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Patterns of Deregulation of Insulin Growth Factor Signaling Pathway in Pediatric and Adult Gastrointestinal Stromal Tumors

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Abstract

Background—Data regarding the patterns and the mechanisms of deregulation of the insulin growth factor (IGF) pathway in adult and pediatric gastrointestinal stromal tumors (GISTs) are limited.

Methods—We investigated the expression profiling of the genes encoding the main components of the IGF signaling pathway in 131 GISTs (106 adult, 21 pediatric and 4 young adult) and 25 other soft-tissue sarcomas (STS) using an Affymetrix U133A platform. *IGF2* was investigated for loss of imprinting (LOI) whereas *IGF1R* was analyzed for copy number aberration and mutation.

Results—*IGF2* was the most highly overexpressed gene of the IGF pathway in GIST. *IGF2* expression was also significantly higher than in other STS. *IGF2* expression was correlated to the age onset and mutational status of GIST. Indeed, *IGF2* expression was significantly higher in the “adult” group than in the “pediatric” and “young adult” groups. Among adult GIST, *IGF2* expression was higher in tumors lacking *KIT* or *PDGFRA* mutations in comparison with mutated cases. A trend for a higher expression of *IGF2* in resistant GIST in comparison to responsive GIST was also found. Overexpression of *IGF2* was not related to LOI. Conversely, the expression of the *IGF1R* gene was significantly higher in the pediatric group than in the adult group. No copy number gains or mutations of *IGF1R* were observed.

Conclusion—The IGF pathway is deregulated in GIST with distinct patterns according to age onset and mutational status. The IGF pathway may represent a therapeutic target in patients with primary or secondary resistance to imatinib.

Keywords

GIST; gastrointestinal stromal tumor; IGF2; IGF1R

INTRODUCTION

The majority of gastrointestinal stromal tumors (GIST) are characterized by gain-of-function mutations of the *KIT* or the *PDGFRA* genes resulting in the activation of the downstream

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Conflict of interest statement

None declared

pathways Ras/MAPK, JAK/STAT3, and PI3K/AKT, playing a crucial role in tumorigenesis (1-2). The discovery of KIT as the biologic driver of GIST favored the rapid development of targeted therapies in the adjuvant and metastatic settings. Currently, imatinib is the only drug approved for first-line therapy of metastatic GIST. Disease control is achieved in approximately 85% of patients with a median progression-free survival (PFS) of 20 to 24 months (3). However, 10-15% of patients exhibit primary resistance, and acquired secondary resistance is ultimately observed in the majority of patients who initially experienced clinical benefit from imatinib (3). Although secondary gene mutation in the KIT kinase domain may represent the most common mechanism of secondary resistance to imatinib in GIST, activation of alternative signaling pathways that bypass the requirement for KIT kinase activity may also play an important role (4). Recent studies have suggested that besides *KIT* or *PDGFRA* mutation, aberrations of the insulin-like growth factor signaling pathway may play an important role in GIST tumorigenesis (5-11). This pathway consists of insulin-like growth factor (IGF1 and IGF2), IGF receptors 1 (IGF1R) and 2 (IGF2R), and IGF-binding proteins (12). Similar to KIT, IGF1R induces signaling through the MAP kinase and PI3 kinase cascades, whereas IGF2R has no signal transduction capacity and acts as a scavenger receptor, down-regulating IGF2 (12). The ligand IGF1 functions primarily by activating the IGF1R, whereas IGF2 can act through either the IGF1R or through the insulin receptor A isoform. Particularly high expression of IGF2 was reported in GIST (5,7,10) and was correlated with a high rate of metastatic relapse in patients treated for a localized disease (7). This overexpression is considered as the primary mechanism of the paraneoplastic hypoglycemia reported in several cases of GIST (13-18), but also in several other tumor types including solitary fibrous tumors (Doege-Potter syndrome) (19-20). The *IGF2* gene is imprinted and active only on the paternally inherited allele. We and others have reported that loss of imprinting (LOI) was the main mechanism responsible for *IGF2* overexpression in several subtypes of carcinomas and mesenchymal tumor (21-23). However, there are no data about the mechanism of overexpression of *IGF2* in GIST. We hypothesized that this overexpression was also related to LOI. Moreover, since there is a major overlap between KIT and IGF1R downstream cascades, we hypothesized that IGF2 overexpression was associated with resistance to imatinib. We performed this study in order to address these two hypotheses.

Patients and Methods

Patients

One hundred thirty one patients with a diagnosis of GIST, identified from the Memorial Sloan-Kettering Cancer Center (New York, NY, USA) sarcoma database, were included in this study on the basis of available material for molecular experiments. Patient characteristics are described in Table 1. The proportion of small bowel GIST was slightly higher than that of GIST originating from the stomach. This reflects the poorer prognosis of small bowel GIST patients which are more likely to relapse and to receive imatinib. Pediatric and young adult cases were defined by an age at diagnosis of ≤ 18 years and >18 but ≤ 30 years, respectively. In all cases, the pathologic diagnosis was confirmed using standard H&E staining and immunoreactivity for CD117 (DAKO Corp., Carpinteria, California; 1:500) on formalin-fixed paraffin-embedded tissue. *KIT* (exons 9, 11, 13, 14, and 17) and *PDGFRA* (exon 12, 14, 18) genotyping was performed as previously described (24). Response to imatinib was assessed according to CHOI criteria (25). "Responsive" samples were obtained by surgical resection of the tumor before any radiological progression of the disease with imatinib treatment. "Resistant" samples were obtained by surgical resection of a lesion with evidence of radiological progression under imatinib treatment. The study was approved by the Institutional Review Board (IRB-protocol 02-060).

Microarray analysis

Total RNA was extracted from frozen tumor tissues with RNeasy Mini Kit (Qiagen, Valencia, CA) and followed by RNase-free DNase treatment (Qiagen, Valencia, CA) to remove DNA contamination shortly before RT. The quality of each RNA sample was tested by using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Microarray analysis was performed by using the Human Genome U133A expression array (Affymetrix, Inc., Santa Clara, CA; containing 22,000 transcripts) as previously described (26). One hundred thirty one GIST tumors and a control group of 25 sarcomas (five leiomyosarcomas, six fibrosarcomas, four clear-cell sarcomas, four undifferentiated round cell sarcomas, four synovial sarcomas and two dedifferentiated liposarcomas) were analyzed. The gene expression data on the soft tissue sarcoma control group has been previously published, which is publically available at: <http://cbio.mskcc.org/Public/SFT> (22). Affymetrix probeset used for expression analysis were 202410_x_at and 210881_s_at for *IGF2*, 203627_at, 203628_at and 208441_at for *IGF1R*, 203851_at for *IGFBP6* and 211972_x_at, 201033_x_at, 208856_x_at, 211720_x_at for *RPLP0*. For each gene, a mean for the probesets of interest was calculated. Results are presented as ratios: (data relevant gene/average data RPLP0) $\times 100$ to compare expression between samples.

Bisulfite sequencing analysis

Bisulfite treatment of genomic DNA was performed by using an Epitect Bisulfite Kit (Qiagen, Valencia, CA). The *IGF2* differentially methylated region (DMR) is located in the 5' flanking region of the H19 gene and 90kb downstream of *IGF2*. Nested PCR of bisulfite-converted genomic DNA was performed using the following primers: 1st Sense: 5'-gggaatgtttattatgtatgaag-3', 1st Antisense: 5'-taaaacacctccacctcc-3', 2nd Sense: 5'-taattatttaggtgtgtgtt-3', 2nd Antisense: 5'-tccaaacacccccaccttaa-3'. The protocols are available on request. Direct sequencing was performed and the methylation status was determined. For all cases examined, the bisulfite sequences could be divided into two patterns of *IGF2* DMR methylation: (i) hypomethylation, whereby all 3 CpG loci were converted to thymidines, indicating that all 3 CpG loci of both alleles were demethylated; and (ii) normal methylation, in which the overlapping of both thymidine and cytosine peaks on a sequencing chromatogram was detected for all 3 CpG loci, indicating that both methylated and demethylated *IGF2* DMR alleles are present.

Determination of informative cases and of LOI status

The presence of a polymorphic pattern was examined in each case using ApaI polymorphism for *IGF2*, as previously described. This restriction enzyme site is found in both genomic DNA and at the cDNA level, and was previously associated with *IGF2* loss of imprinting (26). Genomic DNA and cDNA were amplified by polymerase chain reaction (PCR) and the PCR product was digested with 10u ApaI endonuclease for 6 hours at 37°C, then separated on a 3% agarose gel containing ethidium bromide. Genomic DNA samples with two bands of appropriate size (a: 292bp, b: 231bp) were defined as informative. Fragment 'a' was the undigested fragment (292 bp, lack of polymorphic sites), and fragments 'b' were digested fragments (231 bp and 61 bp, with polymorphic sites). The presence of both fragment 'a' and 'b' of genomic DNA defined a case as informative. The presence of both fragments 'a' and 'b' of cDNA were identified as biallelic expression or LOI; while the presence of only fragment 'a' or 'b' was identified as monoallelic expression or imprinting. To confirm the results, direct sequencing of the amplified genomic DNA and cDNA was performed.

Fluorescence in Situ Hybridization (FISH) for IGF1R copy number analysis

FISH on interphase nuclei from paraffin-embedded 4-micron sections was performed applying custom probes using bacterial artificial chromosomes (BAC), covering and flanking IGF1R: SP.G-RP11-147O9, SP.G-RP11-802L22, SP.R-RP11-1087J7 and SP.R-RP11-320L10, as well as a centromeric chromosome 15 probe, SP.O-15a. BAC clones were chosen according to USCS genome browser (<http://genome.uscs.edu>). The BAC clones were obtained from BACPAC sources of Children's Hospital of Oakland Research Institute (CHORI) (Oakland, CA) (<http://bacpac.chori.org>). DNA from individual BACs was isolated according to the manufacturer's instructions, labeled with different fluorochromes in a nick translation reaction, denatured, and hybridized to pretreated slides. Slides were then incubated, washed, and mounted with DAPI in an antifade solution, as previously described (28). Two hundred successive nuclei were examined using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems).

Full-Length Sequencing of IGF1R and mutation detection

Genomic DNA extracted from 15 tumors with available frozen tissues was used to amplify putative exonic regions of IGF1R, which were broken into target regions of 500 bp or less, and specific primers were designed using Primer 3. Purified PCR reactions were sequenced bidirectionally with M13 primer and Big Dye Terminator Kit v.3.1 (Applied Biosystems). Bidirectional reads and mapping tables were subjected to a QC filter which excludes reads that have an average Phred quality score of < 10 for bases 100–200. Passing reads were assembled against the PTPRD reference sequence using command line Consed 16.0 (29). Assemblies were passed on to Polyphred 6.02b (30) and Polyscan 3.0 (31).

Real-Time Quantitative Reverse Transcription-PCR

One µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) at 25°C for 10 min, 37°C for 120 min, 85°C for five min and hold at 4°C. Twenty ng/µl of resultant cDNA was used in a quantitative-PCR using an 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and pre-designed TaqMan ABI Gene expression Assays (Hs00171254_m1* for IGF2, Hs00609566_m1* for IGF1R, Hs00181853_m1* for IGFBP6). Amplification was carried out at 95°C for 10 min, and 40 cycles (95°C for 15 sec, 60°C for one min). To calculate the efficiency of the PCR and to assess the sensitivity of each assay, we also performed a five point standard curve (80, 26.67, 8.88, 2.96, and 0.98 ng/ul). Triplicates CT values were averaged and amounts of target were interpolated from the standard curves and normalized to GAPDH (reference gene).

RESULTS

GIST show overexpression of *IGF2* compared to other soft tissue sarcomas

We analyzed the expression of 12 genes encoding for the main components of the IGF signaling pathway, which were available on the U133A chip: the two ligands (IGF1, IGF2), the two IGF receptors (IGF1R and IGF2R), the seven binding proteins (IGFBP1-7) and the acid-labile subunit IGFBP3. In GIST, *IGF2* was highly overexpressed by 11 to 375 orders of magnitude above the other signaling molecules of the IGF pathway. *IGF2* expression was also significantly higher than in other sarcomas subtypes (fold-change: 10.4, $p < 0.0001$).

Expression of IGF2 correlates with the age at diagnosis and is inversely associated with IGF1R expression

The pattern of expression of *IGF2* was correlated to the age at onset and mutational status of GIST. Indeed, *IGF2* expression was significantly higher in the “adult” group than in the

pediatric and the young adult groups (fold change: 2.7, $p=0.004$) (Figure 1). This result was also confirmed by quantitative real time RT-PCR (fold change: 13). Among adult GIST, *IGF2* expression was higher in tumors lacking *KIT* or *PDGFRA* mutations in comparison with mutated cases (fold change: 1.9, $p=0.04$) (Figure 1). Conversely, the expression of the *IGF1R* gene was significantly higher in the pediatric group than in the adult group (fold change: 3.2, $p<0.0001$). This result was confirmed by quantitative real time RT-PCR (fold change: 9). Since *IGF1R* overexpression in pediatric GIST has been previously correlated with an increase in the *IGF1R* gene copy number (Tarn et al., 2008), we analyzed *IGF1R* status by FISH in 18 cases (12 pediatric GIST and six adult wild-type GIST) selected on the basis of high *IGF1R* expression. However, no copy number alterations were detected in any of the samples. Additionally, we performed full-length sequencing of the *IGF1R* cDNA in 15 cases (12 pediatric, 2 wild-type young adults and one young adult with a *KIT* exon 9 mutation) and did not detect any mutations. Besides *IGF1R*, *IGF2* expression is also negatively correlated with *IGFBP6* expression ($p<0.0001$).

Acquired imatinib-resistant GIST show marginal IGF2 overexpression

GIST tumor cells can replace the lack of signal due to *KIT*/*PDGFRA* inhibition by activating alternative pathways. We hypothesized that *IGF2* overexpression and the resulting activation of IGF signaling may represent a possible mechanism of secondary resistance to imatinib in advanced GIST. We compared *IGF2* expression in tumor groups according to imatinib status (responsive or resistant). We found a 1.5 fold *IGF2* overexpression in resistant GIST compared to responsive GIST (Figure 1), however, it did not reach statistical significance ($p=0.15$), results being also confirmed by qRT-PCR (fold change: 2.2).

IGF2 overexpression in GIST is associated with *IGF2* DMR hypomethylation, but not loss of imprinting

To investigate whether *IGF2* overexpression in a subset of GIST is the result of the methylation status of the *IGF2* DMR, we investigated this event in 86 of the 131 GIST cases. Hypomethylation of the three CpG loci of the *IGF2* DMR was detected in 20 cases (23%). *IGF2* mRNA expression was significantly higher in hypomethylated cases than in normal cases (fold-change: 1.6; $p=0.005$) (Figure 2). Methylation changes of the DMR have been associated with loss of imprinting in several solid tumors. We decided to investigate if this mechanism was involved in GIST. We identified eight informative cases (heterozygous for the Apal polymorphism in exon 9 of *IGF2*) among the 21 cases with available RNA. All eight cases had monoallelic expression of *IGF2*, including five cases with *IGF2* DMR hypomethylation.

DISCUSSION

There is a growing interest in IGF signaling for the development of anticancer therapies due to the crucial role of this pathway in tumorigenesis. We report here the first study assessing the general patterns of deregulation of the IGF pathway in adult and pediatric GISTs. Previous studies have demonstrated that *IGF2* was highly overexpressed in GISTs and that this overexpression was associated with poor prognostic features. Our results confirm the overexpression of *IGF2* in GIST, and provide additional insights regarding this abnormality. Previous series did not include pediatric and young adult GIST (7, 10). We showed here that the IGF pathway is deregulated in GIST with distinct patterns according to the age onset of the disease. Indeed, we observed that pediatric GISTs are associated with very low levels of *IGF2* and high levels of *IGF1R*. Conversely, adult GISTs express high levels of *IGF2* and low level of *IGF1R*. Among adult GIST, we found that the level of expression of *IGF2* is higher in GIST without detectable *KIT* or *PDGFRA* mutations, than in mutated tumors. Moreover, by analyzing the expression of the 12 genes encoding the main components of the

IGF signaling pathway, we also found that IGF2 expression in GIST is also negatively correlated with IGFBP6 expression ($p < 0.0001$). Although IGFBPs-1-5 binds IGF1 and IGF2 with a similar affinity, IGFBP-6 has a 20 to 100-fold higher affinity to IGF2 than to IGF1 (32-33). IGFBP-6, whose expression is regulated by P53, sequesters IGF2 and thus acts as a tumor suppressor gene in a large variety of cancers. It inhibits growth in lung cancer (34), colorectal cancer (35), prostate cancer (36), neuroblastoma (37) and rhabdomyosarcoma (38), whereas its expression is lower in malignant or metastatic tumors than in benign or non-metastatic tumors (39). Our finding showing a coordinated overexpression of *IGF2* and downregulation of its specific inhibitor *IGFBP-6* in GIST, strongly suggests that IGF2 signaling plays an important role in GIST tumorigenesis.

Altogether, our results suggest that at least a subset of GIST are characterized by a paracrine or an autocrine loop, which may, through the overexpression of IGF2 (particularly wild-type adult GIST) or IGFR1 (pediatric and young adult GIST), induce an auto-enhancing activation of MAPK and PI3K pathways and therefore tumor cell proliferation. The activation of the IGF pathway in wild-type GIST and its ability to bypass KIT signaling may explain at least in part the lesser sensitivity of these tumors to imatinib. However, further functional studies are needed to confirm these hypotheses.

The mechanisms by which *IGF2* and *IGFR1* become deregulated in GIST remain to be identified. Genomic imprinting is a mechanism by which there is preferential expression of a gene based on the parental origin of the allele. *IGF2* and *H19* (which does not encode any protein) are reciprocally imprinted genes, located on human chromosome 11p15.5 (1). Although *H19* does not encode any protein, its expression is associated with silencing of *IGF2* (2). *IGF2* is exclusively expressed from the paternal allele, whereas *H19* is only expressed from the maternal allele. The transcription of the *IGF2* gene is regulated by a differentially methylated region (DMR) located in the 5' flanking region of the *H19* gene and 90kb downstream of *IGF2*. The DMR on the maternal allele is unmethylated, while the DMR on the paternal allele is methylated (40-44). This methylation of the paternal allele ICR blocks the transcription factor CCCTC-binding factor (CTCF) from binding and creating a physical barrier that stops downstream enhancers from augmenting *IGF2* promoters. This effectively silences the maternal allele (45-47). Hence, to assess whether the increased expression of *IGF2* in GIST was due to LOI, we selected samples heterozygous for ApaI polymorphism for evaluating biallelic expression of *IGF2*. Strikingly, all the informative cases had monoallelic expression of *IGF2* including five cases with *IGF2* DMR hypomethylation. Obviously, we cannot exclude that LOI occurs in a minority of cases, however, our results suggest that enhanced paternal allele transcription might be responsible for the increased *IGF2* expression in the majority of GIST. Our results are in line with previous studies showing that changes in DNA methylation of the DMR are not associated with biallelic expression in some tumor models (48). However, further studies are needed to investigate the mechanisms involved in the regulation of DNA methylation in GIST. In regards to *IGF1R* overexpression, our results confirmed more recent reports showing no increase in copy number of the *IGF1R* (11), as it was firstly suggested in a small series indicating low level amplification in size of wild-type GIST (8). Hence, it appears that that *IGF1R* amplification is not a common event in GISTs, and that *IGF1R* overexpression is caused by other mechanisms than copy number abnormalities or activating mutations.

As *IGF2* overexpression was previously shown to correlate with poor prognostic features in GIST, in this study we chose to focus on its potential role in resistance to imatinib in patients with advanced disease. Preliminary *in vitro* data suggested that the IGF pathway may mediate imatinib response or resistance through IGFBP-3 (49). In our series, we found no difference in terms of *IGFBP3* expression between responsive, resistance or naïve GIST (data not shown). However, we found a marginal downregulation of *IGF2* in responsive

lesions and a statistically significant higher expression of IGF2 in wild-type adult GIST which are known to be less responsive to imatinib. The most promising future perspectives of our results are first to study the biological role of IGF2 in GIST using *in vitro* and *in vivo* models. If preclinical functional studies demonstrate the pathogenetic role of IGF2 in GIST and its involvement in imatinib resistance, therapies targeting the IGF pathway may be beneficial in patients with primary or secondary resistance to imatinib.

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Figure 1. *IGF2* and *IGF1R* mRNA expression in GIST. A: *IGF2/IGF1R* ratio according to age onset; B: *IGF1R/IGF2* ratio according to age onset; C: *IGF2/RPLP0* ratio according to mutational status; D: *IGF2/RPLP0* according to sensitivity to imatinib.

Figure 2.
IGF2 mRNA expression according to the methylation status of the *IGF2* differentially-methylated region.

TABLE 1

Patients characteristics (N=131)

	No. of Patients	%
Age onset		
Adult (≥ 30 years)	106	81.0
Young adult (< 18 and < 30 years)	4	16.0
Children (<18 years)	21	4.0
Sex		
Male	70	53.5
Female	61	46.5
Tumor site		
Small bowel	57	76.5
Stomach	42	15.0
Other	32	8.5
Mutational status		
<i>KIT</i> Exon 11	66	50.5
<i>KIT</i> Exon 9	23	17.5
<i>KIT</i> Exon 13	1	0.5
<i>PDGFRA</i>	9	7.0
Wild-type	32	24.5
Treatment status		
Imatinib-naïve	78	59.5
Imatinib-responsive	10	7.5
Imatinib-resistant	43	33.0