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Molecular pathogenesis of multiple gastrointestinal stromal tumors in NF1 patients

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract. *KIT* and *PDGFRA* activating mutations are the oncogenic mechanisms in most sporadic GISTs. In addition to sporadic occurrences, GISTs are increasingly being recognized in association with neurofibromatosis type 1 (NF1), yet the underlying pathogenic mechanism remains elusive. To gain an insight into the mechanisms underlying GIST formation in NF1 patients, we studied seven GISTs from three NF1 patients with a combination of different techniques: mutation analysis (*KIT*, *PDGFRA* and *NF1*), western blotting, array CGH and *ex vivo* imatinib response experiments. We demonstrate that (i) the NF1-related GISTs do not have *KIT* or *PDGFRA* mutations, (ii) the molecular event underlying GIST development in this patient group is a somatic inactivation of the wild-type *NF1* allele in the tumor and (iii) inactivation of neurofibromin is an alternate mechanism to (hyper) activate the MAP-kinase pathway, while the JAK-STAT3 and PI3K-AKT pathways are less activated in NF1-related GIST compared with sporadic GISTs. In conclusion, we report for the first time the molecular pathogenesis of GISTs in NF1 individuals and demonstrate that this type of tumor clearly belongs to the spectrum of clinical symptoms in NF1.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract (1). They occur predominantly in the stomach (70%) and small intestine (10–20%) and originate from primitive cells with characteristics of the interstitial cells of Cajal (ICCs), the autonomic pacemaker cells which regulate peristalsis in the digestive tract (1–3). Most sporadic GISTs contain gain-of-function mutations in *KIT* (80–85%) (4,5) and strongly express the constitutively activated protein (6). Some GISTs contain gain-of-function *PDGFRA* mutations as an alternate oncogenic mechanism (7,8). Both receptor tyrosine kinases transduce their signals through the downstream PI3K-AKT (9), MAP-kinase (10,11) and JAK-STAT3 signaling cascades (12–16). Therapeutic targeting with the tyrosine kinase inhibitor imatinib (Gleevec, Novartis, Basel, Switzerland) shows a clinical benefit in up to 80% of patients with sporadic GIST (17,18).

GISTs are increasingly recognized in adults with neurofibromatosis type 1 (NF1), an autosomal dominant genetic disorder caused by alterations of the *NF1* gene and affecting 1/3500 individuals worldwide. Neurofibromin, the protein encoded by *NF1*, functions as a GTPase-activating protein for Ras by catalyzing the hydrolysis of active Ras-GTP to an inactive Ras-GDP (19–23). The primary clinical features of the disease are café-au-lait spots, freckling and benign peripheral nerve sheath tumors or neurofibromas (24).

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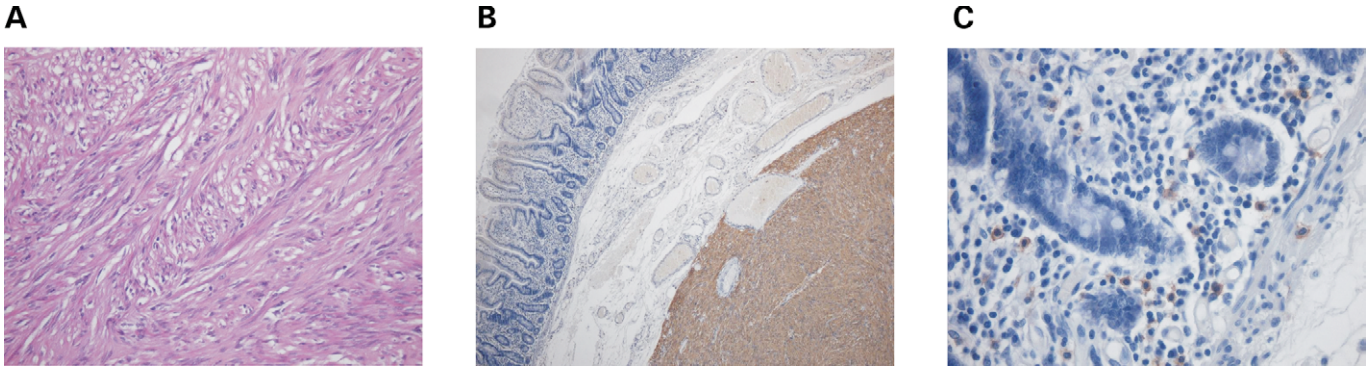

Figure 1. Histopathological analysis of NF1-related GISTs. (**A**) Low power view, showing the spindle cell character of the NF1-related GIST (H&E stain, ×200). (**B**) KIT staining shows strong expression in the tumor (immunoperoxidase stain for CD117, ×50). (**C**) Detail of the mucosa. Only the mast cells are KIT positive (immunoperoxidase stain for CD117, ×350).

Neurofibromin deficiency in neurofibromas results in hyperactivation of the MAP-kinase pathway. On the basis of a single Swedish study of 70 NF1 patients, it is estimated that adults with NF1 might have a risk for GISTs as high as 7% (25). GISTs in NF1 patients tend to be multiple and are located predominantly within the small intestine (26–28). Morphologically and immunohistochemically, GISTs occurring in the NF1 patients are similar to sporadic GISTs (26–29). Little is known about the molecular basis underlying GIST formation in NF1, and whether there is a difference in the molecular pathogenesis of sporadic and NF1-related GISTs. It remains an intriguing question why NF1 patients are at an increased risk for this type of tumor.

RESULTS

Histopathological analysis

The NF1-related intestinal tumors corresponded to bona fide spindle cell type GISTs (Fig. 1A). The tumor cells were strongly positive for CD117 (Fig. 1B and 1C). The mast cells served as positive control for CD117, whereas the other tissue elements of the mucosa and submucosa were negative (Fig. 1B and 1C). There was a variable positivity for alpha smooth muscle actin, CD34 and S-100 protein, whereas desmin was negative.

Mutation analysis

KIT/PDGFRA. Analysis of the entire *KIT* and *PDGFRA* coding region did not reveal any activating mutation in the NF1-related GIST specimens (Table 1). One single nucleotide polymorphism (SNP) in the 3′-UTR region of *KIT* (rs2213181, http://www.ncbi.nlm.nih.gov/SNP) was revealed in all GISTs derived from patient NF1-1. In the GIST of patient NF1-3, one silent polymorphism was found in *KIT* (p.K546K) and five alterations were found in *PDGFRA*: three silent mutations (p.N204N, p.G313G and p.A603A), one 3′-UTR SNP (rs7680422, http://www.ncbi.nlm.nih.gov/SNP) and one missense mutation [c.1825T>C (p.S478P)]. The missense mutation was not predicted to have an impact on protein activity (Polyphen algorithm, http://tux.embl-heidelberg.de/ramensky) and was found in 19/98 (19%) control individuals. All detected variants were also present in DNA extracted from peripheral white blood cells of the respective patients. The detection of heterozygous polymorphisms indicates the presence of both *KIT* alleles in one out of seven tumors and both *PDGFRA* alleles in four out of seven tumors.

NF1. Mutation screening of peripheral blood revealed *NF1* germline mutations in all three patients (Table 1). The somatic alterations in the *NF1* gene region detected in six of the seven GISTs (Table 1) were three nonsense mutations, one donor splice site mutation, a multi-exon deletion encompassing *NF1* exons 10c–21 and one loss of heterozygosity (LOH) event including the *NF1* gene. All *NF1* mutations are assumed to have an inactivating effect (Table 1). Analysis of the nonsense mutation p.R2616X in different cell types derived from tumor II of patient NF1-2 demonstrated that the somatic *NF1* mutation was exclusively present in the cells cultured with the medium selective for ICC culture and not in fibroblasts grown from the same tumor.

Western blotting

All seven NF1-related tumors showed only rudimentary neurofibromin expression in comparison with the control GIST882 cell line harboring the *KIT* p.K642E mutation and the sporadic GIST with the *KIT* p.557–558delWK mutation (Fig. 2). NF1-related GISTs demonstrated normal total KIT expression but low levels of constitutive KIT autophosphorylation in comparison to the control GIST882 and sporadic GIST cells (Table 2). Total MAPK and MAPK phosphorylation was ubiquitously more pronounced in NF1-related GIST tissue. On the basis of the densitometric quantification of autoradiographic signals, the relative p-MAPK expression in NF1-related GIST was 3–18-fold higher than in sporadic GIST (Table 2). Expression of total AKT was variable in NF1-related GISTs and not associated with protein phosphorylation. Moreover, NF1 tumors did not express STAT3 protein.

Table 1. Molecular analysis of seven GISTs derived from three NF1 patients

Patient	Tumor	Material	*KIT*	*PDGFRA*	*NF1*		
					Germline	Somatic	Mutation effect
NF1-1	I	F	−	−	c.4269+1G>T	c.5546+2T>A	Frameshift leading to PTC
	II	F	−	−		c.5242C>T (p.R1748X)	PTC
	III	F	−	−		ND	
NF1-2	I	F	−	−	c.6791dupA	c.279T>A (p.C93X)	PTC
	II	ICC + F	−	−		c.7846C>T (p.R2616X)	PTC
	III	ICC + F	−	−		del [ex10c−21]	Truncating
NF1-3	I	F	−	−	c.7807delG	LOH	Loss wild-type *NF1* allele

F, frozen; −, negative; ND, no mutation detected; PTC, premature termination codon; ICC, interstitial cells of Cajal.

Array CGH experiments

Array CGH experiments on NF1-related GISTs showed similar losses of chromosome regions as seen in sporadic GISTs (i.e. losses of chromosomes 11, 14, 22 and 1p) in addition to several extra alterations (Table 3). These additional chromosomal abnormalities seem not to be recurrent in the different tested NF1-related GISTs. Array CGH confirmed LOH of the *NF1* gene in the large tumor of patient NF1-3 [loss of the clones containing the *NF1* gene (RPCI5-926B9 and RP11-229K15)] while no other aberrations were observed (Table 3 and Fig. 3).

Phenotype of cultured cells

After 3 days in culture, the primary cells of tumor III from patient NF1-2 exhibited spindle shape type of growth and 95% were KIT immunopositive. The Ki-67 labeling index was 0.5 and 8.7% in ligand unstimulated and ligand stimulated cultures, respectively (Fig. 4).

Imatinib assay

As shown in Figure 5, KIT phosphorylation of primary NF1-related GIST cells seeded in culture is ligand-dependent as shown by very limited KIT phosphorylation in the absence of stem cell factor (SCF). SCF-dependent KIT phosphorylation of NF1-related GIST cells is totally inhibited by exposure to imatinib at concentration of 0.5 μM. The same inhibition is observed in the control autophosphorylated GIST882 cell line harboring the *KIT* p.K642E mutation. The level of MAPK phosphorylation of primary NF1-related GIST cells was moderately decreased after exposure to imatinib but the inhibition was neither complete nor dose-dependent. Imatinib decreased in a dose-dependent way the level of MAPK autophosphorylation at concentrations lower than 1.0 μM in the GIST882 cell line but not at any tested doses in control fibroblasts. The relative p-KIT and p-MAPK expressions, based on the densitometric quantification of autoradiographic signals, are shown in Table 4.

DISCUSSION

Although activating mutations of *KIT* and *PDGFRA* are known to be the most frequent genetic events in the development of sporadic GISTs, the underlying pathogenic mechanism leading to GIST formation in the NF1 setting remains elusive. In this study, total screening of *KIT* and *PDGFRA* in seven GISTs from three NF1 patients did not reveal any activating mutation. Several sequence variants were detected: three silent mutations in *PDGFRA* (p.N204N, p.G313G and p.A603A), 1 silent mutation in *KIT* (p.K546K), one SNP in the 3′-UTR region of both *KIT* and *PDGFRA* and one missense mutation in exon 10 of *PDGFRA* (p.S478P) that was not predicted to have an impact on protein activity (Polyphen algorithm) and that is a frequent polymorphism (19/98 controls, 19%) in the examined population. All variants were also present in normal control tissue (white blood cells) of the respective patients. In conclusion, the alterations do not correspond to known mutation hot spots in sporadic GISTs, are not tumor specific and have not been demonstrated to result in activation of the tyrosine kinases. This finding is in line with published reports (27–31). Of the 113 NF1-related GISTs previously published, only eight showed *KIT*/*PDGFRA* alterations. Takazawa *et al*. (2005) (30) reported three *KIT* (p.L558L, p.P627L and p.I653T) and two *PDGFRA* (p.P589S and p.R822S) mutations in 34 NF1-related GISTs. None have been demonstrated to result in activation of the protein nor have they been previously reported in sporadic or familial GIST. Yantiss *et al*. (2005) (29) detected an identical point mutation in *KIT* exon 11 in three tumors from one patient. As control tissue was not analyzed, this patient may have had a germline *KIT* mutation. Taken together, these results indicate that *KIT* or *PDGFRA* mutations are not playing a role in the development of GISTs in NF1 patients.

In accordance with Knudson's two-hit hypothesis (32), somatic inactivation of the *NF1* tumor suppressor gene leads to tumor formation in NF1 (33–36). In line with this concept, we detected somatic *NF1* alterations in six of seven NF1-related GISTs (Table 1). Analysis of different cell types derived from tumor II of patient NF1-2 demonstrated that the somatic *NF1* mutation was only present in tumoral cells cultured using specific conditions for ICCs (37). This is the first time that somatic *NF1* inactivation is demonstrated

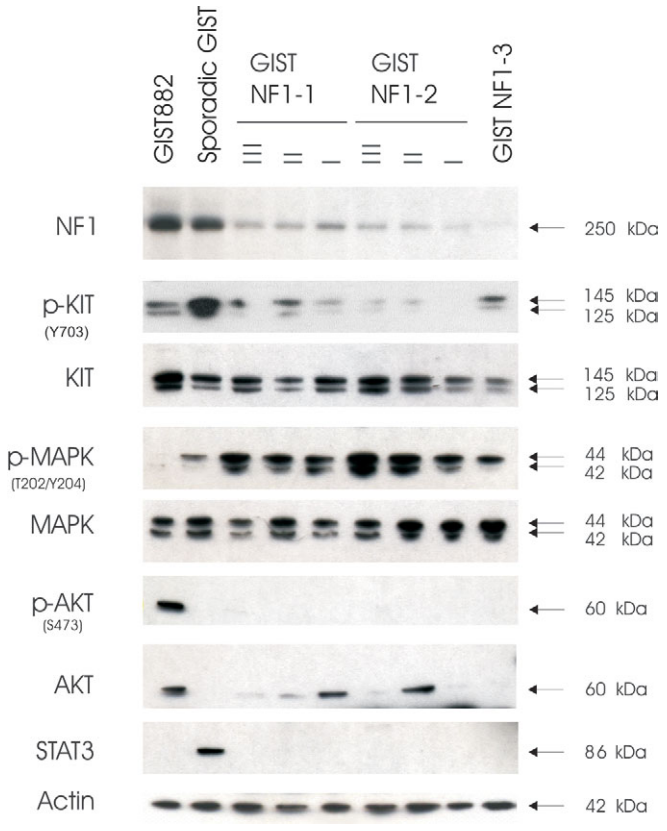

Figure 2. Analysis of KIT/PDGFRA downstream signaling pathways in two sporadic [GIST882 cell line (*KIT* p.K642E) and sporadic GIST (*KIT* p.557–558delWK)] and seven NF1-related GISTs by western blotting. Equal protein loading was demonstrated with anti-actin staining. On the basis of the densitometric quantification of autoradiographic signals, the relative p-MAPK expression in the NF1-related GISTs was 3–18-fold higher than in the sporadic GIST. Because of short exposure time, phosphorylation of MAPK of GIST882 is less visible.

in neoplastic GIST cells and it illustrates that the lack of functional neurofibromin, in the absence of *KIT* or *PDGFRA* mutations, can lead to GIST formation.

Neurofibromin functions as a negative regulator of the MAP-kinase pathway by catalyzing the hydrolysis of active Ras-GTP to an inactive Ras-GDP. In this light, inactivation of neurofibromin in NF1-related GISTs is the mechanism leading to tumor formation. To further elucidate this hypothesis, we evaluated the expression of KIT, neurofibromin and several downstream signaling molecules. Clear differences were observed between sporadic and NF1-related GISTs (Fig. 2). First, NF1-related GISTs demonstrated comparable total KIT expression but low levels of constitutive KIT autophosphorylation. Secondly, in contrast to sporadic tumors, NF1-related GISTs showed a dramatically reduced level of neurofibromin, confirming the inactivating effect of the *NF1* somatic mutations. Thirdly, strong expression and activation of MAPK in NF1-related GISTs illustrated increased signaling through the MAP-kinase pathway when compared with sporadic GIST cells. Fourthly, the JAK-STAT3 and PI3K-AKT pathways were shown to be less active in NF1-related GISTs. These data suggest that activation of the MAP-kinase cascade is a common cause of GIST development in both NF1 and non-NF1 patients, although there are important differences in the mechanism of activation (inactivation of *NF1* versus activation of *KIT/PDGFRA*).

Recurrent cytogenetic aberrations in sporadic GISTs include deletion of 14q, 22q, 1p, 11p and 9p and gain of 8p and 17q (reviewed in 5). These chromosomal changes can be viewed as secondary events acquired after the oncogenic mutations in the receptor tyrosine kinases and are associated with clinical progression. While deletion of chromosome arms 14q (38–43) and 22q (39,42–45) are frequent findings in sporadic GISTs and likely represent sites for tumor suppressor genes that play a role early in GIST formation, losses on chromosomes 1p, 9p and 11p are more significantly associated with malignancy (40,46–48). Gains on chromosomes 8q and 17q are associated with metastatic behavior (41,42,49). The array CGH experiments on NF1-related GISTs showed similar gains and losses of chromosome regions as seen in sporadic GISTs, i.e. losses of chromosomes 11, 14, 22 and 1p, in addition to several extra alterations (Table 3). These additional chromosomal abnormalities seem not to be recurrent in the different tested NF1-related GISTs. Remarkable is the array CGH profile of the very large tumor from patient NF1-3 where only the clones containing the *NF1* gene are deleted while no other aberrations are observed (Fig. 3). Taken together, the genomic changes observed in the analyzed NF1-related GISTs were similar to those described in sporadic GISTs and did not indicate any change that could be associated particularly with NF1-related GISTs, except for the deletion of the *NF1* gene region. It remains elusive why clinically symptomatic GISTs arise in only a minority of NF1 patients and why, in contrast to sporadic tumors, they tend to be multiple and predominantly located in the small intestine. The presence of multiple GISTs in NF1 might reflect a distinct rate-limiting step in oncogenesis compared with sporadic GIST. While a broad spectrum of inactivating genetic mechanisms might lead to suppression of the wild-type *NF1* allele and hence GIST formation in NF1 patients, only a limited set of specific activating mutations in *KIT/PDGFRA* will result in sporadic GIST. Moreover, genotypes at modifying loci, not detectable by array CGH, might trigger the development, location and number of multiple GISTs in NF1 patients.

On the basis of the array CGH profiles of the three tumors from patient NF1-2 and tumors I and II from patient NF1-1 (Table 3), one might argue that clustered GISTs are clonal. In this light, the identical chromosomal alterations in the tumors of each patient (loss 1pter→1p12 and 14 for NF1-2; loss 1pter→1p12, 14 and 22 for NF1-1) would reflect a common precursor lesion, whereas the tumor-specific alterations (cytogenetic aberrations and *NF1* somatic mutation) could be seen as secondary events. However, as illustrated by the same alterations in the tumors from two different patients, deletion of chromosomes 1p, 14 and 22 are recurrent cytogenetic aberrations in GISTs. Moreover, the apparently identical breakpoints at chromosome band 1p12 can be explained by the poor clone coverage at the centromere [21 Mb gap between RP11-418J17 (1p12) and RP11-417J8 (1q12)]. Overall, different tumors derived from the same

Table 2. Relative p-KIT and p-MAPK expression based on densitometric quantification of autoradiographic signals obtained by western blotting (data corresponding to Fig. 2)

	Sporadic GIST		NF1-related GIST						
	GIST882	Sporadic GIST	GIST NF1-1			GIST NF1-2			GIST NF1-3
			III	II	I	III	II	I	
p-KIT/KIT	0.44	3.05	0.64	0.67	0.30	0.22	0.18	0	0.81
p-MAPK/MAPK	0.10	0.11	1.78	0.82	1.50	1.73	1.46	0.98	0.32

Table 3. Summary of array CGH experiments on seven GISTs derived from three NF1 patients

Patient	Tumor	Array CGH	
		Gain	Loss
NF1-1	I	No gain	1pter→1p12, 2pter→2p11.2, 6q12→qter, 11pter→11p11.2, 13, 14, 18, 22
	II	10q25.1→10qter	1pter→1p12, 3q11.2→3qter, 11, 14, 19q13.11→19qter, 22, X
	III	6, 10, 20, 20	no loss
NF1-2	I	9q21.13→9qter	1pter→1p12, 14
	II	9q21.11→9qter	1pter→1p12, 14, 15, 22
	III	Xq22.1→Xqter	1pter→1p12, 14, 15
NF1-3	I	No gain	17q11.2 only RPCI5-926B9 and RP11-229K15 (=*NF1*)

patient bear different *NF1* somatic mutations and, with the exception of the typical GIST-related chromosomal alterations, array CGH profiles are quite diverse. Therefore, it is most likely that multiple GISTs in NF1 patients are independent tumors.

The tyrosine kinase inhibitor imatinib (Gleevec, Novartis) has tremendously improved the treatment of advanced sporadic GISTs, showing a clinical benefit in up to 80% of patients (17,18). To determine whether this drug could also be effective in NF1-related GISTs, we performed an *ex vivo* imatinib response experiment. As illustrated in Figure 5, ligand-dependent KIT autophosphorylation of primary NF1-related GIST cells and the control GIST882 cell line is totally inhibited by the exposure to imatinib at a concentration of 0.5 μM. In contrast to control fibroblasts, the level of MAPK autophosphorylation of primary NF1-related GIST cells was lowered after exposure to imatinib, although the inhibition was neither complete nor dose-dependent as clearly illustrated in the GIST882 cell line. These experiments thus suggest that imatinib might only have a mild effect on NF1-related GISTs. It will be important to determine whether this *ex vivo* observation can be translated into clinical benefit for NF1 patients with GISTs. As it was proven by *in vitro* studies (18), wild-type KIT is equally sensitive to imatinib as mutated KIT protein. Nevertheless, this observation does not translate to clinical response of patients with tumors that do not harbor *KIT* mutations (who poorly respond to the treatment). Therefore, *in vitro* assays may have only restricted predictive value.

In conclusion, we report for the first time the molecular pathogenesis of GISTs in NF1 individuals. Somatic *NF1* inactivation in the ICCs seems to be the molecular event underlying GIST development in the NF1 setting. Inactivation of neurofibromin in the absence of *KIT* or *PDGFRA* mutations is an alternate mechanism leading to GIST formation. Moreover, NF1-related GISTs show an increased signaling through the MAP-kinase pathway when compared with sporadic GIST cell lines. These findings clearly position GISTs in the range of clinical symptoms seen in NF1. It will be important to determine whether the tyrosine kinase inhibitor Gleevec used in the treatment of sporadic GIST also has an effect on NF1-related GIST.

MATERIAL AND METHODS

Patient material

Studies were performed on seven GISTs derived from three consenting NF1 patients. Peripheral blood was available for all patients as well as frozen tissue from the seven tumors and two tumor cell cultures from patient NF1-2. Control GIST cells included a GIST882 cell line harboring a *KIT* p.K642E mutation (50) and a sporadic GIST with a *KIT* p.557–558delWK mutation.

Phenotype NF1 patients

Patient NF1-1 is a 72-year-old female patient with widespread cutaneous neurofibromas. At the age of 59 years, a carcinoid tumor originating from the duodenum was resected. A GIST in the small intestine was diagnosed at the age of 68 and another in the retroperitoneal area adjacent to the bladder 4 years later. During surgery, both tumors were resected as well as a third not previously noted. The patient died postoperatively due to pulmonary complications. Her mother had had NF1 and had died due to complications related to a GIST at the age of 40.

Patient NF1-2 is a 59-year-old male with family history of neurofibromatosis type 1 and multiple cutaneous neurofibromas. Multiple GISTs of the duodenum were diagnosed by endoscopy following massive upper gastrointestinal tract bleeding. The episode of bleeding was preceded by the ingestion of a non-steroidal anti-inflammatory drug (nimesulide). During surgery, tumors were palpated at the serosal side of the duodenum and the proximal jejunum. No tumors could be palpated in the rest of the intestinal tract. Twelve small tumors (diameters of 3–22 mm) were removed from the duodenum and the proximal jejunum. A large lesion was localized in D2 and protruded at the anti-mesenterial side (diameter 20 mm). Two other large lesions (diameters 20 and 22 mm)

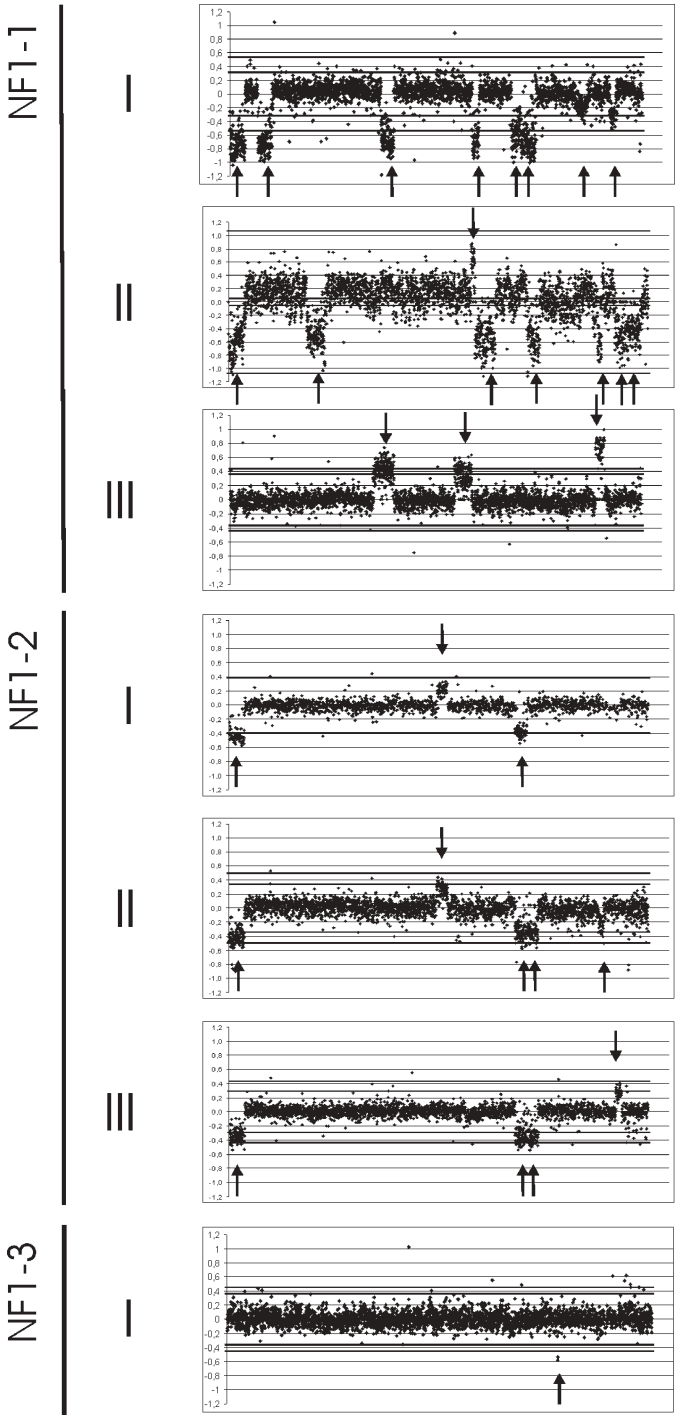

Figure 3. Array CGH profiles of seven NF1-related GISTs. Profiles are composed of numerically ordered autosomes (1–22) followed by X and Y. Arrows below and above profiles mark losses and gains, respectively.

showed mucosal ulcerations and were located in D2–D3. Pathological examination showed very few mitoses (less than 5 per 50 high-power fields). There were no post-operative complications, and post-operative follow-up at 2 years was uneventful. He did not receive adjuvant therapy.

Patient NF1-3 is a male with a severe thoraco-lumbar kyphoscoliosis and multiple cutaneous neurofibromas. At the age of 55, surgery was performed for a growing pelvic mass with nycturia and frequent micturition. The large tumor fills the pelvic cavity completely and is connected to the ileum over a distance of 4 cm. The large tumor was removed from the peritoneum, the bladder and the mesenterial side of the rectum and subsequently resected together with 7 cm of jejunum. The tumor measured $110 \times 110 \times 50\ mm^3$ and showed central necrosis. Pathological examination showed only one mitosis per 50 high-power fields. In the resected fragment of the jejunum, a tumor with a diameter of 20 mm was present (no mitoses detected) and three additional smaller GISTs (diameter 3–5 mm, no mitoses). The post-operative evolution was uneventful and the patient did not receive adjuvant therapy. At follow-up 2.5 years later, he is free of symptoms and a CT-scan of the abdomen did not reveal any mass.

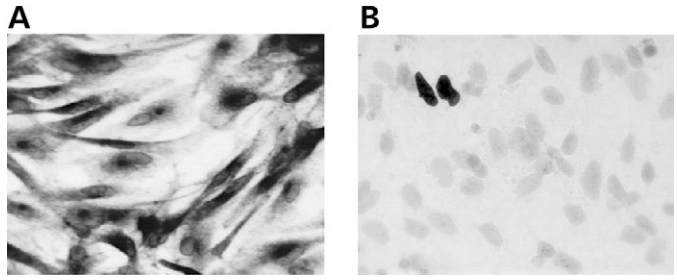

Figure 4. Phenotype of cultured cells. (**A**) After 3 days in culture, the primary cells of tumor III from patient NF1-2 exhibited spindle shape type of growth and 95% were KIT immunopositive. (**B**) The Ki-67 labeling index was 0.5 and 8.7% in ligand unstimulated and ligand stimulated cultures, respectively.

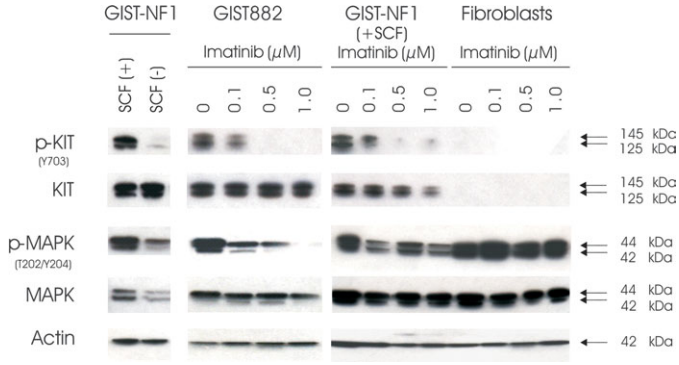

Figure 5. *Ex vivo* effect of imatinib on the phosphorylation of KIT Y703 and MAPK T202/Y204 residues in control GIST882 cell line (*KIT* p.K642E), primary NF1-related GIST cells cultured in medium supplemented with stem cell factor (SCF) and control human fibroblasts determined by western blot assay. Equal protein loading was demonstrated with anti-actin staining. The relative p-MAPK expression was evaluated on the basis of the densitometric quantification of autoradiographic signals. In contrast to control GIST882 cells with activating *KIT* mutation, the phosphorylation of wild-type *KIT* in primary NF1-related GIST cells under *in vitro* condition is ligand-dependent. Imatinib treatment resulted in only partial and dose-independent inhibition of MAPK posphorylation of NF1-GIST cells cultured under ligand stimulation.

Histopathology

Histopathological sections (5 μm) were cut from tumor paraffin blocks for routine H&E staining. Immunohistochemical

Table 4. Relative p-KIT and p-MAPK expression based on densitometric quantification of autoradiographic signals obtained by western blotting (data corresponding to Fig. 5)

	GIST-NF1		GIST-882				GIST-NF1 (+SCF)				Fibroblasts			
	SCF(+)	SCF(−)	Imatinib (μM)				Imatinib (μM)				Imatinib (μM)			
			0	0.1	0.5	1.0	0	0.1	0.5	1.0	0	0.1	0.5	1.0
p-KIT/KIT	1.01	<0.01	0.90	0.21	0	0	1.02	0.33	< 0.1	< 0.1	/	/	/	/
p-MAPK/MAPK	1.79	0.45	1.13	0.37	0.12	0	1.05	0.76	0.84	0.78	1.10	1.15	1.14	1.10

/: not applicable.

staining was conducted using the avidin–biotin–peroxidase complex method. Commercially available monoclonal (mc) or polyclonal (pc) antibodies specific for CD117 (pc, 1:250; DAKO, Glostrup, Denmark), CD34 (mc, 1:10; Becton Dickinson, San Jose, CA, USA), α-Smooth Muscle Actin (mc, 1:100; DAKO), desmin (mc, 1:20; ICN, Aurora, OH, USA) and S-100 (pc, 1:300; DAKO) were used. The CD117 immunostaining was performed without antigen retrieval and the presence of mast cells served as an internal control.

KIT/PDGFRA mutation analysis

Genomic DNA was extracted from snap-frozen tumor tissue of NF1-related GISTs using the High Pure PCR Template Preparation Kit (Roche). The entire *KIT* and *PDGFRA* coding region and the splice junctions were amplified by PCR. All amplicons were bidirectionally cycle sequenced using the ABI3730XL machine (Applied Biosystems).

NF1 mutation analysis

Germline *NF1* mutation analysis was performed (51). An improved strategy for *NF1* somatic mutation detection was developed (Maertens *et al*., manuscript in preparation) and applied specifically to the primary GIST cell cultures. Briefly, the second passage of ICC cell cultures from tumors II and III from patient NF1-2 was treated with puromycin before RNA extraction (RNeasy kit, Qiagen). Genomic DNA was extracted with the PureGene DNA purification kit (Gentra). The entire *NF1* cDNA was sequenced. Mutations found at the cDNA level were confirmed on gDNA. Detection of LOH was performed by genotyping microsatellite markers telomeric (3′NF1-3, 3′NF1-1) (52) and within (Alu, IVS27AC33.1, IVS38GT53.0, IVS27TG24.8) (53–56) *NF1*. Genomic DNA from paired frozen tissue or primary GIST cell cultures and lymphocyte cultures was investigated by multiplex PCR and analyzed on the ABI3100 genetic analyzer (Applied Biosystems) with the Genescan software (Applied Biosystems). Loss of the wild-type *NF1* allele was confirmed by at least one of the following techniques: FISH, array CGH, MLPA or SNP analysis. Frozen tumor specimens negative for LOH were subsequently submitted to PCR amplification and cycle sequencing of all *NF1* exons.

Western blotting

Proteins were isolated from snap-frozen tumor specimens and western blotting was performed as described in Debiec-Rychter *et al*. (57). In short, equivalent amounts of protein (30 μg) from clarified lysates were resolved with SDS–PAGE, transferred to PDVF membranes and immunoblotted sequentially with antisera specific for anti-NF1 (sc-67; 1:200; Biotechnology, Santa Cruz, CA, USA), anti-phospho-KIT (Y703) (1:250; Zymed, San Francisco, CA, USA), anti-KIT (1:500; DAKO), anti-phospho MAPK (T202/Y204) (1:1000; Cell Signaling Technology Inc., Beverly, MA, USA), anti-MAPK (1:1000; Zymed), anti-phospho AKT (S473) (1:500; Cell Signaling Technology Inc.), anti-AKT (1:500; Cell Signaling Technology Inc.), anti-STAT3 (1:500; Zymed) and anti-actin (1:500; Sigma, Saint Louis, MI, USA). After washes, membranes were probed with anti-rabbit or anti-goat immunoglobulin-HRP conjugate and incubated with ECL substrate (Pierce, Rockford, IL, USA). On the basis of the densitometric quantification of autoradiographic signals, the relative p-MAPK expression was determined in NF1-related GISTs.

Array CGH

Array CGH was performed as published previously (58). Tumor DNA was compared with DNA extracted from blood lymphocytes of a sex-matched control individual. Every case was analyzed twice and tumor DNA was once labeled with Cy3 and once with Cy5. Clones that gave the same result twice were taken into account.

Primary GIST cell culture and imatinib assay

The GIST specimen used for this experiment was tumor III of patient NF1-2. Primary cells were obtained from a disaggregated tumor specimen seeded at 60–70% confluence in 100 mm cell culture dishes and grown for 3 days in DMEM supplemented with 10% FBS, 20 ng/ml SCF, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). As a control, the same NF1-2 primary GIST cells, GIST882 cell line and normal human fibroblasts were grown in similar conditions but without the SCF ligand. For evaluation of the phenotype, NF1-2 primary GIST cells from tumor III were seeded at 60–70% confluence in slide chambers and grown for 3 days in the same conditions as above. After fixation in 4%

paraformaldehyde, immunohistochemical staining using the polyclonal rabbit antihuman antibody against CD117 (1:250; DAKO) and Ki-67 antibody (1:250; DAKO) was performed by the avidin–biotin–peroxidase complex method. The proliferation index was determined as the percentage of Ki-67 positive cells of the total number of cells. Imatinib mesylate was provided by Novartis Pharmaceuticals Corporation. The compound was dissolved at 10 mM in 100% DMSO (Sigma). Controls were performed with solvent (DMSO) dilutions. The effect of imatinib on the autophosphorylation of the KIT Y703 and the MAPK T204/Y202 residues in cultured primary NF1-related GIST cells (either ligand stimulated or unstimulated), the control GIST882 cell line and human fibroblasts was determined by western blot assay. In short, cells were exposed to either vehicle alone or to imatinib mesylate within a range of doses (0.1, 0.5 and 1.0 μM) for 90 min, washed with ice-cold PBS and lysed. Lysates were incubated for 30 min at +4°C and then centrifuged for 20 min. Supernatants were removed and used for SDS gel electrophoresis and immunoblotting as described previously (57). Equivalency of loading intracellular proteins was demonstrated by stripping the membrane and re-blotting using anti-actin antibody (1:500; Sigma).

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