Clinical Cancer Research

Quadruple-Negative GIST Is a Sentinel for Unrecognized Neurofibromatosis Type 1 Syndrome

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Abstract

Purpose: The majority of gastrointestinal stromal tumors (GIST) are driven by *KIT*, *PDGFRA*, or, less commonly, *BRAF* mutations, and *SDH* gene inactivation is involved in a limited fraction of gastric lesions. However, about 10% of GISTs are devoid of any of such alterations and are poorly responsive to standard treatments. This study aims to shed light on the molecular drivers of quadruple-negative GISTs.

Experimental Design: Twenty-two sporadic quadruple-negative GISTs with no prior association with Neurofibromatosis Type 1 syndrome were molecularly profiled for a panel of genes belonging to tyrosine kinase pathways or previously implicated in GISTs. For comparison purposes, 24 GISTs carrying *KIT*, *PDGFRA*, or *SDH* gene mutations were also analyzed. Molecular findings were correlated to clinicopathologic features.

Results: Most quadruple-negative GISTs featured intestinal localization, with a female predilection. About 60% (13/22) of quadruple-negative tumors carried *NF1* pathogenic mutations, often associated with biallelic inactivation. The analysis of normal tissues, available in 11 cases, indicated the constitutional nature of the *NF1* mutation in 7 of 11 cases, unveiling an unrecognized Neurofibromatosis Type 1 syndromic condition. Multifocality and a multinodular pattern of growth were common findings in *NF1*-mutated quadruple-negative GISTs.

Conclusions: *NF1* gene mutations are frequent in quadruple-negative GISTs and are often constitutional, indicating that a significant fraction of patients with apparently sporadic quadruple-negative GISTs are affected by unrecognized Neurofibromatosis Type 1 syndrome. Hence, a diagnosis of quadruple-negative GIST, especially if multifocal or with a multinodular growth pattern and a nongastric location, should alert the clinician to a possible Neurofibromatosis Type 1 syndromic condition. *Clin Cancer Res; 23(1); 273–82. ©2016 AACR.*

Introduction

Gastrointestinal stromal tumors (GISTs) are the most frequent mesenchymal neoplasm of the digestive tract, with an incidence of around 1.5 per 100,000/year. GISTs are thought to arise from the interstitial Cajal cells and are typically considered to be KIT/PDGFRA-driven tumors (1). In fact, about 85% of sporadic GISTs are characterized by activating mutations of either *KIT* or *PDGFRA* tyrosine kinase receptor genes, which account for their sensitivity to the kinase inhibitor imatinib. *KIT* and *PDGFRA* mutations result in constitutive activation of the RAS–RAF–MAPK pathway. In about 1% of *KIT*/*PDGFRA* wild-type cases, the same pathway is activated as a result of *BRAF* mutations (1, 2), and we have recently reported the involvement of the *ETV6-NTRK3* gene fusion (3). About 15% of sporadic GISTs are devoid of *KIT*/*PDGFRA*/*BRAF* mutations and are sometimes referred to as triple-negative GISTs. Triple-negative GISTs can be observed in the context of rare hereditary syndromes, including succinate dehydrogenase (SDH) protein complex-related syndromes (4), and, although not comprised in the diagnostic criteria, also in the context of Neurofibromatosis Type 1 (NF-Type 1; refs. 5, 6). Recent studies indicate that SDH-deficient GISTs represent about one third of triple-negative GISTs (7). SDH-associated GISTs are typically gastric, often multifocal, and affect young people, especially females (1, 7–9). They frequently arise in the context of the Carney–Stratakis Syndrome (GIST and paraganglioma dyad), characterized by germline inactivating mutations in any of the four genes encoding the SDH complex (*SDHA-D*; refs. 10, 11), or in the Carney Triad (GIST, paraganglioma, chondroma), associated with *SDHC* promoter hypermethylation (12, 13).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-16-0152

Translational Relevance

About 10% of gastrointestinal stromal tumors (GIST) are devoid of canonical *KIT*, *PDGFRA*, *BRAF*, or *SDH* mutations. These quadruple-negative GISTs, currently orphans of driver alterations, are poorly responsive to standard treatments. The lack of knowledge on their genetics prevents the implementation of targeted treatments. This work demonstrates that a significant fraction of apparently sporadic quadruple-negative GISTs arise in the context of unrecognized Neurofibromatosis Type 1 (NF-Type 1) syndrome. About 60% of the quadruple-negative GISTs analyzed bore pathogenic mutations of the *NF1* gene. A relevant proportion of these mutations were constitutional. *NF1*-mutated GISTs featured distinctive clinicopathologic characteristics. Thus, a diagnosis of a quadruple-negative GIST, especially if multifocal or with a multinodular growth pattern and a nongastric location, should alert the clinician to a possible NF-Type 1 syndromic condition. These patients should be referred to specialists for definitive individual and familial risk assessment.

In the remaining two third of triple-negative sporadic GISTs, hereafter referred to as "quadruple-negative" GISTs (KIT/PDGFRA/BRAF mutation-negative/SDH-proficient), no oncogenic driver alteration has been yet identified. Quadruple-negative GISTs represent an ill-defined and likely heterogeneous category of tumors which overall respond poorly to standard treatments (1). Hence, a better molecular characterization of these neoplasms, which account for about 10% of all GISTs, could pave the way to novel therapeutic avenues.

To shed light on this issue, we sought to investigate a series of 22 quadruple-negative sporadic GISTs arisen in patients with no prior association to NF-Type 1 (no diagnosis or recorded clinical manifestations of NF-Type 1 nor familial history for the disease).

The mutational status of a panel of genes either belonging to the receptor tyrosine kinase pathway or previously associated with GIST or GIST-including syndromic conditions was analyzed. For comparison purposes, 24 consecutive GISTs carrying mutations in either *KIT*, *PDGFRA*, or *SDH* (*KIT*/*PDGFRA*/*SDH*^{m}) were also profiled.

Intriguingly, 13 of 22 (59%) quadruple-negative GISTs analyzed turned out to carry pathogenic *NF1* gene mutations; in 7 of the 11 cases for which normal matched DNA was available, we were able to ascertain the constitutional nature of the alteration. These results indicate that NF1 plays a relevant role in the pathogenesis of quadruple-negative GISTs, and, more importantly, a significant fraction of apparently "sporadic" quadruple-negative GISTs actually arise in undiagnosed NF-Type 1 patients.

Materials and Methods

Patients population and result communication

The series consisted of 22 GIST negative for *KIT*/*PDGFRA*/*BRAF*/*SDH* mutations (quadruple-negative GIST) submitted as sporadic cases by the referral clinicians. Fifteen of these tumors were consultation cases of one of the authors (A.P. Dei Tos), who is the reference pathologist for the Italian Sarcoma Group and for the Italian Rare Cancer Network. For comparison purposes, 24 consecutive sporadic GISTs carrying mutations in KIT, PDGFRA, or SDH were also analyzed (*KIT*/*PDGFRA*/*SDH*^{m} group). The study was approved by the Institutional Review Boards, and informed consent was provided by all living patients.

GIST diagnosis was based on morphology and CD117 expression as assessed by immunohistochemistry (IHC). Site, size, and mitotic count per 5 mm^2 were recorded in most cases. Multifocality, tumor growth pattern (multinodular with fibromuscular septa vs. a single expansive nodule), cell type, degree of cellularity, and presence of skenoid fibers were recorded in all quadruple-negative GISTs.

KIT, *PDGFRA*, *BRAF* mutational status was originally determined by Sanger sequencing. In *KIT*/*PDGFRA*/*BRAF*-negative cases, SDH deficiency was assessed by IHC for SDHB, followed by sequencing. Of the 24 GISTs of the *KIT*/*PDGFRA*/*SDH*^{m} group, 16 cases harbored mutations in KIT, 3 in PDGFRA, 2 in SDHA, and 2 in SDHB; one case (# 46) had a concomitant KIT and SDHA mutation. No tumor included in the study carried BRAF mutations.

Demographic and pathologic features of the series are summarized in Supplementary Table S1. Clinical data, including indications of syndromic conditions, concurrence of other diseases, familial history, and follow-up information, were obtained from review of medical records and interview of referring physicians. No patient with familial history or genetic diagnosis of NF-Type1, or recorded clinical manifestations diagnostic of NF-Type 1 (6, 14), was included in the study. Pediatric GISTs were also excluded from the study.

The results of the mutation screening were returned to the referring clinician. In the presence of ascertained constitutional mutations, namely *NF1* constitutional mutations, living patients were referred to a NF-Type 1 expert. The specialist, according to international guidelines (14–16), integrated molecular data with patient's clinical findings and family history, and informed the patient about the characteristics of the disease and the probability of transmission to the offspring. Further tests and checkups were eventually prescribed, as appropriate.

Massive parallel sequencing and mutation validation

DNA from frozen or formalin-fixed paraffin-embedded (FFPE) tumors and matched normal samples was extracted using the EZ1 biorobot (QIAGEN) or the QIAamp DNA FFPE Tissue Kit (QIAGEN). Massive parallel sequencing (MPS) libraries were prepared with a TruSeq Custom Amplicon v1.5 panel (Illumina) targeting the coding sequence and a 5-nt flanking intronic region of the following genes: *KIT*, *PDGFRA*, *BRAF*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *HRAS*, *KRAS*, *NRAS*, *NF1*, *NF2*, *HIF1A*, *PTEN*, *RAF1*, *RUNX1*, *SMARCB1*, *VHL*, *CDKN2A*, *PIK3CA*, *RB1*, *SPRED1*, and *TP53*.

MPS libraries were sequenced on the MiSeq platform (Illumina) using a v3 kit 2 × 150 cycles. Data were analyzed with the Miseq Reporter software v2.5, using the custom amplicon workflow and somatic variant caller. Mean amplicon coverage was 4367. Variants were analyzed with VariantStudio software (Illumina) and filtered with 100x coverage threshold, and allelic fraction was ≥20%. Neutral population polymorphisms, as well as pseudogene sequences, were filtered out.

In the case of the *NF1* gene, regions with low coverage were also PCR-amplified and sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Mutations detected by MPS in

tumor DNA were validated by PCR-sequencing; where available, matched normal DNA was also analyzed. PCRs were optimized to avoid amplification of pseudogenes.

To assess *NF1* copy-number variations, the MPS depth obtained for a given amplicon was compared with the mean sequencing depth obtained in the other samples of the run for the same amplicon, normalized against the sequencing efficiency of the test sample. Multiplex ligation-dependent probe amplification (MLPA NF1 probemix P081 and P082; MRC Holland) was used to validate the deletions detected in cases # 5 and 19. In case # 5, carrying a large *NF1* deletion, the copy-number loss was also validated by qPCR. To this end, DNAs extracted from FFPE normal specimens from 5 healthy individuals were used as reference. Amplicons were centered on *NF1* exon 14, comprised in the deletion, and exon 53, external to the deletion. *ADA*, *KIT*, *RET*, and *IGF1R* were used as internal normalization controls. Primers and PCR conditions are provided in Supplementary Table S2. Reactions were performed in triplicate with SsoFast EvaGreen Supermix (BioRad) on a CFX96 Real-Time System (Bio-Rad). Relative levels were normalized to the geometric average of the four control genes by the comparative Ct ($\Delta\Delta$Ct) method using the Bio-Rad CFX manager software. Reduction of the signal greater than 80% was considered indicative of homozygous deletion; reduction of the signal to about 50% was considered as suggestive of loss of one allele.

The expression of *NF1*-mutated alleles was investigated on the cDNA of 5 GISTs for which RNA was available (cases # 8, 9, 13, 28, and 38). RNA extraction and cDNA synthesis were as previously described (3).

The Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk) was interrogated to assess for previous association with NF-Type 1 syndrome of the identified mutations. Moreover, five different prediction algorithms, Provean (http://provean.jcvi.org; ref. 17), SIFT (http://sift.bii.a-star.edu.sg; ref. 18), Polyphen2 (genetics.bwh.harvard.edu/pph2; ref. 19), MutationAssessor (http://mutationassessor.org/; ref. 20), and PaPI (http://papi.unipv.it; ref. 21), were used to predict the effect of coding nonsynonymous variants. The Human Splicing Finder software (http://www.umd.be/HSF3/) was used to evaluate splice site mutations (22).

Statistical analyses

Categorical variables were compared by the Fisher two-tailed exact test. Continuous data were compared between groups by the unpaired Student *t* test.

Results

Molecular findings

The series of 46 sporadic GISTs analyzed in this study included 22 quadruple-negative GISTs and 24 tumors belonging to the *KIT/PDGFRA/SDH*^{m} group. MPS analysis confirmed the mutations in *KIT*, *PDGFRA*, or *SDH* genes previously identified by Sanger sequencing (Supplementary Table S1). No mutation in *BRAF*, *SDHC*, *SDHD*, *RB1*, *HRAS*, *KRAS*, *NRAS*, *NF2*, *HIF1A*, *PTEN*, *RAF1*, *RUNX1*, *SMARCB1*, *SPRED1*, and *VHL* was detected in any of the cases analyzed. Three tumors carried missense nucleotide changes in the coding sequence of *CDKN2A*, *TP53*, or *PIK3CA*, respectively (Supplementary Table S3).

Intriguingly, 15 tumors turned out to carry *NF1* mutations (Table 1). These included 13 quadruple-negative GISTs (13/22; 59%) and 2 tumors of the *KIT/PDGFRA/SDH*^{m} group (2/24; 8.3%).

NF1 mutations detected in the quadruple-negative group consisted of small changes (missense mutations, nonsense, frameshift-induced protein truncation, mutations in proximity to splicing sites) as well as large deletions. Most of these mutations had previously been associated with NF-Type 1 (HGMD database) and were predicted to be deleterious (Table 1). The mutation detected in case #17 (His1374Tyr) has been previously reported as a germline mutation of uncertain significance in a subject affected by a not otherwise specified cancer-predisposing syndrome (SCV000215178.1 in ClinVar database; http://www.ncbi.nlm.nih.gov/clinvar). Three of the five prediction algorithms used in this study classified it as damaging or possibly damaging. The *NF1* missense variants detected in the two KIT-mutated GISTs (case # 28, Ala456Val; # 38, Ala1676Thr) were classified as nonpathogenic by at least four of five prediction tools.

Differently from the two *NF1*-mutated GISTs of the *KIT/PDGFRA/SDH*^{m} group, most quadruple-negative/*NF1*-mutated tumors bore a second *NF1* mutation (4 cases) or the mutation was homo/hemizygous (6 cases), suggestive of biallelic *NF1* inactivation (Table 1).

The actual expression of *NF1*-mutated alleles was confirmed in five GISTs for which RNA was available (cases # 8, 9, 13, 28, and 38).

Matched normal DNA was available for 12 of 15 *NF1*-mutated cases (11 quadruple-negative and 1 KIT-mutated GIST). The *NF1* alteration detected in the tumor was also detected in the matched normal tissue, indicative of its constitutional nature, in 7 of 11 (64%) quadruple-negative and in the KIT-mutated case analyzed (Table 1). In case # 19, the *NF1* alteration (a constitutional intragenic deletion encompassing exon 3) was detected in histologically tumor-free normal tissue adjacent to the tumor but was absent in peripheral blood cells, suggesting a postzygotic mosaicism.

Clinicopathologic correlations

Quadruple-negative versus KIT/PDGFRA/SDH^m GISTs. Comparison of the clinical characteristics of quadruple-negative versus *KIT/PDGFRA/SDH*^{m} groups revealed a trend toward female predominance in the quadruple-negative group (male-to-female ratio, 8:14 vs. 14:10; $P = 0.15$; Fisher exact test). The two groups did not significantly differ in terms of age (median, 60 vs. 64.5 years), history of other malignancies (3/22 vs. 3/24), mitotic index (median 8 in both groups), and size (6.0 vs. 5.4 cm), but were instead heterogeneous in terms of anatomical location. The stomach was the prevalent site in the *KIT/PDGFRA/SDH*^{m} group (75%), in line with literature data (1, 8, 23), whereas was uncommon in the quadruple-negative group (19%; $P < 0.001$, Fisher exact test). In this latter group, the majority of the tumors developed in the intestine (71%; 12 small intestine; 2 duodenum; 1 in the sigmoid colon) or other sites (1 peritoneum and 1 retroperitoneum).

In the quadruple-negative group, 8 patients were either metastatic at diagnosis (4 cases) or developed subsequent metastases (4 cases); 5 patients were dead of disease at the last follow-up. In the *KIT/PDGFRA/SDH*^{m} group, 3 of 20 assessable cases developed distant metastases (liver, peritoneum, bone) and 1 patient (*SDH*-mutated) presented at diagnosis with lymph node invasion; only

Table 1. *NF1* gene mutations

Case #	Group (mutated gene)	*NF1* gene mutation	Mutant variant frequency (%)	Biallelic inactivation	Nature of *NF1* mutation	*NF1* mutation consequence	NF1 protein change	HGMD report of NF-Type 1	Prediction of pathogenicity					
									Provean	*SIFT*	*PolyPhen2*	*Mutation assessor*	*PaPI*	*Human splicing finder*
2	Quadruple-negative	c.6855C>A	96	Yes	U	Stop gained	p.Tyr2285Ter	CM981382; CM972796	Deleterious	–	–	–	–	–
3	Quadruple-negative	c.4725-2A>G	32	No	U	Splice acceptor variant	?	CS086424	–	–	–	–	–	Alteration of the wt acceptor site, most probably affecting splicing
5	Quadruple-negative	exon 2-28 del	>80	Yes	C	Deletion	?	CG060178	–	–	–	–	–	–
7	Quadruple-negative	c.1105C>T	51	Yes	C	Stop gained	p.Gln369Ter	–	Deleterious	–	–	–	–	–
		c.6380delT	35		S	Frameshift variant, truncation	p.Asn2128Ile fsTer22	–	Deleterious	–	–	–	–	–
8	Quadruple-negative	c.4600C>T	89	Yes	C	Stop gained	p.Arg1534Ter	CM941093	Deleterious	–	–	–	–	–
9	Quadruple-negative	c.1658A>G	95	Yes	C	Missense variant	p.His553Arg	HM0703	Deleterious	Damaging	Probably damaging	Medium	Damaging	–
11	Quadruple-negative	c.2514delC	34	Yes	S	Frameshift variant, truncation	p.Asn839Thr fsTer2	–	Deleterious	–	–	–	–	–
		c.1641+1G>T	36		S	Splice donor variant	?	CS000896	–	–	–	–	–	Alteration of the wt donor site, most probably affecting splicing
12	Quadruple-negative	c.6148-1G>A	48	Yes	S	Splice acceptor variant	?	CS086428	–	–	–	–	–	Broken wt donor site, activation of an intronic cryptic acceptor site, potential alteration of splicing
		c.7869+1G>T	48		S	Splice donor variant	?	CS031796; CS086439	–	–	–	–	–	Alteration of the wt donor site, most probably affecting splicing
13	Quadruple-negative	c.2511G>A	50	No	S	Stop gained	p.Trp837Ter	CM076345	Deleterious	–	–	–	–	–
17	Quadruple-negative	c.4120C>T	50	No	C	Missense variant	p.His1374Tyr	–	Neutral	Damaging	Possibly damaging	Low	Damaging	–
18	Quadruple-negative	c.2272_2273delAG	89	Yes	C	Frameshift variant, truncation	p.Arg758Ser fsTer9	CD000965	Deleterious	–	–	–	–	–
19	Quadruple-negative	exon 3 del	42	Yes	C	Deletion	?	–	–	–	–	–	–	–
		c.1398_1399insA	34		S	Frameshift variant, truncation	p.Thr467Asn fsTer3	CI031910	Deleterious	–	–	–	–	
21	Quadruple-negative	c.5430_5431insA	72	Yes	S	Frameshift variant, truncation	p.Thr1811AsnfsTer8	–	Deleterious	–	–	–	–	
28	*KIT* p.Val559_Glu561del	c.1367C>T	48	No	U	Missense variant	p.Ala456Val	–	Neutral	Tolerated	Benign	Low	Tolerated	–
38	*KIT* p.Val560Asp	c.5026G>A	51	No	C	Missense variant	p.Ala1676Thr	–	Deleterious	Tolerated	Benign	Low	Tolerated	–

NOTE: *NF1* mutation. Reference gene sequence: NM_001042492.2; Reference protein sequence: NP_001035957.1.

Nature of *NF1* mutation: U, undetermined; C, constitutional; S, somatic, detected in tumor only.

HGMD report of NF-Type 1 association: http://www.hgmd.cf.ac.uk/ac/gene.php?gene=NF1.

Table 2. Clinical characteristics of quadruple-negative GIST patients

Case #	Group	Nature of *NF1* mutation	Sex	Age	Disease status at diagnosis	Other malignancies	Relapse	Time to relapse (months)	GIST Medical therapy	Status at last follow-up (months)
1	*NF1* wild-type	—	F	58	NA	NA	NA		NA	NA
4		—	F	69	Localized	No	Yes (liver)	67	Imatinib (at relapse)	AWD (67)
6		—	M	53	Localized	NA	No		NA	NA
10		—	F	77	Localized	No	No		No	NED (15)
14		—	F	68	Localized	No	No		No	NED (72)
15		—	M	68	Localized	No	No		No	NED (72)
16		—	F	70	Localized	No	Yes (liver + peritoneum)	38	Imatinib (at relapse), sunitinib	DOD (89)
20		—	M	60	Localized	No	No		No	NED (26)
22		—	F	30	Localized	No	No		No	NED (13)
2	*NF1* mutated	U	M	35	NA	NA	NA	NA	NA	NA
3		U	F	76	Localized	No	NA	NA	NA	NA
5		C	F	73	Metastatic (peritoneum)	Breast carcinoma	Yes (liver)	25	No imatinib (chemotherapy for breast cancer)	DOD (39)
7		C	F	60	Localized	Breast carcinoma	Yes (peritoneum)	122	Imatinib (at relapse)	DOD (129)
8		C	F	31	Metastatic (peritoneum)	No	Yes (liver)	12	Imatinib (ab initio)	DOD (16)
9		C	F	73	Localized	No	Yes (peritoneum)	15	Imatinib (at relapse), sunitinib	DOD (39)
11		S	M	59	Localized	Chromophobe renal cell carcinoma; colon adenocarcinoma	No		No	NED (6)
12		S	M	50	Localized	No	No		No	NED (24)
13		S	F	86	Localized	No	No		No	NED (26)
17		C	F	42	Metastatic (peritoneum)	No	Yes (peritoneum)	7	Imatinib (ab initio), sunitinib, regorafenib	AWD (59)
18		C	M	56	Localized	No	No		No	NED (27)
19		C	F	67	Localized	No	No		No	NED (3)
21		S	M	36	Metastatic (peritoneum)	No	No		No	NED (12)

NOTE: Nature of *NF1* mutation: U, undetermined; C, constitutional; S, somatic, detected in tumor only.
Clinical information: NA, not available; AWD, alive with disease; DOD, dead of disease; NED, no evidence of disease.

1 patient (*KIT*-mutated gastric GIST) was dead of disease at the last follow-up.

NF1-mutated versus NF1-wild-type quadruple-negative GISTs. To understand whether the mutation of *NF1* was associated with distinctive biological features, *NF1*-mutated and *NF1*-wild-type quadruple-negative tumors were compared for a number of clinicopathologic characteristics (Tables 2 and 3).

Neither personal/familial history of NF-Type 1 nor dermatological signs or neoplasias referable to NF-Type 1 were documented in the clinical records of any patient prior inclusion in this study. Although we failed to retrieve complete clinical history of 1 patient (case # 2), this case was submitted for consultation as a sporadic GIST.

No statistical difference between *NF1*-mutated and *NF1*-wild-type quadruple-negative cases was observed in terms of sex (male-to-female ratio, 5:8 vs. 3:6), age (median, 59 vs. 68 years), mitotic index (median, 8 vs. 5), tumor size (median, 7 vs. 5.8 cm), and distribution of tumor locations.

Although no other malignancies were reported in the *NF1*-wild-type group, 3 *NF1*-mutated patients had a concurrent malignancy. Specifically, 2 patients with a *NF1* constitutional mutation (cases # 5 and 7) had a history of breast carcinoma; patient # 11, with a somatic *NF1* mutation in the GIST, presented with 2 other concomitant tumors (chromophobe renal cell carcinoma and colon cancer).

Multifocality at diagnosis was observed only in the *NF1*-mutated group. Two patients (# 3 and # 19) presented with synchronous small intestinal lesions. An unusual pattern of progression was noticed in two other cases: patient # 7 developed two subsequent GISTs within the small intestine wall seven years after first surgery for a low-risk small intestinal GIST; patient # 9, with a primary peritoneal GIST, relapsed twice with a predominant small intestinal GIST associated with smaller peritoneal nodules. All these cases carried a *NF1* constitutional defect.

A multinodular growth pattern with fibromuscular septa, similar to that described for SDH-deficient GISTs, was evident in four cases, all with constitutional *NF1* alterations (Fig. 1).

NF1-wild-type tumors exhibited spindle morphology in four cases, mixed in two, and epithelioid in three.

The morphology in the *NF1*-mutated group was spindle in seven cases, mixed in three, and epithelioid in two (Fig. 1). Interestingly, six of seven *NF1*-mutated GISTs with spindle cell morphology showed features typical of GISTs arising in the context of NF-Type 1: they were hypocellular throughout and featured abundant extracellular collagen with skenoid fibers. Areas with such features were also found in one of the three GISTs with mixed morphology. Unusual features were detected in

Table 3. Pathologic features of quadruple-negative GISTs

Case #	Group	Nature of *NF1* mutation	Tumor multifocality	Growth pattern	Site of primary GIST	Size	Mitotic index	Morphology	Degree of cellularity	Presence of skenoid fibers
1	*NF1* wild-type	—	No	Single nodule	Small intestine	6.0	2	Spindle	Moderate	No
4		—	No	NA	Small intestine	NA	21	Mixed	High	NA
6		—	No	Single nodule	Retroperitoneum	8.0	20	Epithelioid	High	No
10		—	No	Single nodule	Stomach	15.0	29	Spindle	High	No
14		—	No	Single nodule	Stomach	5.5	5	Epithelioid	NA	No
15		—	No	Single nodule	Duodenum	2.3	1	Spindle	NA	No
16		—	No	Single nodule	Small intestine	11.5	87	Epithelioid	High	No
20		—	No	Single nodule	Small intestine	4.5	3	Spindle	Low	Yes
22		—	No	Single nodule	Small intestine	3.2	3	Mixed	Moderate	No
2	*NF1* mutated	U	NA	NA	NA	NA	NA	NA	NA	NA
3		U	Yes	Single nodule	Small intestine (Ileum + Jejunum)	1.5+2.5	1	Spindle	Low	Yes
5		C	No	Multinodular	Duodenum	8.0	18	Mixed	Moderate (spindle cell areas of low cellularity)	Yes
7		C	Yes (metachronous[a])	Multinodular	Small intestine	6.0	2	Spindle	Low	Yes
8		C	No	Multinodular	Small intestine	9.0	83	Mixed	High	No
9		C	Yes (metachronous[b])	Single nodule	Peritoneum	16.0	30	Epithelioid/ Plasmacytoid	High	No
11		S	No	Single nodule	Small intestine	1.5	0	Spindle	Low	Yes
12		S	No	Single nodule	Small intestine	6.0	1	Spindle	Low	Yes
13		S	No	Single nodule	Small intestine	8.0	8	Spindle	Low	Yes
17		C	No	NA	Sigmoid colon	8.0	39	Epithelioid/ Small round cell	High	No
18		C	No	Single nodule	Stomach	3.2	8	Mixed	Moderate	No
19		C	Yes	Multinodular	Small intestine	3 + other[c]	2	Spindle	Low	Yes
21		S	No	Single nodule	Stomach	11	10	Spindle	High	No

NOTE: Nature of *NF1* mutation: U, undetermined; C, constitutional; S, somatic, detected in tumor only.

Abbreviation: NA, not available.

[a]This patient developed two subsequent GISTs of small size and low mitotic index in the small intestinal wall, seven years after first surgery.

[b]This patient relapsed twice, 15 and 30 months after first surgery, with a predominant small intestinal tumor and smaller peritoneal nodules.

[c]This patient presented with a predominant tumor (3 cm) and 6 additional smaller subserosal nodules (size range, 0.2–1.2 cm).

the two *NF1*-mutated GISTs with epithelioid morphology: one case displayed a striking plasmacytoid phenotype with eccentric vesicular nuclei, evident nucleoli, and abundant eosinophilic cytoplasm; the other was a small round cell tumor. Both these cases featured a very high mitotic index (Fig. 1).

Disease status at diagnosis was known for 12 *NF1*-mutated and 8 *NF1*-wild-type cases. All *NF1*-wild-type patients presented with localized disease; two of them developed metastasis 38 and 67 months after surgery. Among *NF1*-mutated patients, 4 had peritoneal metastases at diagnosis; progression was reported in 2 cases localized at the diagnosis, at 15 and 122 months, respectively.

Data on the clinical therapy were available for 7 *NF1*-wild-type and 11 *NF1*-mutated cases (Table 2). Imatinib was administered to 2 metastatic patients *ab initio*, both *NF-1* mutated, and upon relapse in 4 cases, 2 *NF1*-wild-type and 2 *NF1*-mutated. One *NF1*-wild-type and 2 *NF1*-mutated cases were shifted to second- and third-line therapy. For *NF1*-mutated cases, median time to progression under imatinib was 9.5 months (range, 7–18 months).

Regarding patients' outcome, follow-up information was available for 7 *NF1*-wild-type patients and 11 cases with *NF1* mutation. Within the *NF1*-wild-type group, patient # 16 died of disease (89 months); patient # 4 was alive with disease (67 months after surgery); 5 patients were disease-free at the last follow-up. Within the *NF1*-mutated group, 4 patients died of disease (median time to death 39 months), 1 patient was alive with disease 59 months after surgery, and 6 patients were disease-free at the last follow-up.

NF-Type 1 expert re-evaluation of patients with constitutional NF1 mutation. Of the 7 patients with ascertained constitutional *NF1* mutation, 4 were deceased and the 3 living patients agreed to be referred to a NF-Type 1 expert. According to the specialist, patient #17 failed to fulfill NIH diagnostic criteria for NF-Type 1 (6). A diagnosis of NF-Type 1 was instead eventually made for patients # 18 and #19 who had a negative family history for NF-Type 1. Patient # 18 presented with axillary/inguinal freckling, several neurofibromas, and 14 *café-au-lait* macules greater than 15 mm. The patient suffered also of severe vision impairment attributed to *Retinitis pigmentosa*. In patient # 19, the medical geneticist detected small cutaneous neurofibromas in the trunk, 2 *café-au-lait* spots and skin-fold freckles, consistent with mild

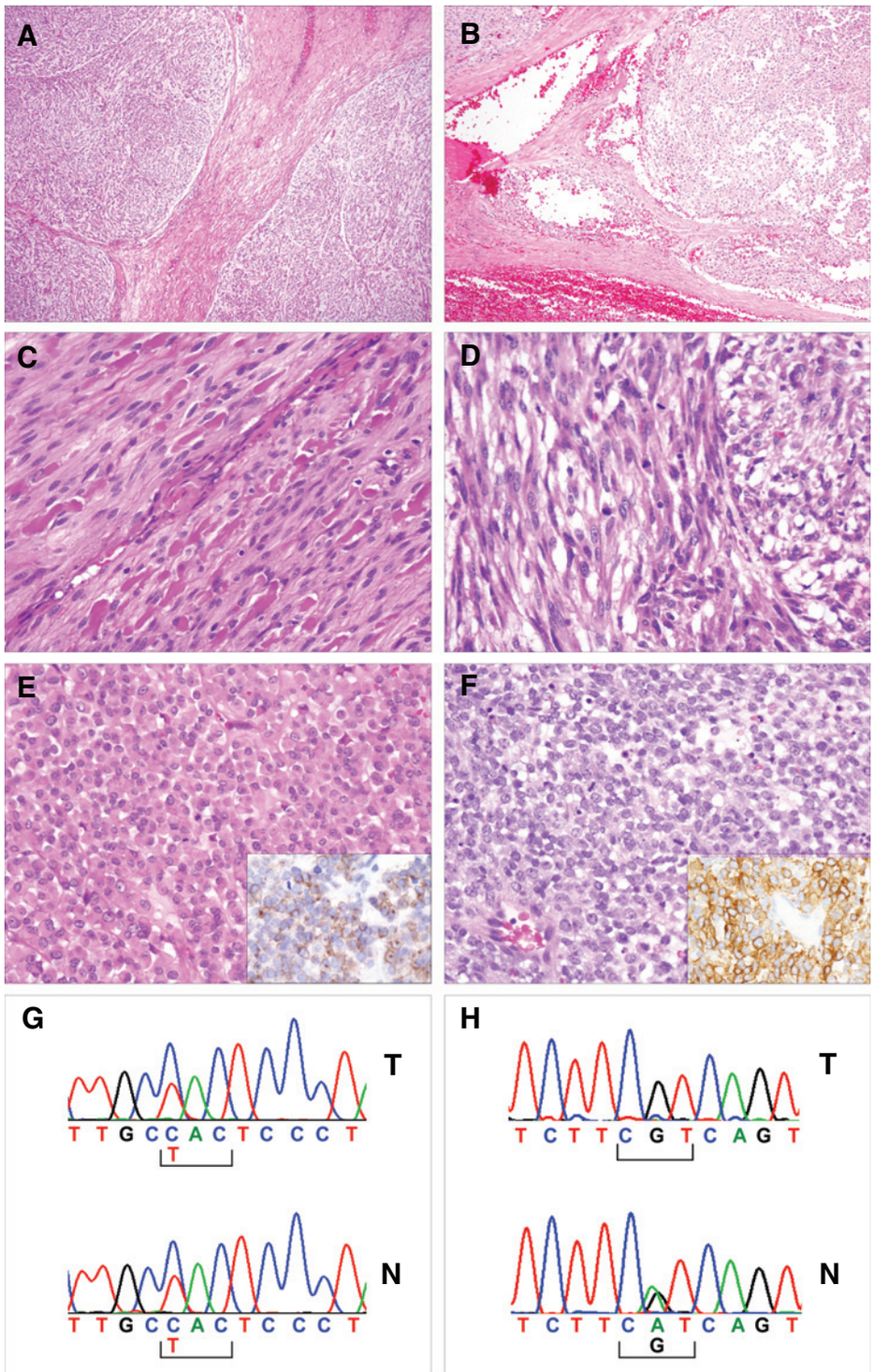

Figure 1.
Pathologic and molecular features of *NF1*-mutated GISTs. **A** and **B,** Multinodular growth pattern with thick fibrous septa in cases # 8 (**A**) and # 5 (**B**). **C,** Spindle cell morphology with abundant skenoid fibers, hypocellularity, and low mitotic activity in case # 3. **D,** Hypercellularity, nuclear atypia, and high mitotic index in case # 8. **E,** Plasmacytoid morphology and high mitotic index in case # 9. CD117 expression was diffused with a membranous and dot-like pattern of staining (inset). **F,** Small round cell morphology and high mitotic index in case # 17. CD117 expression was diffused with a membranous and cytoplasmic pattern of staining (inset). **G,** Case # 17 carried a constitutional C to T transition, resulting in a His to Tyr amino acid substitution at codon 1374. Both tumor (T) and normal tissue (N) were heterozygous for the mutation. **H,** Case # 9 carried a constitutional A to G transition, resulting in a His to Arg amino acid substitution at codon 553. The wild-type allele was lost in the tumor (T).

form of NF-Type 1. Intriguingly, the intragenic deletion encompassing *NF1* exon 3 detected in the proband's tumor and in adjacent, histologically tumor-free, normal tissue was absent in peripheral blood cells, supporting a postzygotic mosaicism. No hallmarks of NF-Type 1 were detected in the patient's daughter (44 years).

Discussion

This study aimed at gaining insight into the genetics of quadruple-negative GISTs, representing a fraction of tumors currently "orphan" of oncogenic driver alterations. To this end, we collected 22 quadruple-negative sporadic GISTs arisen in patients with no

prior association with NF-Type 1. For comparison purposes, 24 consecutive GISTs with *KIT*, *PDGFRA*, or *SDH* alterations were also analyzed. The majority of the quadruple-negative GISTs turned out to be nongastric tumors. This finding may be explained by the fact that a large fraction of wild-type gastric GISTs rely on SDH inactivation as an alternative to *KIT/PDGFRA* oncogenic mutation (8), thus leaving the driver alteration of nongastric GISTs undetermined. This supports the notion that GISTs arising in different sites feature different genetic backgrounds.

The molecular profiling of our GIST series revealed an unexpected high frequency of *NF1* mutations, particularly in the quadruple-negative group (13/22; 59%). *NF1* encodes neurofibromin, a GTPase-activating protein that binds to active GTP-bound RAS, and works as an off signal for all members of the RAS GTPase family. Similarly to *KIT*-, *PDGFRA*-, or *BRAF*-activating mutations, loss of NF1 leads to the unleashing of the MAPK cascade (5). The detection of a high frequency of pathogenic *NF1* mutations in "sporadic" quadruple-negative GISTs indicates that the triggering of the RAS/RAF/MAPK pathway by means of NF1 inactivation plays a relevant role in the pathogenesis of GIST devoid of canonical tyrosine kinase receptor mutations.

Another and perhaps the most intriguing finding of our study is that *NF1* mutations in quadruple-negative GISTs were constitutional in 7 of 11 cases tested, and often associated with biallelic inactivation. We would like to emphasize that the design of our study excluded *a priori* all patients with documented familial history, genetic diagnosis, or recorded diagnostic stigmata of NF-Type 1. Thus, our results demonstrate that a significant fraction of patients presenting to surgical and medical oncology clinics with quadruple-negative "sporadic" GIST are *de facto* affected by unrecognized forms of the NF-Type 1 syndrome.

This is reminiscent of the SDH "saga," where *SDH* germline mutations were originally described in the Carney–Stratakis syndrome (familial paraganglioma and GIST; refs. 10, 11, 24, 25) and only subsequently acknowledged to contribute to a significant fraction of apparently "sporadic" GISTs (8, 24–28). It is also in line with recent reports that susceptibility to cancer due to unsuspected syndromic conditions is more frequent than commonly believed (29).

The association between NF-Type 1 syndrome and cancer is well known, but *NF1* mutation analysis has long been challenging because of the large gene size (58 exons), multiple alternatively spliced isoforms, and existence of multiple pseudogenes (5). The recent advent of MPS technologies is not only facilitating the molecular diagnosis of NF-Type 1 probands, but is also revealing that somatic *NF1* mutations are involved in up to 12% of sporadic tumors, including glioblastomas, lung, breast, ovarian, Merkel cell carcinomas, melanomas, and sarcomas (5, 30–32). Our report highlights that quadruple-negative "sporadic" GISTs often arise in the context of unrecognized NF-Type 1 syndrome.

NF-Type 1 is the most common autosomally dominant inherited disorder in humans, with an incidence of about 1:3,000 live births in Western Countries (14, 15, 33). *NF1* is one of the genes with the highest mutation rates, and approximately 50% of clinically diagnosed NF-Type 1 patients carry *de-novo NF1* mutations. These mutations can also occur late in embryo development and may therefore be present in a mosaic state, accounting for the segmental forms of the disease (33, 34). NF-Type 1 features a poor genotype–phenotype correlation and an extremely variable expression pattern, with the spectrum and extent of manifestations varying greatly among affected individuals within a single family and even within a single person at different times of life (16). NIH clinical diagnostic criteria comprise *café-au-lait* macules, multiple neurofibromas, axillary/groin freckling, iris hamartomas (Lisch nodules), optic pathway glioma, and distinctive osseous lesions (6). It should be kept in mind that these criteria, established in 1988, were originally aimed at selecting those individuals whose genetic profiling would have eventually lead to the identification of the *NF1* gene. They were therefore purposely stringent, essentially based on visual inspection of the subject and did not include malignant tumors. As a matter of fact, NF-Type 1 patients do have an increased risk of developing tumors of different types, primarily in nervous system (i.e., malignant peripheral nerve sheat tumors, gliomas, plexiform neurofibromas, ganglioneuromas) but also in other sites (33, 35–37). The risk of GIST is also augmented in NF-Type 1 patients (36, 37), although Miettinen and colleagues reported that only a minute fraction (1.5%) of GISTs included in the AFIP files were arisen in patients with a diagnosis of NF-Type 1 (38).

The remarkable frequency of *NF1* constitutional mutation detected in our series of quadruple-negative GISTs prompted a clinical reassessment of the carriers. NF-Type 1 expert re-evaluation of the 3 living patients carrying a constitutional mutation (#17, 18, and 19) confirmed a negative familial history for the disease. No clinical manifestations complying with the NIH diagnostic criteria were identified by the specialist in patient #17. The *NF1* mutation detected in this individual, His1374Tyr, has been previously identified in a subject affected by a not otherwise specified cancer-predisposing condition (ClinVar). Although originally classified as of uncertain significance, three of the five prediction algorithms used in our study indicated a damaging/possibly damaging effect. Overall, these findings are compatible with either a segmental disease or with a low-expressivity form. Interestingly, a 3-bp inframe deletion has been recently reported to be associated with a *forme fruste* of NF-Type 1 that is portrayed, in some individuals, by no other sign but few *café-au-lait* macules (39). It is tempting to speculate that the mutation detected in patient #17 may also convey a very attenuated phenotype. Intriguingly, this patient was diagnosed with a GIST located in the colon, an exceedingly rare site, since the so-called colorectal GISTs predominantly develop in the lower rectum (23, 40). The link between His1374Tyr mutation and GIST location in the colon is worth further investigation.

The clinical geneticist was instead able to identify pathognomonic signs consistent with NF-Type 1 in case #18 and, in a milder form, in case #19, who is a carrier of a post-zygotic mosaic *NF1* mutation. It is noteworthy that the syndromic condition of these patients was essentially overlooked by all the doctors (general practitioner, ophthalmologist, surgeon, oncologist, etc.), the patients have dealt with during their life (67 and 56 years, respectively), and that only the occurrence of a GIST with peculiar characteristics (quadruple negative) brought these cases to the attention of the genetic counselor, who eventually made the diagnosis of NF-Type 1.

The extreme variability of NF-Type 1 clinical presentations and expressivity, the fact that GIST are not considered a common finding in these patients, together with the complexity of the molecular diagnosis of NF-Type 1, likely explain the underestimation of the role of *NF1* in the pathogenesis of "sporadic" GISTs. Currently, in the absence of family history or obvious clinical stigmata, the presence of a quadruple-negative GIST is not considered suggestive of an NF-Type 1 syndromic condition. Nevertheless, besides being commonly devoid of *KIT/PDGFRA/BRAF/*

SDH mutations, *NF1*-associated GISTs are reported to have distinctive clinicopathologic features, including multifocality and hypocellularity, spindle cell morphology with skenoid fibers, preferential location in the small bowel, and onset in the late fifth decade (38, 41–44). These characteristics were also exhibited by most *NF1*-mutated GISTs in our series. Moreover, a multinodular growth pattern was observed in four cases. This pattern has previously been deemed as typical of SDH-deficient GISTs (8, 25, 27), but our findings suggest that it likely reflects a more general trait of GISTs arising in syndromic contexts. Finally, although only four *NF1*-mutated GISTs received imatinib, overall the clinical benefit was unsatisfactory (median time to progression 9.5 months), adding support to the concept that *NF1*-associated GISTs are poorly responsive to this drug (44).

In conclusion, this study unveiled the genetic bases of a significant fraction of quadruple-negative GISTs, identifying the inactivation of *NF1 as* a key driver alteration. Moreover, the finding that the *NF1* mutation was constitutional in an important fraction of these patients suggests a role for GIST as a sentinel tumor for NF-Type 1. Hence, a diagnosis of quadruple-negative GIST, especially if multifocal or with a multinodular growth pattern and a nongastric location, should alert the clinician to a possible NF-Type 1 syndromic condition. These patients should be referred to NF-Type 1 specialists for a thorough search for subtle disease manifestations and to genetic counseling for definitive individual and familial risk assessment.

Disclosure of Potential Conflicts of Interest

A. Gronchi holds ownership interest (including patents) in Novartis and is a consultant/advisory board member for Bayer, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors are grateful to Fabio Canal, Matilde Cacciatore, Laura Papi, Francesco Benedicenti, and Joanne Fleming for their suggestions.

Grant Support

This work was supported by the Italian Association for Cancer Research (AIRC), the Italian Ministry of Health (Ricerca Finalizzata), and by a CRO Aviano National Cancer Institute Intramural Grant.

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Received January 19, 2016; revised May 26, 2016; accepted June 14, 2016; published OnlineFirst July 7, 2016.

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