Familial systemic mastocytosis with germline KIT K509I mutation is sensitive to treatment with imatinib, dasatinib and PKC412

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ABSTRACT

Mastocytosis are myeloproliferative neoplasms commonly related to gain-of-function mutations involving the tyrosine kinase domain of KIT. We herein report a case of familial systemic mastocytosis with the rare KIT K509I germ line mutation affecting two family members: mother and daughter. In vitro treatment with imatinib, dasatinib and PKC412 reduced cell viability of primary mast cells harboring KIT K509I mutation. However, imatinib was more effective in inducing apoptosis of neoplastic mast cells. Both patients with familial systemic mastocytosis had remarkable hematological and skin improvement after three months of imatinib treatment, suggesting that it may be an effective front line therapy for patients harboring KIT K509I mutation.

In a relatively high proportion of SM cases, the clonal nature of the disease can be established on the basis of the demonstration of gain-of-function mutations involving the tyrosine kinase domain of KIT in lesional skin and BM cells [1].

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1. Introduction

Mast cell diseases are myeloproliferative neoplasms characterized by an abnormal proliferation and accumulation of mast cells (MC) in different tissues [1]. Clinical presentation is heterogeneous, ranging from skin-limited disease (cutaneous mastocytosis) to more aggressive variants (systemic mastocytosis, SM) that may be associated with multiorgan dysfunction/failure and shortened survival [2–8]. Although rare, cases of familial mastocytosis affecting two or more generations of the same family have been described, most of them are diagnosed with the cutaneous form of the disease [9–13]. The presence of KIT mutations in familial mastocytosis is variable and may be associated with gastrointestinal stromal tumors (GISTs) [14–17].
**Fig. 1.** Mast cell infiltration in bone marrow and spleen from patient 1. (A) Bone marrow smear showing infiltration by mast cells; (B) Bone marrow histology: intense hypercellularity due to infiltration by large nests of mast cells. Scarce hematopoietic remnants of the erythroblastic and megakaryocytic series are seen (HE, 400×); (C) Spleen histology in detail, dense infiltration of the spleen by mast cells, and some foci of necrosis (HE, 250×); (D) The spleen is diffusely infiltrated by neoplastic mast cells, interspersed with remnants of white pulp (HE, 100×).

**Fig. 2.** Mast cell infiltration in bone marrow and spleen from patient 2. (A) Bone marrow smear showing infiltration by mast cells; (B) bone marrow histology: intense hypercellularity due to infiltration by large nests of mast cells. Scarce hematopoietic remnants of the erythroblastic and megakaryocytic series are seen (HE, 400×); (C) skin histology: infiltration of superficial dermis by groups of mast cells in a perivascular pattern (HE, 250×); (D) skin histology in detail, infiltrating neoplastic cells display round nuclei, and abundant granular cytoplasm (HE, 1000×).
the good response of both patients to three months of imatinib treatment.

2. Materials and methods

2.1. Patients

Two patients (case 1 [mother], and case 2 [daughter]), and the parents of case 1 were included in the study. Written informed consent was obtained from subjects and from the guardians on behalf of the minor enrolled in the study. The Institutional Review Board approved this research and the consent procedure.

2.2. Primary hematopoietic cells and tyrosine kinase inhibitors

Bone marrow samples from patients 1 and 2 before treatment were submitted to Ficoll Hypaque density gradient. CD3+ cells were sorted using anti-CD3 monoclonal antibody and MACS® Magnetic Cell sorting technique (Miltenyi Biotec, Bergisch Gladbach, Germany). BM mononuclear cells were treated in vitro with imatinib (5 μM), dasatinib (80 nM) and PKC412 (100 nM) diluted in DMSO, or with DMSO only (control cells) for 4, 8 and 12 days. Imatinib mesylate and PKC412 were kindly provided by Novartis (Basel, Switzerland); dasatinib was obtained from Bristol-Myers Squibb (New Jersey, USA).

2.3. KIT, SF3B1, TET2, DNMT3A and ASXL1 sequencing analysis

Genomic DNA was obtained from total BM cells, CD3+ BM cells and oral mucosa of cases 1 and 2, and from PB of all individuals using the phenol: chloroform method. Direct sequencing was performed on all coding exons of KIT (exons 1–21) and TET2 (exons 3–11), and hotspot regions of DNMT3A (exons 18–23), ASXL1 (exon 12), and SF3B1 (exons 13–16). Primer sequences are described in Supplementary Table 1. Bidirectional sequencing was performed by standard techniques using an ABI 3500 DNA analyzer (Life Technologies, Carlsbad, USA).

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2014.07.010.

2.4. Assessment of cell growth by methylthiazol tetrazolium (MTT) assay

A total of 5 × 10^4 BM mononuclear cells per well were plated in a 96-well plate in Alpha MEM supplemented with 5 ng/ml IL-3 and 20 ng/ml SCF at different concentrations of imatinib (5 μM), dasatinib (80 nM), and PKC412 (100 nM) or DMSO for 4, 8 and 12 days. 10 μL of a 5 mg/ml solution of MTT was added to the wells and incubated at 37°C for 4 h. The reaction was stopped by using 100 μL of 0.01 N HCl in anhydrous isopropanol. Cell growth was evaluated by measuring absorbance at 570 nm. All conditions were tested in six replicates.

2.5. Assessment of apoptosis by Annexin-V/PI staining

A total of 2.5 × 10^5 BM mononuclear cells per well were plated in a 12-well plate in alpha MEM supplemented with 5 ng/ml IL-3 and 20 ng/ml SCF at different concentrations of imatinib (5 μM), dasatinib (80 nM), PKC412 (100 nM) or DMSO for 4, 8 and 12 days. Cells were washed twice with PBS and resuspended in binding buffer containing 1 μg/ml APEL labeled Annexin-V. All specimens were analyzed on a FACSCalibur after incubation for 15 min at room temperature in a light-protected area. Ten thousand events were acquired for each sample. All conditions were tested in triplicates.

2.6. Western blotting

Equal amounts of protein obtained from BM mononuclear cells of patients 1 and 2 treated with imatinib (5 μM), dasatinib (80 nM), PKC412 (100 nM) or DMSO for 12 days were submitted to Western blot and immunoblotting analysis with specific antibodies against phospho-P70S6K and phospho-P70S6K (Santa Cruz Biotechnology; Santa Cruz, CA, USA) and ECL™ Western Blotting Analysis System (Amersham Pharmacia Biotech; Buckinghamshire, England), as described [27].

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Instat 5 (GraphPad Software, Inc., San. Diego, CA, USA). For comparisons, a Student’s t-test was used. A p-value <0.05 was considered as statistically significant.

3. Results

3.1. Patient’s clinicopathological features

Case 1 was a 33-year-old woman with a chronic history of pruritic skin rash who was referred to our outpatient service for evaluation of massive splenomegaly (25 cm in length by palpation below the left subcostal margin) and pancytopenia. She had neither comorbidities nor any familial history of hematological malignancies. The patient had no siblings and had only one daughter (case 2). Skin biopsy revealed extensive mast cells infiltration, and BM biopsy section revealed 70% of mast cells. BM aspirate showed 51% of mast cells, with less than 10% of atypical and spindle shaped elements (Fig. 1A and B). Bone marrow cytogenetics was normal (46, XX). Tryptase level was 165.0 μg/L (reference range: 1.0–11.0 μg/L). During follow up, the patient presented with spontaneous splenic rupture, and spleen histology revealed dense infiltration of mast cells (Fig. 1C and D). The patient had no coagulopathy and underwent an emergency open splenectomy, with subsequent resolution of cytopenias. Treatment with Cladribine was started, but after two cycles of chemotherapy, there was neither clinical response nor reduction in bone marrow mast cells infiltration. Fourteen months later, the patient was started on imatinib treatment (400 mg daily). Her daughter (case 2), a 17-year-old woman, was also evaluated for an insidious history of diffuse skin rash and pruritis. Skin biopsy showed moderate mast cells infiltration, with 70% of mast cells in BM biopsy section. Bone marrow aspirate revealed 17% of mast cells with only 3% of atypical elements (Fig. 2A–D), and normal karyotype. Tryptase level was 130.0 μg/L. She had no organomegaly and her blood counts were normal. The patient was started on imatinib treatment (400 mg daily).

Both patients fulfilled the 2008 World Health Organization (WHO) criteria for the diagnosis of systemic mastocytosis [6]: the major criterion (multifocal, dense infiltrates of MC in bone marrow sections) plus one minor criterion (total tryptase persistently exceeds 20 ng/ml). Patient 1 was diagnosed with aleukemic
mast cell leukemia: she had massive splenomegaly with hypersplenism, pancytopenia, significant BM infiltration by MC with decreased normal hematopoiesis and no mast cells in the peripheral blood. Patient 2 was diagnosed with indolent systemic mastocytosis (no “C” findings, only one “B” finding [30% infiltration by MC in BM biopsy], and no evidence of associated non-MC lineage clonal hematological malignancy). Patients 1 and 2 suffered from the morphologic subvariant called well differentiated SM (WDSM), characterized by mature phenotype with a round shape, fully granulated cytoplasm, and a centrally located round nucleus [3,28,29]. Patients’ clinical characteristics are shown (Table 1).

3.2. Identification of KIT K509I mutation

Sequencing analysis revealed an A>T substitution at position 1547 in exon 9 of KIT, resulting in a change from lysine to isoleucine at amino acid 509 (c.1547 A>T, p.K509I). The mutation was found in DNA samples obtained from oral mucosa, CD3+ and total cells from peripheral blood and total bone marrow cells from cases 1 and 2 (Fig. 3A and B). No other mutations in KIT, including KIT D816V, were found. Patients were wild type for TET2, DNMT3A, ASXL1, and SF3B1. The parents of case 1 were wild type for KIT, as detected by sequencing analysis (Fig. 3C and D). These results indicate that the KIT K509I was a germ line mutation acquired de novo by patient 1, which was subsequently transmitted to her daughter (patient 2).

3.3. Sensitivity of KIT K509I bone marrow cells to tyrosine kinase inhibitors

In vitro treatment of primary bone marrow mononuclear cells harboring the KIT K509I mutation from patients 1 and 2 resulted in variable effects according to the drug used and treatment duration, as evaluated by MTT and Annexin-V/PI assays. Imatinib treatment resulted in significant reduction in cell growth (days 4, 8 and 12 of culture) and an increase in apoptosis (days 8 and 12) in both patients (all p ≤ 0.03). Dasatinib resulted in a statistically significant decrease in cell growth in both patients at days 8 and 12 (all p ≤ 0.05), and there was also a higher apoptosis ratio at day 12 of culture in patient 1 (p = 0.03), PKC412 significantly decreased cell growth at day 8 in patient 1 (days 4 and 8) and in patient 2 (day 4) (all p ≤ 0.03), but no effect in apoptosis ratio was seen (Fig. 4A–B).

To further evaluate the effect of tyrosine kinase inhibitors treatment on PI3K/Akt/P70S6K signaling pathway, that is characteristically stimulated by SCF/KIT, total protein extract obtained
from the KIT K509I cells treated or not with imatinib, dasatinib and PKC412 for 12 days were tested for total and phosphorylated P70S6K. A reduced protein phosphorylation was observed for all drugs in both patients (Fig. 4C).

3.4. Clinical response to imatinib mesylate

Patients were started on imatinib treatment (400 mg daily), and after three months of treatment, both patients had a marked reduction of skin rash and mast cells percentage in bone marrow aspirate; 1.5% and 0.5% of mast cells, with no atypical elements, on BM aspirates for cases 1 and 2, respectively. Photographs of the skin from patient 1 before and after imatinib treatment are shown (Fig. 5A–D). Also, tryptase levels normalized after imatinib treatment: 1.28 μg/L and 1.15 μg/L in patients 1 and 2, respectively. According to 2003 treatment response proposed criteria for aggressive systemic mastocytosis [5], patient 1 had major response/complete remission. Patients remain stable following one year of treatment.

4. Discussion

We herein provide a report of KIT K509I germ line mutation in familial systemic mastocytosis. The mutation was acquired de novo by patient 1 and was subsequently transmitted to her only daughter (patient 2). Zhang and colleagues [30] have first described this mutation in a family with systemic mastocytosis, in which the K509I mutation was present exclusively in the affected individuals. Also, they obtained a negative screening for KIT K509I in 217 control individuals, showing that it is a mutation than a previously undescribed polymorphism. Recently, Chan et al. characterized KIT K509I mast cells as showing a well-differentiated phenotype, with enhanced proliferation, granulation, and activation [31].

Tyrosine kinase inhibitors are treatment choices for mastocytosis, but clinical response is variable and is associated with the specific KIT mutation found and with the conformational changes that it provokes, affecting or not drug binding site. Zermati et al. [23] have shown that imatinib can inhibit KIT wild type kinase activity, but not KIT D816V mutation [32]. Dasatinib has in vitro activity...
against KIT D816V mutation, but no significant clinical response was found in patients harboring this mutation [23,32–34]. The multi-targeted TK inhibitor PKC412 has growth-inhibitory effects in cells exhibiting KIT D816V mutation [35], but clinical responses were variable and it is not clear which subgroups of mastocytosis patients could benefit from the treatment [8].

Due to the heterogeneity in drug response in mastocytosis patients, we aimed to test the sensitivity of the KIT K509I mast cells to the three different TK inhibitors. Our study provides evidence of P70S6K inhibition in primary cells harboring KIT K509I mutation after imatinib, dasatinib and PKC412 treatments. P70S6K is a protein involved in cell proliferation that is activated after SCF/KIT stimulation of the PI3K/Akt/P70S6K pathway [36], suggesting that the TK inhibitors tested may be capable of reducing cell proliferation. In our in vitro studies, imatinib, dasatinib and PKC412 were able to induce apoptosis and reduce cell viability of cells harboring KIT K509I mutation, but imatinib was more effective in inducing apoptosis in vitro. This difference could be secondary to conformational changes induced by KIT K509I mutation that may alter drug binding sites, as described for other KIT mutations [23,33,37,38]. The effect of TKI therapy could be better evaluated in sorted mast cells. However, due to sample and technical limitations, we could not perform functional studies on sorted mast cells. Patients' remarkable clinical and laboratory response to imatinib corroborate our in vitro results. Recent studies have described that additional genetic lesions can be present in patients with mastocytosis [25,26]. Genetic or epigenetic changes associated with KIT mutations might be capable of altering susceptibility to certain drugs. However, we did not observe hotspot mutations in TET2, DNMT3A, ASXL1 and SF3B1. Imatinib has induced good clinical responses in both patients studied, reducing tryptase levels, bone marrow and skin mast cell infiltration. A satisfactory clinical response to imatinib was also shown in the work by Zhang et al. [30], suggesting that this drug may be a good first choice for the treatment of patients harboring KIT K509I mutation.

The identification of specific KIT mutations in patients with familial mastocytosis and the study of mutations' sensitivity to TK inhibitors may contribute to the better understanding of the mechanism of disease and in defining treatment choices. Although rare, the screening for KIT K509I mutation should be considered in all cases of familial mastocytosis.

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Conflict of interest statement

The authors declare that they have no competing interests.

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Contributors: PMC selected the patients, carried out the experiments and participated in the analysis of the results and in the writing of the manuscript; JAMN carried out the experiments, the analysis of results, the statistical analysis, and edited the manuscript. RSR and ASSD provided technical assistance with the experiments, and edited the manuscript; FFCB, JV and ILM helped in conducting patients’ diagnosis, bone marrow aspirates and biopsies analyses, and edited the manuscript; VV, AT and RVT helped with sequencing analyses, and in the writing and editing of the manuscript; HJR performed tryptase measures and edited the manuscript; FFC contributed to the analysis of the results, and edited the manuscript; STOS contributed to the analysis of the results and in the writing and editing of the manuscript. FT was the principal investigator and contributed to all steps.

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