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Stromal Cell-Derived Factor-1 Chemokine Gene Variant in Blood Donors and Chronic Myelogenous Leukemia Patients

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Chronic myelogenous leukemia (CML) is a malignant myeloproliferative disorder that originates from a pluripotent stem cell expressing the *bcr-abl* oncogene. It is characterized by an abnormal release of the expanded, malignant stem cell clone from the bone marrow into the circulation. The stromal cell derived factor-1 (SDF-1) gene contains a common polymorphism, termed SDF1-3′A, in an evolutionarily conserved segment of the 3′ untranslated region (UTR). In this work the SDF-1 genotypes of 25 patients (9–82 years old) who had been clinically and hematologically diagnosed with CML were compared with those of 60 healthy donors. In addition, the nature of *bcr-abl* hybrid mRNA and the association between demographic and hematological parameters were analyzed in cells from 12 CML patients (five women and seven men). All patients underwent blood collection during the chronic phase of disease after they received chemotherapy. b3a2 mRNA was detected in samples from eight of the CML patients and b2a2 mRNA was observed in four cases. An association between basophils and hemoglobin parameters was observed in that hemoglobin levels were higher in b2a2-expressing patients, and mean basophil levels were higher in patients expressing b3a2. Four of the CML patients (16%) were homozygous for 3′A allele. Of the patients who showed the presence of *bcr-abl* transcripts (N = 12), three presented the wt/wt genotype and nine were SDF1-3′A carriers. Three of the latter were homozygous for this mutation. It is possible that the *bcr-abl* fusion gene and the SDF1 genotype for 3′A allele have important implications for the pathogenesis of CML. J. Clin. Lab. Anal. 21:49–54, 2007.

Key words: chronic myeloid leukemia (CML); SDF1 chemokine; *bcr-abl*

INTRODUCTION

Chronic myelogenous leukemia (CML) is a malignant myeloproliferative disorder that originates from a pluripotent stem cell expressing the *bcr-abl* oncogene. It is characterized by an abnormal release of the expanded, malignant stem cell clone from the bone marrow into the circulation.

The *bcr-abl* oncogene is known to transduce its oncogenic signals through several pathways, which are then constitutively activated in a leukemic cell (1). The signal transduction cascades involved in cellular

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processes affected by the deregulated kinase activity of *bcr-abl* include Ras, mitogen-activated protein kinase (MAPK) and its downstream effectors MEK and Erk, and phosphatidylinositol-3 kinase and its downstream effector Akt (2,3).

Research on chemokines has progressed rapidly during the last 3 years, and the list of new chemoattractants that are capable of supporting the in vitro migration of malignant lymphoblasts is growing (4–6). However, limited information is available with regard to the biological and clinical importance of the locomotion of leukemia cells.

It is known that the chemokine stromal cell-derived factor-1 (SDF1/CXCL12) and its monogamous receptor CXCR4, a GTP-binding protein (G-protein)-coupled 7-transmembrane receptor, are involved in the trafficking of B-cells and hematopoietic progenitors. Conditioned medium from bone marrow stromal cells has been shown to contain chemotactic factors that act not only on mature leukocytes, but also on immature hematopoietic progenitor cells (5,7). SDF1 has been identified as the predominant chemotactic factor produced by bone marrow stromal cells (7–9).

Sequence analysis of the human SDF1 gene (GenBank accession number L36033) revealed a common variant containing a G→A transition in an evolutionarily conserved segment of the 3′ untranslated region (UTR). This polymorphism, which is designated SDF1-3′A, is present in the SDF-1β transcripts. Because this variant eliminates the *MspI* restriction enzyme site, polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis can be used for rapid identification of genotypes. The SDF1-3′A allele may play an important regulatory role by increasing the production of SDF-1, which binds to CXCR4 from T-cells (10).

SDF-1 is constitutively produced by stromal cells (8). Moreover, SDF-1 is released not only in the bone marrow, but also in other tissues (11). This suggests that the biological function of SDF-1 is not limited to hematopoietic stem cell homing. SDF-1 most probably contributes to extravasation of leukocytes in the absence of inflammation, which is important for lymphocyte trafficking (12).

In this study the SDF-1 chemokine gene variant was investigated in blood donors and chronic myelogenous leukemia (CML) patients, and the presence of 3′A carriers associated with *bcr-abl* transcripts was verified.

MATERIALS AND METHODS

Patients

With the approval of the Human Ethics Committee of Londrina State University, peripheral blood was collected from 25 patients with a clinical and hematological diagnosis of CML. All of the patients were attending the University Hospital of Londrina and Institute of Cancer in Londrina, Paraná State, Brazil. Samples of normal blood donors were obtained from Ribeirão Preto Blood Center, University of São Paulo, Brazil.

Genotype Analysis for SDF

PCR–SDF

Genomic DNA was isolated from peripheral blood cells as described by Kirby (13). DNA (200 ng) was then analyzed by means of PCR with specific primers for SDF 3′UTR-F1 (sense 5′-CAGTCAACCTGGG CAAAGCC-3′) and SDF 3′UTR-R2 (antisense 5′-CCTGAGAGTCCTTTTGCGGG-3′) (GenBank accession number L36033). Samples were amplified using the kit buffer plus 1.25 U of Taq polymerase (Invitrogen, Life Technologies, Sao Paulo, Brazil). The PCR conditions were as follows: 5-min denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; and 10-min elongation at 72°C in a thermocycler (PCR Sprint ThermoHybaid; Biosystems, Sao Paulo, Brazil). Amplicons of 293 base pairs (bp) were analyzed by electrophoresis in a 2% agarose gel and visualized using UV fluorescence after staining with ethidium bromide.

SDF-1 genotyping

PCR products were subjected to restriction digestion by incubation with *HpaII* (Invitrogen, Life Technologies) for 3 hr at 37°C and then subjected to electrophoresis in 2% agarose gels. SDF-1 wild-type alleles (SDF1-wt) yielded 100 and 193-bp products, while SDF1-3′A alleles yielded a 293-bp product.

Molecular Analysis of *bcr-abl* mRNA

Nucleic acid preparation and reverse transcriptase reaction

Leukocytes were prepared from peripheral blood samples after the application of erythrocytes lysis buffer B (solution A: 0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100). Total cellular RNA was extracted from peripheral white blood cells with Trizol (TRIzol LS; Invitrogen Life Technologies, Sao Paulo, Brazil) according to the manufacturer's instructions. The RNA was resuspended in 20 μl of sterile water treated with diethylpyrocarbonate (DEPC; Invitrogen, Sao Paulo, Brazil). cDNA was generated from 6 μL of total RNA with the use of a specific outer antisense primer and a first-strand cDNA synthesis kit (GeneAmp

RNA PCR kit, part no. N8 08-0017; Perkin Elmer, Foster City, CA).

PCR (nested)–*bcr-abl*

The oligos were designed based on their sequence (GenBank accession number AJ131466) and targeted to amplify the *bcr-abl* gene (LM1 outer sense 5′-TTCAGA AGCTTCTCCCTG-3′; LM2 outer antisense 5′-CTCC ACTGGCCACAAAAT-3′; LM3 inner sense 5′-TT CAGAAGCTTCTCCCTGACATCCG-3′; LM4 inner antisense 5′-CTCCACTGGCCACAAAATCATACA G-3′). The LM1 and LM3 sense oligos were derived from exon b2 of M-*bcr*, and the LM2 and LM4 antisense oligos were derived from exon a2 of *abl*. After two rounds of PCR these oligonucleotides specified a 328-bp fragment from b3a2 mRNA or a 253-bp fragment in the absence of exon 3 (b2a2). The reaction conditions for both PCR rounds were the same (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, and 1.25 U of Taq polymerase) and consisted of an initial denaturing step of 93°C for 2 min followed by 35 cycles of 93°C for 40 sec, 60°C for 30 sec, 72°C for 40 sec, and a final extension of 72°C for 7 min on a thermocycler (PCR Sprint ThermoHybaid; Biosystems, Brazil). Amplicons of 328 or 253 bp were analyzed by electrophoresis on a 10% acrylamide gel visualized by silver staining. A negative control (water instead of cDNA) was included in all reactions. When no amplification was obtained, the cDNA was tested by PCR with β-actin oligonucleotides as a control to assess the integrity of the RNA molecule.

Molecular Analysis of β-actin mRNA

Reverse transcription and PCR for β-actin cDNA were performed as described by Amarante et al. (14).

Statistical Analysis

SDF1-3′A allele frequency was calculated as: $[1 \times (h + 2H)]/2N$, where h represents the heterozygous genotype, H is the homozygous genotype, and N is the sample size for each population. Genotype data were analyzed by the chi-square (χ^2) test with the level of significance set at $P < 0.05$. Student's *t*-test was used to evaluate the demographic characteristics, and Fisher's exact test was used for categorical parameters, analyzed by Statistica 6.0 (StatSoft Inc. Tulsa, OK).

RESULTS

In the present study we employed PCR-RFLP techniques using the *HpaII* restriction enzyme to examine SDF-1 genotypes in 60 normal blood donors. These healthy control subjects had normal hematological values and negative cytological assays for leukemia.

Twenty-five CML patients (11 women and 14 men, 9–82 years old) from North Parana State, Brazil, were also investigated. Diagnosis of disease was based on the clinical and hematological criteria of the University Hospital of Londrina. All patients underwent blood collection during the chronic phase of disease after they received conventional hydroxyurea (N = 19) or Imatinib mesylate (STI571) (N = 6) chemotherapy. The genotypes of these patients are shown in Fig. 1. Of the CML patients, 60% (15/25) were 3′A allele carriers.

The difference in allelic frequency for SDF1-3′A between blood donors and CML patients was statistically significant. For all samples, the genotype did not differ from the theoretical distribution given by the Hardy-Weinberg equilibrium (HWE) (Table 1).

All CML patients were assessed for *bcr-abl* transcripts. Twelve of 25 samples (48%) from patients who had received chemotherapy were positive. All samples demonstrated integrity for β-actin mRNA expression.

TABLE 1. Genotypic and allelic frequencies of wild-type (wt) and 3′A alleles between blood donors and CML patients

	Number of samples	Genotype			Allelic frequency[a]	
		wt/wt	3′A/wt	3′A/3′A	wt	3′A
Blood donors	60*	39	18	3	0.80	0.20
CML patients	25**	10	11	4	0.62	0.38

*χ^2 in HWE = 0.234; $P > 0.05$.
**χ^2 in HWE = 0.109; $P > 0.05$.
[a]Blood donors × CML patients: $\chi^2 = 4.517$ (1 degree of freedom; $P < 0.05$).
HWE, Hardy-Weinberg equilibrium.

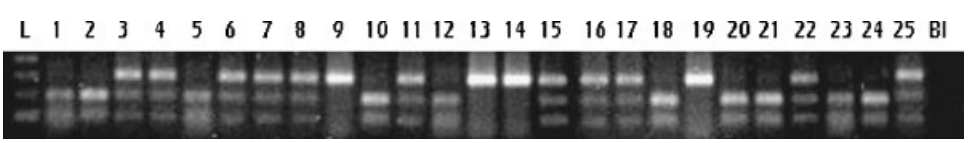

Fig. 1. SDF-1 genotyping. PCR products were subjected to enzymatic digestion by incubation with *HpaII* (Invitrogen) for 3 hr at 37°C and then submitted to electrophoresis in 2% agarose gels. Wild-type alleles (SDF1-wt) yielded 100- and 193-bp products, while SDF1-3′A alleles yielded a 293-bp product. L, ladder; Bl, blank reaction.

b3a2 mRNA was detected in samples from eight CML patients, and b2a2 mRNA was observed in four cases (Fig. 2). Moreover, the samples were tested twice and there were no cases with both types of transcripts.

Three of the patients who expressed *bcr-abl* mRNA presented the wt/wt genotype. Nine were 3′A carriers, and three of these patients were homozygous for this mutation. In the b3a2 transcripts six samples were 3′A carriers ($P<0.04$, χ^2 test; $P<0.05$, Mann-Whitney U-test).

The data for the hematological, demographic, and molecular diagnoses are presented in Table 2. There were significant associations between the mRNA type and hematological parameters for hemoglobin and basophils.

DISCUSSION

In most cases, translational control mechanisms result from the interaction of RNA-binding proteins with 5′ or 3′ UTRs of mRNA. A multispecies analysis has shown that in most vertebrates 3′-UTRs are substantially longer than their 5′ counterparts, indicating a significant potential for regulation. In addition, the fact that the average length of 3′-UTR sequences has increased during evolution suggests that their utilization may contribute to organism complexity (15). The mRNA 3′-UTR of many genes has been identified as an important regulator of both the translated product and the mRNA transcript itself (16).

SDF-1 was initially identified as a bone marrow stromal cell-derived factor and plays an important role in the homing of hematopoietic stem cells to the bone marrow (9). The SDF-1 gene variant, which has a G→A transition in the 3′-UTR, may have an important regulatory function regarding increases in the production of SDF-1 (10). In this study we assayed the genotype for SDF. The percentage of 3′A gene carriers differed significantly between normal donors and CML patients (Fig. 1). Of the CML patients, 40% (10/25) were homozygous for wt, 44% (11/25) had the hetero-

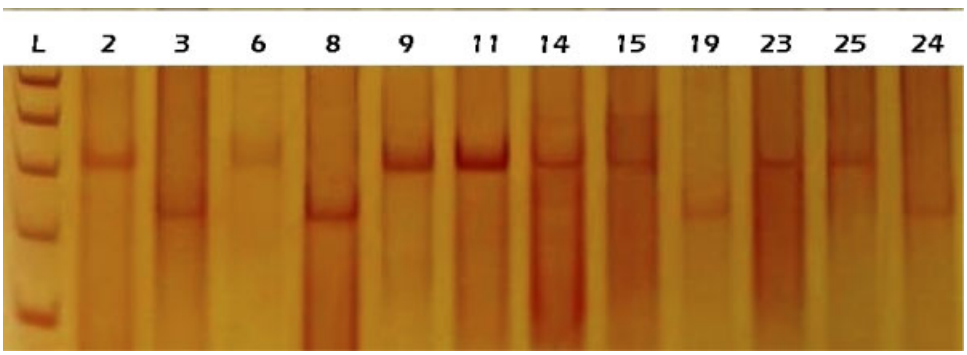

Fig. 2. Expression of *bcr-abl* mRNA in peripheral blood cells from CML patients. PCR products were submitted to electrophoresis in 10% silver-stained acrylamide gels. Amplicons of 328 bp (2, 6, 9, 11, 14, 15, 23, and 25) and 253 bp (3, 8, 19, and 24) correspond to b3a2 and b2a2 transcripts, respectively. Three of the patients who showed *bcr-abl* transcripts (12) presented the wt/wt genotype, and nine were SDF1-3′A carriers. L, ladder.

TABLE 2. Association between demographic, hematological, and molecular analysis of 12 chronic myeloid leukemia patients

	Type of mRNA				
Parameters[a]	b3a2	b2a2	Total	Statistical test[b]	*P*
Age (years)	53.4 (45–67)	46.7 (41–54)	51.2 (41–67)	S	0.213
Sex (M/F)	5/3	2/2	7/5	F	0.575
Hb (g/dL)	9.9 (6.3–13.2)	12.8 (12.2–13.7)	10.8 (6.3–13.7)	S	0.017*
WBC ($\times 10^{-3}/\mu L$)	9.4 (2.3–31.9)	6.1 (3.4–12.1)	8.3 (2.3–31.9)	S	0.538
Platelets ($\times 10^{-3}/\mu L$)	407 (10–734)	193 (131–244)	336 (10–734)	S	0.102
Blasts[c] (%)	2.7 (0–15)	0.5 (0–1)	2.0 (0–15)	S	0.427
Basophils[c] (%)	4.0 (0–8)	0.2 (0–1)	2.7 (0–8)	S	0.021*
Duration CP (months)	37 (0–72)	37 (12–63)	37 (0–72)	S	0.981

*Statistically significant association. All *P* values are two-tailed.
[a]Parameters are reported as means with the range in parentheses.
[b]Statistica 6.0 Program.
[c]Blasts and basophils were evaluated in peripheral blood.
Hb, hemoglobin; WBC, white blood cells; CP, chronic phase; S, Student's *t*-test; F, Fisher's exact test.

zygous genotype, and 16% were homozygous for mutation allele (3′A). Since both groups shared the same ethnic origin, the difference observed between them was not due to a common variation among various ethnic groups.

It has been shown that cells expressing high levels of *bcr-abl* (p210) exhibit an important signaling defect associated with a reduction in CXCR4 expression at the transcriptional level (17). At the molecular level, depending on the BCR gene breakpoint, two types of mRNA (b3a2 or b2a2) are generated. In this work, three of the patients who expressed *bcr-abl* mRNA presented the wt/wt genotype and nine were 3′A carriers. Three of the latter were homozygous for this mutation.

It is known that chemokine SDF-1 and its receptor CXCR4 play essential roles in the mediation and regulation of stem-cell homing and repopulation (9). While a small pool of undifferentiated stem cells with the potential to repeat the entire process in serially transplanted recipients is maintained within the bone marrow, maturing cells are continuously released into the circulation. It has been suggested that Ph^+CD34^+ cells have impaired abilities to interact with the bone marrow endothelial/stromal cells and extracellular matrix in response to SDF-1. These deficiencies could affect their homing to the bone marrow and retention within the stromal microenvironment (18).

The variant allele 3′A of the SDF1 gene may have an important regulatory function by increasing the production of SDF-1, which could prevent or maintain the homing state of blasts. Studies of murine models genetically deficient for SDF-1 (19) have demonstrated the essential role of this chemokine in maintaining hematopoietic progenitor cells within the bone marrow microenvironment. SDF-1 has also been shown to exert strong chemotactic activity on human progenitors cells in vitro (7). It has been verified that p210 *bcr-abl* regulates the expression of several key proteins involved in the cell cycle and inhibits SDF-1 chemotactic response via alteration of CXCR4 signaling and downregulation of CXCR4 expression. The p210 *bcr-abl* effect on CXCR4 is a dose-dependent event that requires a certain threshold of p210 *bcr-abl* expression to take place (17). Given the fact that SDF-1 is constitutively produced by stromal cells from the bone marrow (8,11), we speculate that it may contribute to marrow infiltration of leukemic blasts. It is unknown whether typical sites of leukemic infiltration are characterized by a greater expression and production of SDF-1 compared to other tissues that are not invaded by blasts.

Cashman et al. (20) demonstrated a potential physiological role of SDF-1 in regulating the cell-cycle status of primitive hematopoietic cells, and suggested that the deregulated cycling activity of primitive of CML cells is due to the *bcr-abl*-mediated disruption of a pathway shared by multiple chemokine receptors.

The observation that production of *bcr-abl* is the initiating event in CML has focused attention on the survival signals triggered by this oncogene (21). The translocation results in the fusion of the *abl* gene located at the long arm of chromosome 9 with the *bcr* gene located at the long arm of chromosome 22. The *bcr*/*abl* fusion gene encodes a chimeric protein with elevated tyrosine kinase activity, which plays an important role in the pathogenesis of the disease. The detection of the t(9;22)(q34;q11) translocation and *bcr*/*abl* fusion gene is predictive for the diagnosis of CML and is recommended for evaluations of therapeutic effect.

Some reports have suggested a role for exon b3 in the evolution of CML (22). The presence of exon b3 has been associated with abnormal blood parameters, such as high platelet counts (23), a low percentage of circulating blast cells, and high leukocyte counts (24).

In this work we evaluated 12 patients with CML. Since we used oligos for b3a2 and b2a2 transcripts remove were used, we did not identify mRNA with any other junctions or in rare cases in which the breakpoint was outside M-bcr. Several studies have shown that classifying CML according to mRNA type does not produce homogenous hematological data. Although the variance of each parameter was large among patients for each mRNA type, there were no significant differences between the groups, except with regard to basophils and hemoglobin. The statistical analysis for SDF-1 genotype and clinical parameters was significant only for basophils. There were increased levels of basophils in the 3′A/3′A genotype compared to wt/wt and 3′A/wt patients ($P < 0.049$ by analysis of variance (ANOVA)). In contrast, the hemoglobin levels were higher in b2a2-expressing patients. It is known that SDF-1 induces a strong migratory response in cultured basophils. Expression of CXCR4 is affected by cytokines, and thus may represent an alternative mechanism for control of cell-specific, biological responses to SDF-1 (25). However, a prognostic value for the type of chimeric mRNA *bcr-abl* (b3a2 or b2a2) is still controversial (26).

The present work suggests that the SDF1 polymorphism and the translocation product of chimeric fusion for protein *bcr-abl* may have implications for our understanding of homing to the bone marrow and retention of these cells within the stromal microenvironment.

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