Molecular Analysis of *PDGFRA* and *PDGFRB* Genes by Rapid Single-strand Conformation Polymorphism (SSCP) in Patients with Core-binding Factor Leukaemias without *KIT* or *FLT3* Mutation

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Abstract. *Background: Mutations involving KIT and FLT3 genes, encoding tyrosine kinase (TK) membrane receptors, are detected in core-binding factor leukaemia (CBFL) patients. PDFGRA and PDGFRB encode class III TK receptors and are involved both in physiological processes and in the pathogenesis of haematological and solid tumours. The aim of this study was to investigate if PDGFR mutations are involved in CBFL. Patients and Methods: In order to detect PDGFR mutations in CBFL, 35 patients without KIT or FLT3 mutations patients were screened by rapid and sensitive single-strand conformation polymorphism (SSCP) analysis. Sequence analysis was performed in polymerase chain reaction (PCR) products showing altered mobility in SSCP analysis in order to determine the nucleotide changes. Results: Three types of single-nucleotide polymorphism (SNP) were detected in the PDGFRA gene (exon 12, exon 13 and exon 18) while no mutation of PDGFRB was detected in the tested CBFLs. Conclusion: These data showed that no pathogenic mutations in PDGFRA and PDGFRB were detected in the context of CBFL without KIT and FLT3 mutations. Thus, PDGFR genes do not seem to be involved in CBFL and future studies are needed to establish the genetic causes of the disease in these particular patients.*

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Core-binding factor leukaemias (CBFLs) resulting from anomalies of the CBF a and b subunits represent two of the most prevalent types of acute myeloid leukaemia (AML) with recurrent cytogenetic abnormalities (1). Translocation t(8;21)(q22;q22) and inv(16)(p13q22) occur in 7 to 8% and 4-5% of adult cases, respectively (2, 3). According to the French-American-British (FAB) classification, AML associated with t(8;21) typically shows M2 morphology, with a minority of cases showing M1 or M4 morphology, and has secondary cytogenetic changes, including the loss of a sex chromosome (LOS) or the loss of part or even all of 9q (4-7). AML M2 FAB exhibits a granulocytic maturation along the neutrophil pathway and rarely exhibits eosinophilia and mastocytosis (8).

AML associated with inv(16) more often has FAB M4Eo morphology and is less likely to have secondary cytogenetic changes. AML M4Eo has a specific abnormal eosinophil component as the bone marrow shows abnormalities in that

Table I. *Primers used for amplification of PDGFRA and PDGFRB by PCR.*

Gene	Fragment (Exon)	Size[a] (bp)	Forward	Reverse
PDGFRA	9	296	5'-agttgtgaactcatattcca-3'	5'-atcatttgtgtcaagggag3'
	11	254	5'-gcatgtctgccaggaaactt-3'	5'-agctccttctctgtgccaag3'
	12	373	5'-tggagtgaacgttgttgg-3'	5'-agttcttactaagcacaagc3'
	13	357	5'-gacacgatgacttggaggag3'	5'-agctgcatgattttgagaaa3'
	14	270	5'-tctgagaacaggaagttggtagc3'	5'-tggaggatttaagcctgattg3'
	15	315	5'-gcaggacaattcatggcttt3'	5'-caggacatgggtctttccat3'
	17	243	5'-catgcctctgcaacctgat3'	5'-cgtccacactccactcactg3'
	18	232	5'-tacagatggcttgatcctgagt3'	5'-agtgtgggaggatgagcctg3'
	19	298	5'-tgctgtggatcatcagtgag3'	5'-cacaccaggttatcttaaca3'
	20	270	5'-catgccaagtgtttcagcaa3'	5'-cacaggggaagtctcagg3'
PDGFRB	12	282	5'-cctagacggacgaacctaa3'	5'-ggaccagacctcagagagt3'
	18	332	5'-tcctccaagagcacacca3'	5'-agccacactggtcaggag3'

[a]Size represents the length of the amplified fragment.

compartment (4).

Clinically, both with t(8;21)(q22;q22) and inv(16)(p13q22), the disease is usually associated with a good response to chemotherapy, showing a high remission rate and long-term disease-free survival (9-13). Because CBFLs have relatively favourable prognoses, they are often treated similarly (14-25). Recent advances in molecular biology suggest that leukaemogenesis in AML is the result of two genetic events: mutations of class I which lead to reduced apoptosis and/or increased proliferative advantage in leukaemic cells such as in *KIT, FLT3, RAS* and *c-FMS*, and mutations of class II which involve haematopoietic differentiation (*e.g.* CBF fusion genes) (26, 27).

CBFLs are considered as good examples for such two-event mechanisms. Activating mutations of *FLT3*, described in AML, are both internal tandem duplications (ITDs) and point mutations such as Asp835 and Ala680Val (28-30). *KIT* Asp816 activating loop mutations have been reported in patients with CBFL, while an association between *KIT* exon 8 mutations and inv(16) AML has been documented (31, 33).

Receptor tyrosine kinases (RTKs) are a family of proteins with more than 518 putative protein kinase genes that play a fundamental role in signal transduction (34). Platelet-derived growth factor receptor (*PDFGR*) A and *PDGFRB* encode class III TK receptors and are involved both in physiological processes, such as fibrosis, and in the pathogenesis of haematological and solid tumours. Mutations in *PDGFRA* are found in gastrointestinal stromal tumours (GIST), rarely in synovial sarcomas (SSs) and in malignant peripheral nerve sheath tumours (MPNST), whereas the *FIP1L1-PDGFRA* fusion product occurs in systemic mastocytosis associated with eosinophilia, in idiopathic hypereosinophilic syndrome, in chronic eosinophilic leukaemia and in polycythemia vera patients (35-38). Many different *PDGFRB* chimeras are described in *BCR-ABL*-negative chronic myeloproliferative disorders (39). In general, point mutations detected in KIT and *FLT3* are mutually exclusive (40).

In view of these findings, we screened a significant number (n=35) of patients with CBFL, who had previously tested negative for *KIT* and *FLT3* mutations, for *PDGFRA* and *PDGFRB* mutations with a quick and reliable modified single-strand conformational polymorphism (SSCP) method.

Patients and Methods

Patient selection. Bone marrow samples of 21 AML patients with t(8;21) and 14 patients with inv(16) from six Italian centers (Ferrara, Milan, Naples, Pavia, Verona and Vicenza) were collected and cryopreserved at diagnosis. All patients underwent mutational screening for *KIT* and *FLT3* previously, and no mutations were detected.

Primary leukaemic cells and DNA isolation. Mononuclear bone marrow leukaemic cells were collected after informed consent was given by the patients and were isolated by standard Ficoll-Hypaque (Lymphoprep™, Axis Shield PoC AS, Norway) density gradient centrifugation. Genomic DNA was extracted using standard procedures (Roche Diagnostics, Germany).

Polymerase chain reaction (PCR). Primers for DNA amplification were designed according to human *PDGFRA* and *PDGFRB* gene sequences (GeneBank accession number NM_006206 and NM_002609). The sequences of the primers used for PCR are reported in Table I.

For the analysis of the juxtamembrane and TK domains of *PDGFRA*, exons 9, 11-15 and 17-20 were amplified. For analysis of the TK domain of *PDGFRB*, amplifications of exon 12 and 18 were performed. PCR conditions were as follows: initial denaturation at 95˚C for 10 min, 35 cycles of 95˚C for 40 s, annealing temperature ranging between 48˚C and 59˚C for 40 s and 72˚C for 40 s followed by elongation at 72˚C for 7 min (Mastercycler, Eppendorf, USA).

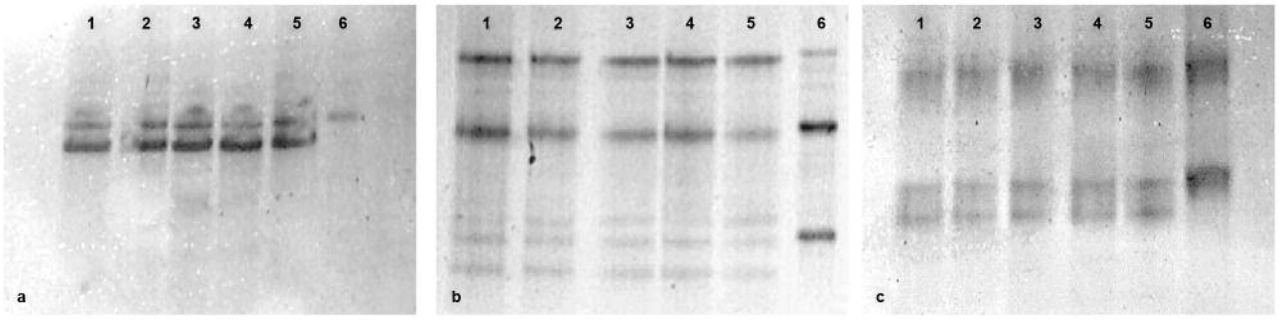

Figure 1. *(a) Silver-stained SSCP gel of exon 12 of the PDGFRA gene. Lanes 1-5 show a polymorphism in heterozygous form as they display bands with abnormal mobility compared to a reference sample with a wild-type genotype (lane 6). (b) SSCP analysis of exon 13 of PDGFRA reveals an heterozygous pattern of polymorphism in lanes 1-5; lane 6 shows wild-type DNA; (c) SSCP analysis of exon 18 of PDGFRA reveals a homozygous pattern in lanes 1-5 compared to the wild-type genotype (lane 6).*

Negative controls for each PCR were routinely coamplified.

SSCP analysis. Mutation analysis was carried out by a sensitive and rapid SSCP. Five μl of PCR product were mixed with 5 μl of formamide denaturing dye mixture (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), heated at 95˚C for 3 min and then placed on ice. Eight μl of the mixture were loaded into each well of 36-well nondenaturing polyacrylamide gradient gels (5-20%) in 0.5X Tris-borate-EDTA buffer (TBE; pH 8.4) containing glycerol (5%). Each gel was electrophoresed (Multiphor II; Pharmacia Biotech, Amersham, UK) at 350 V for 18-20 h (overnight). The gels were run at two different temperatures (12˚C and 23˚C) by thermostatic circulation. Following adequate running times, gels were stained using the PlusOne DNA silver stain kit (Pharmacia Biotech) on a Hoefer automated gel stainer (Pharmacia Biotech). Samples that showed an abnormal SSCP pattern underwent sequencing studies with forward and reverse primers.

Sequence analysis. Direct DNA sequencing was performed with an ABI310 automated sequencer using the Big Dye™ Terminator Cycle Sequencing kit (Applied Biosystems, UK). Numbering of nucleotides is according to the full length *PDGFRA* cDNA (GeneBank accession number NM_006206).

Results

SSCP analysis. We investigated 35 *KIT* and *FLT3* mutation-negative CBFL patients. In *PDGFRA*, altered SSCP patterns were seen in twelve patients in the following three exons: the first was in exon 12 in five out of 35 patients, the second was in exon 13 in 5 out of 35 patients, and the last was in exon 18 in 5 out of 35 patients (Figure 1). Moreover, three patients showed polymorphisms both in exon 13 and in exon 18 as shown in Table II. In exons 12 and 18 of the *PDGFRB* gene, no abnormal SSCP patterns were observed.

Sequencing analysis. The PCR fragments with alterations in the SSCP analysis were sequenced in order to determine the nucleotide change responsible for their mobility shift.

Sequence analysis of the abnormal migrating bands in *PDGFRA* showed polymorphisms: a CCA>CCG transition at codon 567 (Pro) in exon 12, a GCG>GCA transition at codon 603 (Ala) in exon 13, and a GTC>GTT transition at codon 824 (Val) in exon 18. These three variants were previously described as SNPs with reference SNP ID, rs1873778, rs10028020, and rs2228230, respectively. While the single-nucleotide variations in exon 13 and exon 18 were always present in heterozygous form, the SNP in exon 12 was present in homozygous form in 5/35 cases. Exon 13 and exon 18 polymorphisms were present together in 3 patients. No mutation of *PDGFRB* was detected in the tested CBFLs (Table II).

Discussion

The class III RTKs, which include FMS, KIT, FLT3, PDGFRA and PDGFRB, play an important role in normal hematopoiesis (41-44). The chromosomal location and genomic structure of the class III RTKs suggests a close evolutionary relationship. The *KIT* and *PDGFRA* genes, for example, are both located on chromosome 4q11-q13 and have structural similarities with the other *PDGFR* family members (45, 46). KIT, PDGFRA and PDGFRB are transmembrane glycoproteins that belong to the PDGFR subfamily of tyrosine kinases by virtue of their shared amino acid sequence homology in juxtamembrane and intracellular kinase domains.

Intriguing associations between RTK and CBFL have been documented. A substantial proportion of patients with CBFL carry mutations in the *KIT* gene such as Asp816Tyr in patients with t(8;21) or a loss of Asp419 in patients with AML-M4Eo and inv(16) (47, 48). *FLT3* ITD mutations and activating mutations, such as Asp835, are largely documented in CBFL (40). The aim of our study was the

Table II. *Polymorphisms of PDGFR genes in CBFL.*

Patient	Gender	Age (years)	Cytogenetic analysis	Polymorphism (SNP ID)	
				PDGFRA	*PDGFRB*
LA 09	M	53	46, XY, t(8;21)(q22;q22)/45, (IDEM)X-Y/46, XY	rs1873778	ND
LA 11	F	42	46, XX, inv(16)(p13;q22)	rs1873778	ND
LA 12	F	56	46, XX, inv(16)(p13;q22)	ND	ND
LA 16	F	28	45, X, -X, t(8;21)(q22;q22), add(4)(p16), -9, + mar/ 46, XX	ND	ND
LA 25	F	60	46, XX, inv(16)(p13;q22)	rs10028020	ND
LA 32	F	37	46, XX, inv(16)(p13;q22)	rs1873778	ND
LA 33	F	63	45, X-X, t(8;21)(q22;q22)	ND	ND
LA 37	M	32	45, X, -Y, t(8;21)(q22;q22), del 9(q22)	rs1873778	ND
LA 38	M	45	46, XY, inv(16)(p13;q22)	rs2228230	ND
LA 39	M	66	46, XY, t(8;21)(q22;q22), dup(17)(q12), add(18)(q23)	ND	ND
LA 40	M	39	47, XY, inv(16)(p13;q22), +6	rs2228230+rs10028020	ND
LA 41	M	25	45, X, -Y, t(8;21)(q22;q22)/ 46, XY, t(8;21)(q22;q22)/ 46, XY	ND	ND
LA 42	F	88	47, XX, inv(16)(p13;q22), +8	ND	ND
LA 43	F	38	46, XX, t(8;21)(q22;q22)	ND	ND
LA 44	F	49	46, XX, t(8;21)(q22;q22)	rs1873778	ND
LA 45	F	51	46, XX, t(8;21)(q22;q22)	rs2228230+rs10028020	ND
LA 48	M	24	46, XY, t(8;21)(q22;q22)	ND	ND
LA 49	F	70	46, XX, t(8;21)(q22;q22)	ND	ND
LA 51	M	43	46, XY, t(8;21)(q22;q22)	ND	ND
LA 52	M	26	45, X, Y, t(8;21)(q22;q22), del15(q22), der(21), t(8;21)(q22;q22)/ 46, XY	ND	ND
LA 55	M	35	46, XY, inv(16)(p13;q22)	ND	ND
LA 56	M	62	46, XY, t(8;21)(q22;q22)	ND	ND
LA 57	M	64	46, XY, t(8;21)(q22;q22)	ND	ND
LA 58	M	46	46, XY, inv(16)(p13;q22)	ND	ND
LA 59	M	35	46, XY, inv(16)(p13;q22)	rs10028020	ND
LA 60	F	37	45, X, -X, t(8;21)(q22;q22)/ 46, XX	ND	ND
LA 61	F	68	46, XX, t(8;21)(q22;q22)	ND	ND
LA 62	F	42	46, XX, inv(16)(p13;q22)	rs2228230	ND
LA 63	M	37	46, XY, t(8;21)(q22;q22)	ND	ND
LA 64	M	52	46, XY, inv(16)(p13;q22)	rs2228230+rs10028020	ND
LA 65	M	70	47, XY, inv(16)(p13;q22) del(7)(q31), +22	ND	ND
LA 66	M	72	46, XY, t(8;21)(q22;q22)	ND	ND
LA 67	M	48	46, XY, t(8;21)(q22;q22)	ND	ND
LA 68	F	51	46, XX, t(8;21)(q22;q22)	ND	ND
LA 69	M	51	46, XY, inv(16)(p13;q22)	ND	ND

Characteristics of the 35 investigated patients included age at diagnosis, chromosomal aberrations and polymorphisms detected in exons 12, 13 and 18 of *PDGFRA*; no base pair change was detected in exon 12 or exon 18 of *PDGFRB*. ND: not detected.

search for mutations in tyrosine kinase genes which could be associated with CBFL. We decided to investigate *PDGFR* mutations in order to assess a pathogenetic role in CBFL patients without *KIT* and *FLT3* mutations.

For this purpose, we investigated the juxtamembrane and TK domains of *PDGFRA* and the TK domain of *PDGFRB* as they share strong sequence homology with *KIT* domains. It has been demonstrated that mutations in these regions of *KIT* and *FLT3* result in a constitutive activation of their signaling cascades leading to ligand-independent growth and contributing to malignant transformation (49-50).

We used a particular SSCP for mutation detection which is rapid and has higher sensitive in comparison with standard DNA-SSCP method. The performance and quality assessment of this modified SSCP was determinated by different conditions. The search for mutations was based on the evaluation of electrophoretic mobilities of single-stranded DNA molecules in nondenaturing polyacrylamide gels. Conditions influencing separation of the bands include fragment length, base composition, buffer, gel conditions and temperature. The presence of glycerol within the gel and the long time of runs (18-20 h) allowed a better separation of PCR fragments into bands. Moreover, analysis of PCR fragments under two different temperature conditions (12˚C and 23˚C respectively) increases the rate of detectable mutations based on optimal conditions determined empirically. Altered

sequences may change the intramolecular folding and, hence, the rate of migration of these DNA molecules in gels. Furthermore, silver staining techniques had been used to detect DNA fragments with high sensivity on polyacrylamide gels.

The mutational screening of *PDGFRA* and *PDGFRB* detected three types of single-nucleotide alterations which were previously described as SNPs. Detection of the SNPs in the analyzed region of *PDGFRA*, confirmed the sensitivity of this SSCP method for detection of sequence variation.

Regarding the allelic frequencies of the identified SNPs, for rs10028020 (*PDGFRA* exon 13), no data have been reported in public databases. For rs2228230 (*PDGFRA* exon 18), our results are in agreement with the distribution reported in a Caucasian population, while for rs1873778 (*PDGFRA* exon 12) our data differ from those reported in a Caucasian population at the NCBI website (http://www.ncbi.nlm.nih.gov/ sites/entrez/).

In particular, our results show that the A allele (p=0.86) is the most frequent in the 35 Italian patients studied, whereas data reported earlier indicated the G allele as being the most frequent (p=0.98) in a Caucasian population (48 individuals).

Moreover at the NCBI website (http://www.ncbi.nlm.nih.gov/ SNP/snp_ref.cgi?rs=1873778P) a minor frequency for the G allele (p=0.79) in Sub-Saharan African populations with respect to Caucasias is indicated. The Caucasian population reported belongs to western and northern Europe; since our population belongs to the southern part of Europe, we cannot speculate as to whether the observed discrepancy of allelic frequency might be explained as a genetic gradient.

In conclusion, the present study suggests that mutations in *PDGFR* genes, in contrast to *KIT*, do not occur in CBFL, thus *PDGFR* genes do not seem to be involved in CBFL. Moreover, molecular studies of *PDGFRA* and *PDGFRB* genes reported that no pathogenic mutations were detected in CBFL (51-53). Since the central role of receptor tyrosine kinases in the development of haematological malignancies is well-known, our future plan is to develop a careful search for activating mutations in other RTK genes in CBFL patients whom tested negative for *KIT, FLT3, PDGFRA* and *PDGFRB* mutations.

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