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

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Abstract
Gastrointestinal stromal tumors (GIST) lacking mutations in 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 (~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/PDGFRA-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/PDGFRA/SDH/RAS-P WT, quadruple WT

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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.² 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 for approximately 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 SDH-, 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).

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### TABLE 1  Patients and tumor characteristics

| Patient id | Sex | Age | Site | Size (cm) | Mitotic count | Risk classification | Lymph node metastasis | Distant metastasis | Relevant mutations | Tissue Type | FGF4 gain | FGF4 expression |
|------------|-----|-----|------|----------|---------------|---------------------|----------------------|-------------------|-------------------|------------|----------|----------------|
| GIST127    | F   | 63  | Ileum| 5-10     | 6-10         | High               | No                   | Yes               | CTNND2 p.5996fs   | 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                | FGF1 p.N546K     | FF         | Negative | Negative        |
| GIST409    | M   | 45  | Ileum | 13       | <5           | High               | No                   | No                | MEN1 p.L83fs      | FFPE       | Positive | High           |
| GIST320    | M   | 73  | Ileum | 8.5      | 2            | Intermediate       | No                   | No                | NF1 p.R2573L      | FFPE       | Negative | Negative        |
| GIST219    | M   | 44  | Duodenum| 6.5     | >5           | High               | No                   | Yes               | Unknown           | FFPE       | Positive | High           |
| GIST268    | M   | 50  | Ileum | 8.5      | 2            | Intermediate       | No                   | No                | NF1 p.R2573L      | FFPE       | Negative | Negative        |

*Exome 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 al. and GSE20709 from Astolfi et al. 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/PDGFRα and 4 SDHx mutated GIST analyzed with SNP6.0 array. Moreover, SNP6.0 array data of additional 10 KIT/PDGFRα 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 XRRA1 (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 XRRA1 and with a normal diploid sample (calibrator). All experiments were performed in triplicate on the 5 quadruple WT cases vs 10 KIT/PDGFRα 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/PDGFRα mutant samples. FF samples were analyzed as described in the previous publication. 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 x 80 bp on NextSeq500 instrument (Illumina), producing an average of 50 x 10^6 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/PDGFRα 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’- CCAGCCCGTTCTCTGTG-3’; FGF4_Rev 5’- ATCGGTGAA GAAGGGCGGAG-3’; GAPDH_Fw 5’-CGGAAGCTTGTCATCAAT-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 (#83391, 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 through put 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-PPFIA1 and PL2G16-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. 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/PDGFRα/SDHx mutant GIST (9 CytoScan HD and 35 SNP6.0 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).
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/PDGFRα 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/PDGFRα 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/PDGFRα/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).
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 cells and it is usually not expressed in human adult tissues (with the exception of testis). It was firstly identified as an oncogene in gastric cancer and Kaposi’s sarcoma 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. 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. 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.

Noteworthily, while FGF4 was absent or slightly expressed in KIT/PDGFRA/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 CTNNND2 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,⁷,¹⁰ 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,¹⁰¹¹ 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/PDGFRα 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/PDGFRα/SDH1/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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