Multiple Primary Sporadic Gastrointestinal Stromal Tumors in the Adult: An Underestimated Entity

Daniela Gasparotto, Sabrina Rossi, Italo Bearzi, et al.

Gastrointestinal stromal tumors (GIST) are mesenchymal tumors most often originating in the muscular wall of the gastrointestinal tract. The estimated annual incidence is 12 to 14 per million, as recently reported in two European studies (1, 2). Stomach and small intestine are the most commonly affected anatomic sites, accounting for about two thirds and one third of the cases, respectively (3–5). Rare cases of primary GISTs originating outside the gastrointestinal tract have also been reported, although the actual existence of extragastrointestinal GISTs is still debated (4, 6–9).

GISTs are considered KIT signaling-driven mesenchymal tumors. In fact, a distinctive feature of GISTs is the expression of the KIT protein (CD117 antigen), which is immunohistochemically detectable in ~95% of the cases. Accordingly, the presence of activating mutations (missense mutations or small in-frame deletions) of the KIT gene represents the most frequent genetic aberration in GIST (10). Moreover, transgenic mice carrying KIT germ-line mutations develop hyperplasia of interstitial Cajal cells and GISTs (11, 12). Less frequent, and mutually exclusive, is the detection of activating mutation of the PDGFRα gene (13).

Although most GIST patients present with localized disease, ~10% are metastatic at the diagnosis with peritoneum and/or liver being the most common sites of dissemination (1). GISTs are generally considered solitary tumors and the occurrence of multiple primary neoplasms is considered an exceptional event, restricted to familial GISTs (14–19), pediatric forms (20), or distinct syndromes such as type 1 neurofibromatosis (NF1; refs. 21–23) or Carney's syndrome (24). All these are well-defined entities that can be easily distinguished from common sporadic GISTs based on their peculiar clinicopathologic features. Moreover, tumors in these cases generally develop at early age
Translational Relevance

This study reports that a considerable fraction of adult sporadic gastrointestinal stromal tumor (GIST) patients with multifocal manifestations are actually affected by multiple primary GISTs. This finding supports the possibility that widespread tumor priming of GIST precursor mesenchymal cells may be implicated in these patients. The existence of tumor multiplicity in the context of adult GIST suggests that, in the presence of multifocal presentation, molecular assessment of clonal relationship of the different tumor lesions should be done for proper patient staging and planning of therapy.

and either carry germ-line KIT or PDGFRA mutations (familial GISTs) or are devoid of KIT/PDGFRA mutations (6, 25, 26). Beyond these well-defined situations, the detection of multifocal disease, irrespective of the number, size, and location of the lesions, is commonly viewed as the result of the metastatic dissemination of a single primary GIST. Based on this axiom, patients with multifocal GISTs are by default classified as advanced stage and treated as such.

This paradigm has been recently challenged by two articles, which suggested the existence of sporadic multiple primary GISTs (MPG) in adult patients. These articles reported one and four cases, respectively, in which, beside a major mass, one to three additional GIST lesions, apparently independent one to the others because of different molecular and pathologic features, could be identified (26, 27).

The recognition of tumor multiplicity poses obvious problems of diagnosis and patient staging. To shed light on the actual relevance of these phenomena in the context of sporadic adult GIST, 442 consecutive cases collected in three Italian institutions were retrieved and 26 patients presenting at the adult GIST, 442 consecutive cases collected in three Italian institutions (General Hospital of Treviso, Ancona University Hospital, and San Raffaele Institute Milan). About 6% of these patients (26 cases) presented at pathologic diagnosis with tumor multifocality and, accordingly, were clinically diagnosed as advanced disease. Among these, to molecularly characterize the full spectrum of tumor lesions surgically removed from each patient, we sought to restrict our study to those cases that presented at diagnosis with a limited number of distinct nodules (arbitrarily set up to 3), irrespective of their location (gastrointestinal tract or peritoneum/omentum) and size. Five of these 26 patients met our criteria for enrollment (Table 1, cases G1-G5).

A second set (“selected set”), represented by five additional cases matching our inclusion criteria (G6-G10), were contributed by centers with specific expertise in diagnosis and treatment of GIST (Brigham and Women’s Hospital and Leuven Catholic University). All patients were adult individuals (ages 48-85), with no clinicopathologic signs of NF1 (28) or familial history for GIST, NF1, or Carney’s syndromes.

No patients had received radiotherapy/chemotherapy or imatinib before surgery. Five of 10 patients (G3, G4, G6, G8, and G9) received imatinib treatment right after surgery. The follow-up of these patients ranges from 16 to 50 mo, with no evidence of disease (G4, G6, G8, and G9) or death from disease (G3). One patient (G1) started imatinib after having developed liver metastases, but because of unacceptable toxicity, the patient was subsequently put under sunitinib treatment, which induced stabilization of the disease (follow-up, 34 mo). Three patients

**Table 1. Clinicopathologic characteristics and mutation pattern**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>Tumor pairs</th>
<th>Localization</th>
<th>Size (cm)</th>
<th>Cytomorphology</th>
<th>Mitotic count (50 HPFs)</th>
<th>Risk category</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>85/M</td>
<td>G1-a</td>
<td>Stomach</td>
<td>6.2</td>
<td>Spindle</td>
<td>2</td>
<td>Intermed</td>
<td>KIT p.I478fsX2 + W557G</td>
</tr>
<tr>
<td>G2</td>
<td>71/M</td>
<td>G2-a</td>
<td>Stomach</td>
<td>3.0</td>
<td>Spindle</td>
<td>1</td>
<td>Low</td>
<td>KIT p.V560D</td>
</tr>
<tr>
<td>G3</td>
<td>74/M</td>
<td>G3-a</td>
<td>Small intestine</td>
<td>8.0</td>
<td>Spindle</td>
<td>35</td>
<td>High</td>
<td>KIT p.A502_Y503dup</td>
</tr>
<tr>
<td>G5</td>
<td>59/F</td>
<td>G5-a</td>
<td>Stomach</td>
<td>16.0</td>
<td>Mixed</td>
<td>6</td>
<td>High</td>
<td>PDGFRA p.V561D</td>
</tr>
<tr>
<td>G6</td>
<td>69/F</td>
<td>G6-a</td>
<td>Small intestine</td>
<td>1.9</td>
<td>Spindle</td>
<td>1</td>
<td>Very low</td>
<td>KIT p.V559del</td>
</tr>
<tr>
<td>G7</td>
<td>83/F</td>
<td>G7-a</td>
<td>Stomach</td>
<td>14.0</td>
<td>Spindle</td>
<td>1</td>
<td>Low</td>
<td>KIT p.V559G</td>
</tr>
<tr>
<td>G8</td>
<td>53/F</td>
<td>G8-a</td>
<td>Small intestine</td>
<td>6.0</td>
<td>Spindle</td>
<td>30</td>
<td>Very low</td>
<td>KIT p.LS76P</td>
</tr>
<tr>
<td>G9</td>
<td>81/M</td>
<td>G9-a</td>
<td>Small intestine</td>
<td>4.5</td>
<td>Spindle</td>
<td>4</td>
<td>Low</td>
<td>KIT p.V559D</td>
</tr>
<tr>
<td>G10</td>
<td>68/M</td>
<td>G10-a</td>
<td>Stomach</td>
<td>0.6</td>
<td>Spindle</td>
<td>0</td>
<td>Very low</td>
<td>No mutation</td>
</tr>
</tbody>
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(G2, G7, and G10) did not receive imatinib therapy, and only partial follow-up was available for these patients. Patient G2 and G10 were still disease-free 24 and 36 mo after surgery, respectively, whereas patient G7 was in progression 45 mo after surgery. For one patient (G5), no information on the follow-up was available.

**Histopathologic diagnosis and immunohistochemistry.** For all the cases included in the study, formalin-fixed, paraffin-embedded tumor and matched nonpathologic surrounding tissues were available.

Diagnosis of GIST was reconfirmed for all cases based on the combination of histologic evaluation and CD117 immunopositivity. Mitoses were counted in 50 consecutive high-power fields (HPF) for each sample and mitotic index was defined as low for ≤ 5 mitoses/50 HPF, intermediate for >5 and ≤10 mitoses/50 HPF, and high for >10 mitoses/50 HPF. The risk category was assessed according to the 2002 NIH classification (Table 1).

Tumor sections were immunostained for CD117. In five cases (G1-G5), additional sections were also immunostained for DOG1, CD34, smooth muscle actin, S100, and desmin. Where required, antigen retrieval was done using a 30'-microwave oven (MW) pretreatment (750 W) in 10 mmol/L citrate buffer at the indicated pH. All immunostainings were done by an automated immunostainer (Dako Autostainer, DakoCytomation) using the following primary antibodies: CD117 (1:100, no antigen retrieval, polyclonal; DakoCyto- tmation), CD34 (1:50, no antigen retrieval, clone QBEnd/10; DakoCyto- tmation), DOG1 (1:1,000, pH 6 MW, polyclonal; Neo-Markers), smooth muscle actin (prediluted, no antigen retrieval, clone 1A4; NeoMarkers), S100 (1:8,000, Pronase, polyclonal; DakoCyto- tmation), and desmin (prediluted, pH 6 MW, clone D33; NeoMarkers). Standardized 3,3'-diaminobenzidine development times allowed accurate comparison of all samples. Substitution of the primary antibody with PBS served as a negative control.

**Molecular analysis.** DNA was extracted from formalin-fixed/paraffin-embedded tissues of both tumor and surrounding nonpathologic tissues. Several 10-μm-thick sections were deparaffinized by serial xylene/ethanol washings. DNA extraction was done using the EZI Biorobot (Qiagen GmbH). Exons 9, 10, 11, 13, 14, and 17 and intron 10 of KIT gene and PDGFRA exons 12 and 18 were amplified by PCR and both strands were sequenced using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). PCR conditions were as follows: an initial denaturation step at 95°C for 3 min followed by 42 cycles at 95°C/30 s, 58°C/1 min, and 72°C/1 min. The primers used were the following: KIT ex9fw, TTTCCTAGAATAGACCGG; KIT ex9rev, ACAGAGCCTAAATACCCCT; KIT ex10fw, GTGGAACGCCTGACCTC- CAC; KIT ex10rev, TGGTTCATATGCTCTCACCAC; KIT ex11fw, TCTCTCTCAGAGTCTGCTTAC; KIT ex11rev, AAGGAGGC- CACTGAGAACC; KIT ex13fw, TGGATCTGACCTGACATC; KIT ex13rev, AGAGCCGCGACGGCCT; KIT ex14fw, GCCAGC- CACTGGAAGCT; KIT ex14rev, ACCCCGATGAGCTCGTGTC; KIT ex17fw, TGGCTTTCTTTTCTCTCCACC; KIT ex17rev, GCAG- GACTGTAACAGAGGA; PDGFRA ex12fw, TCCAGCTGACTGCTG- GCTT; PDGFRA ex12rev, GACACGAAAAGGGGCTT; PDGFRA ex18fw, TCAGCTCAGATGCTGTAGCT; and PDGFRA ex18rev, TGAAGGAGTACCGTCC.
truncation. In particular, tumor G1-a, beside a missense mutation at codon 557, carried a 29-nucleotide deletion in exon 9, which introduced a premature stop codon (Fig. 1). Similarly, in case G4, both tumor G4-a and G4-b displayed a point mutation in exon 11, but in the large intestinal mass (tumor G4-a), part of the tumor population gained a further KIT alteration in exon 14, resulting in a stop codon at residue 719. These specific KIT alterations have never been reported before. Frameshift/stop codon mutations have been rarely described in GISTs, but, intriguingly, both in previously reported (32–34) and in our series, these mutations occurred as a secondary event after a typical KIT mutation. Thus, as recently suggested (31, 35), protein truncation may represent a mechanism for tumor progression aimed at reducing the tumor to homozygosity for the activating mutation.

Microsatellite analysis. Microsatellite allelic imbalance proved highly informative for tracking tumor-specific chromosome losses. To corroborate our conclusions on the clonal relationship of paired tumors based on KIT/PDGFRA mutation pattern, some cases were further analyzed for loss of heterozygosity at microsatellite loci that are frequently involved in GISTs, but, intriguingly, both in previously reported (32–34) and in our series, these mutations occurred as a secondary event after a typical KIT mutation. Thus, as recently suggested (31, 35), protein truncation may represent a mechanism for tumor progression aimed at reducing the tumor to homozygosity for the activating mutation.

Microsatellite patterns. Loss of heterozygosity at microsatellite loci frequently involved in GISTs was assessed in all tumors. Overall, KIT/PDGFRA and microsatellite analyses confirmed the metastatic nature of tumor multifocality in cases G3, G4, and G5. In contrast, the presence of different genetic lesions in paired tumors of cases G1, G2, G6, G7, G8, and G9 supported the notion of tumor multiplicity.

Comparative clinicopathologic and mutational evaluations. Overall, KIT/PDGFRA and microsatellite analyses confirmed the metastatic nature of tumor multifocality in cases G3, G4, and G5. In contrast, the presence of different genetic lesions in paired tumors of cases G1, G2, G6, G7, G8, and G9 supported the notion of tumor multiplicity.

We then analyzed the clinicopathologic and immunophenotypic features of the tumors within these two categories, in search for variables that could readily predict the nature of distinct lesions.

In the category of truly metastatic tumors (G3–G5), the two masses were markedly different in size (one at least four times larger than the other). Moreover, the matched lesions showed...
the same morphology and the mitotic index was concordant (intermediate or high). These data seem an argument for the metastatic nature of the peritoneal mass.

Nevertheless, also in the category of MPGs, cases in which one tumor was significantly larger than the other could be identified (see for instance cases G7 and G8), cautioning on the adoption of tumor size comparison as a suitable variable to establish clonal relationship. Moreover, although discordant morphology could be observed among MPGs, also primary tumors of independent origin could share, similar to metastatic tumors, the same morphology (see for instance cases G6, G7, and G8), arguing against the use of tumor cell shape as a discriminating factor for metastasis versus second primary tumor. Rather, because the mitotic index of most of the tumors that turned out to be second primary GISTs was concordantly low or markedly different for the paired lesions (G8), in the presence of this evidence, tumor multiplicity should be considered.

Discussion

The majority of GISTs are sporadic and tumor multiplicity is considered an exceptional finding limited to specific conditions: MPGs may be observed in pediatric patients or in individuals affected by hereditary GIST, NF1, or paraganglioma/sarcoma and Carney’s triad syndromes (5, 14–24). Beyond these entities, the occurrence of multiple distinct tumors is conventionally interpreted as indicative of metastatic spread from a primary lesion.

In contrast with this common view, two recent studies revealed the existence of the phenomenon of tumor multiplicity in the context of adult sporadic GISTs. Considering that KIT or PDGFRA mutations are frequent, occur early during GIST development, and are relatively polymorphic, Kang and coworkers (27) used these genes as markers of tumor clonality and screened their own surgical pathology files for GIST patients who presented with at least two tumors in the gastrointestinal tract (stomach and small intestine). Molecular analysis confirmed metastatic dissemination in four of the five selected cases, whereas one patient turned out to be affected by two independent jejunal GISTs. Similarly, Haller and collaborators (26) analyzed four cases of gastric GIST in which, during surgical or pathologic examination, one to three small additional tumor lesions were incidentally discovered in the proximity of the primary lesion. A different KIT or PDGFRA mutation pattern was found in the different paired lesions, indicating that these patients actually developed multiple primary gastric GISTs.

Both articles described the existence of MPGs within the same organ, either stomach or small intestine. No cases of multiple GISTs affecting distant gastrointestinal structures have been reported thus far. Moreover, although still debated, also the peritoneum has been suggested to be a site of origin of primary GIST (7, 9). Thus, whether peritoneal nodules may represent multiple GISTs is still unknown.

With the intent of shedding light on these issues and providing an assessment of the significance of tumor multiplicity in the context of sporadic adult GIST, we sought to screen a series of 442 consecutive cases collected by three Italian institutions. Twenty-six patients presented at diagnosis with apparently disseminated disease. We sought to analyze only those cases with a limited number of distinct GIST nodules (up to three). Five of these 26 cases met these established inclusion criteria. Five further cases with similar features were contributed from collaborating institutions.

Fig. 2. Molecular characterization of case G6. A different KIT mutation pattern was found in the two intestinal lesions of case G6. A, tumor G6-a carried a three-nucleotide deletion at exon 11, resulting in the loss of codon 559. B, instead, tumor G6-b carried a valine to glycine substitution at the same codon. A different microsatellite pattern further supported the independent origin of the two lesions. C, tumor G6-a showed loss of the larger allele at D1S449 locus, whereas G6-b lost the shorter allele. D, similarly, G6-a was deleted for the shorter allele at D1S435 locus, whereas G6-b displayed loss of the larger one. Arrow, allelic losses.
In contrast with two previous reports, in only 4 of 10 patients did the paired neoplasms occur in the same organ. In the remaining six cases, the matched lesions involved the stomach and the small intestine, the stomach and the peritoneum, or the small intestine and the peritoneum.

A combined KIT/PDGFRA and microsatellite analysis supported a metastatic nature of the secondary neoplasm in three patients. In all these cases, the secondary lesion was located in the peritoneum or omentum and was much smaller than the primary one (four to eight times). In one case, because of the lack of KIT or PDGFRA mutations, we were unable to assess the clonal relationships between the matched lesions by this means.

Instead, in six cases, a different KIT mutation pattern was observed in the two matched lesions, indicating an independent origin for these tumors. The lack of relation between the two masses was further corroborated by a divergent microsatellite configuration. In most cases, the two lesions had comparable size, but in one case, the diameter of the two masses differed significantly. In two patients, the synchronous tumors affected different organs: stomach and small intestine, and intestine and peritoneum. This latter case is particularly interesting because it not only supports the existence of primary GIST of the peritoneum but also calls into question the concept of considering peritoneal localizations as metastatic a priori.

A focused analysis of the consecutive set of cases allows us some epidemiologic considerations. Although the limited number of patients analyzed prevents us to draw any definitive conclusion on the prevalence of tumor multiplicity in the context of adult, nonsyndromic GISTs, the fact that two of five patients clinically diagnosed as advanced disease were in fact affected by MPGs is quite impressive. In addition, because we sought to focus our analysis on carriers of just two to three distinct GIST nodules, it cannot be ruled out that MPGs may also affect patients with more disseminated localizations as well as patients who develop metachronous lesions/recurrences.

From a biological standpoint, the finding of GIST patients with tumor multiplicity suggests that, in these subjects, GIST precursors (Cajal cells) or pluripotent mesenchymal stem cells may be somehow primed toward tumorigenic conversion. This may recall the phenomenon of “field cancerization,” reported for the mucosa of the upper respiratory tract (37). In agreement with this hypothesis, Agaimy and coworkers (38) have recently reported that multiple minute (≈4 mm), hyalinizing spindle cell lesions are a common finding in the stomach of adult individuals (≈20%) and that these nodules, named GIST “tumorlets,” are CD117 positive and carry KIT/PDGFRA gene mutations. The authors suggest that GIST tumorlets represent early mesenchymal cell lesions that may eventually, under endogenous or exogenous stimuli, evolve into clinically overt GIST. Indeed, microscopic hyperplastic areas of CD117-positive spindle cells are often detected in the proximity of sporadic GISTs (39, 40) and multifocal hyperplasia of Cajal cells is typical of familial GIST, Carney’s triad, NF1 patients, and mouse GIST models (11, 12, 21, 22, 25, 41–46).

Table 2. Molecular assessment of clonal relationship

<table>
<thead>
<tr>
<th>Case</th>
<th>KIT/PDGFRA mutation pattern (comparison of the paired GIST lesions)</th>
<th>Microsatellite pattern (loss of different alleles/total informative markers)</th>
<th>Concordant mutations test (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Different</td>
<td>2/6</td>
<td>1.0000</td>
</tr>
<tr>
<td>G2</td>
<td>Different</td>
<td>1/3</td>
<td>1.0000</td>
</tr>
<tr>
<td>G3</td>
<td>Same</td>
<td>0/4</td>
<td>0.0417</td>
</tr>
<tr>
<td>G4</td>
<td>Same background + additional alteration</td>
<td>0/6</td>
<td>0.0357</td>
</tr>
<tr>
<td>G5</td>
<td>Same</td>
<td>0/4</td>
<td>0.2500</td>
</tr>
<tr>
<td>G6</td>
<td>Different</td>
<td>2/4</td>
<td>1.0000</td>
</tr>
<tr>
<td>G7</td>
<td>Different</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G8</td>
<td>Different</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G9</td>
<td>Different</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G10</td>
<td>No mutation</td>
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</tr>
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</table>

In summary, our study points out that a significant fraction of adult sporadic GIST patients with multifocal manifestations are actually affected by MPGs. This finding supports the possibility that widespread priming of GIST precursor mesenchymal cells, similar to the “field cancerization” described for the mucosa of the aerodigestive tract, may be implicated in these patients. The biological basis of such a phenomenon and the impact of tumor multiplicity in GIST epidemiology will require further investigation. Nevertheless, the existence of tumor multiplicity in the context of adult GIST suggests that, in the presence of multifocal presentation, an accurate molecular characterization of the different tumor localizations should be taken into account for proper patient staging and planning of therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
References


