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Pediatric Mastocytosis Is a Clonal Disease Associated with $D^{816}V$ and Other Activating c-*KIT* Mutations

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Adult mastocytosis is an incurable clonal disease associated with c-*KIT* mutations, mostly in exon 17 ($D^{816}V$). In contrast, pediatric mastocytosis often spontaneously regresses and is considered a reactive disease. Previous studies on childhood mastocytosis assessed only a few patients and focused primarily on codon 816 mutations, with various results. In this study, we analyzed the entire c-*KIT* sequence from cutaneous biopsies of 50 children with mastocytosis (ages 0–16 years). A mutation of codon 816 (exon 17) was found in 42% of cases, and mutations outside exon 17 were observed in 44%. Unexpectedly, half of the mutations were located in the fifth Ig loop of c-KIT's extracellular domain, which is encoded by exons 8 and 9. All mutations identified in this study were somatic and caused a constitutive activation of c-KIT. There was no clear phenotype–genotype correlation, no clear relationship between the mutations and familial *versus* spontaneous disease, and no significant change in the relative expression of the c-KIT GNNK+ and GNNK isoforms. These findings strongly support the idea that, although pediatric mastocytosis can spontaneously regress, it is a clonal disease most commonly associated with activating mutations in c-*KIT*.

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Abbreviations: GIST, gastrointestinal stromal tumor; ITD, internal tandem duplication; SCF, stem cell factor; WT, wild type

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INTRODUCTION

Mastocytosis is a heterogeneous group of clinical disorders characterized by the abnormal accumulation of mast cells in various tissues, especially in the skin and hematopoietic organs (Valent *et al.*, 2003b; Akin and Metcalfe, 2004). Mastocytosis usually appears in infancy or early adulthood. In most pediatric cases, the disease is limited to the skin, but it can be associated with systemic symptoms due to the release of mediators from mast cells, even when there is no systemic infiltration. The typical presentation of childhood-onset mastocytosis includes cutaneous manifestations (that is, mastocytoma, urticaria pigmentosa, and, less commonly, diffuse cutaneous mastocytosis), and it usually has a good prognosis, with substantial improvement or spontaneous resolution before puberty (Kiszewski *et al.*, 2004; Ben-Amitai *et al.*, 2005). In a few cases, particularly in the neonatal diffuse cutaneous form, the disease can be fatal as a result of hypovolemic shock, although infrequent, systemic forms can occur in children. In these cases, the disease may remain active through adolescence as a systemic adult mastocytosis. Clinical or biological factors that predict the evolution and severity of the disease in affected children have not yet been identified.

In contrast to childhood-onset mastocytosis, adult-onset mastocytosis often persists for the lifetime of the patient. Adult-onset mastocytosis is also more likely to be a severe

and systemic disease involving numerous organs. In some cases, it is associated with a clonal hematologic non-mast-cell lineage disease, such as a myelodysplastic or myeloproliferative disorder. Adult-onset mastocytosis can also lead to the rare mast cell leukemia, which carries a high risk of mortality (Valent *et al.*, 2003a).

It has now been clearly demonstrated that adult mastocytosis is related to the clonal proliferation of mast cells secondary to a gain of function of the proto-oncogene c-*KIT* (Longley *et al.*, 1996, 1999). Thus, in most cases adult mastocytosis is now considered a clonal myeloproliferative disorder. The *c-KIT* gene encodes a type III transmembrane tyrosine kinase that is the receptor for stem cell factor (SCF) (Broudy, 1997). The protein possesses an extracellular ligand-binding domain composed of five Ig-like regions, followed by a transmembrane domain, a negative regulatory juxtamembrane intracellular domain, and a cytoplasmic protein tyrosine kinase domain split by an insert into adenosine triphosphate–binding and phosphotransferase regions (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The two KIT isoforms differ in the presence or absence of four amino acids (GNNK) in the juxtamembrane region of the extracellular domain. Importantly, tyrosine autophosphorylation of the GNNK isoform is more rapid and gives rise to greater phosphorylation and activation of MAP kinase than is seen with the GNNK+ form. Ordinarily, the GNNK− form predominates (Caruana *et al.*, 1999). A variety of mutations have been found in c-*KIT*. Although it is not clear how heterogeneity in c-*KIT* mutations is involved in adult mastocytosis, it might account for the diversity of clinical presentations. Most patients with adult-onset mastocytosis have been reported to have the $D^{816}V$ mutation. The $V^{560}G$ mutation is also found but is less frequent. Both the $D^{816}V$ and the $V^{560}G$ mutations constitutively activate c-KIT, resulting in the clonal proliferation of mast cells (Longley *et al.*, 2001). Constitutively activated KIT is involved in other proliferative disorders, such as in gastrointestinal stromal tumors (GISTs), in which imatinib mesylate was found to be effective. In GISTs, most of the c-KIT mutations lie within the juxtamembrane intracellular domain. The location of the mutation is important in that the presence of a mutation in the phosphotransferase domain ($D^{816}V$ mutation) confers a spontaneous resistance to imatinib mesylate as in most of adult-onset mastocytosis.

The frequency and role of c-*KIT* mutations in childhood-onset mastocytosis and whether it is a clonal disease remain a matter of debate. A study by Longley *et al.* (1996) of six typical pediatric mastocytosis patients found that all lacked mutations in codon 816 but that three of the six had a dominant inactivating mutation, $K^{839}E$. Similarly, Buttner *et al.* (1998) reported that of 11 patients with childhood-onset mastocytosis, none had codon 816 mutations, suggesting that childhood mastocytosis is a reactive rather than a clonal disease. In contrast, in more recent studies, Yanagihori *et al.* (2005) found missense mutations in codon 816 in 10 of 12 Japanese children with mastocytosis, and Verzijl *et al.* (2007) found that 25% of children with urticaria pigmentosa had the $D^{816}V$ mutation.

Thus, most of the studies on the role of c-*KIT* mutations in childhood mastocytosis have focused on codon 816 and included relatively few patients. In this study, we screened the entire coding sequence of c-*KIT* mRNA from cutaneous lesions of 50 children between 0 and 16 years of age with sporadic or familial mastocytosis. Overall, 86% of the patients had mutations in c-*KIT*. The $D^{816}V$ mutation was present in 35% of the children, including two of four children with a familial form of the disease. We also found two patients with a $D^{816}Y$ mutation and one with a $D^{816}I$ mutation. Importantly, 44% of the children had mutations in exons 8, 9, and 11 that were mutually exclusive of the codon 816 mutations. All the detected mutations activated the c-KIT tyrosine kinase. Finally, we did not find any clear phenotype–genotype correlations, any notable differences in the relative expression of c-KIT GNNK isoforms, or a clear relationship between the genotype and familial *versus* sporadic forms of the disease. These findings indicate that, despite the high rate of spontaneous regression, childhood mastocytosis is most often a clonal disease associated with activating mutations in c-*KIT*.

RESULTS

Clinical characteristics of patients

We performed a multicenter study to examine the association of c-*KIT* mutations with childhood-onset mastocytosis. The study included 65 patients with childhood-onset mastocytosis. The mean age was 7.0 ± 4.7 years. A case report form was completed for 54 of the 65 patients, and mutation screening was performed in 50 cases. The total population consisted of 38 (58%) males and 27 (42%) females, with an equal distribution of the different forms of mastocytosis in males and females. The type of skin lesion was urticaria pigmentosa in 80% of the patients, mastocytoma in 11%, and diffuse cutaneous mastocytosis in 9%. In 13% of cases, another member of the family also suffered from mastocytosis. Lesions appeared before 2 years of age in 85% of the patients, between 3 and 9 years in 13%, and around 14 years of age in the remaining 12%. The overall mean age of onset was 3.0 ± 3.5 years. The serum tryptase levels determined in 17 cases were within the normal range ($<15\ \mu g\ l^{-1}$) (Table 1). A bone marrow biopsy was collected from only one case (patient M099) (Table 1). Our ethics committee prohibited the collection of blood or additional biopsy samples from the patients except when necessary for therapeutic decisions. It was possible to collect a bone marrow biopsy from patient M099 because, being 16 years old at the time of analysis, he was considered an adult patient in France. This patient had shown diffuse cutaneous involvement since birth, along with severe systemic manifestations, hepatomegaly, splenomegaly, and adenopathy. Histological analysis of the bone marrow biopsy showed infiltration by pathological mast cells (>10%), confirming systemic disease.

Analysis of c-*KIT* codon 816

The $D^{816}V$ mutation of c-*KIT*, which lies within and activates the kinase domain, is frequently associated with adult mastocytosis (Longley *et al.*, 2001). One previous study of children with mastocytosis did not find this mutation (Longley

Table 1. Summary of patient characteristics and c-*KIT* mutation status

No.	Sex	Type of skin lesion	Age at onset	Age at biopsy	Serum tryptase (ng ml^{-1})	Familial case	c-*KIT* sequence–RNA[1]			c-*KIT* sequence–DNA[2]
							Mutation	Exon	Exon 10 sequence	
E001	M	UP	1 week	3 years	3.2	No	$D^{816}V$	17	$M^{541}L$	ND
E015	M	UP	9 months	3 years	4.9	No	$D^{816}V$	17	$M^{541}L$	ND
E033	M	UP	2 months	10 years	ND	No	$D^{816}V$	17	$M^{541}L$	ND
E047	F	UP	2 months	2 years	ND	No	$D^{816}V$	17	$M^{541}L$	ND
E009	F	UP	Birth	10 years	5	No	$D^{816}V$	17	WT	ND
E012	F	UP	Birth	3 years	ND	No	$D^{816}V$	17	WT	ND
E024	M	UP	Birth	4 years	14.3	No	$D^{816}V$	17	WT	ND
E025	F	UP	1 month	3 years	ND	No	$D^{816}V$	17	WT	WT
E027	F	UP	2 years	7 years	3.4	Yes	$D^{816}V$	17	WT	ND
E031	F	UP	9 months	9 years	6.1	No	$D^{816}V$	17	WT	WT
E034	M	UP	3 months	11 years	ND	No	$D^{816}V$	17	WT	WT
E035	F	UP	16 years	18 years	ND	No	$D^{816}V$	17	WT	WT
E036	M	UP	4 months	2 years	ND	No	$D^{816}V$	17	WT	ND
E049	M	UP	4 months	2 years	ND	No	$D^{816}V$	17	WT	ND
E004	M	DCM	3 months	7 years	5.9	No	$D^{816}V$	17	WT	ND
M081	M	NA	Birth	3 years	ND	No	$D^{816}V$	17	WT	ND
M084	M	NA	Birth	9 years	ND	Yes	$D^{816}V$	17	WT	WT
E006	M	NA	NA	NA	ND	No	$D^{816}V$	17	WT	ND
E018	M	UP	5 weeks	5 years	ND	No	$D^{816}Y$	17	$M^{541}L$	WT
M099	M	DCM[3]	Birth	16 years	ND	No	$D^{816}Y$	17	WT	ND
E010	M	DCM	Birth	7 years	2.7	No	$D^{816}I$	17	WT	ND
E005	M	UP	Birth	2 years	4	No	$InsFF^{419}$	8	$M^{541}L$	WT
E008	F	UP	4 months	8 years	1.4	No	Δ^{419}	8	$M^{541}L$	ND
E050	F	UP	4 months	5 months	ND	No	Δ^{419}	8	$M^{541}L$	ND
E022	F	UP	2 months	2 years	4.5	No	Δ^{419}	8	WT	ND
E038	M	UP	5 months	6 years	ND	No	Δ^{419}	8	WT	WT
E030	M	DCM	2 months	3 years	7.2	No	Δ^{419}	8	$M^{541}L$	ND
E026	F	NA	Birth	9 years	10	No	Δ^{419}	8	$M^{541}L$	WT
E061	F	NA	Birth	1 year	ND	No	Δ^{419}	8	WT	WT
E011	F	MC	Birth	4 years	ND	No	$\Delta^{417-419}InsY$	8	WT	ND
E017	M	MC/UP	3 months/12 months[4]	6 years	ND	No	$C^{443}Y$	8	WT	WT
E032	F	UP	4 m	5 years	ND	No	$S^{476}I$	9	WT	ND
E054	M	NA	NA	NA	ND	No	$ITD^{501-502}$	9	$M^{541}L$	ND
E013	M	UP	1 year	10 years	ND	No	$ITD^{501-502}$	9	$M^{541}L$	WT
E002	M	UP	Birth	1 year	ND	No	$ITD^{502-503}$	9	WT	ND
E020	M	DCM	1 year	5 years	7.1	No	$ITD^{502-503}$	9	WT	ND
E041	F	MC	2 months	3 years	ND	No	$ITD^{505-508}$	9	WT	ND
E007	M	UP	7 years	9 years	4.6	No	$K^{509}I$	9	$M^{541}L$	ND
E014	F	UP	3 months	1 year	3.9	No	$K^{509}I$	9	WT	WT
E040	F	UP	1 year	2 years	ND	No	$K^{509}I$	9	WT	ND
E044	M	UP	10 months	2 years	ND	No	$K^{509}I$	9	WT	ND
E053	F	UP	3 months	2 years	ND	No	$K^{509}I$	9	WT	ND

Table 1 continued on the following page

Table 1. Continued

No.	Sex	Type of skin lesion	Age at onset	Age at biopsy	Serum tryptase (ng ml^{-1})	Familial case	c-*KIT* sequence–RNA[1]			c-*KIT* sequence–DNA[2]
							Mutation	Exon	Exon 10 sequence	
E003	M	NA	3 years	10 years	ND	No	$D^{572}A$	11	$M^{541}L$	ND
E037	M	UP	4 years	12 years	ND	No	WT	—	WT	ND
E042	M	UP	3 years	13 years	ND	No	WT	—	WT	ND
E043	F	UP	3 years	4 years	ND	Yes	WT	—	WT	ND
E060	F	MC	Birth	3 years	ND	No	WT	—	WT	ND
E048	M	NA	Birth	1 years	ND	No	WT	—	WT	ND
M123	M	NA	2 years	8 years	ND	Yes	WT	—	WT	ND
M216	F	NA	3 years	11 years	ND	No	WT	—	WT	ND

DCM, diffuse cutaneous mastocytosis; F, female, M, male; MC, mastocytoma; UP, urticaria pigmentosa; WT, wild type.
[1]The presence of mutations was assessed by reverse transcription–PCR using RNA isolated from skin biopsies, and the mutations are detailed in Table 2.
[2]The presence of mutations was assessed by PCR using DNA isolated from blood samples.
[3]Patient diagnosed with systemic mastocytosis as indicated by bone marrow and liver involvement.
[4]Patient 017 was first diagnosed with a mastocytoma at 3 months and then with UP at 12 months.
The table includes the 50 patients on whom mutation screening was carried out.
ND indicates not done (that is, blood sample was not available); NA, skin involvement unknown because the case report form was not available.

et al., 1996; Buttner *et al.*, 1998), although one study found this mutation in 7% (Longley *et al.*, 1999), another found it in 25% (Verzijl *et al.*, 2007) of patients, and a third found it in 64.3% (Yanagihori *et al.*, 2005). Therefore, we first examined the frequency of this mutation in our panel of children with mastocytosis. We found that 29 of the 50 children (58%) had the wild-type sequence (Asp) at codon 816 (Figure 1 and Table 1). Eighteen (36%) of the remaining children had the $D^{816}V$ mutation, and two (4%) had a $D^{816}Y$ mutation, including the patient diagnosed with systemic mastocytosis. Also, one patient (2%) had a previously undescribed $D^{816}I$ mutation. We did not have sequence data from bone marrow because the collection of bone marrow biopsies from the children in this study was prohibited by our ethical committee, except when necessary for therapeutic decisions. Importantly, bone marrow samples were not needed because analysis of the cutaneous biopsies was sensitive enough to detect the mutations.

We next carried out restriction-length polymorphism analysis to confirm the presence or absence of mutations at codon 816. The cDNAs generated from the cutaneous biopsy samples were amplified by PCR using fluorescent primers, and the resulting amplimers were digested with *Ple*I or *Bsm*A1, which specifically cut the $D^{816}V$-containing sequence and the wild-type sequence, respectively. This method was sensitive enough to detect the mutated allele when it was present in as few as approximately 2% of the cells in the sample. The results of this analysis confirmed the presence or absence of the mutations that had been identified by sequencing (data not shown).

Identification of mutations in other codons

Because a substantial portion of our patient population (58%) lacked changes in codon 816, we investigated whether they had mutations at other sites in c-*KIT* or whether the technique was not sufficiently sensitive to detect the $D^{816}V$ mutation in these cases. We therefore sequenced the entire c-*KIT* coding region for all patients lacking the $D^{816}V$ mutation. Five PCR fragments were amplified covering the entire c-*KIT*-coding sequence: nucleotides 1–792 (exons 1–4), 729–1288 (exons 4–7/8), 1197–2121 (exons 7–14), 1934–2517 (exons 13–17/18), and 2413–2991 (exons 17–21) (Figure 1). Direct sequencing of these PCR amplimers was carried out for each patient sample, and identified mutations were confirmed by performing a new reverse transcription reaction.

Using this method, we identified several new mutations in c-*KIT* in our patient population. These alterations were mainly located in exons 8 and 9. The mutations in exon 8 included an insertion of FF at codon 419 ($InsFF^{419}$, one case (2%)), a single deletion of D^{419} (Δ^{419}, eight cases (16%)), a 2-bp deletion with a 1-bp substitution between codons 417 and 419 ($\Delta^{417-418}D^{419}Y$, one case (2%)), and a substitution of a cysteine at codon 443 with a tyrosine ($C^{443}Y$, one case (2%)) (Tables 1 and 2 and Figure 1). In exon 9, there was a substitution of a serine at codon 476 with an isoleucine ($S^{476}I$, one case (2%)), an internal tandem duplication of the $A^{502}Y^{503}$ pair ($ITD^{502-503}$, two cases (4%)), and two other internal tandem duplications leading to insertions near these two residues at codons 501 and 505 ($ITD^{501-502}$ and $ITD^{505-508}$, three cases (6%)). In addition, we identified five cases (10%) that had $K^{509}I$ substitutions. The $C^{443}Y$ and $S^{476}I$ mutations are new substitutions that lie nearby and between hot spots at codons 417–419 and 501–509. Furthermore, in 14 patients (28%), we found a single type of substitution at codon 541 ($M^{541}L$), which lies in exon 10. This KIT polymorphism was reported in mastocytosis and in the normal population and seems to enhance the response to only low levels of SCF (Foster *et al.*, 2008). Interestingly, none

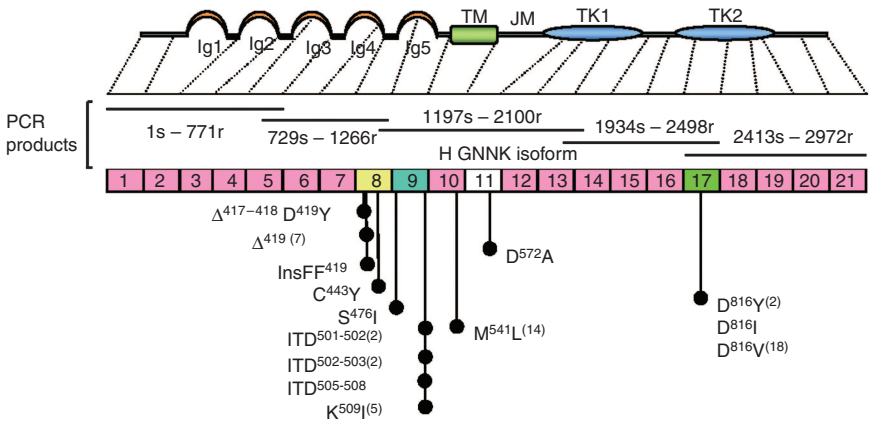

Figure 1. Summary of c-KIT mutations found in the patients with childhood mastocytosis. Schematic diagram of the KIT receptor protein and the corresponding location of the 21-exon coding sequence. The exon numbers are shown in boxes, and specific mutations are indicated in the corresponding regions. The numbers of identical mutations found in this study appear in parentheses. Also shown is a schematic map of PCR products (primers used are listed in Supplementary Table 1). The position of GNNK+ isoform is indicated at the end of exon 9. Ig, Ig loop-like domain; JM, juxtamembrane intracellular domain; TK1 and TK2, tyrosine kinase domain regions 1 and 2, respectively; TM, transmembrane domain.

Table 2. Summary of *c-KIT* mutations found in this study

Mutation	Nucleotide sequence (5′→3′)[1]	Position[2]	Changes in amino acid sequence
$D^{816}V$	agagTcatc	2247	Asp^{816} to Val
$D^{816}Y$	agaTacatc	2446	Asp^{816} to Tyr
$D^{816}I$	agaATcatc	2446	Asp^{816} to Ile
$InsFF^{419}$	acttacTTCTTCgac agg	1255	Insertion of PhePhe at amino acid 419
$\Delta^{417-419}$insY	gaaatcctg — — Tacaggctc	1249	Replacement of $Thr^{417}Tyr^{418}Asp^{419}$ with Tyr
Δ^{419}	acttac — aggctc	1255	Deletion of Asp^{419}
$C^{443}Y$	ttttAtcca	1328	Cys^{443} to Tyr
$S^{476}I$	cagaTttct	1427	Ser^{476} to Ile
$ITD^{501-502}$	acttctgcTTCTGcctatttt	1500	Duplication of Ser^{501}Ala
$ITD^{501-502}$	acttctgcctCTGcCtatttt	1502	Duplication of Ser^{501}Ala
$ITD^{502-503}$	tctgcctatGCCTATtttaac	1504	Duplication of Ala^{502} Tyr
$ITD^{505-508}$	tattttaactttgcatttAACTTTGCATTTaaagag	1525	Duplication of Asn^{505}PheAlaPhe
$K^{509}I$	tttaTagag	1526	Lys^{509} to Ile
$D^{572}A$	atagCccca	1715	Asp^{572} to Ala
$M^{541}L$	gtgCtgatt	1621	Met^{541} to Leu

[1]Altered sequences are indicated in capital letters.
[2]Nucleotide positions are indicated according to the c-*KIT* sequence in GenBank (accession number X06182).

of the patients with the wild-type codon 816 sequence (aspartate) carried the $M^{541}L$ substitution. To assess whether the M541L mutation was located on the same allele as the c-*KIT* D816V mutation, we further genotyped 11 of 14 M541L-positive patients. We found no difference in the frequency of expression of M541L in the allele bearing the activating mutations (5 of 11) *versus* that in the wild-type allele (6 of 11). We did not find clinical differences between both groups, but one child among the five of the former group had diffuse cutaneous mastocytosis. Eventually, we performed sequence analysis for the entire *c-KIT* coding region for all patients. Surprisingly, all the newly identified mutations were mutually exclusive of mutations in codon 816 (data not shown). Overall, 43 of the 50 patients (86%) in this study had mutations in c-*KIT*.

Analysis of genomic DNA for c-*KIT* mutations

Blood samples were available for 13 patients, making it possible to analyze genomic DNA for the presence of c-*KIT* mutations. One of these 13 samples was from a patient (M084) with a familial form of mastocytosis. Mutations in c-*KIT* were not found in any of these samples (Table 1), indicating that in these cases the mutations were somatic rather than germline.

Effect of mutations on KIT activity in Cos cells

Activation of KIT by the $D^{816}V$ mutation is frequently found in adult mastocytosis (Buttner *et al.*, 1998). We therefore examined whether the mutations identified in this study also caused ligand-independent activation of KIT. Cos cells were transiently transfected with plasmids expressing wild-type KIT or KIT containing the $\Delta^{417-418}D^{419}Y$, $C^{443}Y$, $S^{476}I$, $ITD^{502-503}$, $K^{509}I$, $D^{572}A$, $D^{816}V$, $D^{816}Y$, or $D^{816}I$ mutation. After treatment of cells with or without 250 ng ml^{-1} SCF, the tyrosine phosphorylation of KIT was assessed by western blotting. As shown in Figure 2, SCF stimulated the tyrosine phosphorylation of wild-type KIT, and, as expected, the $D^{816}V$ mutation constitutively activated KIT tyrosine phosphorylation. Importantly, all the other tested mutations resulted in a ligand-independent tyrosine phosphorylation of KIT (Supplementary Figure 1), except for the M541L variant (Supplementary Figure 2).

Expression ratio of the GNNK+ and GNNK isoforms in patients

The GNNK (short (S)) and GNNK+ (long (L)) isoforms of KIT have been shown to signal through different pathways and to have distinct transforming activities (Caruana *et al.*, 1999); moreover, deregulation of the expression ratio of these isoforms may participate in mastocytosis. Therefore, we examined the expression ratio of the GNNK and GNNK+ isoforms (S/L ratio). This analysis was performed for 47 patients. In cases in which patients had an insertion or deletion mutation, both short and long wild-type and mutant isoforms could be detected, allowing a direct assessment of the effect of the mutation on mRNA splicing. There was little or no difference in the S/L ratios for the wild-type or mutant-length isoforms for these patients (Table 3). It was not possible to calculate the S/L ratios for patient E041, who had a 12-nucleotide ITD that caused the short mutated isoform to be the same length as the wild-type long form. For patients with point mutations in c-*KIT*, the lengths of the isoforms were the same as those of the wild type. The mean S/L ratio for the entire population was 10.8 ± 2.8 ($n = 47$), and the S/L ratio for patients with an insertion or deletion mutant of c-*KIT* was not significantly different for patients with wild-type KIT (9.1 ± 3.8 ($n = 13$) vs. 10.2 ± 3.4 ($n = 7$)).

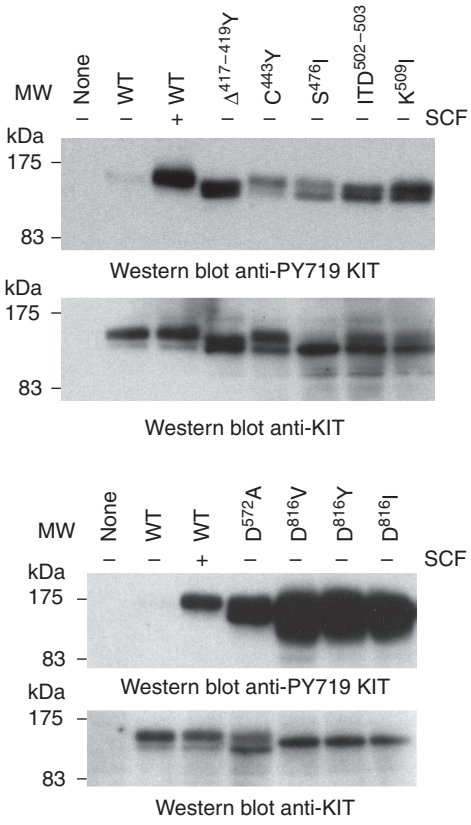

Figure 2. Ligand-independent autophosphorylation of KIT. Cos cells were transiently transfected with pcDNA plasmids for expressing wild type or mutants of KIT and treated with (+) or without (−) 250 ng ml^{-1} stem cell factor (SCF). Western blotting was carried out using an antibody against KIT phosphorylated on Tyr^{719} (anti-PY719 KIT) or with an anti-c-KIT antibody (anti-KIT).

Genotype–phenotype correlation and comparison of familial *versus* sporadic cases

Analysis of genotype–phenotype relationships was possible in 44 cases (Table 4). There was no clear correlation between the phenotypes (cutaneous involvement) and the genotypes of the patients, except for an absence of mutations in codon 816 in children whose mastocytosis occurred between the ages of 3 and 16 years (data not shown). Also, for 48 cases, we had information on whether the disease was familial or sporadic. Again, there was no clear correlation between the genotype and familial *versus* sporadic disease, except for an absence of exon 8, 9, and 11 mutations in patients with a familial form of the disease. Meaningful statistical analysis by phenotype or familial *versus* sporadic disease was not possible because of insufficient numbers of patients in several of the categories. In addition, none of the patients had an aggressive form or a tryptase level above 200 μg l^{-1}.

DISCUSSION

We performed a multicenter study to examine the association of c-*KIT* mutations with childhood-onset mastocytosis. Sequencing of *c-KIT* was performed in 50 children between 0 and 16 years of age. This was the largest study of mutations in childhood-onset mastocytosis to date.

We found the $D^{816}V$ mutation (exon 17), which is frequently associated with adult mastocytosis (Longley *et al.*, 2001), in 18 of 50 (36%) children in this study. Three additional patients (6%) had other mutations in codon 816, including two with a $D^{816}Y$ mutation and one with a previously unreported $D^{816}I$ mutation. As with the $D^{816}V$ mutation, we found that both the $D^{816}Y$ and $D^{816}I$ mutations cause a ligand-independent activation of KIT.

We also identified several genetic alterations in c-*KIT* in our patient population, some of which have not previously been identified in childhood or adult mastocytosis. These alterations were located mainly in exons 8 and 9 (22 of 50 patients (44%); Figure 1), which encode the fifth Ig (D5) domain and the extracellular region near the transmembrane domain, regions that have previously been shown to be

Table 3. Expression of GNNK isoforms and S/L ratios

		Area under the curve					
		Wild-type size		Mutated size		S/L ratio	
Patient	Genotype	GNNK−	GNNK+	GNNK−	GNNK+	Wild-type size	Mutated size
E001	$D^{816}V$	69795	5507	0	0	13	NA
E015	$D^{816}V$	66707	7841	0	0	9	NA
E033	$D^{816}V$	ND	ND	ND	ND	ND	ND
E047	$D^{816}V$	66526	5973	0	0	11	NA
E009	$D^{816}V$	67032	8706	0	0	8	NA
E012	$D^{816}V$	70751	8826	0	0	8	NA
E024	$D^{816}V$	69836	5905	0	0	12	NA
E025	$D^{816}V$	64939	4957	0	0	13	NA
E027	$D^{816}V$	69350	5509	0	0	13	NA
E031	$D^{816}V$	66869	4732	0	0	14	NA
E034	$D^{816}V$	ND	ND	ND	ND	ND	ND
E035	$D^{816}V$	72160	5464	0	0	13	NA
E036	$D^{816}V$	69483	8044	0	0	9	NA
E049	$D^{816}V$	68531	5819	0	0	12	NA
E004	$D^{816}V$	68904	10487	0	0	7	NA
M081	$D^{816}V$	65330	5106	0	0	13	NA
M084	$D^{816}V$	60394	5212	0	0	12	NA
E006	$D^{816}V$	67922	5577	0	0	12	NA
E018	$D^{816}Y$	66245	8212	0	0	8	NA
M099	$D^{816}Y$	ND	ND	ND	ND	ND	ND
E010	$D^{816}I$	28780	1783	0	0	16	NA
E005	$InsFF^{419}$	10664	1688	8040	1202	6	7
E008	Δ^{419}	13733	1016	12833	943	14	14
E050	Δ^{419}	14727	1089	12829	979	14	13
E022	Δ^{419}	13266	1803	12213	1717	7	7
E038	Δ^{419}	67019	7748	57624	7192	9	8
E030	Δ^{419}	14429	1762	11259	1416	8	8
E026	Δ^{419}	59224	7741	21743	2692	8	8
E061	Δ^{419}	12045	1484	12434	1575	8	8
E011	$\Delta^{417-419}InsY$	62184	4197	67474	4863	15	14
E017	$C^{443}Y$	66178	5882	0	0	11	NA
E032	$S^{476}I$	73569	6956	0	0	11	NA
E054	$ITD^{501-502}$	14083	938	13226	1519	15	9
E013	$ITD^{501-502}$	13469	1512	11147	1639	9	7
E002	$ITD^{502-503}$	34023	3324	32606	2807	10	12
E020	$ITD^{502-503}$	65419	4268	54359	3144	15	17
E041[1]	$ITD^{505-508}$	44131	30290	0	0	NC	NA
E007	$K^{509}I$	63898	7419	0	0	9	NA
E014	$K^{509}I$	50404	4751	0	0	11	NA
E040	$K^{509}I$	23766	1954	0	0	12	NA
E044	$K^{509}I$	66224	3909	0	0	17	NA
E053	$K^{509}I$	65933	6508	0	0	10	NA

Table 3 continued on the following page

Table 3. Continued

		Area under the curve					
		Wild-type size		Mutated size		S/L ratio	
Patient	Genotype	GNNK−	GNNK+	GNNK−	GNNK+	Wild-type size	Mutated size
E003	$D^{572}A$	66258	7454	0	0	9	NA
E037	Wild type	24654	2719	0	0	9	NA
E042	Wild type	68563	10774	0	0	6	NA
E043	Wild type	68893	7190	0	0	10	NA
E060	Wild type	69413	4040	0	0	17	NA
E048	Wild type	50468	5672	0	0	9	NA
M123	Wild type	57655	9215	0	0	6	NA
M216	Wild type	62026	8657	0	0	7	NA

NA, not applicable; ND, not done.
[1]NC, not calculable because the short mutated form contained a 12-nucleotide difference, so that it could not be distinguished from the wild-type long isoform allele.

Table 4. c-*KIT* mutations by phenotype

		Type of skin lesion			Familial vs sporadic	
Mutation	All, *n* (%) (*n*=50)	Urticaria pigmentosa, *n* (%) (*n*=31)	Diffuse cutaneous mastocytosis, *n* (%) (*n*=4)	Mastocytoma, *n* (%) (*n*=2)	Familial, *n* (%) (*n*=4)	Sporadic, *n* (%) (*n*=44)
$D^{816}V$	18 (36)	14 (45)	1 (25)	0 (0)	2 (50)	15 (34)
Any codon 816	21 (42)	15 (48)	2 (50)	0 (0)	2 (50)	18 (41)
Exons 8, 9, or 11	22 (44)	13 (42)	2 (50)	2 (100)	0 (0)	21 (48)
Any mutation	43 (86)	28 (90)	4 (100)	2 (100)	2 (50)	39 (89)
None (wild type)	7 (14)	3 (10)	0 (0)	0 (0)	2 (50)	5 (11)

affected in core-binding factor-acute myeloid leukemia and in GISTs, respectively (Gari *et al.*, 1999; Lux *et al.*, 2000). Interestingly, alterations in these two exons have been only rarely found in pediatric or adult forms of mastocytosis. The mutations in exon 8 included an insertion of FF ($InsFF^{419}$) and a single deletion at D^{419} (Δ^{419}), as well as a 2-bp deletion with a 1-bp substitution in the same area ($\Delta^{417-418}D^{419}Y$). The latter insertion has been reported in core-binding factor-acute myeloid leukemia (Gari *et al.*, 1999; Goemans *et al.*, 2005), and the Δ^{419} mutation has been identified in kindreds with familial GISTs and mastocytosis (Hartmann *et al.*, 2005).

In exon 9, there was an internal tandem duplication of the $A^{502}Y^{503}$ pair, which has been described mainly in GISTs (Lux *et al.*, 2000), as well as insertions around these two residues at codons 501 and 505. In addition, we identified a substitution in codon 509 ($K^{509}I$). This mutation was previously reported in three siblings with familial mastocytosis, but its effect on KIT activity was not examined (Zhang *et al.*, 2006). Although this is at the 3′-limit of exon 9, the relative ratio of GNNK+ to GNNK isoforms was unchanged by this mutation, indicating that it did not alter mRNA splicing. We also identified substitutions $C^{443}Y$ (exon 8) and $S^{476}I$ (exon 9) for the first time. According to these results, it seems that there are two hot spots for mutations in childhood-onset mastocytosis at codons 417–419 and 501–509 (exons 8 and 9, respectively; Figure 1). Substitutions $C^{443}Y$ and $S^{476}I$ lie near and between these two hot spots.

Interestingly, all the tested mutations in exons 8 and 9 ($\Delta^{417-418}D^{419}Y$, $C^{443}Y$, $S^{476}I$, and $ITD^{502-503}$, and $K^{509}I$) activated the tyrosine phosphorylation of KIT. Indeed, mutations at or near Asp^{419} and Ala^{502}–Tyr^{503} are known to activate KIT, and a very recent crystallographic study revealed that these residues lie at an interface between D5 domains in the ligand-induced dimers of KIT, suggesting that oncogenic mutations at these sites act by enhancing homotypic interactions between D5 domains (Yuzawa *et al.*, 2007). The activation of KIT by the $C^{443}Y$ mutation supports the idea that this residue is involved in the proper folding of the D5 domain (Broudy *et al.*, 2001), so that its mutation could also enhance the association of D5 domains. Although the importance of S^{476} is not currently clear, it could act in the same manner. Overall, these results strongly support the idea that residues 417–419 and 501–509 have key roles in KIT activation, and, furthermore, that they and other activating mutations in exons 8 and 9 are selectively involved in childhood-onset mastocytosis.

Surprisingly, we found only one substitution in exon 11 ($D^{572}A$; patient E003). This residue is part of a peptide

sequence that includes Tyr^{570}, which, when phosphorylated, binds several SH2-domain-containing proteins, such as the tyrosine phosphatases SHP1 and SHP2 (Kozlowski *et al.*, 1998), members of the Src family kinases (Lennartsson *et al.*, 1999), and SOCS6 (Bayle *et al.*, 2004). Thus, this amino acid could control the recognition of SH2 domains by KIT or regulate the autoinhibition of its tyrosine kinase activity (Ma *et al.*, 1999a; Mol *et al.*, 2004). Indeed, we found that, *in vitro*, this mutation activated KIT tyrosine phosphorylation. Several genetic alterations were reported in this peptide sequence in a large proportion of GISTs (Heinrich *et al.*, 2003a), as well as in dog mast cell tumors (Longley *et al.*, 1995), and it has been reported at the analogous site in other receptor tyrosine kinases, such as in FLT3 in acute myeloid leukemia (Gilliland and Griffin, 2002) and platelet-derived growth factor-α receptor in GISTs (Heinrich *et al.*, 2003b). Although the $D^{572}A$ mutation was found less frequently than the other c-*KIT* mutations, it also caused constitutive ligand-independent autophosphorylation of the KIT protein when expressed in Cos cells. Importantly, all the identified extracellular and juxtamembrane intracellular domain mutations are sensitive to imatinib, in contrast to the D^{816} mutation (Y Yang *et al.*, unpublished data).

Interestingly, we did not detect mutations $F^{522}C$, $V^{530}I$, or $A^{533}D$, which have been previously identified in mastocytosis patients and lie in the transmembrane domain of KIT (exon 10) (Akin *et al.*, 2004; O'Brien *et al.*, 2004; Tang *et al.*, 2004). Because each of these was reported in only a single patient, they could represent very rare events not appearing in our panel, or they may be specifically associated with familial and/or adult mastocytosis.

We found a single substitution at codon 541 ($M^{541}L$). This codon lies within exon 10, which encodes the transmembrane domain. The Met-to-Leu substitution is conservative, and because this mutation was previously reported in a normal population and in normal tissues (Nagata *et al.*, 1996), it is considered a polymorphic variation. In a recent article, germline $M^{541}L$ mutations were reported in familial cases of pediatric mastocytosis. This mutation was shown to enhance SCF sensitivity slightly (Foster *et al.*, 2008). However, for one of our $M^{541}L$-expressing patients, a blood sample was available, allowing the analysis of genomic DNA. In this case, the substitution was not present in the genomic DNA, suggesting that this position may be highly affected by somatic variation, as previously reported in patients in the blast crisis of chronic myeloid leukemia (Inokuchi *et al.*, 2002). In addition, when ectopically expressed in murine Ba/F3 cells, KIT containing this mutation mediates cell proliferation to the same extent as the wild-type receptor, although the mutant seems to be slightly more sensitive to SCF than the wild-type receptor—but only to low levels of SCF, as previously reported (Foster *et al.*, 2008) (Supplementary Figure 2). This $M^{541}L$ mutation could act by causing a slight change in the conformation of KIT, resulting in enhanced dimerization and autophosphorylation. In this way, the $M^{541}L$ mutation could increase cell proliferation in chronic myeloid leukemia and thereby reduce the survival of these patients (Inokuchi *et al.*, 2002). In mastocytosis, this hypersensitivity to SCF could increase the survival of $M^{541}L$-expressing mast cells, leading to mast cell accumulation. It is noteworthy that none of the patients with the wild-type codon 816 sequence (Asp) carried the $M^{541}L$ substitution.

Importantly, all the tested mutations in codons 8, 9, 11, and 17 ($\Delta^{417-418}D^{419}Y$, $C^{443}Y$, $S^{476}I$, $ITD^{502-503}$, $K^{509}I$, $D^{572}A$, $D^{816}V$, $D^{816}Y$, and $D^{816}I$) caused a constitutive ligand-independent activation of KIT, with a higher phosphorylation status for exon 17 mutations (Figure 2). Combined with the fact that most (86%) of the patients had mutations in these codons, this strongly supports the idea that, like adult mastocytosis, childhood mastocytosis is a clonal disease. These findings further emphasize the importance of KIT activation in both pediatric and adult mastocytosis.

Yanagihori *et al.* (2005) recently described c-*KIT* mutations in 12 patients with childhood-onset mastocytosis. They reported that children with a $D^{816}F$ mutation developed cutaneous mastocytosis at an earlier age than those with the $D^{816}V$ mutation, although the statistical significance was very low ($P=0.068$). In contrast, in this study we did not find any obvious genotype–phenotype correlations, except for an absence of mutations in codon 816 in children with an onset of mastocytosis between 3 and 16 years of age. We also did not find the $D^{816}F$ mutation in our panel of children with mastocytosis. The difference between our findings and those of Yanagihori *et al.* (2005) may be due to the facts that all of their patients were of different genetic backgrounds and that we included a larger number of patients (50 vs 12) in our study.

This study included four children with a familial form of the disease. Of these, two had exon 17 ($D^{816}V$) mutations without any history of KIT-related tumors (other than mastocytosis). The other two lacked c-*KIT* mutations. In contrast, Longley *et al.* (1996) did not find exon 17 mutations in three cases of familial childhood mastocytosis, and, similarly, Rosbotham *et al.* (1999) did not find exon 17 mutations in three siblings with familial urticaria pigmentosa. Furthermore, the four patients in this study with familial mastocytosis lacked the $A^{533}D$ germline mutation that was previously reported in a kindred with childhood-onset familial mastocytosis (Tang *et al.*, 2004), as well as the Δ^{419} and $K^{509}I$ mutations, which were originally identified in kindreds with a combination of familial GISTs and mastocytosis (Hartmann *et al.*, 2005) and with familial mastocytosis (Zhang *et al.*, 2006), respectively. These four patients also lacked mutations in exons 8, 9, and 11, although the number of patients was too low to reliably assess whether there is any meaningful correlation. In general, these results confirm that familial childhood-onset mastocytosis can occur both in the presence and in the absence of c-*KIT* mutations.

In the 13 patients from whom we could collect blood samples, we were able to examine genomic DNA for the presence of c-*KIT* mutations. Mutations were not found in any of these cases, suggesting that the mutations are somatic rather than in the germline. This included one of the patients with a familial form of mastocytosis, suggesting that it can arise from causes other than germline mutations in *c-KIT*. Interestingly, this was true for several different mutations,

including in the phosphotransferase and regulatory domains. This finding supports the idea that, in most cases, childhood mastocytosis is a clonal disease. Moreover, overall, the mutations did not seem to cause any changes in mRNA splicing, as indicated by the S/L ratio, although there was a less than twofold difference in one case.

We were unable to detect mutations in only 14% (7/50) of the children in this study. This may be attributable to a lack of sensitivity of our direct sequencing technique, but previous studies suggest that it is a result of the absence of genetic alterations within *c-KIT* in some mastocytosis patients (Longley *et al.*, 1999; Valent *et al.*, 2003a). This implies that these patients have alterations in proteins other than KIT that regulate mast cell proliferation and function.

Collectively, the results reveal that, like adult-onset mastocytosis, most cases of childhood-onset mastocytosis are clonal in nature and are associated with activating mutations in c-*KIT*. How the pediatric form can spontaneously resolve if it is a clonal disease is not clear and is a major question that remains to be resolved. The understanding of this process may open new avenues in the treatment of adult mastocytosis and may apply in other cancers as well. In addition, long-term follow-up is needed to determine whether spontaneous resolution is associated with particular c-*KIT* mutations. We propose that *c-KIT* mutation analysis should be performed only in clinical trials and in the few severe cases in which a treatment must be chosen and targeted therapy could be interesting. Our findings suggest that, as in adult mastocytosis, KIT could be a good target in the treatment of pediatric mastocytosis.

MATERIALS AND METHODS

Patients

We assessed patients with childhood-onset cutaneous mastocytosis in a multicenter clinical study. Mastocytosis was suspected by the presence of a positive Darier's sign and was confirmed by histological analyses. A specific clinical records form was specially designed for this multicenter study to record the type of mastocytosis, familial history, and results of a complete clinical examination. For all patients with mastocytosis, the type of skin lesion was classified as mastocytoma (one to three lesions presenting as red-brown elevated plaques or nodules), urticaria pigmentosa (red-brown macules, papules, or nodules with a random distribution), or diffuse cutaneous mastocytosis (diffuse skin infiltration by mast cells and increased erythema, with frequent vesicles) (Tharp, 1985; Longley *et al.*, 1995). When there was evidence of cutaneous and extracutaneous infiltration by mast cells, patients were diagnosed with systemic mastocytosis; however, as stipulated by our ethics committee, extracutaneous biopsies and blood samples could not be collected unless necessary for treating the patient. Therefore, we cannot rule out that some cases might have been classified as systemic if biopsies had been performed. Death was censored. Parents of the patients in all centers signed the informed-consent and authorization forms for the study protocol, including genetic analysis, and 2-mm biopsy samples were collected from a cutaneous lesion after informed consent had been obtained. This study was approved by the ethics committee of Hôpital Necker and was carried out in compliance with the Declaration of Helsinki Principles protocol.

Mutation screening

Total RNA was extracted from cutaneous biopsy samples using an RNeasy Mini Kit (Qiagen S.A., Courtaboeuf, France). RNA was reverse transcribed into cDNA using a StrataScript first-strand synthesis system (Stratagene, Massy, France) and random hexamer primers in a total volume of 25 µl, according to the manufacturer's instructions. Next, the *c-KIT* coding sequence was amplified from 2.5 µl of cDNA by PCR, using HotStarTaq DNA polymerase (Qiagen S.A.) and the primers listed in Supplementary Table 1 for 40 cycles at 94 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 45 seconds. For the specific detection of mutations at codon 816, primers U2 and L1 were used. The PCR products were purified using a GeneClean III KIT (Qbiogene, Illkirch-Graffenstaden, France), and the entire *c-KIT* coding region was directly sequenced using a BigDye terminator v1.1 kit (Applied Biosystems, Courtaboeuf, France), the sequencing primers listed in Supplementary Table 1, and an ABI Prism 3100 sequencer (Applied Biosystems). Because mast cells are the main source of c-*KIT* mRNA in mastocytosis lesions, the presence of both mutant and wild-type sequences indicates either heterozygosity or a nonclonal mast cell population in biopsies. The $D^{816}V$ mutation was also confirmed by PCR amplification of the *c-KIT* coding sequence using the fluorescent primers U2F and L1F (see Supplementary Table 1), followed by restriction digestion using *Bsm*A1 and *Ple*1, which detect the wild-type and mutant forms of *c-KIT*, respectively. The restriction fragments were separated on an ABI Prism 3100 16-capillary sequencer, and the size of the restriction fragments was determined by comparison with the sizes of Genescan ROX 500 markers (Applied Biosystems) using GeneMapper software (Applied Biosystems).

Determination of the GNNK/GNNK + isoform (S/L) ratio

Wild-type and mutant isoforms of c-*KIT* were simultaneously detected using the LAPP method (Theou *et al.*, 2004) using fluorescent primers with the following sequences: forward, 5′-CCTAGTGTCCAATTCTGACG-3′ (corresponding to nucleotides 1176–1195); reverse, 5′-CGATTACGAAACCAATCAGCA-3′ (corresponding to nucleotides 1594–1574). Fluorescent amplicons were separated by capillary electrophoresis and identified according to their size, which was determined by comparison with ILS-600 size markers (Promega, Madison, WI). The short isoform (GNNK) produced a 404-bp fragment, and the long isoform (GNNK+) produced a 416-bp fragment. The length of the PCR fragment obtained from the mutated alleles was calculated by adding or subtracting the number of base pairs inserted or deleted, respectively. The area under the curve for each peak of fluorescence was determined using GeneMapper software (Applied Biosystems). The ratio of short to long isoforms (S/L) was calculated for each patient sample for both wild-type and mutated alleles.

Analysis of genomic DNA

Genomic DNA was purified from blood using the QIAamp DNA Blood Mini Kit (Qiagen), amplified with intronic primers (see Supplementary Table 1), and sequenced as described above (see ''Mutation screening'').

Immunoprecipitation and western blotting

Cos cells were transiently transfected with pcDNA3 plasmids for expressing human wild-type or mutant c-*KIT* and treated with or without 250 ng ml^{-1} SCF (Amgen, Thousand Oaks, CA). Cell lysates

were prepared as previously described (Ma *et al.*, 1999b). Western blotting for KIT tyrosine phosphorylation and KIT protein was performed using an anti-phospho-c-KIT (Tyr^{719}) antibody and a rabbit anti-human KIT polyclonal antibody (Cell Signaling Technology, Danvers, MA), respectively.

Statistical analysis

All criteria were analyzed using descriptive statistics, numbers, and rates for categorical and discontinuous variables, and parametric and nonparametric distribution elements for continuous variables. The correlation between phenotypes and genotypes was examined by comparison of the frequencies using χ^2-test or Fisher's exact test and by comparison of the means using Student's *t*-test or the Mann–Whitney *U*-test.

CONFLICT OF INTEREST

The authors state no competing interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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