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STRONG EXPRESSION OF IGF1R IN PEDIATRIC GASTROINTESTINAL STROMAL TUMORS WITHOUT *IGF1R* GENOMIC AMPLIFICATION

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Abstract

Wildtype (WT) gastrointestinal stromal tumors (GISTs), lacking mutations in *KIT* or *PDGFRA*, represent 85% of GISTs in pediatric patients. Treatment options for pediatric WT GIST are limited. Recently, expression profiling of a limited number of pediatric and adult WT GISTs and more in depth study of a single pediatric WT GIST implicated the insulin like growth factor 1 receptor (IGF1R) as a potential therapeutic target in pediatric WT GIST. We performed immunoblotting, SNP and FISH studies to determine the extent of expression, biochemical activation and genomic amplification of IGF1R in a larger number of pediatric WT GISTs. Pediatric WT GISTs expressed IGF1R strongly, whereas typical adult *KIT* mutant GISTs did not. *IGF1R* gene amplification was not detected in pediatric WT GISTs, and some *KIT*-mutant GISTs had *IGF1R* gene deletion due to monosomy 15. Despite the absence of apparent genomic activation mechanisms accounting for overexpression, clinical study of IGF1R-directed therapies in pediatric WT GIST is warranted.

The insulin-like growth factor 1 receptor (IGF1R) and its ligands, insulin-like growth factors (IGF) 1 and 2 serve crucial physiologic roles in growth and development [1, 2]. The IGF pathway also has important pathophysiologic roles in cancer: IGF1R is required for neoplastic transformation in many experimental systems [3-5]; IGF1R is expressed strongly in a variety of neoplasms; and IGFs promote proliferation of neoplastic cells [6, 7]. Monoclonal antibodies and small molecules targeting IGF1R are currently undergoing clinical

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evaluation. In early phase clinical trials of anti-IGF1R antibodies, adverse effects have been minimal and objective responses have been documented in advanced neuroendocrine tumors, Ewing sarcoma, and osteosarcoma 8-10.

Gastrointestinal stromal tumor (GIST), the most common mesenchymal neoplasm of the gastrointestinal tract, is resistant to conventional cytotoxic chemotherapy 11. Mutations in *KIT* or *PDGFRA* are present in 85% of GISTs occurring in adults. Imatinib and sunitinib, small-molecule inhibitors of the mutant KIT and PDGFRA receptor tyrosine kinases, significantly prolong survival in patients with GIST 12, 13. However, more than 85% of GISTs in children are wild-type (WT), lacking detectable mutations in *KIT* or *PDGFRA*14. Imatinib appears to be less effective against these WT tumors than against GIST harboring activating mutations 15, 16. Sunitinib therapy only rarely results in objective responses in children and adults with WT GIST16, 17.

A recent study demonstrated strong IGF1R expression and low level *IGF1R* gene amplification in several adult WT GISTs and a single pediatric WT GIST. A subsequent study confirmed high IGF1R expression in 2 adult WT GISTs but did not find associated *IGF1R* gene amplification18. These studies implicate IGF1R as a potential therapeutic target in adult WT GIST19-21. However, results in adult WT GIST do not necessarily apply to pediatric WT GIST because in prior studies, pediatric and adult WT GISTs have been found to have distinct clinical and biological features20, 22. Because additional pre-clinical data would help determine whether clinical trials of IGF1R-directed therapy in pediatric GIST are warranted, we evaluated a more sizeable sampling of pediatric GIST specimens for multiple characteristics, including IGF1R protein expression, IGF1R activation, and *IGF1R* gene copy number.

Materials and methods

Western blotting

The level of IGF1R expression in pediatric WT GISTs vs. adult KIT-mutant GISTs was analyzed by western blot. Whole cell lysates were prepared from 14 cryopreserved GISTs for which *KIT* and *PDGFRA* mutation status had already been determined14. Lysates from nine pediatric WT GISTs (cases P1-P9) and five *KIT*-mutant GISTs (cases M1-M5) were subjected to gel electrophoresis as previously described 14, 23. Blots were stained with antibodies to IGF1R (Cell signaling, Beverly, MA) and actin (Sigma, St. Louis, MO). Additional staining of the GIST immunoblots for activated IGF1R expression was performed using an antibody to phospho-IGF1R Y1135/1136 (Cell signaling, Beverly, MA).

SNP array and analysis

We evaluated *IGF1R* gene amplification in pediatric WT GIST using single nucleotide polymorphism (SNP) arrays. Genomic DNA isolated from 14 cryopreserved pediatric WT GISTs, 3 cryopreserved adult *KIT*-mutant GISTs and 4 normal control samples was digested with the StyI restriction enzyme. Digested DNA was then ligated to an adaptor before subsequent PCR amplification using AmpliTaq Gold (Applied Biosystems, Foster City, CA). PCR products were pooled, concentrated, and fragmented with DNase I to a size range of 200-1100 bp. Fragmented PCR products were then labeled, denatured, and hybridized to Affymetrix 250K *Sty* SNP arrays interrogating ~238,000 SNPs. After hybridization, the arrays were washed on the Affymetrix fluidics stations, stained, and scanned using the Gene Chip Scanner 3000 7G and the genotyping software Affymetrix Genotyping Tools Version 2.0. DNA isolation and data analysis were performed as previously described 14, 24.

IGF1R Fluorescence In Situ Hybridization (FISH)

FISH was performed in four μm formalin-fixed paraffin-embedded tissue sections. Hybridization and signal detection techniques were as described previously 25. The *IGF1R* probe was composed of two overlapping BAC clones, RP11-262P8 and RP11-654A16, labeled by random priming with digoxigenin and detected with FITC anti-digoxigenin, and co-hybridized with a spectrum orange-labeled chromosome 15 pericentromeric probe (CEP15 D15Z4; Abbott Molecular). *IGF1R* to chromosome 15 centromeric ratio was determined in 100 nuclei.

Results

High-level IGF1R expression was detected in eight of nine pediatric WT GISTs, whereas all five *KIT*-mutant GISTs lacked IGF1R expression (Figure 1). All pediatric WT GISTs and case M5, a *KIT* mutant GIST lacking IGF1R expression, appeared to express phosphorylated IGF1R (Figure 1b). All of the pediatric WT GISTs featured AKT activation (Figure 1b).

By SNP analysis, none of the pediatric WT GISTs had *IGF1R* gene amplification (Figure 2a). The SNP results were validated by fluorescence *in situ* hybridization (FISH) in pediatric WT GISTs P5 and P7 (Figure 2b). FISH confirmed the absence of *IGF1R* gene amplification in these two pediatric WT GISTs and in one additional pediatric WT GIST (P15) for which there was insufficient fresh frozen specimen for SNP analysis (Figure 2c).

The three *KIT*-mutant cases, M1, M2 and M3 included in the SNP study had decreased copy number at the *IGF1R* locus due to monosomy 15, which is a common cytogenetic finding in adult *KIT*-mutant GIST26. Three additional adult *KIT* mutant cases were analyzed by FISH and had *IGF1R* to chromosome 15 centromeric ratios of 0.98 to 1.54. Notably, Case 5, which had the *IGF1R* to chromosome 15 centromeric ratio of 1.54 did not express IGF1R.

Discussion

Our immunoblotting results demonstrating high IGF1R expression in pediatric WT GISTs are in agreement with transcriptional profiling of 8 pediatric WT GISTs performed by Agaram 20 and with immunohistochemistry of a single pediatric WT GIST performed by Tarn 19. The consistent high-level IGF1R expression in pediatric WT GISTs and the stark contrast with low-level IGF1R expression in *KIT*-mutant GISTs demonstrated in the present study further implicates IGF1R as a potential therapeutic target in pediatric WT GIST. Similar to this study, Tarn and colleagues found a lack of correlation between phosphorylated IGF1R and total IGF1R expression 19. This might result from cross-reactivity of the phospho-IGF1R antibody with the phophorylated insulin receptor, which has close homology to IGF1R. All of the pediatric WT GISTs studied featured AKT activation but we have previously reported KIT activation in these same tumors 14. Hence, it is unclear whether activation of downstream survival and growth pathways in pediatric WT GIST is IGF1R-dependent, KIT-dependent, or both. Therefore, these studies, although consistent with IGF1R activation, are not definitive.

In a prior study, low level *IGF1R* gene amplification was detected in WT GISTs in adults and in a single pediatric GIST 19. However, in a recent publication by Pantaleo and colleagues18 two WT GISTs had high IGF1R expression without *IGF1R* gene amplification. Our SNP array and FISH results demonstrate that the strong IGF1R expression present in pediatric WT GISTs is not due to gene amplification. The *KIT*-mutant GISTs we studied had low-to-absent IGF1R expression. In some of the cases absent IGF1R expression could potentially be due to monosomy 15. However, absent IGF1R expression

was also seen in a *KIT*-mutant GIST with two copies of *IGF1R* and so lack of IGF1R expression in adult *KIT*-mutant GIST cannot be attributed entirely to *IGF1R* gene deletion.

In conclusion, IGF1R is expressed strongly in pediatric WT GISTs but this is not due to detectable gene amplification. Further pre-clinical evaluation of the IGF1R pathway in pediatric GISTs such as drug-response studies in cell lines or animal models are warranted. Unfortunately, attempts to develop such models of pediatric and adult WT GIST have not yet been successful. These results support clinical evaluation of anti-IGF1R antibody therapies in pediatric patients with metastatic WT GIST especially given the relatively mild toxicity profile of anti-IGF1R antibodies and the limited alternative therapeutic options.

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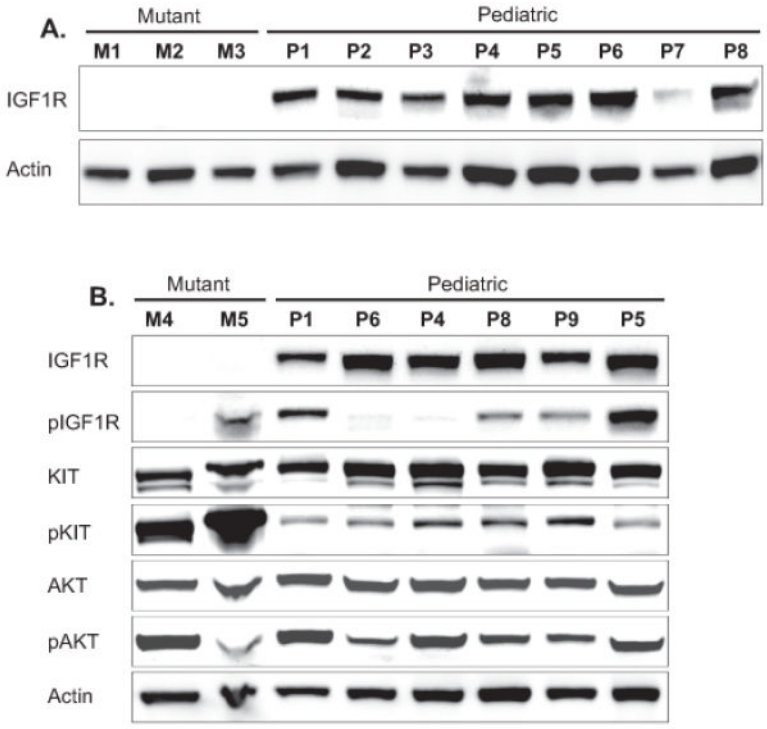
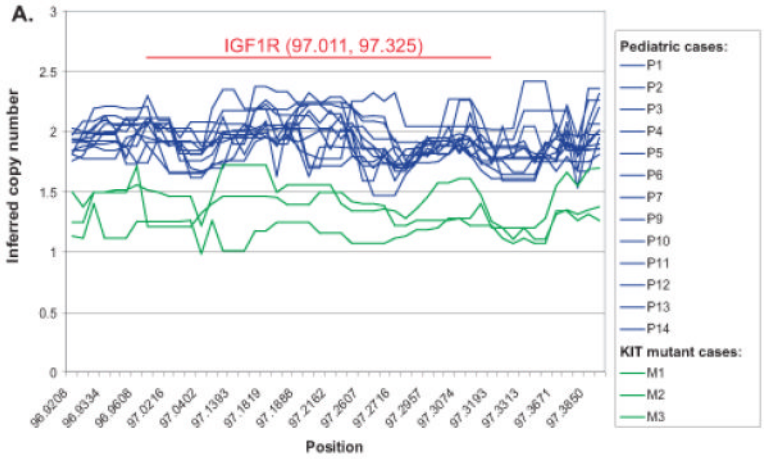


Figure 1.
Western blotting of IGF1R, KIT and AKT shows strong IGF1R expression in pediatric WT GISTs but not in comparison *KIT*-mutant GISTs.



B.

Case	Ratio IGF1R to Ch15 centromere
P5	1.04
P7	1.02
P15	1.11
M5	1.54
M6	1.05
M7	0.98

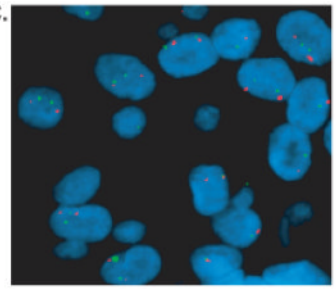


Figure 2.
Copy number at the *IGF1R* locus as determined by SNP array study (a) and FISH (b and c) of *IGF1R* (green) and chromosome 15 centromere (red) demonstrates lack of *IGF1R* gene amplification in pediatric WT GIST.