Screening for diverse *PDGFRA* or *PDGFRB* fusion genes is facilitated by generic quantitative reverse transcriptase polymerase chain reaction analysis

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ABSTRACT

Background
Rapid identification of diverse fusion genes with involvement of *PDGFRA* or *PDGFRB* in eosinophilia-associated myeloproliferative neoplasms is essential for adequate clinical management but is complicated by the multitude and heterogeneity of partner genes and breakpoints.

Design and Methods
We established a generic quantitative reverse transcriptase polymerase chain reaction to detect overexpression of the 3'-regions of *PDGFRA* or *PDGFRB* as a possible indicator of an underlying fusion.

Results
At diagnosis, all patients with known fusion genes involving *PDGFRA* (n=5; 51 patients) or *PDGFRB* (n=5; 7 patients) showed significantly increased normalized expression levels compared to 191 patients with fusion gene-negative eosinophilia or healthy individuals (*PDGFRA/ABL*: 0.73 *versus* 0.0066 *versus* 0.0064, *P*<0.0001; *PDGFRB/ABL*: 196 *versus* 3.8 *versus* 5.85, *P*<0.0001). The sensitivity and specificity of the activation screening test were, respectively, 100% and 88.4% for *PDGFRA* and 100% and 94% for *PDGFRB*. Furthermore, significant overexpression of *PDGFRB* was found in a patient with an eosinophilia-associated myeloproliferative neoplasm with uninformative cytogenetics and an excellent response to imatinib. Subsequently, a new *SART3-PDGFRB* fusion gene was identified by 5´-rapid amplification of cDNA ends polymerase chain reaction (5'-RACE-PCR).

Conclusions
Quantitative reverse transcriptase polymerase chain reaction analysis is a simple and useful adjunct to standard diagnostic assays to detect clinically significant overexpression of *PDGFRA* and *PDGFRB* in eosinophilia-associated myeloproliferative neoplasms or related disorders.

Key words: myeloproliferative neoplasm, *PDGFRA*, *PDGFRB*, RQ-PCR.

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Introduction

The identification of the *BCR-ABL* fusion gene and variable point and length mutations of *JAK2* and *MPL* have highlighted the fundamental role of constitutively activated tyrosine kinases in the pathogenesis of myeloproliferative neoplasms such as chronic myeloid leukemia, polycythemia vera, essential thrombocythemia and primary myelofibrosis.[1,2] In contrast, the majority of underlying molecular aberrations in other and less frequent subtypes of myeloproliferative neoplasms, such as atypical chronic myeloid leukemia, overlap syndromes between myelodysplastic syndrome and myeloproliferative neoplasms, chronic eosinophilic leukemia, hypereosinophilic syndrome, chronic myelomonocytic leukemia and chronic neutrophilic leukemia are largely unknown.

A minority of cases present with acquired chromosomal aberrations or cytogenetically invisible deletions leading to constitutive activation of related tyrosine kinases such as *PDGFRA*, *PDGFRB*, *FGFR1*, *JAK2*, and *FLT3* through fusion to a variety of unrelated partner genes.[1,3] The new World Health Organization classification now includes patients with fusion genes and involvement of *PDGFRA*, *PDGFRB* and *FGFR1* in a separate category.[4] At present, the most common abnormalities are *FIP1L1-PDGFRA* fusions in chronic eosinophilic leukemia resulting from a cytogenetically invisible deletion on 4q12 and the *ETV6-PDGFRB* fusion in chronic myelomonocytic leukemia with a t(5;12)(p12;q31-33).[5,6] However, five other *PDGFRA* fusion partners and more than 20 *PDGFRB* fusion partners have been reported to be associated with eosinophilia-associated myeloproliferative neoplasms including chronic eosinophilic leukemia, chronic myelomonocytic leukemia, atypical chronic myeloid leukemia, and myelodysplastic/myeloproliferative neoplasms.[3] Although these abnormalities are very uncommon, they are associated with excellent responses to imatinib and thus their detection is critical for optimal management of patients.[7-12] Accurate detection is, however, complicated by several factors: (i) bone marrow cytogenetic assessment, which is critical to the detection of 4q (*PDGFRA*) or 5q (*PDGFRB*) rearrangements for all fusions apart from *FIP1L1-PDGFRA*, may fail to yield adequate metaphases; (ii) the size of the clone in peripheral blood may be very small and the abnormality thus escapes detection by cytogenetics; (iii) split apart fluorescence *in situ* hybridization (FISH) may fail to detect small clones or cases with complex rearrangements;[13,14] (iv) the heterogeneity of fusion partners and breakpoints makes it difficult and expensive to develop comprehensive and specific reverse transcriptase polymerase chain reaction (RT-PCR) assays.[15] Although some clinicians consider that a short trial of imatinib might be the best way to identify sensitive cases, this is simply not possible in many countries due to budgetary and prescribing restrictions.

We describe here the development of technically straightforward generic quantitative RT-PCR (RQ-PCR) assays that enable rapid screening of patients with eosinophilia-associated myeloproliferative neoplasms, hypereosinophilic syndrome and reactive eosinophilia for the potential constitutive activation of *PDGFRA* and *PDGFRB* by fusion genes as adjuncts to standard diagnostic tests.

Design and Methods

Patients and samples

A total of 542 peripheral blood samples from 249 patients (170 males, 79 females) and 35 healthy individuals were investigated. The study included diagnostic samples from 45 patients (44 males, 1 females; median age 54 years, range 33–75) with a *FIP1L1-PDGFRA* fusion gene (chronic phase: n=37; blast phase: n=8), six patients (4 males, 2 females; median age 51 years, range 37-71) with diverse *PDGFRA* fusion genes *(BCR-PDGFRA,*[16] n=3; *ETV6-PDGFRA,*[17] n=1; *CDK5RAP2-PDGFRA,*[18] n=1 or *KIF5B-PDGFRA,*[19] n=1) and seven patients (7 males; median age 57 years, range 21-78) with diverse *PDGFRB* fusion genes (*ETV6-PDGFRB,*[6] n=2, CCDC6-*PDGFRB,*[14,20] n=2; *GIT2-PDGFRB,*[21] n=1 *GPIAP1-PDGFRB,*[21] n=1 and *MYO18A-PDGFRB,*[22] n=1). In addition, five patients (3 males, 2 females; median age 46 years, range 36-75) with reciprocal translocations and involvement of chromosome bands 5q31-32 (Table 1) were analyzed, as were 191 diagnostic samples from patients with eosinophilia-associated myeloproliferative neoplasms, hypereosinophilic syndrome or eosinophilia of unknown origin (115 males, 76 females; median age 56 years, range 5-89), which had tested negative for *FIP1L1-PDGFRA*. Seven of these patients without known molecular aberrations had a sustained response to treatment with imatinib and were screened within this series. Informed consent was obtained from all patients according to the Declaration of Helsinki.

Cytogenetic analysis

Bone marrow cells were cultured for 24 or 48 h. Metaphases were analyzed after G-banding or R-banding and karyotypes are described according to the International System for Human Cytogenetic Nomenclature (2005).

Cell and mRNA dilutions

FIP1L1-PDGFRA positive EOL-1 cells were serially diluted in HL-60 cells (both obtained from DSMZ, Braunschweig, Germany). In addition, serial dilutions of RNA from a *FIP1L1-PDGFRA*-positive patient and a CCDC6-*PDGFRB*-positive patient were made with RNA from a healthy donor (Peripheral Leukocytes Total RNA, Clontech, Mountain View, CA, USA).

RNA extraction, cDNA synthesis and reverse transcriptase polymerase chain reaction

Following red cell lysis for the isolation of total leukocytes from peripheral blood using standard procedures, RNA was extracted using Trizol™ reagent (Invitrogen, Karlsruhe, Germany), the RNeasy extraction kit (Qiagen, Hilden, Germany) or cesium chloride gradient ultracentrifugation, as described elsewhere.[23] RNA was reverse transcribed using random hexamer priming and MMLV reverse transcriptase (Invitrogen). Single-step and nested RT-PCR for the detection of *FIP1L1-PDGFRA,*[5] *BCR-PDGFRA,*[16] *ETV6-PDGFRA,*[17] *CDK5RAP2-PDGFRA,*[18] *KIF5B-PDGFRA,*[19] *ETV6-PDGFRB,*[11] *H4-PDGFRB,*[14,20] *GIT2-PDGFRB,*[21] *GIPIAP1-PDGFRB,*[21] and *MYO18A-PDGFRB*[22] fusion genes was performed as previously described. Single-step primers for the detection of the *SART3-PDGFRB* fusion gene were SART2F: 5'-CTGATTATGTGGAGATTTGGCA-3', and PDGFR-C 5'-TGGCTTCTTCTGCCAAAGCA-3'. The reciprocal fusion transcript was amplified with PDB-9F 5'-AGACCTCAAAAGGT-GTCCACG-3' and SART19R 5'-TAGAGACAGCTGCGTC-CTTC-3'. *ETV6-ACSL6* and *NPM1-MLF1* fusion transcripts were amplified as previously described.[24,25] Amplification reactions were undertaken for 32 cycles with an annealing temperature of 60°C.

Real time quantitative reverse transcriptase polymerase chain reaction

The expression of *PDGFRA* and *PDGFRB* was analyzed using the LightCycler instrument 1.5 (Roche Diagnostics, Mannheim, Germany). Each 20 μL reaction mix for *PDGFRA* RQ-PCR contained 4 μL LightCycler Faststart DNA Master^plus Hyb Probes Master Mix (Roche Diagnostics), 2 μL cDNA template or plasmid dilution, 0.5 μM forward primer PDA12F: 5'-CCAAGAGATGGACTAGTGCTTG-3', 0.5 μM reverse primer PDA15R: 5'-TAGCTCCGTGTGCTTTCATCAG-3', 0.25 μM anchor probe PDA15FL: GAATAGGGATAGCTTCCTGAGCCACCA-fluorescein, 0.25 μM sensor probe PDA15LC: LCred640-CCAGAGAAGCCAAAGAAAGAGCTGGA-P, (TIB Molbiol, Berlin, Germany). The *PDGFRB* RQ-PCR reaction mix contained 4 μL LightCycler Faststart DNA Master^plus Hyb Probes Master Mix, 2 μL cDNA template or plasmid dilution, 0.5 μM forward primer PDB13F: 5'-CGTCAAGATGCTTAAATCCACAGC-3', 0.5 μM reverse primer PDB15R: 5'-TGATGATATAGATGGGTCCTCCTTTG, 0.25 μM anchor probe PDB14FL: 5'-GCTGAAGATCATGAGTCACCTTGGGC-fluorescein, 0.25 μM sensor probe PDB14LC: LCRed640-CCACCTGAACGTGGTCAACCTGTTG-P. Cycler conditions were the following: 10 min denaturation at 95°C, 50 cycles of 10 sec at 59°C/60°C (annealing *PDGFRA/PDGFRB*) and 26 sec at 72°C (elongation). A 5 log series of plasmid dilutions (see below) was amplified within the PCR runs for quantification of *PDGFRA* and *PDGFRB*. *ABL* mRNA was quantified as an internal control as previously described.[26] The LightCycler software prepares standard curves using linear regression analysis of the plasmid dilutions and calculates copy numbers of the unknown sample.[27] Values below the lowest standard dilution for *PDGFRA* (4 copies) and for *PDGFRB* (400 copies) were assigned as negative.

Cloning of quantification standards

For plasmid preparation, nested RT-PCR products from sequences of *PDGFRA* and *PDGFRB* were amplified from cell lines (HL-60, *PDGFRA*; SW480, *PDGFRB*) with the following primers (PDGFRA - PDA11F1: 5'-TGGCTGCTGCAGTCCTGGTGCT-3', PDA16R1: 5'-CTGTGTAGTATCAGCCTGCTTC-3', PDA11F2: 5'-AGTCCTGGTGCTGTTGGTGATTGTGA-3', PDA16R2: 5'-AGTATCAGCCTGCTTCATGTCCATGT-3'; PDGFRB - PDB1F: 5'-TGTCAGAGCTGACACTGGTTCG-3', PDB1R: 5'-CCATGTAGTTGGAGGACTCGATG-3', PDB2F: 5'-GCTGACACTGGTTCGCGTGAA-3', PDB2R: 5'-GTTGGAGGACTCGATGTCTGCAT-3'). The Expand high fidelity plus PCR system (Roche Diagnostics) was used. PCR transcripts were cloned into the PCR2.1-TOPO vector and introduced into *E. coli* TOP10F' according to the manufacturer's instructions (Invitrogen). Plasmid DNA containing the desired construct was isolated using the Plasmid Midi and Maxi Kit (Qiagen) and inserts were confirmed by bidirectional direct sequencing. The resulting plasmid was linearized by *XbaI* digestion at 37°C for 2 h followed by heat inactivation at 65°C for 20 min. *ABL* mRNA transcripts were measured as an internal control using a standard plasmid (pME-2) containing *BCR-ABL*, *ABL*, and *GUS* sequences.[28] Dilutions of the linearized plasmid were prepared in 10 mM Tris-HCl pH 8.0; 1 mM EDTA containing 20 μg/mL tRNA (Roche Diagnostics).

5'- rapid amplification of cDNA ends polymerase chain reaction and bubble polymerase chain reaction

Screening for potential *PDGFRA* and *PDGFRB* fusion genes was performed by 5´-rapid amplification of cDNA ends polymerase chain reaction (5´-RACE-PCR) according to the manufacturer's instructions (5´/3´RACE Kit, Roche Diagnostics) as recently described.[21] Bubble-PCR for *PDGFRA* fusion genes was also performed as recently described.[19]

Statistical analysis

Comparisons between two groups of variables were performed using the non-parametric Mann-Whitney U test. Correlations between continuous variables were calculated using the Spearman's rank test. *P* values below 0.05 were considered statistically significant. The diagnostic sensitivity and specificity of the RQ-PCR assay were calculated (GraphPad prism, Version 5.0 software, San Diego, CA, USA).

Results

The generic RQ-PCR assays target the 3´-sequences of *PDGFRA* and *PDGFRB* which are retained in all known fusion genes and which may, therefore, be overexpressed, as described previously.[19]

Precision analysis of quantitative reverse transcriptase polymerase chain reaction assays

Intra- and inter-assay coefficients of variation (CV) were calculated to test the reproducibility of the assays. Two *FIP1L1-PDGFRA* cDNA samples with high and low level PDGFRA expression were tested ten times within one assay (intra-assay variability, "high level" CV 7%, "low level" CV 10%). In addition, the samples were tested ten times in different assays (inter-assay variability, "high level" CV 25%, "low level" CV 44%). Identical testing was undertaken with a *CCDC6-PDGFRB* sample (intra-assay variability, CV 6%, inter-assay variability CV 29%).

Table 1. Patients' characteristics.

Cytogenetic analysis	Fusion gene	Disorder	N.	Age
46,XY (n=36)	*FIP1L1-PDGFRA*	CEL	37	33-74
46,XX (n=1)				
46,XY	*FIP1L1-PDGFRA*	sAML/blast phase	8	40-68
46,XX,t(4;22)(q12;q11)	*BCR-PDGFRA*	CEL	2	37/47
46,XX,ins(9;4)(q34;q21q31)	*CDK5RAP2-PDGFRA*	CEL	1	71
46,XY,t(4;12)(q2?3;p1?2)	*ETV6-PDGFRA*	CEL	1	51
46,XY,del(3)(p21),add(4)(q12), -10,13q?+der(?) (?-cen-?::4q12_ 4q12 _ 4q28.3::10q11.2 _ 10qter)	*KIF5B-PDGFRA*	CEL	1	54
46,XY,t(5;10)(q32;q21)	*CCDC6-PDGFRB*	CEL	2	53/73
46,XY,t(5;12)(q31;p13)	*ETV6-PDGFRB*	CEL	2	21/59
46,XY,der(1)t(1;5)(p34;q33), der(5)(1;5) (p34;q15), der(11)ins(11;5)(p12;q15q33)	*GPIAP1-PDGFRB*	CEL	1	61
46,XY,t(5;12)(q31;q24)	*GIT2-PDGFRB*	CEL	1	78
46,XY,t(5;17)(q33-35;q11.2)	*MYO18A-PDGFRB*	CEL	1	51
46,XY [4]	*SART3-PDGFRB*	CEL	1	46
46,XY,t(5;12)(q31;p13)	*ETV6-ACSL6*	MDS/MPN	1	42
46,XX,t(3;5)(q24;q33)	*NPM1-MLF1*	MPN/blast phase	3	35-36
46,XY,t(2;5)(p23;q31)	?	MDS/sAML	1	75
46,XY	–	HES	125	10-89
46,XX	–	HES	80	22-89

CEL: chronic eosinophilic leukemia, HES: hypereosinophilic syndrome; sAML: secondary acute myeloid leukemia, MDS: myelodysplastic syndrome; MPN: myeloproliferative neoplasm.

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Detection level of quantitative reverse transcriptase polymerase chain reaction assays

The RQ-PCR assays were linear over six orders of magnitude as assessed by analysis of plasmid dilutions. Cell and RNA dilutions were processed and analyzed for the calculation of the maximum detection level over the background. In healthy individuals, *PDGFRA* is only expressed at very low levels (median *PDGFRA/ABL* 0.00068; range, 0-0.0027) while *PDGFRB* is expressed at significantly higher levels (median *PDGFRB/ABL* 5.85; range, 0.97-22.2). A cut-off point for overexpression of *PDGFRA* and *PDGFRB* (mean+3SD) was determined by analysis of a series of 35 healthy volunteers and set at 0.0030 for *PDGFRA/ABL* and 23 for *PDGFRB/ABL*. For *FIP1L1-PDGFRA,* the levels detectable over the background were between 10^{-3} and 10^{-4} for cell line dilutions and between 10^{-2} and 10^{-3} for RNA dilutions. The detection limit of *FIP1L1-PDGFRA* and *CCDC6-PDGFRB* fusion transcripts by nested RT-PCR was between 10^{-4} and 10^{-5}.

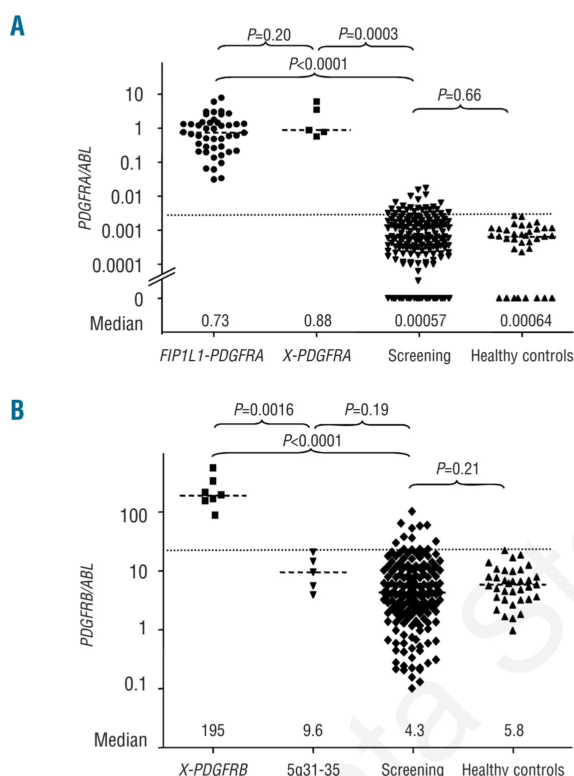


Figure 1. (A) Patients with *PDGFRA* fusion genes showed significantly increased *PDGFRA* expression levels (*PDGFRA/ABL*) as compared to patients with non-reactive eosinophilia without known molecular aberrations (n=191) and healthy controls (n=35). The expression level was not different between patients with *FIP1L1-PDGFRA* (n=45) and alternative *PDGFRA* fusion genes (*X-PDGFRA*: *BCR-PDGFRA*, *CDK5RAP2-PDGFRA*, *ETV6-PDGFRA*, *KIF5B-PDGFRA*). The cut-off point for overexpression of *PDGFRA* was determined for *PDGFRA* at a ratio of 0.030 *PDGFRA/ABL* (35 healthy controls, mean + 3 SD, dotted line). *PDGFRA* expression levels were not different between patients with non-reactive eosinophilia and healthy controls. (B) Patients with different *PDGFRB* fusion genes (*X-PDGFRB* [n=7]: *ETV6-PDGFRB*, *CCDC6-PDGFRB*, *GIT2-PDGFRB*, *GPIAP1-PDGFRB* and *MYO18A-PDGFRB* showed significantly increased *PDGFRB* expression levels compared to patients with non-reactive eosinophilia without known molecular aberrations and healthy controls. In the screening group, 13 patients showed significant overexpression of *PDGFRB*. In one of these patients, with uninformative cytogenetics and an excellent response to imatinib a new *SART3-PDGFRB* fusion gene was identified by 5'-RACE-PCR. No increased *PDGFRB* expression was found in five patients with chromosomal aberrations and involvement of chromosome bands 5q31-32. In four of these cases, alternative fusion genes with involvement of *ETV6*, *NPM1*, *MLF1* and *ACSL6* could be confirmed by RT-PCR. The cut-off point for overexpression was determined at a ratio of 23 *PDGFRB/ABL* (35 healthy controls, mean+ 3 SD, dotted line). *PDGFRB* expression levels were not different between patients with non-reactive eosinophilia and healthy controls.

Diagnosis of PDGFR *overexpression by quantitative reverse transcriptase polymerase chain reaction*

The RQ-PCR assay for overexpression of *PDGFRA* was validated in 15 *FIP1L1-PDGFRA* positive samples by comparison with specific RQ-PCR for *FIP1L1-PDGFRA* (r=-0.66, *P*=0.0004).[10] Overall, patients with *PDGFRA* fusion genes (n=51) showed significantly increased expression of *PDGFRA/ABL* at diagnosis without the levels overlapping with those of normal controls (n=35). The median *PDGFRA/ABL* ratio was 0.73 (range, 0.31-7.77) in patients compared to 0.00068 in healthy controls (*P*<0.0001). The level of expression of *PDGFRA* was not different between patients with a *FIP1L1-PDGFRA* fusion gene and those with other *PDGFRA* fusion genes (*PDGFRA/ABL* 0.73 *versus* 0.88, *P*=0.26, Figure 1). At diagnosis and during the first 12 months on imatinib, the normalized *PDGFRA* ratio was not statistically different in patients in chronic phase (n=37) compared to patients in blast phase or with secondary acute myeloid leukemia (n=8) (Figure 2).

The median *PDGFRB/ABL* ratio in seven patients with *PDGFRB* fusion genes was 195 (range, 68-581) compared to 5.85 in healthy controls (*P*<0.0001). The *PDGFRB/ABL* ratios in five patients with breakpoints at chromosome band 5q31-35 but without a rearrangement of *PDGFRB* were not different from those in normal controls (median 5.8, range 0.97-22.2 *versus* median 9.6, range 0.91-22.1, respectively; *P*=0.19). Specific RT-PCR revealed *NPM1-MLF1* fusion genes (n=3) and an *ETV6-ACSL6* fusion (n=1). The underlying fusion gene in the remaining patient could not be identified.

The ratios of *PDGFRA/ABL* and *PDGFRB/ABL* in patients with hypereosinophilic syndrome/chronic eosinophilic leukemia (n=191) without known molecular aberrations were comparable to those of healthy controls in the vast majority of cases. Twenty-five patients (13%) showed a significantly elevated *PDGFRA/ABL* (median 0.043, 0.0030-0.017 *versus* 0.00068, *P*<0.0001) and 13 (6.8%) showed elevated *PDGFRB/ABL* (median 36.3, 23-102 *versus* 5.8, *P*<0.0001) over the cut-off level determined in healthy controls. None of the patients showed simultaneous overexpression of *PDGFRA* and *PDGFRB*. For *PDGFRA,* none of the patients with elevated expression levels showed levels comparable to cases with *PDGFRA* fusions. For *PDGFRB*, three of the patients showed expression levels comparable to those of patients with *PDGFRB* fusions. In one of these, a novel fusion transcript was identified. Thus, the diagnostic sensitivity and specificity of the described screening test were, respectively, 100% and 88.4% for *PDGFRA* and 100% and 94% for *PDGFRB*.

A SART3-PDGFRB *fusion gene*

Selected cases (based on availability of suitable samples) were analyzed in more detail to determine whether the observed overexpression was a consequence of previously unrecognized *PDGFRA* or *PDGFRB* fusions. Because the genomic breakpoint region within *PDGFRA* is known to be highly restricted to *PDGFRA* exon 12, bubble-PCR with

DNA and reverse primers located immediately downstream of the breakpoint cluster region[19] was employed in five patients with significant overexpression of *PDGFRA*, but no new *PDGFRA* fusions were identified. Genomic breakpoint regions within *PDGFRB* are more heterogeneous and, therefore, 5'-RACE-PCR was used with cDNA samples derived from eight patients with significant overexpression of *PDGFRB*, including two patients who achieved complete clinical and hematologic remission on imatinib. A male patient with an eosinophilia-associated myeloproliferative neoplasm and uninformative cytogenetic analysis due to myelofibrosis (normal karyotype in 4 of 4 metaphases) but rapid achievement of complete remission following treatment with 100 mg imatinib revealed the fusion of a novel sequence to *PDGFRB* exon 12. Sequencing of the RACE-PCR products revealed an in-frame fusion between *SART3* exon 15 (*squamous cell carcinoma antigen recognized by T-cells 3*, Gene Bank accession number: AB020880) and *PDGFRB* exon 12 (Figure 3). A reciprocal fusion gene could be amplified by RT-PCR and revealed a fusion between *PDGFRB* exon 11 and *SART3* exon 16. No cytogenetic or molecular aberration could be identified in the second patient with response to imatinib and *PDGFRB* overexpression.

Discussion

For patients with non-reactive eosinophilia, a major diagnostic and therapeutic breakthrough was achieved by the identification of the *FIP1L1-PDGFRA* fusion gene. Virtually all patients with this fusion gene achieve rapid and sustained complete clinical and hematologic remissions, and the majority even obtain complete molecular remissions, at low toxicity. However, this fusion is only seen in approximately 5-15% of cases with non-reactive eosinophilia and, furthermore, may be difficult to detect in some diagnostic cases.[7,9,13] In contrast, the clinical phenotype of *FIP1L1-PDGFRA*-negative cases is frequently indistinguishable from that in patients in whom the fusion is present, suggesting the presence of alternative fusion genes or as yet unknown molecular mechanisms leading to constitutive tyrosine kinase activation.

In *FIP1L1-PDGFRA* negative cases, cytogenetic analysis

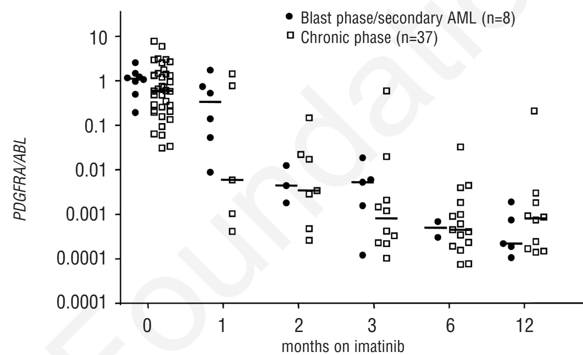
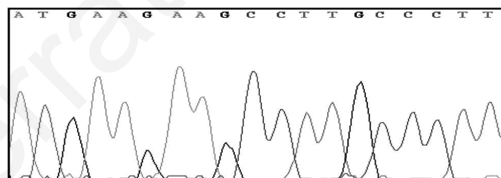


Figure 2. At diagnosis and during the first 12 months on imatinib the normalized *PDGFRA* ratio was not statistically different in patients in chronic phase (n=37) compared to patients in blast phase or with secondary AML (n=8).

SART3-PDGFRB mRNA

SART3 Exon 15 *PDGFRB* Exon 11

GATGATGAGAAAGAGTGGGGCGATGATGAAGAAGCCTTGCCCTTTAAGGTGGTGGTGATCTCAGCCATCCTGGCC
-D--D--E--K--E--W--G--D--D--E--E--A--L--P--F--K--V--V--V--I--S--A--I--L--A-
S



A

B

Figure 3. (A) Junction sequence and corresponding amino acids of the SART3-PDGFRB fusion protein. *SART3-PDGFRB* splice variants contain intron-derived sequences which are spliced in-frame between *SART3* exon 14 and *PDGFRB* exon 10. (B) Structure of SART3, PDGFRB and the predicted fusion protein. TM: transmembrane domain, WW: WW-like domain, TK: tyrosine kinase domain.

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from bone marrow cells is recommended since all other known *PDGFRA* or *PDGFRB* fusions are associated with abnormalities involving chromosome bands 4q12 (*PDGFRA*) and 5q31-33 (*PDGFRB*). Overall, 28 fusion genes are currently known with involvement of *PDGFRA* or *PDGFRB*.[3] Response rates are similar to those seen in *FIP1L1-PDGFRA*-positive chronic eosinophilic leukemia and importantly, primary or secondary resistance is very rare. In contrast, fusion genes with involvement of *FGFR1* and *JAK2* are imatinib-resistant and associated with an aggressive clinical course. Transformation to blast phase/secondary acute leukemia, usually of myeloid phenotype, regularly occurs within 1 or 2 years of diagnosis. For these patients, allogeneic stem cell transplantation remains the only potentially curative treatment option as long as selective inhibitors of FGFR1 and JAK2 are not widely available.

Detection of variant *PDGFR* fusions remains a significant diagnostic challenge. We sought to address this by designing and validating generic quantitative RT-PCR assays which allow rapid screening of fresh or stored peripheral blood or bone marrow material for the possible presence of fusion genes involving *PDGFRA* or *PDGFRB*. All positive controls were correctly identified and significant overexpression of *PDGFRA* or *PDGFRB* was found in 13% and 7%, respectively, of cases with non-reactive eosinophilia lacking *FIP1L1-PDGFRA* or cytogenetic indicators of other *PDGFR* fusions. Among the over-expressors, a single case was found to harbor a *PDGFR* fusion and thus the sensitivity and specificity of the screening test were, respectively, 100% and 88.4% for *PDGFRA* and 100% and 94% for *PDGFRB*.

This screening strategy may be particularly useful as a means to select candidates for exploratory imatinib treatment. Seven patients with hypereosinophilic syndrome without known molecular aberrations and sustained response to treatment with imatinib were screened within this series. None of them showed significant overexpression of *PDGFRA* while two patients showed overexpression of *PDGFRB* comparable to *PDGFRB* fusions. 5'-RACE-PCR was performed in both patients and identified a new *SART3-PDGFRB* fusion gene corresponding to a t(5;12)(q31-32;q23-24) in one of these cases. This reciprocal translocation was not picked up by routine cytogenetic analysis; however, it had only been possible to investigate four metaphases because of myelofibrosis. The lack of overexpression of *PDGFRA* or *PDGFRB* in five imatinib-responsive patients highlights the fact that assays may miss some patients with potential response due to yet unknown targets or off-target activity of imatinib. We screened these patients for abnormalities of *PDGFRA*, *PDGFRB*, *KIT*, *FMS* and also by array comparative genome hybridization but did not find anything.

SART3 (*squamous cell carcinoma antigen recognized by T-cells 3*, synonym: *KIAA0156*, *RP11-13G14*, *p110*) maps to chromosome 12q23-24. It encodes for a RNA-binding nuclear protein that was initially identified from cDNA clones of the myeloid cell line KG-1.[29] The protein is present in almost all tissues analyzed but seems to be highly expressed in tumor tissue and cancer cells.[30] This antigen possesses tumor epitopes capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes in cancer patients suggesting that these proteins could be useful for specific immunotherapy, as shown for colorectal, bladder and breast cancer.[31-33] SART3 associates transiently with U6 and U4/U6 small nuclear ribonucleoproteins during the recycling phase of the spliceosome cycle and is involved in regulation of mRNA splicing.[34,35] The genomic *PDGFRB* breakpoint is located within intron 10, and thus the transmembrane and juxtamembrane domains of PDGFRB are retained in the SART3-PDGFRB fusion protein. Coiled-coil domains are present in the N-terminal region of SART3 suggesting that dimerization of the fusion protein causes constitutive activation of the PDGFRB kinase domain.[3] The majority of known *PDGFRB* fusion genes have genomic breakpoints leading to disruption of the autoinhibitory WW-like domain within the juxtamembrane region that may enhance transformation properties of the chimeric fusion protein.[36,37]

Our data indicate that expression analysis of *PDGFRA* or *PDGFRB* is helpful in cases of eosinophilia-associated myeloproliferative neoplasms which are negative for *FIP1L1-PDGFRA* and which have a normal, insufficient or missing karyotype. It should be emphasized that screening for overexpression of *PDGFRA* and *PDGFRB* may also be useful for patients with eosinophilia-associated primary and secondary acute leukemias which are negative for core-binding factor fusions genes. We have recently reported rapid and sustained complete hematologic and complete molecular remissions in patients with *FIP1L1-PDGFRA*-positive blast phase disease on imatinib as monotherapy or as maintenance after intensive chemotherapy.[9] Of interest, the decline of expression levels was no different between patients in chronic phase or with blast phase/secondary acute leukemia. In addition, this assay is useful in cases presenting with reciprocal translocations and involvement of 5q31-33. *PDGFRB* is clearly not involved in all these cases[38,39] and FISH analysis alone might occasionally miss a rearrangement of *PDGFRB*.[14]

The identification of more than 40 different fusion genes as the consequence of diverse chromosomal abnormalities in eosinophilia-associated myeloproliferative neoplasms has highlighted the fundamental role of constitutively activated tyrosine kinases in the pathogenesis of these disorders. We have shown here that the universal quantification of regions that are retained in all known *PDGFRA* and *PDGFRB* fusion genes is a sensitive assay for the screening of potential fusion genes and serves as a useful adjunct to standard diagnostic procedures, particularly for laboratories that are familiar with routine RQ-PCR analysis. Expanding these techniques to other tyrosine kinases or translocation partners might help to define the molecular pathogenesis of the vast majority of eosinophilia-associated myeloproliferative neoplasms for whom the causative lesion remains unknown.

Authorship and Disclosures

AR was the principal investigator, takes primary responsibility for the paper and co-ordinated the research. AR, FV, GM recruited the patients. PE, MM, AH and NCBC developed the PCR methodology. PE, DG, MM, JR, CW, JM, JS, TE, and CH performed the laboratory work for this study. PE and AR wrote the paper. AH and NCPC revised the manuscript.

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