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Identification of a point mutation in the catalytic domain of the protooncogene c-*kit* in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder

(protein tyrosine kinase/mutation)

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ABSTRACT **Both stem cells and mast cells express c-*kit* and proliferate after exposure to c-kit ligand. Mutations in c-*kit* may enhance or interfere with the ability of c-kit receptor to initiate the intracellular pathways resulting in cell proliferation. These observations suggested to us that mastocytosis might in some patients result from mutations in c-*kit*. cDNA synthesized from peripheral blood mononuclear cells of patients with indolent mastocytosis, mastocytosis with an associated hematologic disorder, aggressive mastocytosis, solitary mastocytoma, and chronic myelomonocytic leukemia unassociated with mastocytosis was thus screened for a mutation of c-*kit*. This analysis revealed that four of four mastocytosis patients with an associated hematologic disorder with predominantly myelodysplastic features had an A → T substitution at nt 2468 of c-kit mRNA that causes an Asp-816 → Val substitution. One of one patient examined who had mastocytosis with an associated hematologic disorder had the corresponding mutation in genomic DNA. Identical or similar amino acid substitutions in mast cell lines result in ligand-independent autophosphorylation of the c-kit receptor. This mutation was not identified in the patients within the other disease categories or in 67 of 67 controls. The identification of the point mutation Asp816Val in c-*kit* in patients with mastocytosis with an associated hematologic disorder provides insight not only into the pathogenesis of this form of mastocytosis but also into how hematopoiesis may become dysregulated and may serve to provide a means of confirming the diagnosis, assessing prognosis, and developing intervention strategies.**

Mastocytosis is a disease characterized by mast cell hyperplasia in the bone marrow, liver, spleen, lymph nodes, gastrointestinal tract, and skin (1). It is classified into four categories: indolent mastocytosis, mastocytosis with an associated hematologic disorder, mast cell leukemia, and aggressive mastocytosis. The most common form of the disease is indolent mastocytosis, which involves the skin, bone marrow, and gastrointestinal tract, and is characterized by symptoms caused by mast cell mediators. Patients with mastocytosis with an associated hematologic disorder exhibit the classic mast cell lesions in their bone marrow but also have evidence of myelodysplastic syndromes, myeloproliferative disorders, acute nonlymphocytic leukemia, or chronic neutropenia. Aggressive mastocytosis is characterized by a rapid increase in mast cells in the liver, spleen, lymph nodes, and bone marrow. Mastocytosis may thus be considered to consist of a heterogenous group of diseases in terms of hematopathologic findings, prognosis, and possibly etiology.

The protooncogene c-*kit* encodes a transmembrane tyrosine kinase receptor (2, 3). Transcripts and protein products of c-*kit* are expressed on hematopoietic stem cells (4–8), mast cells (4, 9), melanocytes (10–12), and germ-cell lineages (10, 13). c-*kit* mediates signals for proliferation and maturation of these cells (14). c-kit ligand, or stem cell factor (SCF), promotes the *in vitro* growth of mast cells from human $CD34^+$ cells isolated from human marrow or peripheral blood mononuclear cells (PBMCs) (15–18). These observations suggest involvement of c-*kit* in mastocytosis. In agreement with its hypothesis, it has been reported that there is increased soluble SCF in the skin of patients with indolent mastocytosis (19), although this observation alone would not explain the diversity in the clinical patterns of disease. It has also been demonstrated that both rodent mast cell lines (20) and a human c-kit^+, $Fc\varepsilon RI^-$ cell line (HMC-1), established from peripheral blood of a patient with mast-cell leukemia (21), exhibit point mutations in the cytoplasmic domain of c-*kit*, which promote ligand-independent autophosphorylation of the mutant receptor (22). It is not clear whether such mutations arise *in vivo* or during adaptation of such cells to culture. Nonetheless, there is no report of c-*kit* abnormalities in cells directly obtained from the mastocytosis population. In the present study, we thus screened for mutations of c-*kit* transcripts expressed in PBMCs of patients with indolent mastocytosis, mastocytosis with an associated hematologic disorder, aggressive mastocytosis, and one patient with solitary mastocytoma.

MATERIALS AND METHODS

Patients. Blood was obtained with informed consent from eight patients with mastocytosis, one with a solitary mastocytoma, and one with chronic myelomonocytic leukemia (CMML). The diagnosis for each patient was based on clinical symptomatology, physical findings, bone marrow biopsy and aspirate, and other appropriate biopsy specimens. Blood or buffy coats were also obtained through the National Institutes of Health Clinical Center Blood Bank from 67 anonymous donors between the ages of 25 and 60 years.

RNA Extraction and PCR. PBMCs were isolated by Ficoll/Hypaque density gradient centrifugation (density, 1.077 g/ml;

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Abbreviations: PBMC, peripheral blood mononuclear cell; SCF, stem cell factor; CMML, chronic myelomonocytic leukemia; SSCP, single-strand conformation polymorphism.

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Sigma), resuspended in phosphate-buffered saline, and counted. Approximately 1×10^7 PBMCs were next distributed into separate tubes, and total RNA was extracted by the acid guanidinium/phenol/chloroform method (23). Total RNA (5 μg) was then reverse-transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase without RNase H activity (Life Technologies, Gaithersburg, MD) and oligo(dT) primer. PCR was performed in 200 nM primers/all four dNTPs (each at 150 μM)/20 mM Tris·HCl, pH 8.4/50 mM KCl/1.5 mM $MgCl_2$/1.5 units of *Taq* DNA polymerase in a 50-μl reaction solution.

Screening of Polymorphisms. To screen for polymorphisms through the entire coding region of c-*kit* cDNA, single-strand conformation polymorphism (SSCP) analysis of PCR products was performed with 22 overlapping primer pairs (24). All nucleotide and amino acid numbers are according to the c-*kit* cDNA sequence (2). Total RNA from PBMCs was reverse-transcribed and amplified in the presence of 30 nM [γ-^{32}P]dCTP. One microliter of the PCR product was mixed with 1 μl of 95% formamide/20 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol. Samples were denatured at 90°C for 3 min and loaded onto 0.4-mm-thick 6% nondenaturing polyacrylamide gels containing 90 mM Tris borate, pH 8.3/2 mM EDTA/10% (vol/vol) glycerol. Electrophoresis was carried out for 4 h at 4°C and 48 W constant power. The gel was dried on Whatman 3MM paper and autoradiographed at −70°C overnight.

Sequencing of DNA. Once a polymorphism was identified by SSCP analysis, the region including the polymorphism was sequenced. To synthesize a template for sequencing, independent PCR was performed with the same primers used for SSCP analysis, and the PCR product was purified by agarose gel electrophoresis. One-twentieth of the purified product was then amplified asymmetrically in both directions with the same primers. Primers were removed by three centrifugations in a Centricon-30 column (Amicon). The asymmetrical PCR products were then sequenced in both directions with a Sequitherm cycle sequencing kit (Epicentre Technologies, Madison, WI) with a primer in a direction reverse to the initial asymmetrical amplification. The method of sequencing was according to the manufacturer's instructions with a primer end-labeled with [γ-^{32}P]ATP.

Analysis of *Hin*fI Digestion. To analyze the A → T substitution at nt 2468, which make a new *Hin*fI restriction site (GANTC), restriction endonuclease digestion of a PCR product was performed. After a 346-bp PCR product was amplified with the primer 5′-ACATAGAAAGAGATGTGACTCCCG-3′ (nt 2261–2284) and the downstream primer of the primer pair nt 2413–2436/nt 2606–2582, the products were digested with *Hae* III and *Hin*fI. Predicted sizes of digested fragments with 171, 127, and 48 bp from the wild type and 157, 127, 48, and 14 bp from the cDNA with the A → T substitution at nt 2468. Digested fragments were then separated on a 20% polyacrylamide gel. cDNAs synthesized from the PBMCs of 67 unselected donors were also screened for the mutation.

Genomic DNA Analysis. Genomic DNA from patients was analyzed for the A → T mutation identified in mRNA. DNA was extracted from PBMCs and PCR-amplified by using primers 5′-TGTGAACATCATTCAAGGCGTAC-3′ and 5′-TGTCAAGCAGAGAATGGGTACTC-3′ in PCR buffer containing 3 mM $MgCl_2$. The primers are complementary to the sequence of introns 16 and 17 of the c-*kit* gene (25). The A → T mutation is located within exon 17 of the gene. A single 322-bp DNA fragment of the expected size was amplified. The PCR product was digested with *Hin*fI and separated on an 8% polyacrylamide gel. Since the mutation creates a new *Hin*fI site, predicted fragments were 271 and 51 bp for the wild type and 257, 51, and 14 bp for the mutation.

Analysis of HMC-1 Cells. HMC-1 cells were also analyzed for A → T mutation and were obtained directly from J. H. Butterfield (Mayo Clinic) (HMC-1_A) and from P. Valent (University of Vienna), who had earlier received the cells from J. H. Butterfield (HMC-1_B). Genomic DNA and cDNA were prepared from each HMC-1 cell population and analyzed for the mutation by PCR amplification using the same primers followed by *Hin*fI digestion.

RESULTS

Clinical Characteristics of Patients with Mastocytosis. Analysis of the clinical and histopathologic features of the 10 patients differentiated them into five groups; (*i*) indolent mastocytosis (group I), (*ii*) mastocytosis with an associated hematologic disorder (group II), (*iii*) aggressive mastocytosis (group III), (*iv*) solitary mastocytoma (group IV), and (*v*) CMML unassociated with mastocytosis (group V) (Table 1) in accordance with established guidelines (1).

Identification of Base Changes in c-*kit*. Three of the 22 primer pairs used in the SSCP screening of PCR products detected polymorphisms in the catalytic domain of c-*kit* downstream to the kinase insert. One primer pair (nt 2289–2311/nt 2493–2467) detected a polymorphism in patient V-1, one primer pair (nt 2413–236/nt 2606–2582) detected a polymorphism in patients II-1, II-2, II-3, and II-4, and a third primer pair (nt 2530–2554/nt 2724–2701) detected a polymorphism in patient IV-1. All polymorphisms were observed in a heterogenous pattern; aberrant as well as normal bands were observed in the same lane. Moreover, abnormal bands amplified with one primer pair (nt 2413–2436/nt 2606–2582) in patients II-1, II-2, II-3, and II-4 were observed at the same level, suggesting that these patients had an identical base change (Fig. 1).

After confirming the results of the first screening with SSCP analysis of independent PCR products, each segment of cDNA that showed a polymorphism was sequenced, which identified

Table 1. Clinical and pathological data on 10 patients examined for c-*kit* mutation

Patient	Diagnosis	Age/sex	Duration of disease, years	Extent of disease
I-1	Indolent mastocytosis	43/F	3	UP, marrow, mild GI
I-2	Indolent mastocytosis	59/M	16	UP, marrow, focal bone, mild GI
I-3	Indolent mastocytosis	16/F	14.5	UP, marrow, focal bone
II-1	Mastocytosis with an AHD	64/F	1.5	UP, marrow, HSM, GI, LN
II-2	Mastocytosis with an AHD	39/M	19	UP, marrow, hepatomegaly, diffuse bone, mild GI
II-3	Mastocytosis with an AHD	46/M	3*	UP, marrow, HSM, extensive GI, portal hypertension, LN
II-4	Mastocytosis with an AHD	64/M	1	HSM, marrow, diffuse bone, LN
III-1	Aggressive mastocytosis	20/F	6	UP, marrow, HSM, diffuse bone, mild GI, LN, pulmonary
IV-1	Solitary mastocytoma	41/F	1	Focal bone
V-1	CMML	69/F	7	Peripheral blood, marrow, HSM

AHD, associated hematologic disorder; UP, urticaria pigmentosa; GI, gastrointestinal disease; HSM, hepatosplenomegaly; LN, lymph node involvement; M, male; F, female.
*Died of infection 3 years after diagnosed.

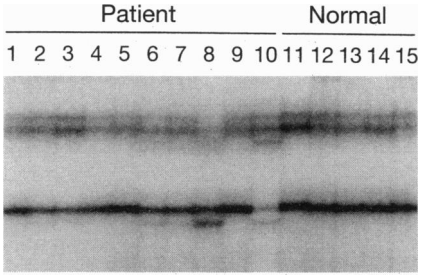

FIG. 1. Polymorphism in the catalytic domain of c-*kit* in four patients with mastocytosis with an associated hematologic disorder. cDNAs made from the PBMCs of 10 patients with mastocytosis, solitary mastocytoma, or CMML and 5 normals were subjected to PCR–SSCP analysis. Primer pair nt 2413–2436/nt 2606–2532 was used. Lanes: 1, patient I-1; 2, I-2; 3, IV-1; 4, I-3; 5, III-1; 6, II-1; 7, II-2; 8, II-3; 9, V-1; 10, II-4; 11–15, normal controls. Samples from patients II-1, II-2, II-3, and II-4 show extra bands and normal bands, indicating that both mutant and wild-type mRNA are expressed. The extra bands in these four patients are at the same level, suggesting an identical mutation.

a changed nucleotide sequence in all six patients. In patient IV-1, nt 2607 consisted of a wild-type guanosine and a mutant cytidine residue (Table 2). In patient V-1, nt 2415 consisted of a wild-type cytidine and a mutant thymidine residue. Amino acid sequences deduced from these base changes in patients IV-1 and V-1 showed no difference from the wild-type sequences. In contrast, in patients II-1, II-2, II-3, and II-4, nt 2468 consisted of a wild-type adenosine and a mutant thymidine residue, deduced to generate the point mutation Asp816Val (Fig. 2), similar to one of the two mutations detected in the HMC-1 cell line (22).

Because the A → T substitution at nt 2468 creates a new restriction site for *Hin*fI (GANTC), we confirmed this base substitution by *Hin*fI digestion of PCR products. Cleavage of the 346-bp PCR products showed an abnormal 157-bp band as well as normal 171- and 127-bp bands in patients II-1, II-2, II-3, and II-4, but no abnormal bands in the other patients (Fig. 3). Screening of PBMCs from 67 adult blood bank donors with this technique showed no abnormal band.

Genomic DNA was analyzed for the A → T mutation. The one patient examined who had the mutation in mRNA showed the same mutation in DNA prepared from PBMCs. The 322-bp PCR product on digestion with *Hin*fI showed 271- and 257-bp bands of approximately equal intensity suggestive of the presence of both the wild-type and mutated gene. Six patients with mastocytosis without an associated hematological disorder did not show the mutation.

Analysis of one population of HMC-1 cells (HMC-1_A) that was available in our laboratory did not show the A → T mutation either in mRNA or in genomic DNA. However, analysis of a second population of HMC-1 cells obtained from another laboratory (HMC-1_B) exhibited the A → T mutation in mRNA and in genomic DNA, again yielding 271- and 257-bp bands of approximately equal intensity.

Analysis of Hematologic Findings Associated with Asp816Val. The peripheral blood findings in patients with indolent disease were unremarkable. Bone marrow biopsies revealed characteristic focal mast-cell lesions (Table 3). The peripheral blood and bone marrow of the four c-*kit*-mutation-positive patients exhibited dysplastic or proliferative abnormalities. Three of four patients showed dysplastic features of one or more cell lineages (patients II-2, -3, and -4). The peripheral blood of patient II-1 showed a leukoerythroblastic profile, a shift to immature forms of the myeloid series, and a low leukocyte alkaline phosphatase level (26). With the fibrosis present in this patient's marrow biopsy, these findings are consistent with a diagnosis of myelofibrosis (27–29). The marrows of all four of these patients showed hypercellularity of hematopoietic elements and diffuse mast cell lesions. Megakaryocytes in this group of patients were generally increased, abnormal in appearance, and clustered. The major hematologic differences between patient III-1 and those patients with hematologic disorders were that patient III-1 had no cytopenias and had an elevated number of white blood cells composed of mature morphologically normal neutrophils with a marked thrombocytosis in addition to an elevated leukocyte alkaline phosphatase level, features consistent with a reactive hematologic diagnosis (26).

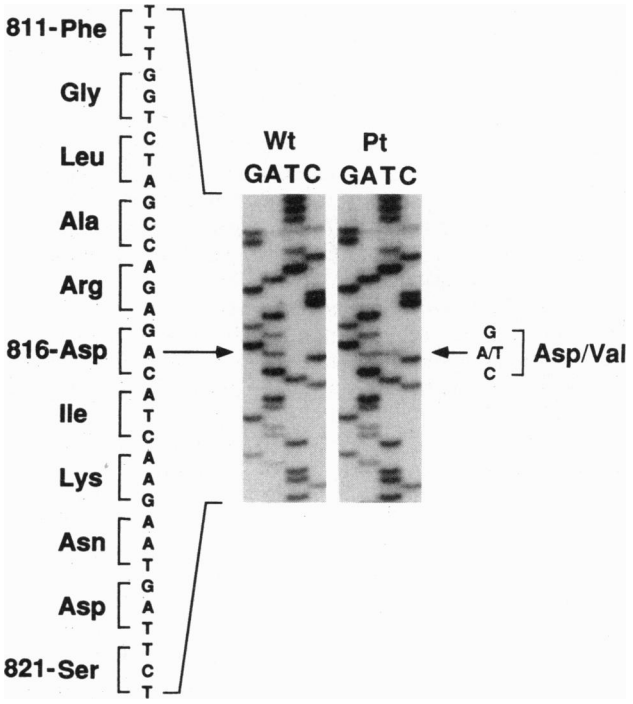

FIG. 2. Base change at codon 816 identified by direct sequencing of PCR products in a patient with mastocytosis with an associated hematologic disorder. cDNA from the PBMCs of patient II-3 was amplified with primer pair nt 2413–2435/nt 2606–2582, and the PCR products were sequenced. Both adenosine and thymidine residues are observed at nt 2468 of c-*kit* in the patient (Pt). Only an adenosine residue is seen at the same level in wild-type (Wt) cDNA. Thus, there is an Asp-816 → Val substitution in the patient's c-*kit*. The same base change was identified in patients II-1, II-2, and II-4.

DISCUSSION

In this study, we screened for a point mutation of c-*kit* transcripts in the PBMCs of patients with mastocytosis (Fig. 1) and identified the point mutation Asp816Val in four patients

Table 2. Polymorphisms identified in patients with mastocytosis, solitary mastocytoma, or CMML

Patient(s)	Primer pair for polymorphism	Change in nucleotide	Change in amino acid
IV-1	nt 2530–2554/nt 2724–2701	G-2607 → C	No change (CTG → CTC; Leu-862)
V-1	nt 2289–2311/nt 2493–2467	C-2415 → T	No change (ATC → ATT; Ile-798)
II-1, II-2, II-3, II-4	nt 2413–2436/nt 2606–2582	A-2468 → T	Asp-816 → Val (GAC → GTC)

Table 3. Hematologic manifestations of patients and Asp816Val mutation

Patient	Asp816Val mutation	Peripheral blood smear	Bone marrow findings: Mast cell lesions	Bone marrow findings: No. of megakaryocytes	Bone marrow findings: Dysplastic lineages	Hematologic diagnosis
I-1	No	Normal	Focal	Normal	None	None
I-2	No	Normal	Focal	Normal	None	None
I-3	No	Normal	Focal	Normal	None	None
II-1	Yes	Leukoerythroblastosis	Diffuse	Increased	None	Myelofibrosis
II-2	Yes	Leukopenia	Diffuse	Increased	None	MDS(RA)
II-3	Yes	Left shift Giant hypogranular platelets, monocytosis	Diffuse	Increased	Myeloid Megakaryocyte	MDS(CMML)
II-4	Yes	Marked RBCs Myeloid dysplasia Giant hypogranular platelets, monocytosis	Diffuse	Increased	Erythroid Myeloid Megakaryocyte	MDS(RA)
III-1	No	Giant hypogranular platelets	Diffuse and dysplastic	Increased	Myeloid Megakaryocyte	Reactive marrow
IV-1	No	Normal	None	Normal	None	None
V-1	No	Left shift Monocytosis, eosinophilia	None	Increased	Myeloid Megakaryocyte	MDS(CMML)

RBC, red blood cell; MDS, myelodysplastic syndrome; RA, refractory anemia; CMML, chronic myelomonocytic leukemia.

with mastocytosis with an associated hematologic disorder that was the result of an A → T substitution at nt 2468 (Fig. 2). We observed two other single-base changes resulting in probable silent mutations, one in a patient with a solitary mastocytoma and one in a patient with CMML unassociated with mastocytosis (Table 2). The Asp816Val mutation was not found in the three patients with indolent mastocytosis, the patient with aggressive mastocytosis, the patient with a solitary mastocytoma, the patient with CMML unassociated with mast cell disease, and 67 control subjects (Fig. 3). These data suggest that the Asp816Val point mutation in c-*kit* is specific for and may contribute to the pathogenesis of mastocytosis with an associated hematologic disorder (Tables 1 and 3). It is unlikely that the mutation is involved in the pathogenesis of similar hematologic disorders in the absence of mastocytosis, as the one patient with CMML unassociated with mast cell disease that we studied did not have the mutation. Also, others have reported absence of any mutations in c-*kit* in patients with hematologic diseases (30).

The point mutation Asp816Val is identical to one of two mutations reported in the HMC-1 cell line (22) that has been shown to cause ligand-independent phosphorylation of c-*kit* (22). Similarly, a homologous Asp residue in the mouse, Asp-814, is mutated in the mouse mastocytoma cell line P-815, with the mutant c-*kit* receptor containing the Asp-814 → Tyr substitution also showing ligand-independent activation (20). The Asp residue conserved in the human and mouse c-*kit* may thus represent a "hot spot" for activating mutations of c-*kit*. It has been suggested that the point mutation Asp816Val in the HMC-1 cell line was present in the patient's circulating cells and did not arise during adaptation of these cells to culture. Against this argument is the finding that not all HMC-1 cell populations have this mutation.

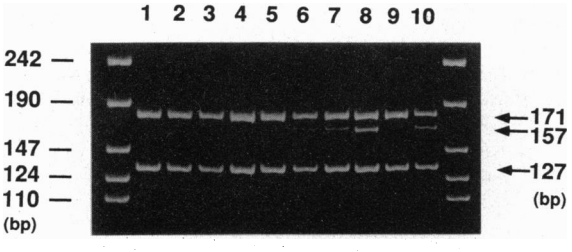

FIG. 3. Creation of a new *Hin*fI restriction site by the base change at nt 2468. The A → T substitution at nt 2468 of c-*kit* generates a new *Hin*fI site. A segment including this site was amplified by PCR and digested with *Hae* III and *Hin*fI. Predicted sizes of digested fragments are 171, 127,and 48 bp in wild-type cDNA and 157, 127, 48, and 14 bp in the mutant cDNA. Lanes: 1, patient I-1; 2, I-2; 3, IV-1; 4, I-3; 5, III-1; 6, II-1; 7, II-2; 8, II-3; 9, V-1; 10, II-4. Patients II-1, II-2, II-3, and II-4 show a 157-bp aberrant band and 171- and 127-bp normal bands. The intensity of the abnormal bands is weaker than normal bands, indicating the level of expression of wild-type mRNA is still higher than that of mutant mRNA in the PBMCs of these patients. Sixty-seven normal control individuals were screened for the mutation by this technique and no abnormality was found.

Cells expressing the mutant form of the gene in the circulation are likely to be $CD34^+$ cells, which represent the majority of cells expressing c-*kit* in blood (4, 6–8). These $CD34^+$, c-kit^+ cells are pluripotent hematopoietic progenitor cells (4, 15–18) and give rise to mast cells in the presence of SCF (30–33). The finding of a mutation in the genomic DNA isolated from total PBMCs of a patient who had the mutation in mRNA and where the PCR products of normal and mutated c-*kit* are approximately equal suggests that the mutation is present in the DNA of all hematopoietic cells. It is likely that $CD34^+$ cells in these patients have the DNA mutation and all cells that develop from the progenitor cell carry the mutation. A study of nonhematopoietic tissues from these patients should further clarify these issues.

Whether or not this single point mutation is able to cause abnormal growth of human mast cells *in vivo* or whether these mast cells express the mutation Asp816Val is also not clear. The culture of $CD34^+$ cells of patient II-3, who had mastocytosis with CMML, *in vitro*, with the addition of recombinant human SCF to the culture medium weekly for 6 weeks has been reported (18). In that study, two to three times the number of $CD34^+$ cells compared with normal controls and six times as many mast cells cultured *in vitro* per $CD34^+$ cell compared with controls at 6 weeks were observed. These observations suggest that the Asp816Val mutation leads to an increased proliferative rate for mast cells (and possibly $CD34^+$ cells) *in vivo* with the end result being mastocytosis and, in some cases, myelodysplastic or myeloproliferative disease.

Nonetheless, a retrospective analysis of our c-*kit*-mutation-negative and -positive mastocytosis patients revealed the latter group had disseminated mast-cell disease, with extensive organ involvement (Tables 1 and 3). Peripheral blood and bone marrow biopsy findings supported these observations. Whereas the c-*kit*-mutation-negative indolent mastocytosis patient group had normal peripheral blood smears with es-

sentially normal bone marrow biopsies other than focal mast cell lesions, c-*kit*-mutation-positive mastocytosis patients exhibited distinct abnormalities in myeloid and/or erythroid lineages, hypercellular bone marrows, and diffuse mast-cell involvement. These findings, based on the FAB (French-American-British) criteria (27), fit into the hematologic diagnosis of myelodysplastic syndrome (patients II-2, -3, and -4) or myelofibrosis (II-1), a myeloproliferative syndrome (26–28). Mastocytosis patients with associated hematologic disorders have a significantly poorer prognosis than patients with indolent systemic mastocytosis (1), where it is possible that local excess production of SCF results in disease (19). The finding of a mutation in the c-kit receptor in mastocytosis patients with associated hematologic disease may provide a basis for a molecular diagnosis. Additionally, some have suggested that mastocytosis represents a spectrum of myelodysplastic disease, where patients with indolent mastocytosis represent a group less affected compared with mastocytosis patients who have obvious hematologic disease. If this is true, it is also possible with time that some patients with indolent disease may also be shown to express this mutation or a similar mutation. Similarly, this mutation giving rise to a single localized clone of cells could have resulted in the local mastocytoma reported in one patient, in which case it would not be identified in PBMCs. A more detailed understanding of the relationship between the c-*kit* mutation and the category of mastocytosis thus warrants further screening of patients and a close follow-up of their clinical course.

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