

RESEARCH ARTICLE

Gain of FGF4 is a frequent event in KIT/PDGFR/SDH/RAS-P WT GIST

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Abstract

Gastrointestinal stromal tumors (GIST) lacking mutations in KIT/PDGFR/SDH/RAS-P pathways and retaining an intact SDH complex are usually referred to as KIT/PDGFR/SDH/RAS-P WT GIST or more simply *quadruple* WT GIST (~5% of all GIST). Despite efforts made, no recurrent genetic event in *quadruple* WT GIST has been identified so far. To further investigate this disease, we performed high throughput copy number analysis on *quadruple* WT GIST specimens identifying a recurrent focal gain in band 11q13.3 (involving *FGF3/FGF4*) in 6/8 cases. This event was not found in the other molecular GIST subgroups. *FGF3/FGF4* duplication was associated with high expression of FGF4, both at mRNA and protein level, a growth factor normally not expressed in adult tissues or in KIT/PDGFR-mutated GIST. FGFR1 was found to be the predominant FGF receptor expressed and phosphorylation of AKT was detected, suggesting that a FGF4-FGFR1 autocrine loop could stimulate downstream signaling in *quadruple* WT GIST. Together with the recent reports of *quadruple* WT cases carrying FGFR1 activating alterations, these findings strengthen the hypothesis of a potential involvement of FGFR pathway deregulation in *quadruple* WT GIST, which may represent a rationale for novel therapeutic approaches.

KEYWORDS

FGF3/FGF4, FGFR inhibitors, FGFR1, gastrointestinal stromal tumours, KIT/PDGFR/SDH/RAS-P WT, *quadruple* WT

Annalisa Astolfi and Maria Abbondanza Pantaleo authors contributed equally.

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1 | INTRODUCTION

Gastrointestinal stromal tumors (GIST) not harboring mutations in KIT or platelet-derived growth factor receptor alpha (PDGFRA) receptors (~10%-15% of adult cases) are often referred to as KIT/PDGFRA wild-type (WT) GIST.¹ Between 20% and 40% of KIT/PDGFRA WT GIST show loss of function of the succinate dehydrogenase complex (SDH), designated as *SDH-deficient* GIST, recognized by the loss of subunit B (SDHB) protein expression.²⁻⁴ Moreover, another subgroup (~15% of KIT/PDGFRA WT GIST) harbours mutations in BRAF/RAS or NF1 and are referred to as RAS-pathway (RAS-P) mutant GIST.⁵⁻⁷ The remaining cases, lacking mutations in the KIT/PDGFRA or RAS pathways, and retaining an intact SDH complex, are usually referred to as KIT/PDGFRA/SDH/RAS-P WT GIST or more simply *quadruple* WT GIST, accounting approximately for 50% of KIT/PDGFRA WT GIST and 5% of all GIST.⁸ These cases show a transcriptome profile that profoundly differs from KIT/PDGFRA- and SDHA-, or possibly all SDH-mutant GIST, indicating that *quadruple* WT GIST could represent another unique group within the family of GIST.^{9,10}

Recently, despite the homogenous transcriptome profile, the extensive molecular characterization of *quadruple* WT GIST failed to detect any recurrent genetic event underlying the disease, conversely providing evidence of a great molecular heterogeneity, with many different and sometimes private mutational events such as fusion genes involving *ETV6-NTRK3* or *FGFR1* and mutations on *FGFR1*, *TP53*, *MAX*, and *MEN1*.¹⁰⁻¹³ However, whether these alterations were *driver* or secondary events is still to be proven, even if the heterogeneity of the mutated genes and the lack of any recurrent genetic feature supports the view that the underlying shared pathway of *quadruple* WT GIST is still to be uncovered. In this study, a deep molecular analysis was performed to further investigate the biological background of this very rare subgroup of disease that extensively differs from other GIST.

2 | MATERIALS AND METHODS

2.1 | Patients and tumor samples

Tissue samples of eight *quadruple* WT, five fresh frozen (FF), and three formalin-fixed paraffin-embedded (FFPE), being negative for mutations in KIT, PDGFRA, SDHx, and RAS-P genes, were centralized at "Giorgio Prodi" Cancer Research Center (CIRC), University of Bologna, within a multicentric collaborative project. Patients and tumor characteristics are listed in Table 1. GIST diagnosis was based on histologic evaluation and on immunohistochemistry of CD117 and DOG1 and was centrally reviewed. In addition to Sanger sequencing performed at the time of diagnosis, the mutational status of *KIT*, *PDGFRA*, *BRAF*, *KRAS*, *SDHx*, and *NF1* was evaluated through a custom amplicon sequencing panel using Truseq Custom Amplicon low input kit (Illumina). Moreover, SDH deficiency was excluded by IHC of SDHB. Seven out of these eight *quadruple* WT cases were also analyzed through whole exome sequencing in a previously published article¹⁰ and relevant alterations were reported in four cases: a truncating mutation of *CTNND2* in GIST127, a homozygous frameshift deletion of *MEN1* and *TP53* mutation in GIST320, a frameshift deletion on *MAX* combined with a germline variant on *NF1* (p.R2573L) in GIST268, an activating mutation (p.N546K) of *FGFR1* in GIST409 (Table 1).

This study was approved by the local institutional ethical committee of Azienda Ospedaliero-Universitaria Policlinico S.Orsola-Malpighi (number 113/2008/U/Tess).

2.2 | Copy number

Whole chromosome gains and losses and copy number aberrations (deletions and duplications) were determined in *quadruple* WT cases using CytoScan HD or Oncoscan CNV Plus array (Thermo Fisher Scientific, Milan, IT) for FF or FFPE specimens, respectively. Copy number data were analyzed and visualized with Chas 3.1 (Thermo Fisher Scientific).

TABLE 1 Patients and tumor characteristics

Patient id	Sex	Age	Site	Size (cm)	Mitotic count	Risk classification	Lymph node metastasis	Distant metastasis	Relevant mutations ^a	Tissue Type	FGF4 gain	FGF4 expression
GIST127	F	63	Ileum	5-10	6-10	High	No	Yes	<i>CTNND2</i> p.S996fs	FF	Positive	High
GIST133	M	57	Duodenum	1.6	<5	Very low	No	No	None	FF	Positive	High
GIST400	M	69	Duodenum	NA	NA	NA	No	No	None	FF	Positive	High
GIST401	F	45	Duodenum	NA	NA	NA	No	No	None	FF	Positive	High
GIST409	M	45	Ileum	NA	NA	NA	No	No	<i>FGFR1</i> p.N546K	FF	Negative	Negative
GIST320	M	73	Ileum	13	<5	High	No	No	<i>MEN1</i> p.L83fs <i>TP53</i> p.V216M	FFPE	Positive	High
GIST219	M	44	Duodenum	6.5	>5	High	No	Yes	Unknown	FFPE	Positive	High
GIST268	M	50	Ileum	8.5	2	Intermediate	No	No	<i>NF1</i> p.R2573L <i>MAX</i> p.K34fs	FFPE	Negative	Negative

^aExome sequencing performed by Pantaleo et al.¹⁰

As reference control dataset we used copy number data available online from other GIST samples: GSE93077 from Schaefer et al¹⁴ and GSE20709 from Astolfi et al¹⁵. GSE93077 is a series of 9 KIT mutated cases for which copy number data produced using CytoScan HD Array was available. GSE20709 is a series of 21 KIT/PDGFR and 4 SDHx mutated GIST analyzed with SNP6.0 array. Moreover, SNP6.0 array data of additional 10 KIT/PDGFR mutated GIST were used. Global copy number alteration were analyzed with Chas 3.1 and FGF3/FGF4 locus was manually checked for putative focal alterations.

2.3 | Copy number Taqman assays

Validation of FGF4 copy number state was performed on FF *quadruple* WT tumor samples, using FAM-labeled TaqMan Copy Number Assays (Thermo Fisher Scientific) targeting *FGF4* (Hs02374436_cn) and *XXRA1* (Hs03782780_cn), respectively, located in chromosome bands 11q13.3 and 11q13.4, were used on ABI Prism 7900HT platform (Applied Biosystems, Foster City, California). TaqMan RNaseP Control Reagent (VIC-labeled; Thermo Fisher Scientific) was used as internal reference control. Estimation of *FGF4* copy number was done using DDcT method in comparison with *XXRA1* and with a normal diploid sample (calibrator). All experiments were performed in triplicate on the 5 *quadruple* WT cases vs 10 KIT/PDGFR mutant GIST.

2.4 | RNA-sequencing (RNA-seq)

RNA-seq data was analyzed on the 8 *quadruple* WT GIST, 5 SDH deficient GIST and 16 KIT/PDGFR mutant samples. FF samples were analyzed as described in the previous publication.¹⁰ For FFPE samples, RNA was extracted using RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific) and cDNA libraries were synthesized from 100 ng total RNA with TruSeq RNA Exome (Illumina) according to the manufacturer's recommendations. Then, libraries were pooled and hybridized to probes specific for the enrichment of coding regions. Libraries were quality-checked and sized with Bioanalyzer 2100 (Agilent Technologies), and then quantified using a fluorimetric assay (QuantIT Picogreen assay, Thermo Fisher Scientific). Paired-end libraries were amplified and ligated to the flowcell by bridge PCR, and sequenced at 2 × 80 bp on NextSeq500 instrument (Illumina), producing an average of 50 × 10⁶ reads per sample. After FASTQ generation and trimming of low quality bases and sequencing adapters, gene expression was quantified using the tool Kallisto (<https://pachterlab.github.io/kallisto/>) adopting the Transcript per Million (TPM) normalization.

2.5 | qRT-PCR

FGF4 qRT-PCR was performed on cDNA synthesized from FF tumor samples (5 *quadruple* WT, 5 SDH deficient, and 18 KIT/PDGFR mutant GIST). cDNA was obtained with First Strand cDNA synthesis kit (Roche) and *FGF4* expression level was evaluated in using quantitative-PCR on Light Cycler 480 instrument (Roche). Fold change was evaluated using DDcT method, using *GAPDH* as housekeeping gene. Primer used were: *FGF4_Fw* 5'- CCAGCCGGTCTTCGTG-3'; *FGF4_Rev* 5'- ATCGGTGAA

GAAGGGCGAG-3'; *GAPDH_Fw* 5'-CGGGAAGCTTGTCATCAAT-3' and *GAPDH_Rev* 5'- GACTCCACGACGTACTCAGC-3'.

2.6 | Western blot

Frozen tumor samples were homogenized in RIPA buffer containing phosphatase and protease inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific) and immunoblotted. The following primary antibodies were used: FGFR1 (#9740, Cell Signaling, Leiden, the Netherlands), FGFR2 (#11835, Cell Signaling), FGF4 (PA5-52804, Thermo Fisher Scientific), phospho-AKT (#9271, Cell Signaling), Phospho-c-KIT (#3391, Cell Signaling) and β-Actin (A1978, Sigma-Aldrich, Milan, Italy).

3 | RESULTS

3.1 | FGF3/FGF4 locus is recurrently duplicated in quadruple WT GIST

High throughput copy number analysis was performed in eight *quadruple* WT GIST, highlighting the presence of a recurrent focal gain of one copy in chromosome 11q13.3 cytoband in six samples. The cryptic copy number gain detected overlapped the FGF3/FGF4 locus, and was present either in tumors with a normal disomic chromosome 11 (Sample GIST400, 401, 133, and 219) and in two other cases (GIST127 and 320), that showed complete loss of the q-arm of chromosome 11 (Figure 1A). In addition GIST127 showed a complex copy number state in the region spanning 11q with the presence of the intrachromosomal rearrangements *MARK2-PPF1A1* and *PLA2G16-ATL3*, involving genes located in close proximity of the 11q13.3 copy number gain (Figure 1B). Conversely, the two cases without 11q13.3 focal gain were known to carry putative pathogenetic alterations (Table 1), since GIST268 harboured a germline rare variant on *NF1* and a somatic frameshift deletion on *MAX* and GIST409 carried an activating mutation (p.N546K) of *FGFR1*.¹⁰

Quantitative PCR on the *FGF4* region was employed to validate the copy number gain in FF *quadruple* WT, confirming the presence of the focal duplication of *FGF4* in the four previously identified cases (GIST127, GIST133, GIST400, GIST401) while, as expected, GIST409 did not show the *FGF4* copy number gain (Figure 1C).

These findings reveal the presence of a recurrent duplication of the 11q13.3 region, encompassing *FGF3/FGF4*, in one of the two alleles at chr11 in *quadruple* WT GIST. To further assess whether 11q13.3 gain was present also in other GIST molecular subgroups, we analyzed previously produced data of 44 KIT/PDGFR/SDHx mutant GIST (9 CytoScan HD¹⁴ and 35 SNP6.0¹⁵ arrays). Among these, four cases showed alterations involving 11q (two cases with trisomy of chromosome 11 and two cases with loss of the entire q-arm), however no focal alteration involving the FGF3/FGF4 locus was detected. GIST133, a *quadruple* WT GIST, was found positive for FGF3/FGF4 gain using both CytoScan HD and SNP6.0 arrays, confirming the sensibility of both array types to detect the cryptic gain (data not shown).

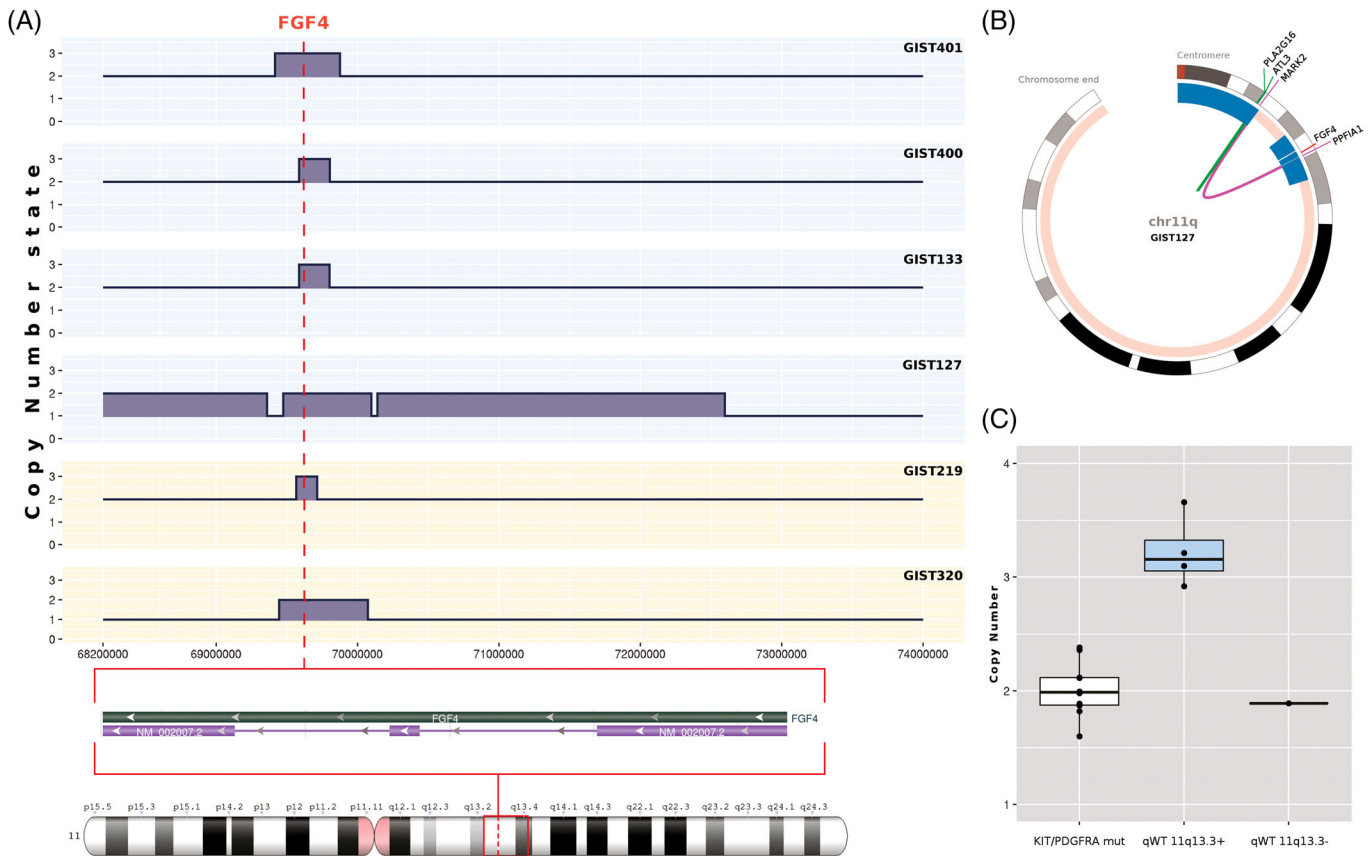


FIGURE 1 Identification of a recurrent focal gain of FGF3/FGF4 locus in quadruple WT GIST. A, Focal gain of chr11q13.3 identified in six cases (four FF light cyan background, two FFPE light yellow background) of quadruple WT GIST through high resolution copy number arrays. At the bottom of the image is showed the position of *FGF4* in the chr11 long arm while at the top a magnification on the *FGF4* region, blue boxes indicate region of copy number gain. B, Circo plot of GIST127 chromosome 11 long arm in which are showed the experimentally-validated rearrangements involving *MARK2-PPF1A1* (in purple) and *PLA2G16-ATL3* (green) that support the complexity of copy number alterations. C, Validation of *FGF4* copy number gain through qPCR using Taqman assays. Four FF *quadruple* WT GIST (in light cyan) carried the gain of *FGF4* (with an estimated copy number = 3), while one *quadruple* WT and 10 KIT/PDGFR α mutant cases were normal diploid (CN = 2; P -value < .01). *FGF4* relative copy number was evaluated in comparison with one normal diploid sample (calibrator) and *XRR1* (located on the adjacent cytoband of *FGF4*, chr11q13.4). P -value was calculated with Mann-Whitney test [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | FGF4 is highly expressed in quadruple WT GIST carrying FGF3/FGF4 duplication

To understand the pathogenic effect of FGF3/FGF4 focal copy number gain, we analyzed the expression profile of 8 *quadruple* WT GIST in comparison with 5 SDH deficient and 16 KIT/PDGFR α mutant GIST. While *FGF3* was generally lowly expressed and not significantly altered among GIST subgroups, *FGF4* was found highly expressed only in *quadruple* WT samples carrying *FGF4* gain (qWT 11q13.3+), with an average expression of 163 TPM. Interestingly, the two *quadruple* WT cases not expressing *FGF4* were those without 11q13.3 gain (GIST268 and GIST409), supporting the correlation between the gain and the overexpression of *FGF4* (Figure 2A). Conversely, *FGF4* was almost not expressed in KIT/PDGFR α mutant cases (average TPM = 0.96; P -value = .0005) and significantly lowly expressed in SDH mutant cases (average TPM = 19; P -value = .0043) with respect to qWT11q13.3+ (Figure 2A). Through qRT-PCR, *FGF4* was confirmed as poorly expressed in an additional cohort of 23 KIT/PDGFR α /SDH mutant GIST in comparison with qWT

11q13.3+, further supporting that the overexpression of *FGF4* is exclusive for *quadruple* WT cases with the focal duplication of the growth factor (Figure 2B).

Expression of *FGF4* receptors was evaluated at the mRNA level, finding two out of four FGF receptors (FGFR1 and 2) expressed in all GIST subgroups, with FGFR1 being the most expressed (FGFR1 average-TPM = 361 vs FGFR2 average-TPM = 47) (Figure 2A). At the protein level FGFR1 was confirmed as the predominant FGF receptor commonly expressed in all GIST, while the ligand *FGF4* was detected only in the *quadruple* WT subgroup (Figure 2C). GIST409, similarly to KIT-mutant GIST, did not express *FGF4* protein (Figure 2C).

We then investigated whether *FGF4* could activate its downstream signaling. Interestingly, while KIT phosphorylation was not detected in *quadruple* WT GIST, phosphorylation of AKT was detected also in *quadruple* WT GIST, suggesting the presence of a *FGF4*/FGFR1 autocrine loop that activates downstream signaling in this subgroup (Figure 2D).

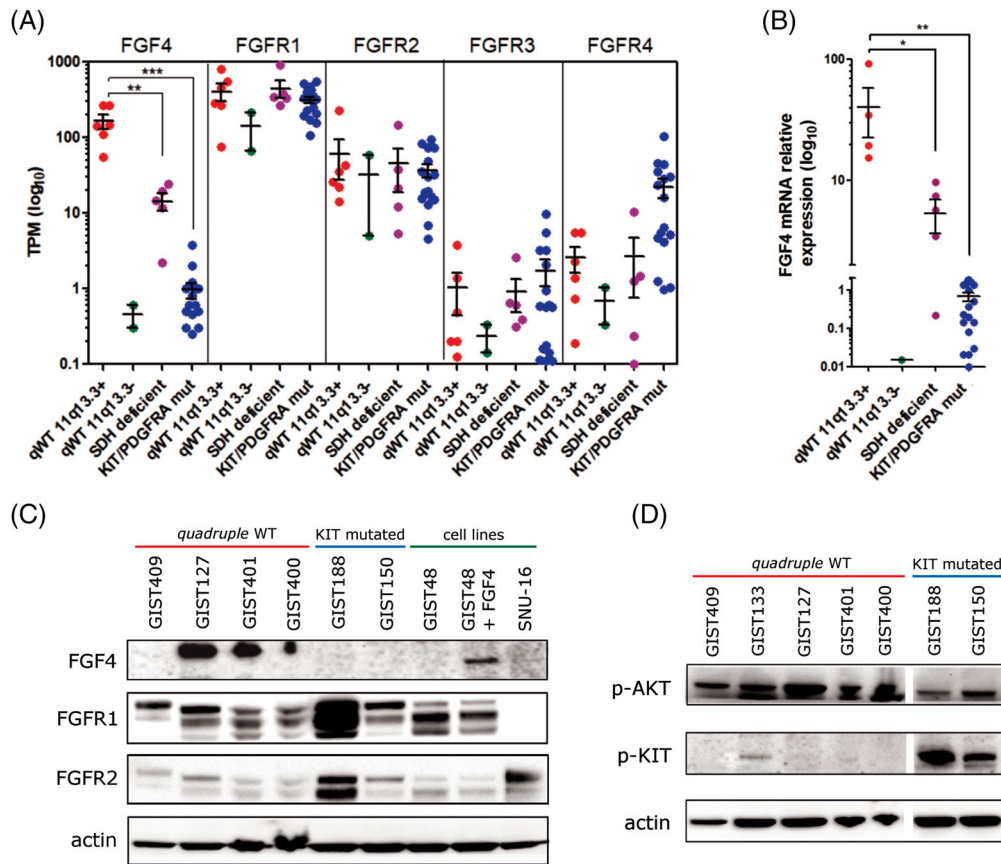


FIGURE 2 Expression of *FGF4* and FGF receptors in *quadruple WT* with respect to *KIT/PDGFR/SDH* mutant GIST. A, Log10 TPM counting, calculated from RNA-sequencing data of 8 *quadruple WT* in comparison with 5 *SDH* deficient and 16 *KIT/PDGFR* mutant GIST. *FGF4* (left panel) was significantly overexpressed in *quadruple WT* GIST carrying 11q13.3 gain. *P*-value was calculated with Mann-Whitney test (**<.01; ***<.001). On the right panel, FGF receptors mRNA expression level is shown: while *FGFR3* and *FGFR4* are lowly expressed, *FGFR1* and *FGFR2* are highly expressed and present in *quadruple WT* GIST at a levels comparable to *KIT/PDGFR/SDH* mutant cases. B, *FGF4* mRNA relative expression evaluated through qRT-PCR on FF tumor samples (5 *quadruple WT*, 5 *SDH* deficient, and 18 *KIT/PDGFR* mutant GIST). C, Western blot evaluation of protein expression level of *FGF4*, *FGFR1* and *FGFR2* in *quadruple WT* GIST. Cell lines lysates were used as positive controls: untreated GIST48 and GIST48 supplemented with 100 μ g/mL of recombinant *FGF4* protein (for *FGFR1* and *FGF4* expression) and untreated SNU-16 (for *FGFR2* expression). β -Actin was used as loading control. D, Evaluation of *KIT* and *AKT* phosphorylation level in GIST tumors. β -Actin was used as loading control [Color figure can be viewed at wileyonlinelibrary.com]

Together these findings suggest that the recurrent 11q13.3 duplication detected in *quadruple WT* is the pathogenic *driver* event, provoking the overexpression of *FGF4* and the activation of *FGFR1* downstream signaling.

4 | DISCUSSION

In the present study we discovered the presence of a recurrent focal copy number gain encompassing the *FGF3/FGF4* locus specifically in *quadruple WT* GIST, which was associated with a high expression of *FGF4*. Fibroblast growth factor 4 has a key role in maintaining the self-renewal potential of normal stem cells¹⁶ and it is usually not expressed in human adult tissues (with the exception of testis).¹⁷ It was firstly identified as an oncogene in gastric cancer and Kaposi's sarcoma^{18,19} and it has been found overexpressed in several malignancies (including germ cell tumors, ovarian cancer, hepatocellular carcinoma, and lung adenocarcinoma), generally associated with aggressiveness and

poorer prognosis.^{17,20–22} In our cases, the cryptic duplication of *FGF3/FGF4* was predominantly found in tumors with a normal disomic chromosome 11, however, it was detected also in cases showing the complete loss of one of the two copies of the q-arm of the chromosome. Both genes are located in the 11q13 cytoband, and together with *FGF19*, *EMS1*, and *CCND1* are frequently amplified in solid cancers including breast cancer, squamous cell carcinoma, esophageal cancer, bladder cancer, and hepatocellular carcinoma.^{23–27} Interestingly, through *in vitro* and *in vivo* studies in hepatocellular carcinoma, *FGF3/FGF4* focal amplification was demonstrated to be related to *FGF3* and *FGF4* overexpression and to an increased sensitivity to sorafenib.²¹

Noticeably, while *FGF4* was absent or slightly expressed in *KIT/PDGFR/SDH*-mutated GIST, it was found highly expressed in *quadruple WT* GIST, supporting a causal role of the gain in the transcriptional activation of the gene. On the other hand, no significant upregulation of *FGF3* was detected, indicating that this gene may not be the biologically-relevant pathogenic event. The mechanism for which the duplication

found in *quadruple* WT GIST leads to the re-activation of *FGF4* transcription is not clear. However, it could be hypothesized that this alteration may cause conformational or epigenetic changes able to disrupt the silencing of the gene, possibly through an escape from the epigenetic repression of *FGF4* transcription.²⁸

The novelty and interest of these findings are that, for the first time, a recurrent event shared by the majority of *quadruple* WT GIST was identified in this subgroup of disease that until now was characterized only by private and heterogeneous molecular events. Therefore, this opens a new molecular path of study in this very rare disease. Predominantly, *quadruple* WT GIST positive for *FGF4* duplication were shown to be negative for any other relevant alterations, with the exception of one case mutated in *MEN1* and one carrying *CTNND2* inactivation. On the other side, the two *quadruple* WT cases negative for *FGF4* expression showed other relevant alterations: *MAX* inactivation and a *NF1* germline variant in one case and a *FGFR1* p. N546K activating mutation in the other.¹⁰

In previous works, *quadruple* WT GIST have been shown to carry a homogeneous signature profile,^{9,10} however, no recurrent genetic alteration has been detected so far.¹³ Interestingly, among the alterations identified, three different events affecting *FGFR1* were detected: *FGFR1* p.N546K mutation^{10,11} and *FGFR1-HOOK3* and *FGFR1-TACC1* fusion genes.¹¹ These events are predicted to constitutively activate *FGFR1* and the downstream signaling pathways. A mechanism of an autocrine *FGF2/FGFR1* activation loop controlling *AKT* signaling has been identified in many cancer histotypes, including non small cell lung cancer and malignant pleural mesothelioma, in which it may be predictive of drug response.^{29,30} Intriguingly, *FGF4* is reported to be a ligand of all four *FGF* receptors, including *FGFR1*, which we have demonstrated to be highly expressed in GIST including *quadruple* WT cases. It could be hypothesized that in these *FGF4*-positive GIST, an autocrine loop between *FGF4* and *FGFR1* is present, supporting tumor growth. Indeed, we demonstrated that signaling through *AKT* is active in *quadruple* WT GIST, suggesting that, in the absence of *KIT* phosphorylation, activation of *FGFR1* through the autocrine loop could stimulate downstream signaling. Interestingly, the case harboring *FGFR1* p.N546K mutation did not show expression of *FGF4*, suggesting that only one alteration in the same pathway is necessary and sufficient to activate downstream signaling. Additional studies further investigating the role of *FGF4* overexpression in tumor growth and in *FGFR* signaling activation will be necessary to confirm these hypotheses.

Altogether, these findings support a potential involvement in disease onset of the *FGFR* pathway deregulation shared by all *quadruple* WT GIST suggesting a possible role for *FGFR* inhibitors. Evaluation of nonselective *FGFR* inhibitors (eg, regorafenib, sorafenib, ponatinib, pazopanib, dovitinib) was well known, however no information on the activity of these treatments are available specifically in *KIT/PDGFR* WT GIST.³¹ Therefore, selective *FGFR* inhibitors (eg, AZD4547, BGJ398) could be considered for the treatment of this subgroup of GIST. Recently, a cross-talk between *KIT* and *FGFR* playing an important role in imatinib resistance was reported and a clinical trial with BGJ398 in combination with imatinib was conducted in advanced

GIST.^{32,33} In our series, no patient had received treatment with *FGFR* inhibitors.

Due to the small number of cases and the clinical heterogeneity, we cannot postulate any definitive consideration regarding the prognostic or predictive significance or clinical association of *FGF3/FGF4* duplication from our series. In fact, in localized cases risk of recurrence ranged from very low to high, while the two metastatic cases did not receive regorafenib. There was no gender or age predominance in patients. The only interesting clinical data were that all *quadruple* WT cases developed GIST in the small bowel and did not present lymph nodes metastases, as these occur mostly in *SDH*-deficient GIST.

In conclusion, for the first time a recurrent event shared by *quadruple* WT GIST is reported in this study, even if these findings should be tested and confirmed in larger series. Gain of *FGF4*, together with *FGFR1* mutations, represents the most frequent molecular alteration identified in this subgroup so far, suggesting that these specific driver events could aid the diagnostic process of *KIT/PDGFR/SDH/RAS-P* WT GIST, that are currently still diagnosed only by exclusion, and, most important, that *FGFR* pathway activation could provide a rationale for targeted therapeutic approaches.

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CONFLICT OF INTEREST

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