Novel, activating KIT-N822I mutation in familial cutaneous mastocytosis

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Objective. We report the rare family in which cutaneous mastocytosis was diagnosed in the father and two children, with urticaria pigmentosa as the only manifestation of the disease. The diagnosis of mastocytosis in the father included bone marrow histopathological and cytological examinations and flow cytometry, and histopathological examination of the skin. In the children, tryptase measurement and skin histopathological examination were performed.

Materials and Methods. Blood, urine, and buccal swab specimens were collected from the family members. HEK293T cells were transiently transfected with plasmids expressing KIT-WT and KIT-N882I. In addition, Ba/F3 cell lines expressing KIT-N822I, KIT-D816V, and KIT-V559D mutants were treated with imatinib and dasatinib. The effect of treatment on proliferation, survival, and signaling was determined.

Results. Germ-line KIT-N822I missense mutation was detected in the affected members of the family. Western blot analysis using HEK293T and Ba/F3 cells expressing KIT-N882I isoform showed that KIT-N882I constitutively activated KIT tyrosine phosphorylation. In vitro assays on KIT-N822I-expressing Ba/F3 cells confirmed that the N822I mutant is resistant to imatinib mesylate. In contrast, a high efficacy of dasatinib toward the KIT-N822I-expressing Ba/F3 cells was observed.

Conclusions. We provided evidence that KIT p.N822I mutation has transforming potential and can cause a constitutive activation of KIT. In addition, we demonstrated that KIT-N822I is resistant to imatinib and sensitive to dasatinib. Finally, our findings support the hypothesis that not only KIT mutations but other additional genetic abnormalities are contributing to more advanced forms of the disease. © 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Mastocytosis is a rare group of diseases characterized by abnormal proliferation and infiltration of mast cells within the tissues. The skin is most frequently involved but mast cells also accumulate in the bone marrow, gastrointestinal tract, lymph nodes, spleen, and liver. Oncogenic mutations within KIT gene are the most common causal genetic abnormalities in sporadic mastocytosis, therefore, KIT tyrosine kinase forms a promising therapeutic target [1]. Multilineage involvement by KIT-D816V has an impact on disease severity and progression [2]. However, it is widely accepted that other cooperating genetic events are contributing to the phenotypic diversity of KIT-positive mastocytosis [3].

In contrast to mastocytosis associated with somatic KIT mutations, the accounts of familial forms of mastocytosis with KIT germline mutations are extremely rare [1]. To date, KIT germline mutations have been reported in four families with mastocytosis [4–7]. Identified mutations were located within extracellular domain or in transmembrane domain. More recently, Bodemer et al. found KIT-D816V mutation in two children with a familial form of the disease [8]. The presence and type of the KIT mutation is important because it is known that sensitivity to imatinib depends on the location of the alteration. Previous studies showed that a subset of mutations in the KIT second
tyrosine kinase domain (activation loop domain) does not respond to imatinib treatment [1].

Dasatinib, formerly known as BMS-354825, is an adenosine triphosphate–competitive, multitarget inhibitor of BCR-ABL and SRC family kinases [9]. Inhibitory activity of dasatinib was also recorded for other protein kinases, such as KIT, platelet-derived growth factor receptors, c-FMS, and EPHA2 receptor [10]. More recently, dasatinib was shown to inhibit the kinase activity of KIT mutants resistant to imatinib [11]. Therefore, use of dasatinib could be considered as a new therapeutic approach to the treatment of patients whose cancer cells carry imatinib-resistant KIT mutant isoforms.

In the present study, we describe a family with urticaria pigmentosa as the only manifestation of the disease. A novel germline KIT mutation in exon 17 (p.N822I) was detected in all affected family members. We have explored the transforming potential of that mutation and tested the efficacy of tyrosine kinase inhibitors, imatinib and dasatinib, for the inhibition of Ba/F3 cell line expressing KIT-N822I isoform.

Materials and methods

Patients

A 33-year-old male was referred to the Department of Allergology, Medical University of Gdansk, with an initial diagnosis of mastocytosis for further examination. The patient suffered from skin lesions (urticaria pigmentosa) and mild itching since early childhood. The changes were present mainly on the trunk and lower extremities and did not affect the patient’s quality of life. The skin biopsy confirmed the clinical diagnosis. Mast cell infiltrate was present in the subepidermal location and presented KIT (CD117) and tryptase expression. CD25 and CD2 antigens were not disclosed in these mast cells. Histological and immunohistochemical findings are presented in Figure 1 (CD25 not shown). There were no symptoms of systemic or aggressive mastocytosis; the patient did not suffer from anaphylaxis and did not present any symptoms of food intolerance or allergy. No abnormalities were found on the chest x-ray and abdomen ultrasound images. The patient has two children. The family anamnesis revealed similar skin lesions (urticaria pigmentosa) in both children of the patient, a 5-year-old daughter and 1-year-old son. The children had no systemic symptoms nor any food intolerance or allergy. No complications were present during pregnancy and delivery. The skin lesions were present since infancy. The diagnosis of mastocytosis was confirmed by histopathology.

The bone marrow analysis was performed in the adult patient, including trephine bone marrow biopsy, aspiration cytology, and cytophotometry with CD2/CD25 evaluation. None of the procedures revealed bone marrow involvement by mastocytosis. Serum tryptase level was 11.6 ng/mL (normal serum tryptase levels: 0–11.4 ng/mL). None of the other members of the family suffered from urticaria pigmentosa or systemic mastocytosis and the family history did not disclose any neoplastic disease in the first-line family members. Lung cancer was present in the distant family, in a heavy smoker, male.

Treatment with H1 antihistamine and rescue kit containing adrenaline, glucocorticosteroids, and H1 blockers was started. During 3-year follow-up, the patient and his children did not show any symptoms of mastocytosis other than urticaria pigmentosa.

Figure 1. Histological biopsy of the skin lesion discloses the nodular infiltrate of the mast cell below the epidermis (A, hematoxylin and eosin, 200×). The mast cell infiltrate expresses tryptase (B) and CD117 (C), while CD2 is absent (D).
Molecular analysis

Buccal swabs, peripheral blood, and urine samples from the father, mother, and two children were collected after obtaining informed consent. The study was approved by the ethical committee of the Medical University of Gdansk. Genomic DNA was extracted using a standard procedure based on ionic detergent lysis and proteinase K digestion, phenol/chloroform extraction, and isopropanol precipitation. The coding sequence of KIT was analyzed as described previously [12]. Polymerase chain reaction products were sequenced using ABI3130 (Applied Biosystems, Foster City, CA, USA) and data were analyzed by Sequencher v.4.7 DNA Software (Gene Codes Corporation, Ann Arbor, MI, USA). The presence of a mutation was confirmed by independent polymerase chain reaction amplification followed by bidirectional sequencing. In addition, to assess the presence of a KIT-D816V mutation, allele-specific polymerase chain reaction assay was applied.

Inhibitors

The inhibitors, imatinib mesylate (Glivec/Gleevec; Novartis, New York, NY, USA) and dasatinib (BMS-354825; Bristol-Meyers-Squibb, New York, NY, USA), were purchased from Selleck Chemicals. 10 mM stock solutions of the inhibitors, dissolved in dimethyl sulfoxide, were stored at −80°C.

Functional analysis

Detailed protocols for generation of the construct, cell culture, retroviral transduction, proliferation, apoptotic, and Western blotting assays were described previously [10,13]. Briefly, the KIT was amplified from proband peripheral blood leukocytes complementary DNA (cDNA) and cloned into the pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) or retroviral pMSCVpuro vector (Clontech, Mountain View, CA, USA). All constructs used in this study were encoding the GNNK isoforms of KIT. HEK293T and Ba/F3 cells were grown in Dulbecco's modified Eagle's medium and RPMI-1640, supplemented with 10% fetal bovine serum. After 24-hour serum starvation, HEK293T cells were stimulated or not with 200 ng/mL stem cell factor (SCF; Sigma-Aldrich, St Louis, MO, USA) at +37°C for 10 minutes. The Celltiter AQeousOne Solution (Promega, Madison, WI, USA) was applied to obtain dose-response curves of transduced Ba/F3 cells, treated with vehicle alone or with varying concentrations of imatinib and dasatinib for 48 hours. Induction of apoptosis of transduced Ba/F3 cells was evaluated by flow cytometry using Annexin-V-FLUOS Staining Kit (Roche Applied Science, Indianapolis, IN, USA). Finally, Western immunoblotting was performed using the following antibodies: anti-α-tubulin (Sigma-Aldrich), anti-KIT (DAKO, Carpinteria, CA, USA), anti-phospho-KIT (pY703) (BioSource, Carlsbad, CA, USA), anti-AKT, anti-phospho-AKT (Ser473), anti-p44/42 mitogen-activated protein kinase, anti-phospho-p44/42 mitogen-activated protein kinase (Erk1/2) (Thr202/Tyr204), anti-RPS6K, and anti-phospho-RPS6K (Ser235/236) (Cell Signaling Technology, Danvers, MA, USA). Quantitative analysis of protein blot images was performed using Scion software.

Results and discussion

KIT mutations within codon p.N822 were detected in a number of pediatric and adult patients with acute myeloid leukemia, sporadic and familial gastrointestinal stromal tumors (GIST), and seminomas. The vast majority of the identified alterations were c.2563T>A point mutations, resulting in p.N822K substitution. In addition, a variety of other amino acid substitutions affecting codon 822 have been reported previously. Point mutation resulting in p.N822H was detected in patients with seminomas and GISTs [14,15]. Shimada et al. identified p.N822T substitution in a pediatric acute myeloid leukemia patient [16]. More recently, a novel KIT germline mutation resulting in the replacement of asparagine by tyrosine (p.N822Y) was found in kindred with familial GISTs [17].

In the present report, we describe a novel germline KIT mutation in codon 822 in the family with cutaneous mastocytosis. In all affected family members, the kinase domain mutant p.N822I was detected in buccal swabs, peripheral blood, and urine samples (Supplementary Figure E1). No other KIT mutation was detected. Recently, such a KIT mutation was reported by McDonnell et al. in a patient with melanoma [18]. In this individual, p.N822I substitution

Figure 2. Western blot of HEK293T cells transfected with plasmids encoding KIT-N822I construct (lanes 1, 2), empty pcDNA3.1 vector (lanes 3, 4) and KIT-WT construct (lanes 5, 6). After 24 hours, cells were treated with 200 ng/mL SCF for 15 minutes. ImmunobLOTS with total lysates were probed with anti-KIT and anti–phospho-KIT[Y703] antibodies. Bottom: P-KIT/KIT ratios were normalized to 1 for the KIT-WT (stimulated). Student's t-test was used for statistical analysis. *p < 0.001. Error bars represent standard deviation (n = 2).
coexisted with p.V559A mutation. In our family, the affected members had urticaria pigmentosa as the only manifestation of the disease. Urticaria pigmentosa has already been reported as an additional symptom in two families with GISTs and a syndrome of diffuse leiomyomatosis-associated achalasia, in which KIT germline mutations were detected [4,19].

Given the lack of enough primary neoplastic cells of the patient to establish a number of primary cultures dishes, the expression vector containing KIT-N822I cDNA was transfected into HEK293T cells to investigate the biological consequences of asparagine for isoleucine substitution. The empty expression vector and vector containing the KIT-WT cDNA were also transfected into the cells and the status of tyrosine phosphorylation of KIT was assessed by Western blot. As shown in Figure 2, autophosphorylation of KIT-WT without SCF stimulation was very weak, but became significant in response to SCF addition. In contrast, high constitutive tyrosine phosphorylation was detected in KIT-N822I-expressing cells in the absence of SCF. However, stimulation of HEK293T cells with SCF resulted in further phosphorylation of KIT-N822I mutant. It is of note that the same phenomena have already been observed for the KIT p.A502_Y503dup and p.K509I mutations [20]. Another characteristic feature of KIT mutant isoforms is greater or equal expression of immature forms (145 kDa) when compared with mature forms (160 kDa), while in the KIT-WT expressing cells, the mature hyperglycosylated form predominates [20]. In line with previously published results, KIT-N822I mutant also showed greater expression of immature form as compared with mature form.

To investigate KIT-N822I oncogenic potential, we designed another construct and expressed it in the interleukin-3–dependent Ba/F3 cells. As controls, imatinib-sensitive KIT-V559D and imatinib-resistant KIT-D816V–transduced Ba/F3 cells were used. Oncogenic activity of the studied KIT mutant isoforms was confirmed because expression of KIT-V559D, KIT-D816V, and KIT-N822I transformed the Ba/F3 cells to interleukin-3–independent growth (Fig. 3A).

During 3-year follow-up, the patient and his children did not suffer from any other symptoms of mastocytosis than urticaria pigmentosa, therefore, they have not required further treatment.

Figure 3. In vitro assays of KIT-V559D, KIT-N822I, and KIT-D816V–expressing Ba/F3 cells. (A) Interleukin-3 (IL-3)–deprivation of KIT-V559D, KIT-N822I, and KIT-D816V–transduced Ba/F3 cells resulted in transformation to IL-3–independent growth. The mean growth ± standard error of mean of three separate measurements over 4 consecutive days are shown. (B) In vitro effect of dasatinib on KIT-V559D, KIT-N822I, and KIT-D816V–expressing Ba/F3 cells. The dose-response proliferation curves of transduced Ba/F3 cells, treated with dasatinib for 48 hours are shown. Points represent the average results of experiment done in triplicate; bars: standard deviation (SD). The calculated IC50 for each cell line is indicated. Graph was plotted with the curve-fitting GraphPad Prism 5 software. (C) In vitro effect of imatinib on apoptosis of KIT-V559D, KIT-N822I, and KIT-D816V–expressing Ba/F3 cells treated for 48 hours. The percentage of apoptotic and necrotic transduced Ba/F3 cells are indicated. (D) Induction of apoptosis in KIT-V559D, KIT-N822I, and KIT-D816V–expressing Ba/F3 cells upon dasatinib treatment.
treatment with tyrosine kinase inhibitors. However, because KIT-N822 mutations have been reported in other tumors, we decided to define the tyrosine kinase inhibitors sensitivity of the KIT-N822I.

We examined the inhibitory effect of imatinib and dasatinib on the ligand-independent KIT phosphorylation in Ba/F3 cells. By proliferation assay, imatinib exhibited a high efficacy only toward the imatinib-sensitive KIT-V559D mutant, with an IC50 of 82 nM (data not shown). Furthermore, significant induction of apoptosis of these cells was recorded at 100 nM imatinib (Fig. 3C). KIT-D816V and KIT-N822I–expressing Ba/F3 cells were resistant to imatinib and no induction of apoptosis was recorded (Fig. 3C). In contrast, dasatinib effectively inhibited the KIT-V559D, KIT-D816V, and KIT-N822I isoforms with an IC50 value of 6 nM, 77 nM, and 68 nM, respectively (Fig. 3B). An apoptosis assay showed the induction of apoptosis in KIT-D816V and KIT-N822I Ba/F3 transformants 48 hours after treatment with 100 nM dasatinib (Fig. 3D). Massive apoptosis of KIT-V559D was recorded already at 50 nM dasatinib (data not shown). Addition of interleukin-3, the normal growth factor for Ba/F3 cells, resulted in near complete rescue, suggesting selective inhibition of transduced Ba/F3 by dasatinib (Supplementary Figure E2).

The effect of the inhibitors on signaling in KIT-V559D, KIT-D816V, and KIT-N822I–expressing Ba/F3 cells was also investigated by Western immunoassays in wide dose range. Imatinib was very efficient in reducing the tyrosine phosphorylation of the KIT-V559D and downstream proteins because low nanomolar concentrations were required to inhibit KIT kinase activity (Fig. 3B). Decrease in phosphorylation status of AKT, ERK1/2, and RPS6K were observed already at 50 nM imatinib. As expected, KIT-D816V and KIT-N822I–expressing Ba/F3 cells were resistant to imatinib (Fig. 3E). Western blot analysis for KIT-V559D, KIT-D816V, and KIT-N822I–expressing Ba/F3 cells confirmed a decrease in KIT activation with an increasing dose of dasatinib, while total protein expression was unaffected (Fig. 3F). Western immunoassays showed that dasatinib inhibited the phospho-KIT already at 50 nM in KIT-V559D–expressing Ba/F3. In KIT-D816V and KIT-N822I–expressing Ba/F3 cells, inhibition of phosphorylation of KIT was recorded at 100 nM. Downstream effectors ERK1/2, AKT, and RPS6K also showed decreased phosphorylation with increasing inhibitor concentrations, while total protein expression remained unaffected (Fig. 3F).

Some of the previously published in vitro experiments have shown that cells carrying p.N822K mutations or other
mutations in the receptor A-loop are sensitive to imatinib [21]. Contrary data illustrating the minimal response of such mutations to imatinib have been presented by other groups [22]. In the current study, we showed that KIT-N822I mutation is resistant to imatinib. Our in vitro observations are consistent with the findings of McDonnell et al. [18]. The patient with melanoma and double KIT mutation, including p.N822I, failed to respond to imatinib.

More recently, Guo et al. reported that KIT exon Ba/F3 cells expressing KIT exon 13 or exon 17 single or double mutants were sensitive to dasatinib inhibition [22]. Our studies were in line with this report and we showed that dasatinib exhibited a high efficacy toward the KIT-N822I mutant on Ba/F3 cells.

The results of phase II study in systemic mastocytosis by Verstovsek et al. showed that dasatinib does not eliminate the disease in systemic mastocytosis patients with KIT-D816V mutation [23]. However, contrary data were presented by Ustun et al. [24]. They presented in vitro and in vivo efficacy of dasatinib in systemic mastocytosis acute myeloid leukemia patient with KIT(D816V) mutation. In addition, authors claimed that no significant adverse effects of dasatinib occurred.

In the current study, functional studies revealed that KIT-N822I mutation is oncogenic and results in a high constitutive tyrosine phosphorylation of KIT. However, we demonstrated that urticaria pigmentosa was the only manifestation of mastocytosis in affected family members. Therefore, our results support previous findings that phenotypic diversity among patients with mastocytosis is due to multiple and complex molecular events.

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Conflict of interest disclosure
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

Author contributions
B.W. designed and performed experiments, analysed the data and wrote the report; M.N. provided clinical data and contributed to the paper; A.P. performed and analyzed molecular data of patients’ samples; M.L., J.R. provided patients’ samples; E.J. revised the article for intellectual content; W.B. performed histopathological examination; M.D.R., J.L. analyzed the data and wrote the report.

References


Supplementary Figure E1. Electropherograms with the fragments of KIT exon 17 sequences of the PCR products. (A) No KIT mutation was detected in proband’s parents and his sister. (B) The heterozygous KIT-N822I mutation was detected in the proband and his children. Alteration is indicated by the arrow.
Supplementary Figure E2. Proliferation assay for KIT-V559D-, KIT-N822I-, and KIT-D816V-transduced Ba/F3 cells, incubated with dasatinib for 48 hours in the presence of 10 ng/mL interleukin-3 (IL-3). Proliferation of the cells became independent of the KIT pathway and was not influenced by dasatinib. In KIT-V559D, KIT-N822I, and KIT-D816V-transduced Ba/F3 cells, dasatinib was not able to overcome IL-3-mediated rescue.