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ORIGINAL ARTICLE

Mast cell leukemia: identification of a new *c-Kit* mutation, dup(501-502), and response to masitinib, a c-Kit tyrosine kinase inhibitor

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Abstract

Objective: Most patients with systemic mastocytosis bear mutations in the tyrosine kinase receptor gene *c-Kit*. Limited treatment options exist for mast cell leukemia, a rare form of systemic mastocytosis associated with a dire prognosis. Our aim was to investigate *c-Kit* mutations associated with mast cell leukemia and find new treatment for this severe form of mastocytosis. *Patient and methods:* We describe here a patient with mast cell leukemia characterized by 42% of circulating mast cells associated with a previously unidentified *c-Kit* mutation in adult mastocytosis: dup(501-502). *Main findings:* This patient was treated with masitinib, a novel c-Kit tyrosine kinase inhibitor, with a dramatic response observed following 3 months of treatment, including clinical improvement, disappearance of circulating mast cells, and decrease in both serum histamine and tryptase levels. *In vitro* and *ex vivo* research was performed on the patient's cells and revealed constitutive c-Kit phosphorylation in mast cell leukemia. *Conclusions:* This case highlights the importance of sequencing all *c-Kit* exons when the classical D816V *c-Kit* mutation is not found, even in adults with SM. It also indicates that masitinib may be safe and effective for the treatment for some mast cell leukemia.

Key words mast cell leukemia; *c-Kit* mutation; treatment; mastocytosis; masitinib

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Mastocytosis is a rare heterogeneous disease characterized by mast cell (MC) accumulation in one or several organs (1–6). A WHO classification described several subcategories of the disease (5–7), broadly divided into localized vs. systemic disease. Systemic disease is subsequently divided into indolent and aggressive disease based on organ injuries. Mast cell leukemia (MCL) is a rare subtype of systemic mastocytosis (SM) defined by leukemic bone marrow (BM) infiltration, atypical neoplastic MCs, and a severe prognosis. BM infiltration can

lead to myelofibrosis with hematopoietic insufficiency, organ dysfunction, bleeding, and death after a median survival time of 6–7 months (5–7). MCL diagnosis criteria include BM MC infiltration (>20%) and peripheral blood-circulating MC (>10%). Most patients survive <1 yr, responding poorly to cytoreductive drugs or chemotherapy with no curative treatment available. Several trials including inhibitors of tyrosine kinase showed promising results in the absence of D816V *c-Kit* mutation (8–12).

The proto-oncogene *c-Kit* encodes for a transmembrane stem cell factor (SCF) receptor tyrosine kinase that is expressed on MC. Adults with SM usually present *c-Kit* tyrosine kinase domain mutations, most frequently D816V. Masitinib is a new tyrosine kinase inhibitor (TKI) with a preclinical profile suggesting greater affinity and selectivity *in vitro* for the wild-type (WT) *c-Kit* and its juxtamembrane mutation than imatinib (13–16). Masitinib inhibits both human and murine wild-type c-Kit *in vitro*, and orally administered masitinib blocks the tumor growth of juxtamembrane-mutated c-Kit-expressing cells in mice (15). This molecule has also proven its efficacy in canine MC tumors (15) and is currently under evaluation in several human clinical trials (17–21). Considering the poor prognosis and absence of efficient MCL treatment, we evaluated *in vivo* and *in vitro* effects of masitinib in a MCL patient with a newly identified $^{dup(501\text{-}502)}$ *c-Kit* mutation.

Patient and methods

Patient

A 66-yr-old woman presented with a history of malaise, low blood pressure, flushes without sweating, profuse diarrhea, and erythematous cutaneous rashes. With the exception of an abdominal pain, there were very few notable physical findings, including neither splenomegaly nor cutaneous rash. Serum tryptase was very high (351 μg/L, (normal < 15)). A cutaneous biopsy was normal. This study was approved by the ethical committee of Necker Hospital and carried out in accordance with the Helsinki convention, and informed consent was obtained.

Peripheral blood mononuclear cell (PBMC) isolation and immunophenotypic and histology study

Blood and BM samples were collected at diagnosis and after 3 months of treatment. PBMCs were isolated by Ficoll-Hypaque, stained using FITC, PE, PerCP, or APC-coupled anti-CD34(581), anti-CD25(M-A2S1), anti-CD14 (MSE2), anti-CD32(3D3), anti-CD2(S5.2), anti-CD4(RPA-T4), anti-CD8(RPA-T8) and anti-CD117(YB5.B8) or with appropriate control, and analyzed by FACSCalibur (all from Becton Dickinson, Franklin Lakes, NJ, USA). Anti-CD117 from DAKO, (Carpinteria, CA, USA) was used for the BM immunochemistry.

Mutation screening

Total RNA was isolated from BM aspirate, and cDNA was synthesized as previously described (15).

Analysis of c-Kit tyrosine phosphorylation

Transfected Cos cells with pcDNA3 plasmids encoding for human WT or mutant *c-Kit* were cultured with or without 1 μM of masitinib and treated with or without 250 ng/mL SCF (AMGEN, Thousand Oaks, CA, USA). For *ex vivo* analysis during masitinib treatment, PBMC and BM aspirates from the patient were lysed, as well as Cos cells, as previously described (16). Immunoblots were performed using anti-phospho-c-Kit (Tyr^{719}) (Cell Signaling Technology, Berverly, MA, USA) with anti-Grb2 antibodies (sc255 from Santa Cruz, Santa Cruz, CA, USA) used as a control for protein loading.

Cells culture and assay of cell proliferation

Ba/F3 cells expressing ^{WT}*c-Kit* were cultured as previously described (16). The medium was supplemented with 0.1 or 1 μM masitinib. Cells were grown for 48 h at 37°C and then incubated with 10 μL/well of WST-1 reagent (Roche Applied Science, Indianapolis, IN, USA) for 3 h at 37°C. Measurements were taken as previously described (16).

Results

Bone marrow smear showed 70% massive infiltration with immature MCs bearing c-Kit by flow cytometry analysis. The karyotype was normal. BM biopsy showed an infiltration by 90% of dysmorphic spindled MCs, as highlighted by c-Kit immunohistochemical staining. These MC were characterized by clear cytoplasm, bilobated nucleus, and granulous elements. BM aspirate revealed heterozygosity for a not previously described *c-Kit* mutation: duplication (501-502) in exon 9. The absence of ^{D816V}*c-Kit* mutation was confirmed by sequencing after a RT-PCR, as previously described (18), except that a more sensitive technique was used; that is, nested PCR was performed using an additional PNA-modified oligonucleotide: PNA-modified primer $K816^{PNA}$ nucleotide position 2436 to 2461 (*c-Kit* sequence in GenBank, accession number X06182) (TCTAGCCAGAG$\underline{A}^{\textbf{PNA-modified}}$CATCAAGAATGATT). These findings were consistent with MCL.

After diagnosis confirmation, she orally received masitinib (6.5 mg/kg/d). After 3 months, symptoms of flush, eruptions, and diarrhea had disappeared; the percentage

of peripheral blood circulating MCs markedly decreased; serum histamine and tryptase levels decreased to approximately normal values; and MC infiltration on the BM biopsy also decreased. Table 1 and Figs 1 and 2 show relevant abnormal studies at baseline and dramatic response after 3 months of treatment. Masitinib was very well tolerated.

Ex vivo, a western blot analysis of phospho-c-Kit on the patient's PBMC before treatment showed a phosphorylated form of c-Kit, without SCF compared to control PBMCs. This c-Kit phosphorylated form showed a gel migration comparable with a mutated form ($dup^{501\text{-}502}$) of the receptor transiently transfected in Cos cells. Furthermore, this $^{dup501\text{-}502}$*c-Kit* showed a constitutive phosphorylation in Cos cell w SCF stimulation (Figure 1E).

Table 1 Patient's laboratory values at baseline and after 3 months of treatment with masitinib

	Pre treatment	Remission
Masitinib quantity per day, mg	0	400
Clinical features		
Weight	66	64
Flush, number per day	2	0
Pruritus	+	0
Abdominal pain	0	0
Diarrhea	0	0
Biology		
Hemoglobin, g/dL	10.6	12
Platelets/mm^3	124 000	247 000
WBC/mm^3	8100	3200
Segmented neutrophils/mm^3	4000	1900
Monocytes/mm^3	1100	200
Lymphocytes/mm^3	2000	1600
Mast cell %	7	0
LDH, UI/L	360	418
Tryptase μg/L, norm < 15	351	23.2
Histamine nmol/L, norm < 700	14 314	52.1
Bone marrow		
% mast cells on aspiration	70	NI
% mast cells on biopsy	90	48
PB phenotype		
% circulating c-Kit^+ cells	46	2
% circulating c-Kit^+CD25^+ cells	0.1	0
Mutation	Dup (501,502)	Dup (501,502)
Caryotype	46, XX	46, XX

NI, not interpretable (hemodilution).

In vitro analysis of WT and $dup^{501\text{-}502}$*c-Kit* tyrosine phosphorylation in transfected Cos cells (Figure 1F,G) showed that c-Kit was not phosphorylated in untransfected cells. ^{WT}*c-Kit* in transfected Cos cells was phosphorylated only in the presence of SCF and was unphosphorylated in the presence of masitinib. In contrast, $^{dup501\text{-}502}$*c-Kit* mutation in transfected Cos cells was phosphorylated independently of SCF, but masitinib remained efficient. Proliferation analysis of the patient's PBMC before treatment (Figure 1H; upper panel) at various concentrations of masitinib or imatinib (0.1 or 1 μM) showed that both are similarly effective for inhibiting proliferation. As a control, we examined transfected Ba/F3 cell growth in the presence and absence of SCF and IL-3 (Figure 1H; center and lower panels). In all cases, IL-3-induced proliferation was not affected by either kinase inhibitor. Ba/F3 cells expressing ^{WT}*c-Kit* were sensitized to TKIs at 1 μM in the presence of SCF. These results show that this mutation induced c-Kit autophosphorylation independently of SCF and that this effect could be inhibited by masitinib at concentrations reachable *in vivo* (20).

Discussion

In our case, a new *c-Kit* mutation located in exon 9 was identified. This exon encodes for the extracellular domain of the c-Kit receptor. Our team recently published that usually, mutations of the extracellular or juxtamembrane regions of *c-Kit* are identified among children and correlate with cutaneous and non-aggressive forms of mastocytosis (22). Such mutations were also described in gastrointestinal stromal tumors. Interestingly, Mital *et al.* reported one case of MCL with non-D816V mutation of *c-Kit* that is a p.A502_Y503dup mutation (23, 24). However, the duplication (501-502) in exon 9 of c-Kit has never previously been reported, especially among an adult without any history of pediatric or cutaneous mastocytosis. This newly described mutation represents a new mechanism of c-Kit autoactivation. This highlights the importance of sequencing the entire *c-Kit* gene when searching for atypical mutations in adults when ^{D816V}*c-Kit* is not found. In summary, this is the first case report of MCL with $^{dup(501\text{-}502)}$*c-Kit* mutation in an adult patient.

This case represents also the first reported successful treatment of MCL using a new TKI, masitinib. Masitinib's effectiveness was demonstrated after 3 months, as evidenced by an improvement in clinical stage, substantial decrease in BM infiltration by MCs and tryptase level, and a normalized peripheral blood phenotype. Masitinib induced remission within 3 months and was well tolerated. Based on the literature, mast cell leukemia may carry D816V mutation of *c-Kit* (25–27). Such

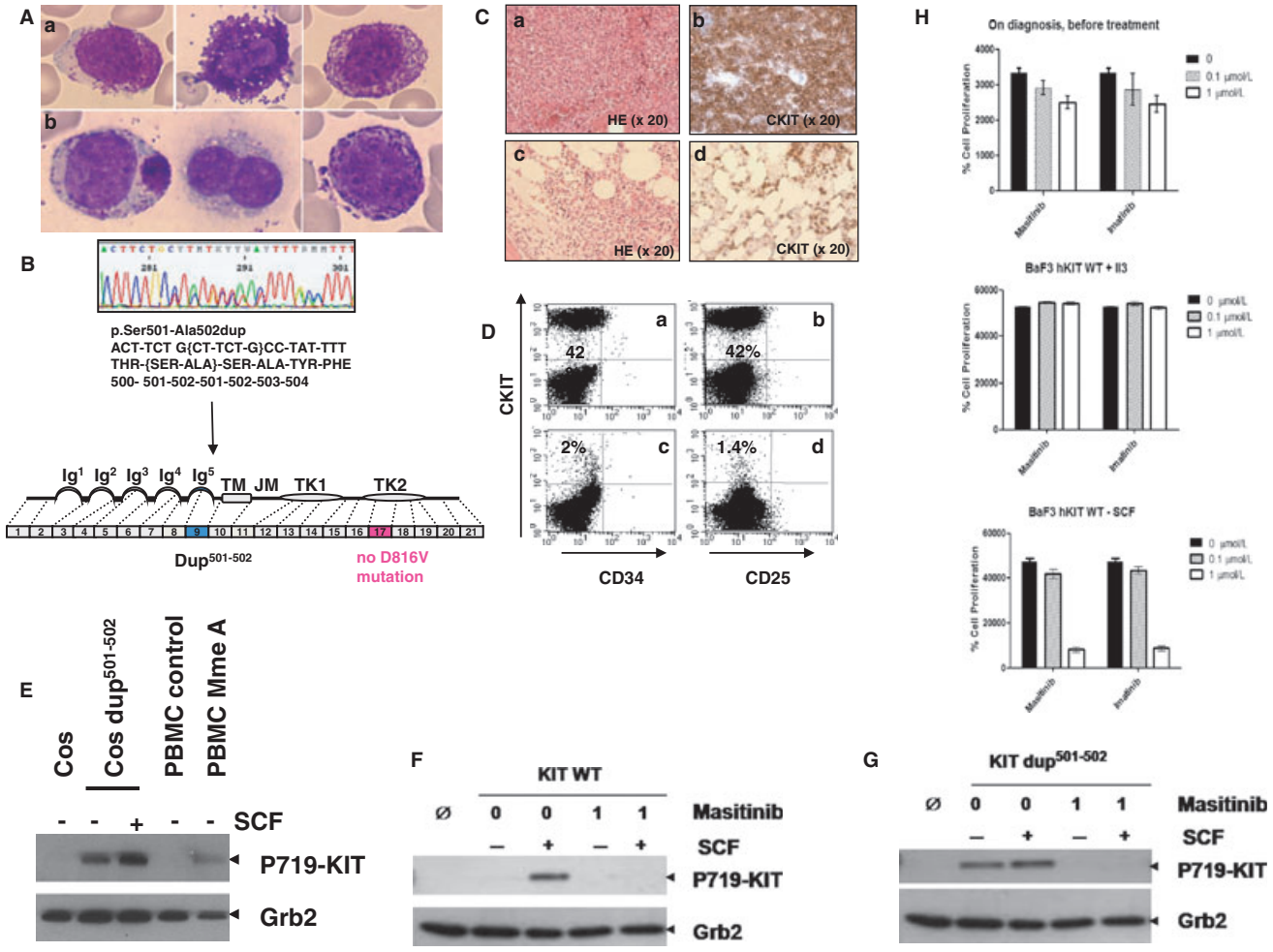

Figure 1 Peripheral blood and bone marrow findings at baseline and after 3 months of treatment with masitinib. (A) a: Circulating dysmorphic mast cell (MC) in peripheral blood smear; b: dysmorphic MC in bone marrow smear; May–Grunwald–Giemsa, 1000× (B) Sequence of mutated *c-Kit* with a duplication of six nucleotides dup(501-502). (C) Hypercellular bone marrow infiltrated by dysplasic MC occupying approximately 90% marrow cellularity; hematoxylin and eosin, 20× (C.a). The majority of MC are highlighted by c-Kit antibody; c-Kit, 40× (C.b). The post-treatment bone marrow shows a decrease in MC infiltration, occupying only 40% marrow cellularity; hematoxylin and eosin, 20× (C.c) as well as a decrease in c-Kit-positive MC; c-Kit, 20× (C.d). (D) Peripheral blood mononuclear cells (PBMC) phenotype: dot plot showing 42% c-Kit^{+}CD34^{-} cells (D.a) with only 0.1% of CD25^{+} cells (D.b). After treatment, there is a dramatic decrease in c-Kit^{+}CD34^{-} cells, representing only 2% of circulating cells (D.c) without CD25^{+} cells (D.d). (E) E*x vivo*: immunoblotting of cell lysates: control PBMC and patient's PBMC before treatment lysates were used after 7 d of culture with or without SCF. Untransfected Cos cells or dup$^{501\text{-}502}$ transiently transfected Cos cells were stimulated with or without SCF as control. Tyrosine phosphorylation of KIT was analyzed by Western blotting using a rabbit anti-Phospho KIT antibody. (F, G) *In vitro* effect of masitinib on ligand-independent activity of mutant KIT. Western blot analysis of either untransfected COS cells or transiently transfected COS with human ^{WT}*c-Kit* (F) or dup$^{501\text{-}502}$*KIT* (G). Cells were serum-starved for 3 h and treated for 5 min with (+) or without (–) 250 ng/mL recombinant SCF. Tyrosine phosphorylation of KIT was analyzed as described above. (H) Proliferation of the patient's PBMC expressing mutant KIT: before treatment (upper panel); Ba/F3 control cells cultivated with either IL3 (center panel) or SCF (lower panel). Cells expressing WT or mutant KIT were plated in 96-well plates and grown for 48 h with RPMI 10 with or without 0.1% conditioned medium from X63-IL-3 cells (*IL*-3) or 250 ng/mL SCF. Cell growth was assessed by measuring mitochondrial conversion of WST-1 into blue formazan dye with a spectrophotometer after 7 h. Experiments were conducted in triplicate.

patients may benefit from a treatment using TKI such as PKC412 (25). However, MCL do not constantly harbor D816V *c-Kit* mutation (8, 24, 28). In these cases, alternative TKI such as imatinib have shown efficiency, despite inconstant (8–12, 24, 28). Here, we report a new TKI efficient in D816V-*c-Kit*-negative MCL case. In this regard, masitinib may represent a new interesting therapeutic option in this specific subset of patients with 'responsive c-Kit mutations', considering the poor prognosis of the disease. In addition, owing to its safety

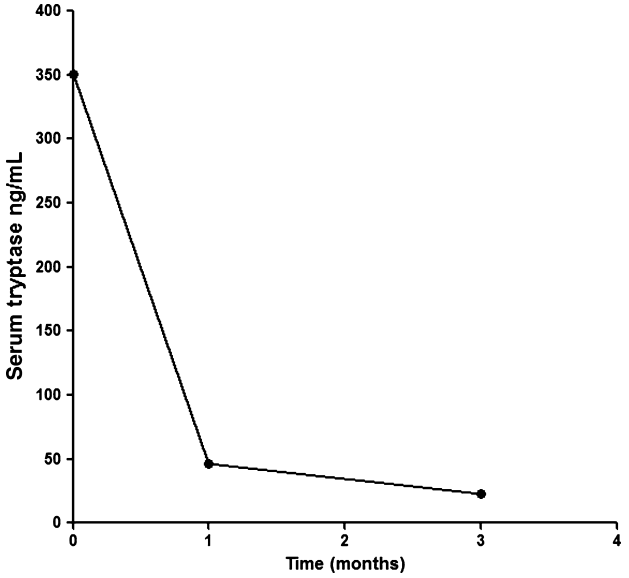

Figure 2 Serum tryptase under masitinib therapy. Dramatic decrease in serum tryptase measurement after 3 months of treatment with masitinib.

profile, selectivity, ability to inhibit mast degranulation (13), and efficiency *in vivo*, masitinib is currently investigated in symptomatic patients with SM having less advanced disease and symptoms related to mast cell degranulation (14, 29). Efficacy of different TKI such as imatinib and masitinib needs to be further studied on larger cohorts of aggressive mastocytosis including MCL to determine whether there exist any complementarities in their efficacy.

Authorship contributions

S.G.-L. wrote the manuscript, carried out experiments, and collected, analyzed, and interpreted data. F.S. treated the patient, performed bone marrow biopsies, helped design the study, and analyzed and interpreted data. Y.L. carried out experiments, collected, analyzed and interpreted data, and assisted in writing the manuscript. L.L., Y.Y., K.H., S.L., C.B., D.C., V.A., and A.R. carried out experiments and collected data. F.F. and L.L. helped design the study and analyzed and interpreted data. O.H., A.M., C.M., M.-O.C., A.A., E.M., and P.G. designed the study, interpreted data, and assisted in writing the manuscript. P.D. carried out experiments, collected, analyzed and interpreted data, and assisted in writing the manuscript. All authors validated the final manuscript.

Conflicts of interest

O.H. and P.D. receive research funding and honorarium from AB Science. A.M., C.D.M., and P.G. are employees of AB Science. Other authors declare no competing financial interests.

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