Activity of imatinib in systemic mastocytosis with chronic basophilic leukemia and a *PRKG2-PDGFRB* fusion

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Funding: this research was supported in part by the Intramural Research Program of the NIH, NIAID and NCI Center for Cancer Research; a grant from the Foundation Against Cancer (a public interest foundation) (J.C.); a Concerted Research Action Grant from K.U. Leuven (J.C., I.W., P.M.), and the NIH/NIAID Division of Intramural Research.

Manuscript received June 12, 2007.
Manuscript accepted August 9, 2007.

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ABSTRACT

Background
Translocations involving region 5q31-32 (*PDGFRB*) have been reported in a variety of myeloproliferative diseases and are often associated with significant peripheral eosinophilia. We report an unusual case of a patient presenting with peripheral basophilia and systemic mastocytosis in whom cytogenetic analysis revealed a t(4;5)(q21.1;q31.3).

Design and Methods
We used molecular analyses to determine the role of *PDGFRB* in this case. The patient was treated with imatinib.

Results
Fluorescence *in situ* hybridization (FISH) documented a breakpoint in *PDGFRB*. In agreement with this, the patient responded very well to imatinib with resolution of clinical symptoms, basophilia, and mast cell disease. Molecular analyses revealed that *PDGFRB*, encoding an imatinib-sensitive tyrosine kinase, was fused to *PRKG2*. The fusion gene incorporates the first two exons of *PRKG2* fused to the truncated exon 12 of *PDGFRB*, resulting in the disruption of its juxtamembrane domain. Functional studies confirmed that the activity and transforming properties of PRKG2-PDGFRβ were dependent on the disruption of the auto-inhibitory juxtamembrane domain.

Conclusions
Our results identify a second case of the *PRKG2-PDGFRB* fusion and confirm the unusual *PDGFRB* breakpoint associated with this fusion. This work also illustrates the use of imatinib for the treatment of specific cases of systemic mastocytosis.

Key words: systemic mastocytosis, chronic basophilic leukemia, PRKG2-PDGFRB, imatinib

Citation: Lahortiga I, Akin C, Cools J, Wilson TM, Mentens N, Arthur DC, Maric I, Noel P, Kocabas C, Marynen P, Lessin LS, Wlodarska I, Robyn J, Metcalfe DD. Activity of imatinib in systemic mastocytosis with chronic basophilic leukemia and a PRKG2-PDGFRB fusion. Haematologica. 2008 Jan; 93(1):49-56. DOI: 10.3324/haematol.11836

Introduction

Systemic mastocytosis (SM) is an uncommon myeloproliferative disease with variable clinical manifestations. Activating mutations of *KIT* (most commonly D816V) are found in more than 90% of cases of SM and are believed to be involved in disease pathogenesis.[1] In about 10-35% of cases, SM is associated with a second hematologic neoplasm. This subtype of SM is referred to as SM with an associated hematologic non-mast cell lineage disease (SM-AHNMD). In order to establish the diagnosis, WHO criteria for both SM and the AHNMD must be met.

SM-AHNMD has been described in a number of case series and while the AHNMD may be of myeloid or lymphoid origin, most often the associated neoplasm is myeloid.[2] The molecular pathogenesis of SM-AHNMD appears to be variable. In one series of patients with SM-AHNMD, *KIT* point mutations were detected in 16 out of 20 cases,[3] while in other cases, SM-AHNMD has been reported in association with a variety of cytogenetic abnormalities including t(8;21)(q22;q22), del 20(q12), and trisomy 9.[2,4]

Other myeloproliferative diseases are also frequently characterized by the presence of mutations or rearrangements of protein tyrosine kinase genes such as *JAK2, FGFR1*, *PDGFRA* and *PDGFRB*.[5,6] A variety of chromosomal translocations involving *PDGFRB* have been identified and reveal that *PDGFRB* can fuse to many different partner genes.[7] The most common fusion is the *ETV6-PDGFRB* (*TEL-PDGFRB*) fusion, for which it has been well documented that homo-oligomerization of ETV6-PDGFRβ is responsible for its constitutive kinase activation.[8] PDGFRβ, as well as PDGFRα, can also be activated by disruption of the inhibitory juxtamembrane region, as recently demonstrated in the case of the FIP1L1-PDGFRα fusion found in patients with chronic eosinophilic leukemia.[9]

Walz and colleagues have recently described three novel *PDGFRB* fusions in myeloproliferative disorders, including a case with a t(4;5;5)(q23;q31;q33) resulting in the fusion of *PRKG2* with *PDGFRB*.[10] We here describe a patient with a novel clinical presentation of SM with associated chronic basophilic leukemia characterized by leukocytosis, dramatic peripheral basophilia, atypical bone marrow mast cells, and a t(4;5)(q21.1;q31.3). Reverse transcriptase-polymerase chain reaction (RT-PCR) showed the presence of a *PRKG2-PDGFRB* fusion transcript, with an unusual breakpoint located within exon 12 of *PDGFRB*.

Design and Methods

Immunophenotypic analysis of bone marrow mast cells by multiparameter flow cytometry

Bone marrow aspirates were washed and the samples were stained using directly conjugated antibodies against CD2, CD25, CD35, CD45 and CD117 (Becton Dickinson, San José, CA, USA). Red blood cells were lysed using FACS Lysing Buffer (Becton Dickinson). After washing, the cells were resuspended in phosphate-buffered saline 1% formaldehyde. Three-color flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson). CELLQuest software was used for acquisition of data and analysis.

Cytogenetics

Cytogenetic studies were performed using standard methods. Metaphase chromosomes were obtained from 24- and 48-hour unstimulated cultures of buffy coat cells from fresh heparinized bone marrow aspirates. Chromosomes were G-banded using Wright's stain. A minimum of 20 metaphase cells were fully analyzed at the 400-550 band level of resolution at all time points; 30 additional cells were scored for chromosome number and chromosomes #4 and #5 at diagnosis. Multiple digital images were captured and karyotyped using a CytoVision™ system (Applied Imaging Corp., San José, CA, USA). Karyotypes were designated according to the ISCN (2005).[11]

Fluorescence in situ hybridization (FISH) studies

FISH was performed according to standard procedures. The applied probes included four cosmid clones flanking the *PDGFRB* gene (c9-4, c12-a, c4-1, c4-6) and three bacterial artificial chromosome (BAC) clones flanking the *PRKG2* gene (RP11-660B13, RP11-22A1 and RP11-29L8). BAC clones were selected from the Human Gene Project map (*www.ensembl.org*) and obtained from the Roswell Park Cancer Institute libraries (*www.chori.org/BACPAC*). The *PDGFRB* probes were kindly provided by Dr. M. Dixon (University of Manchester, Manchester, UK). Cosmid and BAC clones were directly labeled with SpectrumGreen- and SpectrumOrange-dUTP (Vysis, Downers Grove, USA) using random primer reactions (Invitrogen, Carlsbad, USA). FISH experiments were evaluated using an Axioplan 2 fluorescence microscope equipped with a charge-coupled device Axiophot 2 camera (Carl Zeiss Microscopy, Jena, Germany) and a MetaSystems Isis imaging system (MetaSystems, Altlussheim, Germany). Five abnormal metaphases and 200 interphase cells were evaluated in each FISH study.

RNA isolation

Bone marrow or peripheral blood mononuclear cells were obtained by Ficoll-Hypaque gradient centrifugation and cells were lysed in Trizol (Invitrogen). Total RNA was extracted from $1x10^6$ to $1x10^7$ cells using the RNAEasy RNA isolation kit (Qiagen, Valencia, CA USA).

Screening for KIT and interleukin-3 receptor β chain mutations

To screen for D816V *KIT* mutation by restriction frag-

ment length polymorphism (RFLP) analysis, approximately 1 μg of total RNA was reverse transcribed as described above. A 373-bp region of the c-*kit* gene was amplified by PCR using the following primers: primer1 (KITAsp816F 5'-AAA GGA GAT CTG TGA GAA TAG GCT C-3') and primer2 (KITAsp816R: 5'-AGC TCC CAA AGA AAA ATC CCA TAG G-3'). PCR products were digested with the restriction enzymes *Hae* III and *Hinf* I (New England Biolabs, USA) and electrophoresed on a 10% tris-borate EDTA polyacrylamide gel (Invitrogen, San Diego, CA, USA) stained with ethidium bromide. Digestion of the wild-type PCR product with these enzymes yields fragments of 171 bp, 127 bp, 43 bp, and 31 bp. An A to T substitution in *KIT* nucleotide 2468 (within the 171-bp fragment) causing the D816V mutation introduces a new *Hinf* I recognition site resulting in fragments of 157 bp, 127 bp, 43 bp, 31 bp, and 14 bp. Full length coding sequences of *KIT* and IL3 receptor beta genes were analyzed by RT-PCR amplification using overlapping primers followed by direct sequencing of the amplified PCR fragments on a capillary sequencer. Total RNA was reverse transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase without RNaseH activity and oligo (dT) primer (Invitrogen). cDNA was amplified by PCR using overlapping oligonucleotide primers. Resulting PCR products were sequenced directly using the Big Dye Terminator kit (PE Biosystems, Foster City, CA, USA) and analyzed on a capillary automated sequencer (ABI Prism 310 Genetic Analyzer; PE Biosystems).

Reverse transcription PCR for the PRKG2-PDGFRB fusion gene

RNA was reverse transcribed with random hexamers using standard techniques. The *PRKG2-PDGFRB* fusion gene was amplified using a nested PCR with primers for the first round PRKG2-F1: 5´-GAGCGGGAGTAC-CATTTGAA-3´ and *PDGFRB*-R1: 5´-TAAGCATCTT-GACGGCCACT-3´ and primers PRKG2-F2: 5'-GCCATTGCTGAACTCACAGA-3´ and *PDGFRB*-R2: 5'-CTCCACCACCTGCCCAAA-3´ for the second round of amplification.

PDGFRB and PDGFRA constructs

PRKG2-PDGFRB, *PRKG2-JM-PDGFRB* and *myc-W-PDGFRA* constructs were generated by PCR and were cloned into the MSCV-puromycin vector (Clontech).

Cell culture

Ba/F3 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1 ng/mL mouse IL3. Ba/F3 cells expressing the different *PRKG2-PDGFRB* fusions were generated by retroviral transduction and subsequent puromycin selection. To test IL3-independent growth, stable Ba/F3 cell lines were washed three times in PBS and cultured in medium without IL3. The number of viable cells was counted with a Vi-cell XR cell viability analyzer (Beckman Coulter, Fullerton, CA, USA). Retroviral vectors were generated by co-transfection of MSCV vectors with a packaging construct, as described elsewhere.[12]

Western blotting

Total cell lysates were obtained by lysing cells in cold lysis buffer (PBS with 1 mM Na_2EDTA, 1M NaF, 0.1% Triton X-100, 5 mM Na_3VO_4, 200 mM phenylarsine oxide, pH 7.2) (Calbiochem) and complete protease inhibitor tablets (Roche). Thirty micrograms of protein lysate were combined with SDS loading buffer plus DTT (Cell Signaling Technology, Beverly, MA, USA) before electrophoresis on 4-12% Bis-Tris gradient gels (Invitrogen) and transferred to nitrocellulose membranes. The following antibodies were used: anti-PDGFRβ (Santa Cruz Biotechnology), anti-phosphotyrosine (4g10; Upstate Biotechnology, Lake Placid, NY, USA), anti-ERK2 (Santa Cruz Biotechnology), anti-phosphoERK, and anti-mouse peroxidase and anti-rabbit peroxidase (Amersham Pharmacia Biotech).

Results

Clinicopathological features and response to imatinib

The patient is a 61-year old Caucasian male with a history of familial polycystic kidney disease, who received a cadaveric renal transplant in 1991. In 1997 he developed generalized pruritus with an erythematous, reticulated-appearing rash. In 2003 he developed myalgia, abdominal pain, and diarrhea; and was noted to have splenomegaly on examination. A complete blood count showed a white blood cell count of 26,300/mm^3, with 26% basophils, 4% eosinophils, and a platelet count of 118,000/mm^3. Total serum tryptase concentration was 200 ng/mL. His bone marrow was hypercellular with 10% basophils and 2% mast cells. There were some atypical megakaryocytes but normal myeloid and erythroid maturation. Focal interstitial and peri-vascular mast cell aggregates with 11-18% spindle-shaped mast cells were observed (Figure 1A-C). Reticulin staining revealed grades 2-3/4 fibrosis. Flow cytometric analysis of mast cells revealed aberrant surface expression of CD25 and CD35 but not CD2.

RT-PCR followed by RFLP analysis was negative for the *KIT* D816V mutation, and sequencing of the entire *KIT* cDNA revealed no mutations. The *bcr-abl* translocation was not found. Because of the involvement of the IL-3 receptor common β chain in both basophil and eosinophil growth and survival, the entire coding sequence of this gene was also analyzed and no mutations were found. This patient was thought to have SM with chronic basophilic leukemia. Cytogenetics showed a translocation, t(4;5)(q21.1;q31.3), in 47 (94%) of 50 bone marrow metaphases analyzed (Figure 2).

Due to the lack of a codon 816 *KIT* mutation, imatinib mesylate treatment was initiated empirically at a dose of 200 mg daily. The pruritus, myalgia and abdominal complaints improved dramatically within 1-2 weeks after start-

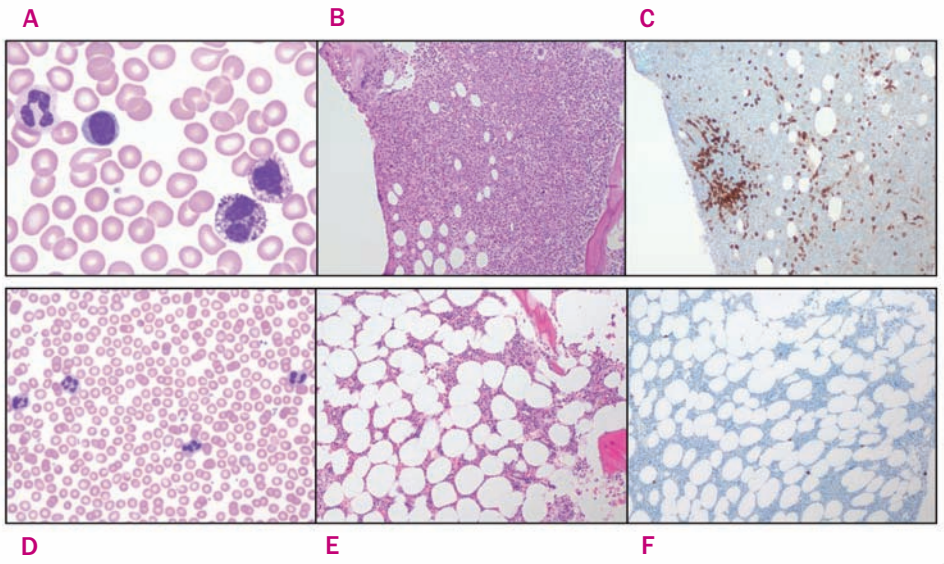


Figure 1. Pre-treatment and post-treatment peripheral blood smears and bone marrow biopsies. (**A**) Pre-treatment peripheral blood smear showing marked basophilia with partially degranulated basophils. (**B,C**) Pre-treatment bone marrow biopsy stained with hematoxylin and eosin (**B**) and tryptase immunostaining (**C**) showing a hypercellular marrow with mast cell aggregates and spindle-shaped mast cells. (**D**) Post-treatment peripheral blood smear showing disappearance of circulating basophils. (**E,F**) Post-treatment bone marrow biopsy stained with hematoxylin and eosin (**E**) and tryptase immunostaining (**F**) showing a mildly hypocellular marrow with scattered tryptase-positive cells, mostly round in shape.

ing imatinib. Basophilia and elevated serum tryptase levels normalized over the next 3-4 weeks (Figure 3A-B). A repeat bone marrow analysis 1 month after initiating imatinib showed only scattered mast cells, mostly round in shape (Figure 1D-E). The dose of imatinib was increased to 300 mg/day. Cytogenetic analysis 4 months after the dose increase showed persistence of the t(4;5)(q21.1;q31.3) in ten (50%) of 20 metaphase cells analyzed. Cytogenetic remission was documented 1 year after diagnosis. The patient remains in clinical and cytogenetic remission 3 years after imatinib was initiated.

Molecular analyses of the t(4;5)(q21.1;q31.3)

A variety of chromosomal translocations involving the *PDGFRB* gene in chromosome band 5q31-32 have been reported.[7] Using FISH, we investigated whether *PDGFRB* was also involved in this case. FISH analysis using probes flanking the *PDGFRB* gene documented a breakpoint within *PDGFRB* in this case, with the 5' part of *PDGFRB* translocated to chromosome 4 (Figure 4).

Walz and colleagues recently identified three novel *PDGFRB* fusion partners in myeloproliferative diseases, including a case with a complex t(4;5;5)(q23;q31;q33). This case harbored a *PRKG2-PDGFRB* fusion with an unusual breakpoint within exon 12 of *PDGFRB*.[10] Since our case showed similar breakpoints on chromosome 4, we investigated whether our case also harbored the *PRKG2-PDGFRB* fusion. RT-PCR confirmed an in-frame fusion between exon 2 of *PRKG2*, a 5 bp insert derived from intron 2 of *PRKG2*, and a truncated version (last 33 bp) of exon 12 of *PDGFRB* (Figure 5A).

This was similar to the fusion described by Walz *et al.*, in which there was a fusion between exon 5 of *PRKG2*, a 17 bp insert derived from intron 5 of *PRKG2*, and a truncated version (last 41 bp) of exon 12 of *PDGFRB*. In both fusions, the region encoding the catalytic domain of PDGFRβ was retained, with *PRKG2* contributing a coiled-coil domain that may potentially lead to dimerization and constitutive activation of the fusion protein.

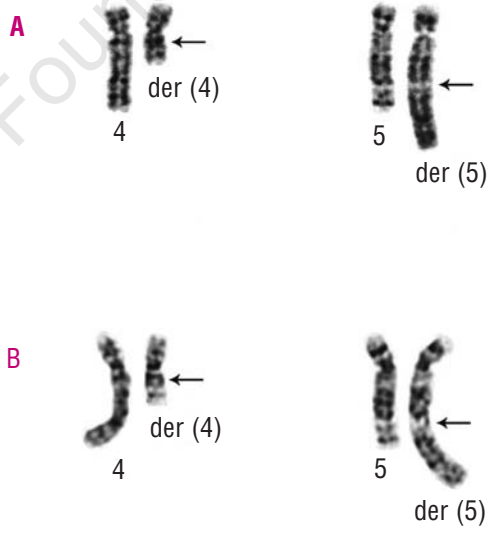


Figure 2. Cytogenetic analysis of the patient. Composite of chromosomes 4 and 5 from two G-banded metaphase cells (**A**, **B**) showing the t(4;5)(q21.1;q31.3).

Interruption of the juxtamembrane domain of PDGFRβ is required for kinase activation and for the transforming properties of PRKG2-PDGFRβ

To test whether the interruption of the juxtamembrane was required for constitutive activation and the transforming properties of *PRKG2-PDGFRβ*, we generated two constructs. The first construct represented the *PRKG2-PDGFRB* fusion, as identified in the case described in this report with the juxtamembrane domain interrupted. A second construct was generated represent-

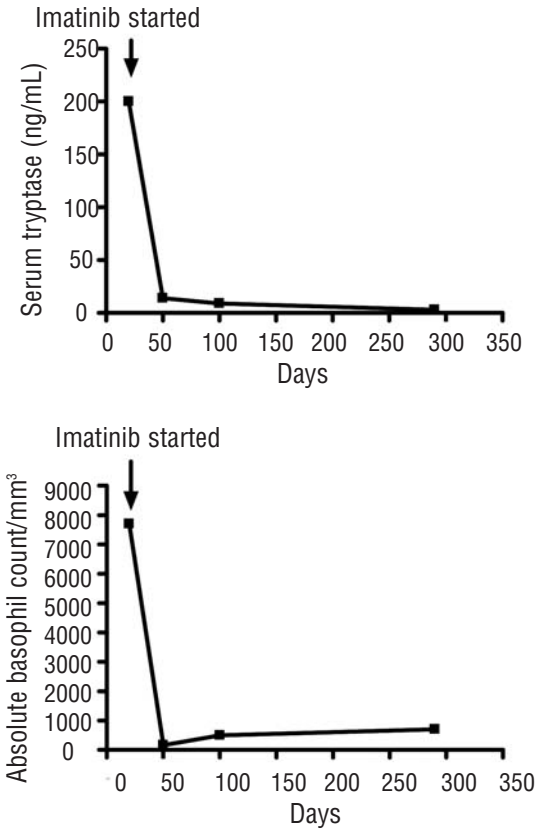


Figure 3. Decrease in basophil count and serum tryptase levels over time post-treatment. Decrease in total serum tryptase (A) and absolute basophil count (B) over time after initiation of imatinib (arrow).

ing the *PRKG2-JM-PDGFRB* fusion, with the complete transmembrane and juxtamembrane regions present, as usually found in almost all *PDGFRB* fusions (Figure 5B).

Ba/F3 is a murine hematopoietic cell line that requires IL3 for proliferation and survival. It has been shown that Ba/F3 cells can be transformed by activated forms of PDGFRα and PDGFRβ.[9]

Here we used an activated form of PDGFRα (Myc-PDGFRα) as a positive control for Ba/F3 cell transformation. Expression of PRKG2-PDFGRβ also transformed Ba/F3 cells to IL3-independent growth. Western blot analysis confirmed autophosphorylation of PRKG2-PDGFRβ and activation of the downstream signaling proteins ERK1/2 (Figure 5C-D). In contrast, expression of PRKG2-JM-PDFGRβ did not result in transformation of the Ba/F3 cells or autophosphorylation of the fusion protein (Figure 5C-D).

Discussion

SM is a myeloproliferative disease with diverse clinical features which has been sub-classified by the WHO into four variants: indolent, aggressive, SM-AHNMD and mast cell leukemia. While the prognosis of indolent SM is usually good, such that most patients do not require cytoreductive therapy, patients with aggressive forms of the disease have a much worse prognosis and may survive for only a few years. The variant of mastocytosis known as SM-AHNMD occurs in 10-35% of patients and is believed to be associated with a less favorable prognosis. While peripheral blood eosinophilia is not uncommonly seen in association with SM-AHNMD, basophilia has been rarely described. The association of SM and

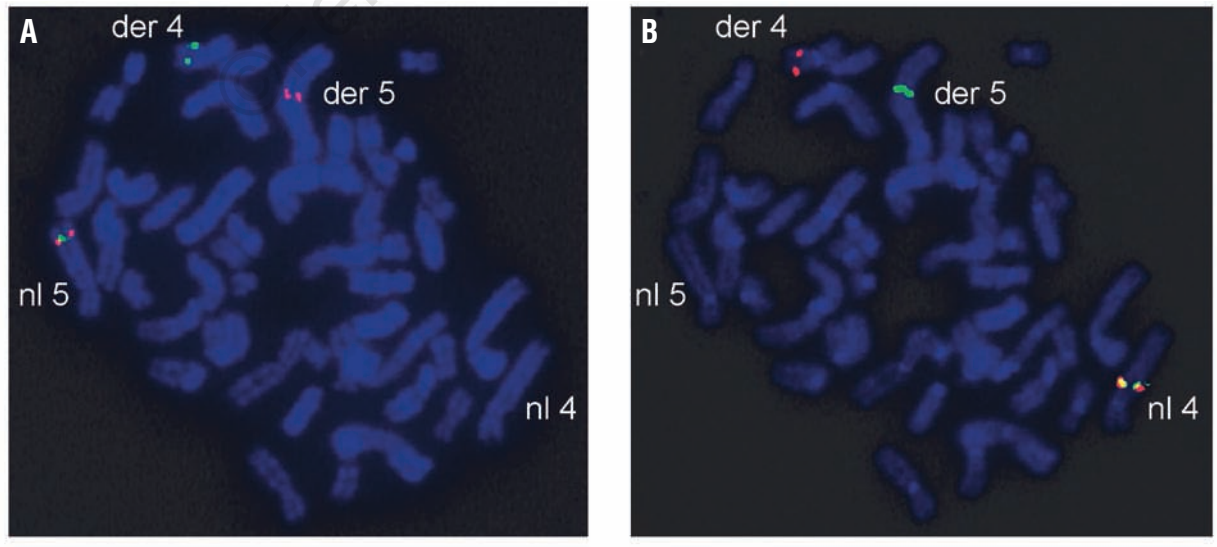
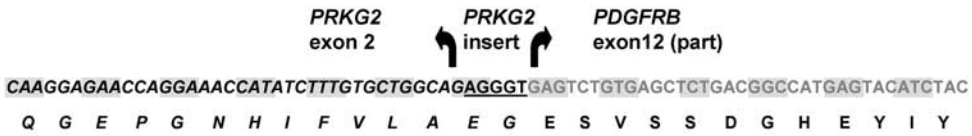
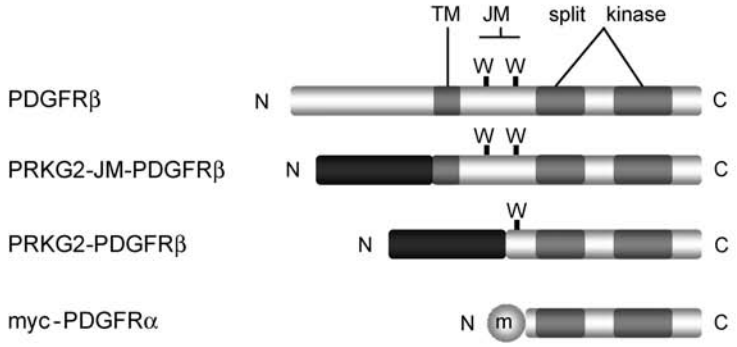


Figure 4. Analysis of the breakpoints on chromosomes 4q and 5q by FISH. Examples of FISH analysis showing the 5q33 and 4q21 breakpoints within the *PDGFRB* (A) and *PRKG2* loci (B), respectively. The applied flanking assays consisted of (A) c9-4/c12-a (green) and c4-1/c4-6 (red) and (B) RP11-660B13 (red) and RP11-22A1/RP11-29L8 (green). The same metaphase could be used to analyze both the *PDGFRB* and *PRKG2* loci.

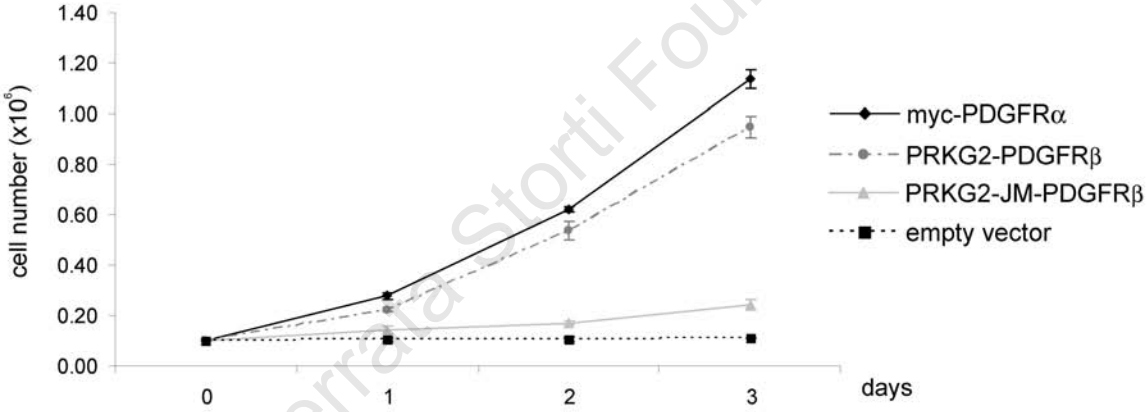
A



B



C



D

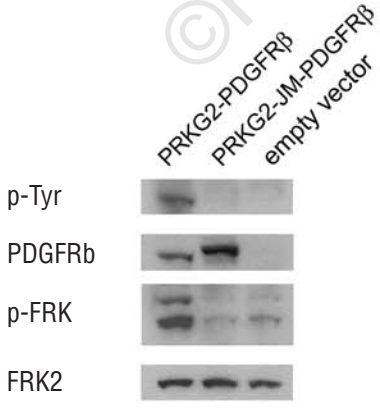


Figure 5. Molecular characterization and *in vitro* functional studies of the *PRKG2-PDGFRB* fusion. (A) Sequence of the identified *PRKG2-PDGFRB* fusion. *PRKG2* sequences are shown in black and *PDGFRB* sequences in gray. The location of the breakpoints is represented with arrows. (B) Schematic representation of the proteins studied in this work. JM: juxtamembrane domain; TM: transmembrane domain; *m* indicates the myc epitope tag. (C) Transformation assay of Ba/F3 cells in the absence of IL-3 reveals that expression of PRKG2-PDGFRβ, but not of PRKG2-JM-PDGFRβ transforms Ba/F3 cells to growth factor-independent growth. (D) Phosphorylation of the PRKG2-PDGFRβ, PRKG2-JM-PDGFRβ, and ERK1/2 proteins in Ba/F3 cells. Anti-ERK was detected as a control for loading.

chronic basophilic leukemia has not been reported.

Chronic basophilic leukemia is a rare disorder, which was first described in 1966.[13] A detailed retrospective review at the Mayo Clinic revealed only four cases of chronic basophilic leukemia over a 28-year period.[14] Symptoms related to basophil mediator release, including ulcers of the gastrointestinal tract, diarrhea, coagulopathy, pruritis and urticaria, were noted. An abnormal infiltrate of atypical mast cells was present in two of these cases. Cytogenetics were normal in two patients, one had trisomy 8 along with 11q- and the fourth had monosomy 7. Two of the four patients in this series died, one after progression to acute leukemia, and the second as a result of complications after bone marrow transplantation.

Unfortunately, options for cytoreductive treatment in most cases of systemic mastocytosis are limited. The *KIT* D816V mutation found in most patients with SM appears to confer resistance to imatinib.[15,16] Rare patients with novel *KIT* mutations have shown responses to imatinib, including a patient with a novel transmembrane mutation in *KIT*.[17] A recent report described a patient with aggressive SM and eosinophilia with a fusion gene involving *PDGFRB* who showed a clinical response to imatinib.[18] The pathogenetic role of *PDGFRB* or its 5' fusion partner was not identified in this case. Earlier, published descriptions of responses to imatinib in patients with SM associated with eosinophilia[19] prompted a series of clinical trials. The molecular basis of response was ultimately determined to be a novel fusion gene, *FIP1L1-PDGFRA*,[20,21] and these patients appear to have a disease that is both clinically and molecularly distinct from *KIT* D816V-associated SM.[22]

Durable responses to imatinib have been described in rare patients with atypical myeloproliferative diseases. In most instances these imatinib-responsive diseases have been associated with fusion genes involving *PDGFRB* and a total of 15 *PDGFRB* fusion partners have been described to date: *ETV6* (*TEL*, 12p13), *TRIP11* (*CEV14*, 14q32), *HIP1* (7q11), *CCDC6* (*H4*, 10q21), *RABEP1* (17p13), *PDE4DIP* (1q22), *SPECC1* (*HCMOGT-1*, 17p11), *NIN* (14q24), *KIAA1509* (14q32), *TP53BP1* (15q22), *NDE1* (16p13), *TPM3* (1q21), *GIT2* (12q24), *GPIAP1* (11p13) and *PRKG2* (4q21).7 The breakpoints in most of these fusion genes are upstream of exon 10, joining the transmembrane domain of PDGFRβ to the 5' partners, in each case containing putative oligomerization domains.

The case we describe here is unusual in many respects. This is the first demonstration of an auto-activating rearrangement of PDGFRβ in mast cell disease and the second report of a *PDGFRB*-associated fusion gene in SM. Furthermore, this is the second reported case of a fusion of *PDGFRB* with *PRKG2*, which encodes a type II, cyclic GMP-dependent protein kinase that seems to be involved in neuronal adaptation, smooth muscle relaxation and growth, intestinal water secretion, bone growth and renin secretion.[10] In both cases with the PRKG2-PDGFRβ fusion, the juxtamembrane domain of PDGFRβ was interrupted. This is in contrast to all other known PDGFRβ fusions in which the entire juxtamembrane domain of PDGFRβ is present.[9] In most characterized examples of fusion tyrosine kinases, oligomerization domains of the fusion partners have been shown to be necessary for both kinase activation and for transforming properties of the fusion proteins.[6,8,23,24] Although the PRKG2 moiety contains a predicted coiled-coil domain, a recent study has shown that disruption of the juxtamembrane domain of PDGFRα or PDGFRβ alone is sufficient to cause constitutive activation of kinase activity irrespective of the presence of a fusion partner that mediates oligomerization.[9] Indeed, the juxtamembrane domain has been shown to have an auto-inhibitory role in receptor tyrosine kinases.[25] We show that in the context of the PRKG2-PDGFRβ fusion, the interruption of the juxtamembrane domain of PDGFRβ is required for the constitutive kinase activity and transforming properties.

Our study illustrates the importance of searching for *PDGFRB* rearrangements in patients with SM associated with myeloproliferative disorders, as this subgroup responds well to imatinib. This disease variant should be considered in patients without codon 816 *KIT* mutations and with cytogenetic abnormalities involving 5q31-32. The prevalence of this variant of SM is unclear at present, but this case illustrates the importance of cytogenetic analysis when evaluating patients with SM-AHNMD.

Authorship and Disclosures

CA, TMW, PN, LSL, JR and DDM provided clinical care; IL, CA, JC, TMW, NM, DCA, IM, CK, IW and JR performed experiments and analyzed data and IL, CA, JC, IM, PN, PM, LSL, IW, JR and DDM contributed to writing the paper.

The authors reported no potential conflicts of interest.

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