Characterization of three new imatinib-responsive fusion genes in chronic myeloproliferative disorders generated by disruption of the platelet-derived growth factor receptor β gene

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Funding: this study was supported by the 'Deutsche José Carreras Leukämie-Stiftung e.V.' (grant no. DJCLS R06/02), Germany, the Competence Network "Acute and chronic leukemias", sponsored by the German Bundesministerium für Bildung und Forschung (Projektträger Gesundheitsforschung; DLR e V. - 01GI9980/6), Germany, the Leukaemia Research Fund, UK, and the "European LeukemiaNet" within the 6th European Community Framework Programme for Research and Technological Development.

Manuscript received October 30, 2006. Accepted January 2, 2007.

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ABSTRACT

Background and Objectives

We sought to identify new fusion genes with involvement of the platelet-derived growth factor receptor β gene (*PDGFRB*) in three patients presenting with various subtypes of chronic myeloproliferative disorders associated with chromosomal aberrations involving chromosome bands 5q31-33.

Design and Methods

We performed 5' rapid amplification of cDNA ends (5'-RACE)-polymerase chain reaction (PCR) with RNA/cDNA derived from a patient (case #1) with a t(5;12)(q31-33;q24) and a second patient (case #2) with a complex rearrangement involving chromosomes 1, 5 and 11. A newly developed DNA-based 'long-distance inverse PCR' (LDI-PCR) was performed on a third patient (case #3) with a t(4;5;5)(q23;q31;q33).

Results

In cases #1 and #2, we identified mRNA fusions between *GIT2* exon 12 and *GPIAP1* exon 7, respectively, and *PDGFRB* exon 11. In case #3, LDI-PCR revealed a fusion between *PRKG2* exon 5 and a truncated *PDGFRB* exon 12. The region encoding the catalytic domain of PDGFRβ is retained in all three cases, with the partner contributing a coiled-coil domain (GPIAP1, PRKG2) or an ankyrin protein interaction motif (GIT2) that may potentially lead to dimerization and constitutive activation of the fusion proteins. Treatment with imatinib (400 mg/day) has led to sustained complete hematologic remission in all three patients.

Interpretation and Conclusions

These data provide further evidence that numerous partner genes fuse to *PDGFRB* in *BCR-ABL* negative chronic myeloproliferative disorders. Although these fusion genes occur rarely, their identification is essential in order to detect patients in whom targeted treatment with tyrosine kinase inhibitors is likely to be successful.

Key words: *GPIAP1, GIT2, PRKG2, PDGFRB*, imatinib.

Haematologica 2007; 92:163-169

Chronic myeloproliferative disorders (CMPD) are clonal stem cell diseases characterized by excess proliferation of cells from one or more myeloid lineages accompanied by relatively normal maturation. Patients with clinical features of chronic myeloid leukemia (CML) who lack the Philadelphia (Ph) chromosome and/or the *BCR-ABL* fusion gene are referred to as having atypical CML, or more broadly unclassifiable myeloproliferative or myelodysplastic/myeloproliferative disease (MDS/MPD).

The molecular pathogenesis of these *BCR-ABL*-negative CMPD is incompletely understood and most individuals display a normal or aneuploid karyotype in cytogenetic analysis. However, a small proportion of affected patients present with acquired reciprocal chromosomal translocations involving recurrent breakpoint clusters at chromosome bands 4q12, 5q31-33, 8p11, 9p24 and 13q12. Molecular analysis demonstrated that these clusters are linked to the tyrosine kinase genes *PDGFRA, PDGFRB, FGFR1, JAK2* and *FLT3*, respectively. Chromosomal translocations involving tyrosine kinases produce fusion genes encoding novel chimeric proteins with constitutive kinase activity in the absence of the natural ligands, resulting in deregulation of hematopoiesis in a manner that is similar to BCR-ABL in CML.[1,2] In addition, 10-20% of cases are characterized by the presence of the *JAK2* V617F mutation or activating *NRAS* mutations.[3] In this study, we sought to identify new fusion genes in three patients presenting with variant CMPD subtypes and chromosomal aberrations involving chromosome bands 5q31-33, suggestive of a rearrangement of the *PDGFRB* gene.

Design and Methods

Case reports

Case #1. A 67-year old male patient presented with a 5-year history of eosinophilia in the peripheral blood and cutaneous itching. The peripheral blood revealed a leukocytosis of 21×10^9/L with a normal hemoglobin level (14.1 g/dL) and platelet count (276×10^9/L). The otherwise normal differential blood count displayed prominent eosinophilia (44%). There were no signs of hepatosplenomegaly. The bone marrow was hypercellular with prominent and dysplastic eosinophils accounting for 20-30% of the cells but no increase of atypical mast cells. A trephine biopsy was not performed. Cytogenetic analysis revealed a t(5;12)(q31-33;q24)[6];46,XY[4].

Case #2. The peripheral blood of a 59-year old male patient showed normal leukocytes (10×10^9/L) with 47% eosinophils, a normal platelet count (187×10^9/L) and a slightly reduced hemoglobin level (11.0 g/dL). There were no signs of lymphadenopathy or hepatosplenomegaly. The trephine biopsy revealed argyrophilic fibrosis, dysplastic megakaryocytes and spindle-shaped $CD2^+$ and $CD25^+$ mast cells (5%). The level of serum tryptase was elevated to 17 ng/mL (normal value <11.4 ng/mL). Cytogenetic analysis revealed a complex der(1)t(1;5)(p34;q33),der(5)(1;5)(p34;q15),der(11)ins(11;5)(p12;q15q33)[23];46,XY[1]. Involvement of chromosomes 1, 5 and 11 was confirmed by multicolor-fluorescence *in situ* hybridization (FISH) analysis (*data not shown*). Four months after starting treatment with hydroxyurea, the patient presented with massive global cardiac decompensation and Löffler's endocarditis was diagnosed subsequently. The patient developed an acute Leriche syndrome with consecutive incomplete *conus-cauda* syndrome in association with spinal ischemia and flaccid paresis of the right leg following multiple cardiac embolisms.

Case #3. A 45-year old female patient presented with a leukocytosis of 34×10^9/L. The otherwise normal differential blood count repeatedly revealed basophilia between 20 and 40%. The platelet count and hemoglobin level were within the normal ranges. The spleen diameter was enlarged to 18 cm. The bone marrow displayed marked fibrosis, abnormal aggregates of dysplastic megakaryocytes and an increase of eosinophils and spindle-shaped $CD2^+$ and $CD25^+$ mast cells. The serum tryptase level was elevated to 138 ng/mL. Conventional cytogenetics revealed a t(4;5;5)(q23;q31;q33)[20]. Subsequent treatment with imatinib resulted in the appearance of normal karyotype metaphases indicating that the t(4;5;5) was acquired and not inherited.

Rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR)

5´-RACE-PCR was performed using a second generation 5'/3' RACE kit from Roche Diagnostics (Mannheim, Germany) essentially according to the manufacturer´s instructions. Briefly, 2 μg RNA extracted from peripheral blood leukocytes using the RNeasy system (Qiagen, Hilden, Germany) were reverse transcribed using primer PDGFR-D: 5´-GAAGGTGTGTTTGTTGCGGT-3´. Nested PCR was performed with primer PDGFR-F: 5´-TTGACGGCCACTTTCATCGT-3´ in the first step and primer PDGFR-C: 5´-TGGCTTCTTCTGCCAAAGCA-3´ in the second step in conjunction with RACE anchor primers supplied by the manufacturer. Products were cloned using the TOPO cloning kit (Invitrogen, Leek, The Netherlands) and sequenced.

Reverse transcription PCR

RNA was reverse transcribed with random hexamers using standard techniques. The *GPIAP1-PDGFRB* fusion gene was amplified with primers C11-f-1: 5´-ACAGCACCCACAACCACCAGAA-3´ and C5-r-1: 5´-CATCGGATCTCGTAACGTGGCT-3´. The *GIT2-PDGFRB* fusion gene was amplified with primers GIT2/f1: 5´-GGCAGACAGAAGTTAGCTCGGT-3´ and C5-r-1. Nested PCR for the monitoring of residual disease was performed with inner primers GIT2/f2: 5´-GTTCAACGCCCATGAGTTTGCC-3´ and C5-r-2: 5´-AGGGAGATGATGGTGAGCACCA-3´. The *PRKG2-PDGFRB*

fusion gene was amplified with primers PRKG2f/1: 5'-CATGGGCACTAGATCGAGAGGT-3' and PRKG2r/1 5'-AAGCATCTTGACGGCCACTTTCA-3', nested PCR was performed with primers PRKG2f/2: 5'-GGACAGC-CCAAGCTAGAGATGA-3' and PRKG2r/2 5'-ATCGTG-GCCTGAGAATGGCTCA-3'. Primers used for the detection of reciprocal fusion transcripts are available on request. All amplifications were performed for 32 cycles at 60°C annealing temperature.

Long-distance inverse PCR cloning of PDGFRB rearrangements

For the detection of *PDGFRB* rearrangements on the genomic level, LDI-PCR was carried out using a modification of the method described previously for the IGHJ locus and the *BCL6* gene.[4,5] Briefly, 1 µg high molecular weight DNA was digested with *Kpn*I (New England Biolabs, Boston, USA) and purified by phenol:chloroform extraction. The digested DNA was ligated at 15°C overnight in a total volume of 50 µL with 5 U of T4 DNA ligase (Roche, Mannheim, Germany). The self-ligated circular DNA was used as a template for a single-step PCR with primers pKpnI/f: 5´-CCTCAGCCACAGCC-CGCAGCAGTGAGAAGC-3´ and pBr1: 5´-TCAGA-GAGTCTTCCCACCCAACTTGAGTCC-3´. Aliquots of the reaction were run in a 0.8% agarose gel. Any bands in addition to the expected germline band were excised from the gel, purified using Qiaquick spin columns (Qiagen) and sequenced.

Amplification refractory mutation system (ARMS)-PCR for JAK2 V617F

DNA was extracted following standard procedures. ARMS-PCR was used for the identification of the *JAK2* V617F mutation as previously described.[3]

Results

The association of CMPD with chromosomal aberrations that involve chromosome bands 5q31-33 suggested a rearrangement of *PDGFRB*. Because all but one (*NIN-PDGFRB*)[6] of the known genomic *PDGFRB* breakpoints are located within *PDGFRB* intron 11, we selected the reverse primers for 5´-RACE-PCR from sequences located within *PDGFRB* exons 11 and 12. All three patients were negative for the *JAK2* V617F mutation.

A GIT2-PDGFRB fusion gene in a t(5;12)(q31-33;q24)

Sequencing of the RACE PCR products revealed an in-frame fusion between *GIT2* exon 12 (accession no. NM_057169) and *PDGFRB* exon 11. RT-PCR confirmed the presence of the *GIT2-PDGFRB* fusion gene in case #1 but not in controls. A reciprocal fusion gene could be amplified by RT-PCR with a fusion between *PDGFRB* exon 10 and *GIT2* exon 13.

A GPIAP1-PDGFRB fusion gene in a complex translocation t(1;5;11)

Sequencing of the RACE PCR products revealed an in-frame fusion between *GPIAP1* exon 7 (accession no. NM_005898) and *PDGFRB* exon 11. The *GPIAP1-PDGFRB* fusion gene was confirmed by RT-PCR in case #2 but not in controls. No reciprocal *PDGFRB-GPIAP1* fusion gene could be amplified by RT-PCR.

A PRKG2-PDGFRB fusion gene in a t(4;5;5)(q23;q31;q33)

The disease phenotype, the chromosomal abnormalities and a complete cytogenetic response following treatment with imatinib clearly suggested an involvement of *PDGFRB*. However, 5´-RACE-PCR only amplified wild-type *PDGFRB* sequences. We therefore designed a LDI-PCR for the screening of *PDGFRB* fusion genes at the genomic level and identified a fusion between *PRKG2* intron 5 (accession no. NM_006259) and a truncated (-41 bp) *PDGFRB* exon 12. 5´-RACE-PCR failed to identify the fusion gene because the primers that were used were derived from sequences located upstream of this atypical breakpoint. RT-PCR confirmed an in-frame *PRKG2-PDGFRB* fusion involving *PRKG2* exon 5, a 17 bp insert derived from *PRKG2* intron 5 and a 41 bp truncated *PDGFRB* exon 12 in case #3 but not in controls. No reciprocal *PDGFRB-PRKG2* fusion gene could be amplified by RT-PCR. Sequence data of all three mRNA fusion genes and their corresponding exon numbers are shown in Figure 1.

Identification of genomic breakpoints by LDI-PCR

To further assess the value of LDI-PCR for detecting *PDGFRB* rearrangements, we applied this technique to DNA from the patients with *GPIAP1-PDGFRB* and *GIT2-PDGFRB* fusion genes (Figure 2). One single germline band was amplified from DNA of normal controls whereas additional bands (lanes 4, 5, 10-12) of different size were amplified from DNA of patients with the newly identified fusion genes (Figure 3). Sequencing confirmed that the genomic junction sequences were compatible with the mRNA fusions identified by RACE-PCR. On the genomic level, *GIT2* intron 12 was fused to *PDGFRB* intron 10 in case #1 and *GPIAP1* intron 7 was fused to *PDGFRB* intron 10 in case #2.

Response to imatinib in vivo and monitoring of residual disease

All three patients have been treated with imatinib 400 mg/day (median, 14 months, range, 12-30) and cases #1 and #3 achieved rapid and sustained complete hematologic responses within 3 and 4 weeks, respectively. The first complete hematologic response in case #2 was seen after 3 months. There have been no hematologic or non-hematologic dose-limiting toxicities. Follow-up of residual disease by nested RT-PCR could be performed in two patients. The *GIT2-PDGFRB* fusion gene in case

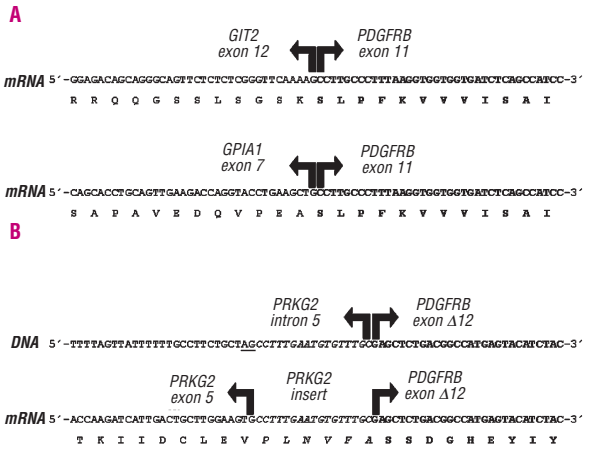

Figure 1. Upper panel (A): The in frame *GIT2-PDGFRB* and *GPIAP1-PDGFRB* junction sequences with corresponding amino acids. *GIT2* and *GPIAP1* sequences are in plain type and *PDGFRB* sequences in bold. Lower panel (B): The genomic fusion sequence of *PRKG2-PDGFRB* and the corresponding mRNA fusion transcript. The 17-bp insert (cryptic AG splice site underlined) is derived from *PRKG2* intron 5 (shown in italics) and fused to a truncated *PDGFRB* exon 12. A similar splicing mechanism and corresponding structure of the fusion sequence is found in other tyrosine kinase fusion genes, e.g. *FIP1L1-PDGFRA*, *CDK5RAP2-PDGFRA* and *PCM1-JAK2*.[25,46,47]

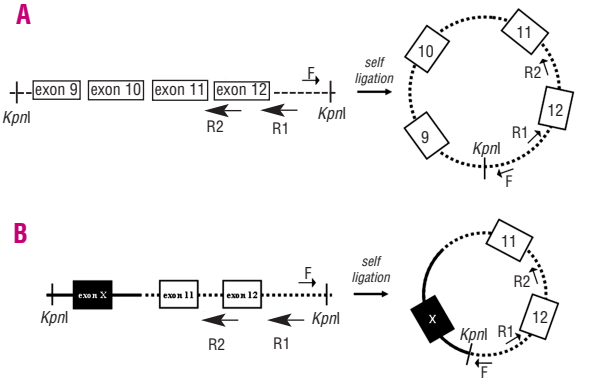

Figure 2. Diagrammatic representation of LDI-PCR. DNA fragments of different sizes were generated by the restriction enzyme *Kpn*I and subsequently self-ligated. PCR was performed with forward primers (F) which are located upstream of the *Kpn*I restriction site and reverse primers which are located downstream of known *PDGFRB* breakpoint regions in intron 11 (R1) and 10 (R2). PCR products that are different in size compared to the normal *PDGFRB* ring (A) are likely to include an *X-PDGFRB* fusion gene (B).

#1 was detected repeatedly by nested RT-PCR up to 16 months after starting imatinib. For case #3, a complete cytogenetic response was seen after 7 months of treatment and a sustained complete molecular response was subsequently achieved.

Discussion

Deregulation and constitutive activation of diverse receptor tyrosine kinases (e.g. PDGFRα, PDGFRβ, FGFR1, FLT3 or KIT) and non-receptor tyrosine kinases (e.g. JAK2 or ABL) as a consequence of single point mutations (e.g. JAK2 V617F or KIT D816V) or fusion genes caused by balanced chromosomal translocations (e.g. *BCR-ABL*), insertions (e.g. *CDK5RAP2-PDGFRA*) or deletions (e.g. *FIP1L1-PDGFRA*) are fundamental to the pathogenesis of CMPD.[7,8] With the exception of the cytogenetically invisible *FIP1L1-PDGFRA* fusion gene, all tyrosine kinase fusion genes have been cloned following the finding of chromosome aberrations involving chromosome bands such as 4q12 (*PDGFRA*), 5q31-33 (*PDGFRB*), 8p11 (*FGFR1*), 9p24 (*JAK2*) or 13q12 (*FLT3*). Cytogenetic analysis is, therefore, still of the utmost importance in the diagnostic work-up of patients with *BCR-ABL* negative CMPD and acts as a pointer towards molecular analysis and the potential use of tyrosine kinase inhibitors.

A consistent feature of all known tyrosine kinase fusion genes is the 3′ location of the region encoding the entire catalytic domain of the kinase and the 5′ location of the partner gene. The partner proteins are generally

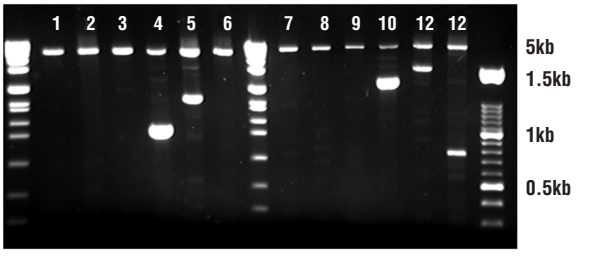

Figure 3. LDI-PCR for the detection of genomic *PDGFRB* fusions. PCR products in lanes 1-6 were amplified with forward primer F and reverse primer R2 for fusion genes involving *PDGFRB* exon 11 and reverse primer R1 for fusion genes involving *PDGFRB* exon 12. *GIT2-PDGFRB* is shown in lanes 4 and 10, *GPIAP1-PDGFRB* in lanes 5 and 11 and *PRKG2-PDGFRB* in lanes 6 and 12. For *PRKG2-PDGFRB*, an additional band is only present in lane 12 but not in lane 6 because of the atypical *PDGFRB* breakpoint within exon 12.

unrelated in sequence but have some structural and functional properties in common. The vast majority contain coiled-coil or other self-association motifs which are required for dimerization or oligomerization of the fusion protein mimicking the normal process of ligand-mediated dimerization and resulting in constitutive activation and transforming activity of the tyrosine kinase moiety in a manner that is similar to BCR-ABL in CML.[1,2,8] The partner proteins may also provide additional functions and some may alter centrosomal function.[9–12]

Currently, 15 distinct fusion partners of *PDGFRB* have been identified: *ETV6* (*TEL*, 12p13), *TRIP11* (*CEV14*, 14q32), *HIP1* (7q11), *CCDC6* (*H4*, 10q21), *RABEP1* (17p13), *PDE4DIP* (1q22), *SPECC1* (*HCMOGT-1*,

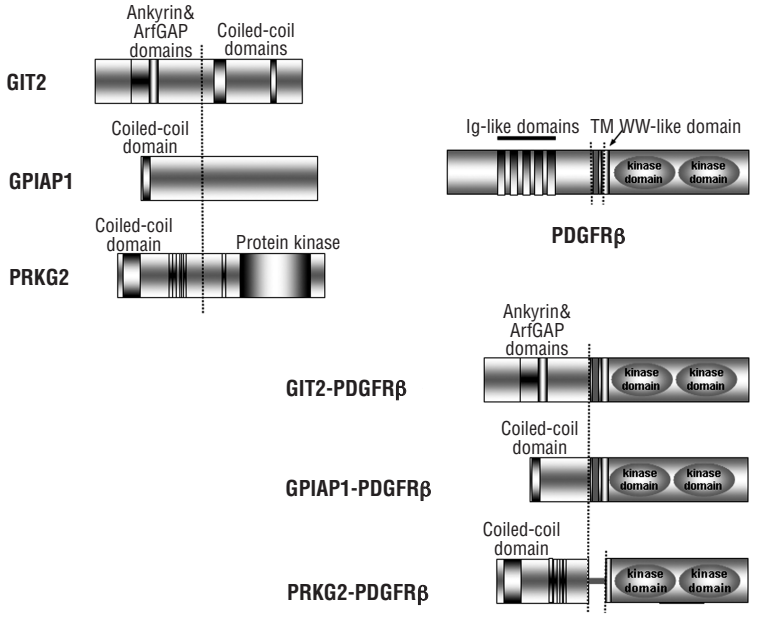

Figure 4. Diagrammatic representation of normal GIT2, GPIAP1, PRKG2 and PDGFRβ proteins and the respective fusion proteins. The transmembrane domain of PDGFRβ is retained in GIT2-PDGFRβ and GPIAP1-PDGFRβ, whereas it is deleted in PRKG2-PDGFRβ due to its atypical breakpoint within PDGFRB exon 12. The autoinhibitory WW-like domain is disrupted in the PRKG2-PDGFRβ fusion protein.

17p11), *NIN* (14q24), *KIAA1509* (14q32), *TP53BP1* (15q22), *NDE1* (16p13), *TPM3* (1q21), *GIT2* (12q24), *GPIAP1* (11p13) and *PRKG2* (4q21).[6,13-23] The newly identified fusion genes are predicted to encode fusion proteins of 742 amino acids (GIT2-PDGFRβ), 803 amino acids (GPIAP1-PDGFRβ) and 845 amino acids (PRKG2-PDGFRβ).

Coiled-coil domains (*http://www.ch.embnet.org/software/COILS_form.html*) were identified within the N-terminal moieties of GPIAP1 and PRKG2 that are retained in the fusions (Figure 4). The PDGFRβ transmembrane domain is lost in the PRKG2-PDGFRβ fusion protein due to the atypical breakpoint within *PDGFRB* exon 12. This breakpoint leads to the disruption of the WW-like domain within the juxtamembrane region, a protein-protein interaction motif that is believed to mediate important autoinhibitory functions. Disruption of WW-like domains was also observed in the *BCR-PDGFRA* and *FIP1L1-PDGFRA* fusion genes.[24,25] Importantly, the disruption of the juxtamembrane domain was recently shown to be sufficient to activate PDGFRβ and PDGFRα, while the process of oligomerization conferred by the partner protein is not mandatory.[26] No coiled-coil domains were found within the retained moieties of GIT2 and also the juxtamembrane domain was not disrupted. However, an ankyrin domain is present in the N-terminal part of GIT2-PDGFRβ which is potentially involved in protein-protein interactions.[27,28] It is, therefore, possible that the ankyrin domain supplies dimerization properties to the GIT2-PDGFRβ fusion protein. GPIAP1 (CAPRIN-1, M11S1) is a cytoplasmic phosphoprotein that plays an uncharacterized role in cellular activation or proliferation.[29,30] It exists in many isoforms that are predicted to exercise different and mostly unknown functions. Of interest, CAPRIN-1 is highly expressed in hematopoietic cells, including erythroid progenitor cells. The CAPRIN-1 isoform contains homology regions-1 and -2, at least one of which seems to be necessary for the formation of multimeric complexes.[30] Of interest, inhibition of proliferation was seen when *CAPRIN-1* was overexpressed in BaF/3 cells.[30,31]

GIT2 (ARF GTPase-activating protein, G protein-coupled receptor kinase-interactor 2, GRK-interacting protein 2, Cool-interacting tyrosine-phosphorylated protein 2, CAT2) is a ubiquitously expressed member of the GIT protein family that is extensively alternatively spliced to yield many distinct isoforms.[32] GIT2 is a GTPase-activating protein for the ADP ribosylation factor family but has also been shown to interact with G protein-coupled receptor kinases and other molecules. Functionally, the GIT2 protein is involved in the dynamic reorganization of adhesion and the cytoskeleton during cell motility.[33]

PRKG2 (cGMP-dependent protein kinase 2, CGK 2, cGKII) belongs to the group of three mammalian cGMP-dependent protein kinases that are encoded by two genes, *PKRG1* and *PKRG2*.[34] These kinases seem to be involved in neuronal adaptation, smooth muscle relaxation and growth, intestinal water secretion, bone growth, and renin secretion. So far, no link has been established between cGMP-dependent protein kinases and the development of hematologic malignancies.

The chromosomal 5q breakpoints underlying *PDGFRB* fusion genes are variable and have been assigned at 5q31-33. Due to the presence of other translocation targets in this region, the involvement of *PDGFRB* can neither be confirmed nor excluded by cytogenetic analysis alone.[35-39] Dual-color FISH analysis can confirm rearrangements of *PDGFRB*, however, the interpretation of results may sometimes be difficult and

potentially false-negative due to complex translocations. RACE-PCR has been very successful in identifying new fusion genes, but this technique may fail to detect some abnormalities as we found for case #3. We demonstrated that LDI-PCR is a complementary technique for rapid screening of underlying *PDGFRB* rearrangements in genomic DNA. Although we were able to identify rearrangements of *PDGFRB* using LDI-PCR in all three of our cases, this strategy would fail in cases in which the ring structure that contains the fusion sequence is too small, too big or of the same size as the germline band. The sensitivity could be improved by using alternative restriction enzymes similar to the procedure which has been applied in the molecular cloning of genomic junction sequences by bubble-PCR.[40-43]

Strikingly, features of the bone marrow histology in cases #2 and #3 were very similar to those in *FIP1L1-PDGFRA*-positive disease with argyrophilic fibrosis and increased numbers of atypical, $CD2^+$ and $CD25^+$ mast cells in addition to marked eosinophilia. Serum tryptase levels were elevated accordingly. As it was recently shown that mast cells carry the *FIP1L1-PDGFRA* fusion gene in *FIP1L1-PDGFRA*-positive disease,[44] it must be speculated that these eosinophlia-associated CMPD represent distinct stem cell disorders rather than a systemic mastocytosis with an associated clonal hematologic non-mast-cell disorder or systemic mastocytosis with associated eosinophilia.[45]

In summary, the combination of established and new cloning strategies led to the identification of three novel imatinib-responsive *PDGFRB* fusion genes in patients with distinct subtypes of CMPD with chromosomal abnormalities of chromosome bands 5q31-33. These findings further support the fundamental role of deregulated tyrosine kinases in the pathogenesis of CMPD and the importance of cytogenetic analysis for the screening of patients amenable to targeted therapy with tyrosine kinase inhibitors.

Authors' Contributions

CW, NCPC and AR designed the study, analyzed the data and wrote the paper. GM, CH, ASG, AF, RH and AH delivered essential support for genetic analyses. VH, OP, SR were involved in patient accrual and delivery of clinical follow-up data. We are grateful to Prof. Torsten Haferlach (MLL Münchner Leukämie Labor, München, Germany) for critical comments on our manuscript and Dr. Bernd Lathan (Gemeinschaftspraxis für Hämatologie und Onkologie, Dortmund, Germany) for providing additional clinical follow-up data.

Conflict of Interest

AH received research support from BMS and Novartis. AR and NCPC received research support from Novartis. None of the other authors has potential conflicts of interest regarding this publication.

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