moderate level of KIT phosphorylation, had minimal AKT and no MAPK phosphorylation, whereas case 12, with no KIT phosphorylation, had a moderate level of AKT and MAPK phosphorylation. One explanation for this observation is the variable participation of additional upstream kinases. A subset of the KIT–wild-type GISTS reported herein were screened with a proteomic technique (10) for strongly activated RTKs. However, other than KIT, no strongly activated RTKs were identified.11

SNP analysis. SNP analyses of three adult KIT-mutant GISTS showed typical cytogenetic alterations, including LOH at 1p and LOH and decreased copy number at 14q and 22q (Fig. 2). Each of these adult KIT-mutant GISTS had additional regions of LOH and copy number change, spanning large numbers of SNPs. The two pediatric KIT-mutant GISTS had chromosomal changes typical of adult KIT-mutant GIST. Case 1 had loss of 1p, 9p, and 14q, whereas case 2 had loss of 1p and 22q. Both of these pediatric KIT-mutant cases, like the adult KIT-mutant cases, had multiple additional large chromosomal regions with LOH and copy number change. In contrast, the pediatric KIT–wild-type cases lacked the typical cytogenetic deletions (1p, 14q, and 22q) seen in KIT-mutant GISTS, and indeed had minimal areas of LOH or copy number change. Large chromosomal region(s) with LOH or copy number change were seen in only three KIT–wild-type pediatric GISTS. Case 9 had 5p LOH with apparent trisomy in this region, consistent with allelic duplication and retention of heterozygosity rather than true LOH. Case 14 had 11q LOH without associated copy number change, consistent with true LOH, followed by allelic duplication. Case 5 had multiple regions of apparent LOH, including 1p, 3q, 5q, and 11 Unpublished data.
chromosomes 13 and 18. There was also 1p LOH but without a
decrease in copy number.

Diploid karyotypes are seen frequently in benign solid tumors
but not in malignant or metastatic solid tumors (12). In a study of
24 "benign" adult GISTs, characterized by <2 mitoses/hpf and lack
of clinical recurrence, all had chromosomal aberrations, and
the most common changes were loss of 1p, 14q, and 22q (23).
Malignant adult primary GISTs and metastatic GISTs have still
more cytogenetic changes with a mean of nine demonstrable
aberrations in metastatic GISTs. In our group of pediatric KIT–
wild-type GISTs lacking chromosomal aberrations, one GIST
(case 12) was high risk (Fletcher classification) based on mitoses,
two GISTs (cases 3 and 6) were metastatic, and two GISTs recurred
after resection (cases 8 and 15). If genetic mechanisms of
progression in pediatric GIST mirrored those in adult tumors,
each of these high-grade pediatric GISTs would be expected to
contain multiple large-scale chromosomal changes.

Notably, our present findings show that pediatric malignant
GISTs are the first clinically aggressive solid tumor, in which
cytogenetic aberrations, even when queried by high-resolution SNP
assays, are undetectable in most cases. The normal genomic profiles
in these malignant pediatric GISTs are representative of the
neoplastic population based on pathologic examination of cryo-
opreserved specimen regions, showing 10% or less nonneoplastic
cells, from which DNAs for SNP assays were extracted (further
details available in Supplementary Material). In addition, protein
and DNA isolates were prepared from the exact same tumor aliquot
in each case, and immunoblotting studies showed variable
expression of the GIST-specific biomarkers KIT and PKCα in all
but one of these samples. Further, concurrent SNP analyses in the
adult GISTs and pediatric KIT-mutant GISTs (which, like pediatric
KIT–wild-type GISTs, are composed primarily of neoplastic cells)
showed numerous large-scale chromosomal deletions in all cases.

The methods used for analysis of the SNP data do not require
matched normal DNA to identify large-scale genetic changes in
heterozygosity or copy number. As a demonstration of this point,
the cases in which SNP analysis identified the greatest extent of
LOH and copy number change were those that lacked matched
normal DNA (see Fig. 2), whereas one case (case 8) with matched
normal DNA had no large-scale genetic changes. SNP (10K)
analysis, without the use of matched normal controls, has been
validated as a highly sensitive method for detecting regions of copy
number change compared with genomic hybridization (CGH) or
karyotyping (24).

Data from CGH array analyses and karyotypes (23, 25) of adult
GIST strongly support the conclusion that chromosomal changes
contribute to clinical progression. Similar chromosomal regions are
affected in most GISTs, and the pattern of genetic changes is
similar in KIT-mutant and PDGFRα-mutant GISTs (10). Finally,
there is similarity between GIST and other oncogene-driven
malignancies, such as chronic myelogenous leukemia, in which
cytogenetic abnormalities, after the acquisition of an oncogene, are
progressively acquired during disease progression.

The markedly different pattern of chromosomal aberrations in
pediatric KIT–wild-type versus pediatric and adult KIT-mutant
GIST suggests that, despite sharing KIT activation, these tumors
likely have quite distinct mechanisms of genetic progression. In
that sense, our findings show that pediatric and adult GISTs differ
genetically and biologically. By CGH analysis, the genetic pattern of
one pediatric GIST in a Carney Triad patient also differed from the
pattern seen in adult tumors (2). Similarly, a cDNA profiling study
has shown that pediatric GISTs cluster separately from adult GISTs
(7). The paucity of chromosomal changes in most KIT–wild-type
malignant pediatric GISTs suggests that substantial accumulation
of additional genetic events does not occur or more likely occurs

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**Figure 2.** SNP LOH display shows few LOH regions in KIT–wild-type pediatric
GISTs compared with KIT-mutant pediatric or adult GISTs. Cases in bold
have companion normal DNAs. Blue, LOH; yellow, retained heterozygosity.
via highly localized genetic alterations, such as intragenic point mutations or balanced cytogenetic rearrangements, which are undetected by conventional high-density SNP assays. It is also possible that GIST genetic progression in pediatric patients results, at least in part, from epigenetic phenomena, such as gene promoter methylation, which are undetected by the genomic evaluations used in this study.

In summary, this study shows that pediatric KIT–wild-type GISTs exhibit KIT activation at levels comparable with KIT–mutant pediatric and adult GISTs although they do not have KIT (or PDGFRA) mutations. In addition, genetic progression seems to be very different in KIT–mutant versus KIT–wild-type pediatric GISTs. KIT–mutant pediatric GISTs have large-scale chromosomal aberrations similar to those seen in KIT–mutant adult GISTS, whereas KIT–wild-type pediatric GISTs have few large-scale chromosomal aberrations. Our findings suggest that targeted therapies for pediatric GIST should focus on inhibitors of KIT activation or signaling molecules downstream of KIT with an emphasis on those agents that strongly inhibit wild-type KIT.

Acknowledgments


Grant support: An anonymous donor, Department of Veterans Affairs merit review grant (M.C. Heinrich), Life Raft Group (M.C. Heinrich, C.L. Corless, and J.A. Fletcher), and Virginia and Daniel K. Ludwig Trust for Cancer Research and GI SPORE P50CA12703-01 (J.A. Fletcher) and NIH grant 5732HL007574-25 (K.A. Janeway).

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