

SHORT REPORT

Insulin-like growth factor 1 receptor expression in wild-type GISTs: A potential novel therapeutic target

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Aberrations of the Insulin-like Growth Factor (IGF) system have been found in association with a variety of cancer types. The potential role of IGF1R has been postulated in a small subset of GISTs, but until now the implications of its aberrations have not been defined. The aim of the study was to examine the IGF1R status in patients with gastric GIST in regard to KIT and PDGFRA genotype. Fresh resection specimens were collected from 8 primary tumours [2 wild-type (WT) and 6 mutant cases]. IGF1R was studied as gene expression profiling with Affymetrix GeneChip HG-U133Plus 2.0 arrays and as genomic copy number with SNP array analysis Affymetrix Genome Wide Human SNP 6.0 arrays, and at protein level with western blotting (WB) and immunohistochemistry (IHC). The unsupervised analysis of gene expression profiling of our patients merged with a data set from gastric GISTs identified 2 patients out of 8 with different expression of IGF1R. The data were confirmed by WB and IHC. In particular, IGF1R was upregulated in 2 young patients (<30-years old), who had both WT disease and metastases at diagnosis. The SNP array analysis showed that none of the tumours had IGF1R amplification. GISTs are characterized by abnormalities of the KIT and PDGFRA receptors that affect prognosis and response to tyrosine kinase inhibitors. Both young adult with WT GIST had the overexpression of IGF1R at mRNA and protein level. These results further confirm the hypothesis that IGF1R may be a potential therapeutic target in GISTs lacking KIT and PDGFRA mutations.

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Key words: gastrointestinal stromal tumours; insulin-like growth factor 1 receptor; KIT receptor; PDGFRA

The Insulin-like Growth Factor 1 receptor (IGF1R) is a tyrosine kinase receptor that binds either IGF1 or IGF2.¹ After ligand binding, the tyrosine kinase domain is activated and stimulates the intracellular signalling pathways that control the proliferation rate and apoptosis. Two key signal transduction networks have been identified: the GPTase Ras-Raf-ERK/MAPK and PI3K-AKT/mTOR.² The IGF system plays a key role in the biology of normal tissues. Aberrations of this molecular pathway such as the overexpression of IGF1R, or elevated plasma levels of IGF1, or genetic polymorphisms of the gene encoding IGF1 have been found in association with a variety of cancers including sarcoma.^{3–5} Recently, some data have been published suggesting a role for IGF1R in the biology of a subset of gastrointestinal stromal tumours (GISTs).^{6–8} As well known, GISTs are characterized by the abnormalities of the KIT and PDGFRA receptors that represent the key oncogenic event as well as the most important therapeutic target.^{9–11} Mutational status of these genes affects prognosis and responsiveness to tyrosine kinase inhibitors.^{12–14} IGF1R has come up as a novel molecular signalling pathway involved in GISTs, but until now its role on the pathogenesis of this disease and the clinical implications of its aberrations have not been defined in regard to KIT and PDGFRA kinase genotype, to clinical

outcome, to prognosis and therapeutic responsiveness. We studied the status of IGF1R in patients affected by GISTs at gene level, as gene expression profiling and genomic amplification using microarray technologies, and at protein level with western blotting (WB) and immunohistochemistry (IHC).

Material and methods

Patients and tissue

We examined the IGF1R axis in 8 patients affected by gastric GIST (3 F/5 M; mean age 59 years, range 28–85 years). The histopathological characteristics, immunoprofile and the clinical details of tumours are described in Table I.

At the diagnosis the tumour was localized in all patients except 2 (GIST_7, GIST_10) in which metastases were detected in the lung and liver. Seven patients underwent surgery at diagnosis, whereas 1 patient (GIST_7) was operated after imatinib and sunitinib treatment. The mutational analysis of KIT and PDGFRA is described in Table II. The patients (GIST_7, GIST_10) with wild-type (WT) tumour were 28- and 30-years old. The age of the other patients with kinase-mutant tumour ranged between 54 and 85 years.

The analyses were conducted on fresh tissue specimens collected during the surgical operation and were obtained from GIST primary lesion in all patients. The specimens were immediately frozen at –80°C and stored until RNA and DNA extraction.

Gene expression analysis

Gene expression profiling was performed with Affymetrix GeneChip HG-U133 Plus 2.0 arrays following manufacturer’s instructions. Raw data (CEL files) were normalized using the “rma” algorithm together with data from a public dataset of gastric GIST gene expression profiles available at Gene Expression Omnibus

Abbreviations: GISTs, gastrointestinal stromal tumours; IGF1R, insulin-like growth factor 1 receptor; IHC, immunohistochemistry; PDGFRA, platelet-derived growth factor receptor Alfa; SNP Array Analysis, single nucleotide polymorphisms array analysis; WB, western blotting; WT, wild-type.

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TABLE I – PATIENTS AND TUMOURS CHARACTERISTICS

ID patient	Ages	Tumour site	Disease status (at diagnosis)	Tumour dimension (cm)	Tumour histological type	Mitotic index (/50HPF)	CD117	CD34	Desmin	S100	Smooth muscle actine	KI-67 (%)	EGFr
GIST_2	85	Stomach	Localized	8	Spindle	<5	Pos	Pos	Neg	Neg	Rare cells	–	Pos
GIST_4	79	Stomach	Localized	5	Spindle	7	Pos	Pos	Rare cells	Neg	Rare cells	6,3	–
GIST_5	68	Stomach	Localized	7	Spindle	4	Pos	–	Neg	Neg	Rare cells	1,4	Pos
GIST_7	28	Stomach	Metastatic	7	Mixed	>5	Pos	Pos	Neg	Neg	Neg	12,5	Pos
GIST_8	62	Stomach	Localized	2	–	4	Pos	Pos	Neg	Neg	Rare cells	2,8	Pos
GIST_9	54	Stomach	Localized	3	Spindle	<5	Pos	Pos	Neg	Neg	Neg	–	–
GIST_10	30	Stomach	Metastatic	7	Mixed	9	Pos	Pos	Neg	Neg	Rare cells	3,8	Pos
GIST_12	66	Stomach	Localized	4,5	Hepiteliod	<5	Pos	Pos	Neg	Neg	Rare cells	3,4	Pos

TABLE II – MOLECULAR CHARACTERISTICS OF KIT AND PDGFRA

ID Patient	Mutational status (KIT exons 9,11,13,17; PDGFRA exons 12,14,18)
GIST_2	KIT exon 11 V560D
GIST_4	KIT exon 9 AY502-503 insertion
GIST_5	PDGFRA exon 12 SPDGHE566-571RIQ
GIST_7	KIT and PDGFRA WT
GIST_8	KIT exon 11 V559D
GIST_9	KIT exon 11 insertion TQLPYDHWKWEFP574–585 at P585
GIST_10	KIT and PDGFRA WT
GIST_12	PDGFRA exon 14 K646E

database (www.ncbi.nlm.nih.gov/geo) with accession ID GSE8167.¹⁵ Normalized data were filtered to exclude probes poorly expressed or with an IQR <10% of mean total IQR. All the analyses were performed with Bioconductor v2.1 on R 2.6.0. Multidimensional scaling was performed with BRB Array Tools software (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). Genes differentially expressed between young age GISTs and all the other samples were selected by significant analysis of microarrays algorithm setting the FDR threshold to 0.2% (False Discovery Rate = number of false positive genes/all significant genes). Genes were represented by hierarchical clustering using Pearson correlation metric and complete linkage clustering using TIGR MeV (<http://www.tm4.org/mev.html>). Pathway analysis of genes differentially expressed between young age GIST and all the other patients was performed with BRB Array Tools using the Gene Set Expression Comparison tool on Biocarta pathways. Pathways were considered significantly different if Efron-Tibshirani's GSA test *p*-value was <0.005.

SNPs array analysis

SNP array analysis of genomic copy number was performed with Genome Wide Human SNP 6.0 arrays (Affymetrix). Genomic copy number deviation from diploid DNA content was analyzed by applying both a segmentation algorithm and HMM algorithm (Partek Genomic Suite software), and compared to a set of 270 normal HapMap samples that served as reference controls.

Protein extraction and WB analysis

Frozen tissues were homogenised using lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM DTT) containing protease inhibitors (10 mg/mL aprotinin and leupeptin, 5 mg/mL pepstatin, 1mM PMSF) and phosphatase inhibitors (50 mM NaF, 10 mM Na₄P₂O₇, 1 mM Na₃VO₄, 3 mM H₂O₂). Homogenates were centrifuged at 13,000g for 15 min at 4°C, and supernatants were stored at –80°C until analysis. Protein lysate from MCF7 cell line was used as a positive control. Total protein extracts (50 µg/lane) were separated on a 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and electro-transferred to a nitrocellulose membrane (Amersham Bioscience, Freiburg, Germany). Anti-IGF-IRβ [1:3,000; (C-20) Santa Cruz Biotechnology, Heidelberg, Germany] and anti-β-actin [1:10,000; (AC-15) Sigma-Aldrich] were diluted in TBST (Tris-buffered saline/Tween0,1%) (5% milk powder) and incubated at 4°C overnight. The appropriate secondary antibody was applied (1:4,000 horseradish peroxidase antirabbit and 1:5,000

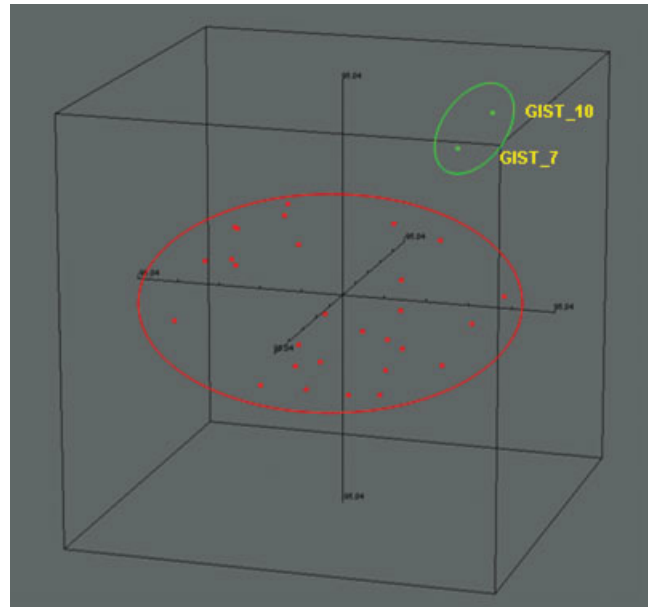


FIGURE 1 – Unsupervised analysis of gene expression profiling of our patients merged with data from a public dataset of gastric GIST gene expression profiles available at gene expression omnibus database (www.ncbi.nlm.nih.gov/geo) with accession ID GSE8167 (Yamaguchi et al.).¹⁵

horseradish peroxidase antimouse) at room temperature for 1 hr. Visualization was performed by enhanced chemiluminescence (Amersham Bioscience, Freiburg, Germany).

Immunohistochemistry analysis

IHC for IGF1R expression was performed on 4–6 µm paraffin embedded tumour sections by using an avidin-biotin peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Briefly, sections were pretreated with a citrate buffer solution (0.01 M citric acid and 0.01 M sodium citrate, pH 6.0) in a microwave oven at 750 W for 3 cycles of 5 min each. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 30 min at room temperature. A blocking step with normal rabbit or goat serum (Vector, Burlingame, CA) was used. This treatment ensured antigen retrieval from samples. The primary anti-IGF-IRβ (C-20) (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) antibody was applied overnight in a moist chamber at 4°C. The following day, tissue sections were incubated with a secondary-biotinylated anti-goat antibody and with an avidin-biotin-peroxidase complex (Vector, Burlingame, CA). The final reaction product was revealed by exposure to 0.03% diaminobenzidine (Sigma, St. Louis, MO), and nuclei were counterstained with Mayer's hematoxylin. Samples were scored separately by 2 independent investigators in a blinded fashion. Specimens with a diffused, high (+++) or moderate

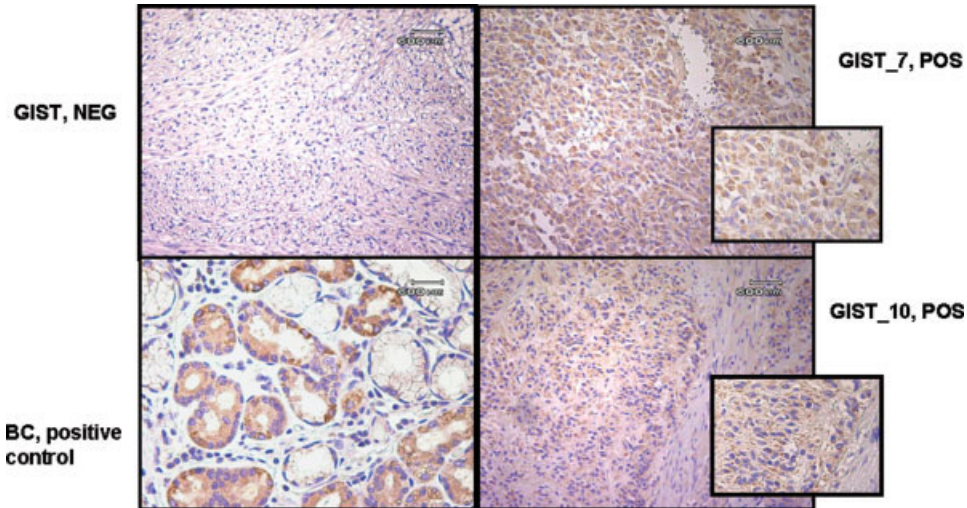


FIGURE 5 – IGF1R immunostaining of the 2 over expressed tumors (GIST_7, GIST_10) and IGF1R immunostaining examples of negative GIST and breast cancer, BC, as positive control.

EGF). An interesting role could be played by AP-2 alpha, a transcription factor expressed in cells of the neural crest, that can bind to consensus sequences present in the IGF1R promoter.¹⁶ This transcription factor is not expressed in KIT or PDGFRA mutant GISTs, while it is highly over expressed in WT GISTs which indeed exhibit a clear neural commitment by gene expression analysis. The tumours from our 2 young adult patients had strongly different gene expression profiles in comparison to the tumours resected from other older patients. Notably, the gene expression profiles of our young adult GIST patients seem similar to reported cases of pediatric GIST that have WT KIT and PDGFRA genotypes.⁷

About the correlation with the clinical course, in our study both patients had metastatic disease at diagnosis and one also received

TK inhibitors. Future studies are needed to investigate if IGF pathway could be considered a salvage pathway in a small subgroup of GIST not showing mutations in KIT and PDGFRA and not a primarily decisive aspect of tumour progression.

In conclusion, both of the young adults (≤ 30 -years old) with WT GIST tumours had over expression of IGF1R at gene mRNA and protein expression. These results further confirm the hypothesis that IGF1R may be a potential therapeutic target in GISTs lacking KIT and PDGFRA mutations.

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