Methylation status of the \textit{SOCS 1} and \textit{JUNB} genes in chronic myeloid leukemia patients

\textit{Padrão de metilação dos genes SOCS 1 e JUNB em pacientes com leucemia mieloide crônica}

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\textbf{Introduction}

Chronic myeloid leukemia (CML) is characterized by reciprocal translocation between chromosome 9 and chromosome 22 t(9;22)(q34;q11), leading to the formation of the Philadelphia chromosome (Ph1).1 This translocation is responsible for the gene fusion of the \textit{ABL} oncogene with the \textit{BCR} gene resulting in the \textit{BCR/ABL}2 hybrid gene. This gene codifies the altered protein tyrosine kinase,3 which plays an important role in the pathogenesis of this disease. Chronic myeloid leukemia is responsible for 15\% to 20\% of all leukemias in adults and incidence rates range from 1 to 2 cases per 100,000 inhabitants.4 Nowadays, the only curative treatment option for CML is the hematopoietic stem cell transplantation (HSCT). One of the complications after HSCT is the graft-versus-host disease (GVHD) in which the T lymphocytes of the donor

\begin{itemize}
\item Alterations in the methylation status of genes may contribute to the progression of Chronic Myeloid Leukemia (CML). In this study, the methylation status in exon2 of SOCS-1 and promoter regions of both SOCS-1 and JUNB were evaluated in CML patients. The methylation status of these genes was analyzed using methylation-specific Polymerase Chain Reaction (MSP) in 30 samples from CML patients, 30 samples from these same patients after hematopoietic stem cell transplantation (HSCT) and 30 samples from healthy controls. The samples of CML patients presented methylation as follows: JUNB gene (3.3\%), promoter region of the SOCS-1 gene (6.6\%) and exon2 of the SOCS-1 gene (46.6\%). The samples of the healthy individuals presented methylation (10\%, P = 0.002) only in exon 2 of the SOCS-1 gene. After transplantation, patients presented alterations in the methylation status of the promoter region of the SOCS-1 gene (6.6\%), exon2 of SOCS-1 (46.6\%) and the promoter region of the JUNB gene (16.6\%). Methylation of the promoter regions of the SOCS-1 gene and the JUNB gene is not a frequent event in CML. In contrast, SOCS-1 gene methylation in exon2 is a frequent event, susceptible to alterations in status after HSCT with possible implications for the progression of this disease. Rev. Bras. Hematol. Hemoter. 2009; 31(3):147-152.

\textbf{Key words:} Chronic myeloid leukemia; methylation; MSP-PCR, SOCS-1, JUNB.
see the antigens of the recipient as foreign. The preventive treatment for GVHD is performed by using immunosuppressants.

DNA methylation in the promoter region is an event which may alter the expression of a specific gene. In mammals, DNA methylation occurs by adding a methyl group on carbon-5 of cytosine. DNA methyltransferase enzymes (DNMTs) catalyze this reaction, specially where cytosine (C) lies next to guanine (G) (5′-CpG-3′).

Alterations in the methylation status, mainly in cancer, may lead to the disease progression by either hypermethylation of the CpGs islands of tumor suppressor genes or hypomethylation of protooncogenes and deacetylation of histones. Many genes in CML, including p53, p16, calcitonin, cadherin 13, ABL, hPER3, JUNB and SOCS-1 have been reported to be inactivated by methylation.

Proliferation and differentiation of hematopoietic precursor cells are regulated by many cytokines. SOCS (suppressor of cytokine signaling) proteins are related to negative regulation of many cytokine signaling pathways, including suppression of JAK-STAT (Janus tyrosine kinase-signal transducers and activators of transcription) signaling, among others. Loss of this regulation may be associated with leukemogenesis and the progression of CML. SOCS-1 transcript is often present in reduced levels in cells and it may rapidly be induced by several kinds of cytokines, hormones and growth factors. SOCS-1 protein has been shown to be an inhibitor of several cytokine signaling pathways, including interferons, interleukins IL-2, IL-3, IL-4, IL-6, erythropoietin, thrombopoietin, among others.

Suppression of the SOCS-1 gene by aberrant hypermethylation has been observed in several diseases such as hepatocellular carcinoma, multiple myeloma, acute myeloid leukemia, ovarian and breast carcinomas, myelodysplastic syndrome, and chronic myeloid leukemia.

JUNB gene is a member of the AP-1 transcription factor complex (activating protein-1). This gene, is constitutively expressed in human granulocytes and its expression promotes myeloid differentiation. JUNB protein can regulate transcription of several genes either in a positive or negative way. Mice lacking JunB protein expression in the myeloid lineage develop a myeloproliferative disease, progressing to blast crisis that resembles human chronic myeloid leukemia.

As mentioned previously, SOCS-1 and JUNB play an essential role on the control of proliferation and cellular differentiation. Loss or suppression of their expressions may alter the methylation status in CML.

In this study, the methylation status in exon2 of SOCS-1 and promoter regions of SOCS-1 and JUNB were analyzed in 30 CML patients using methylation-specific (MSP) polymerase chain reaction (PCR).

Patients and Method

Patient samples

Peripheral blood and bone marrow samples were randomly collected from 30 CML patients (14 male, 16 female) attended at Blood Transfusion Center, in a Medical School Teaching Hospital Unesp, Botucatu/Amaral Carvalho Hospital, Jaú, Brazil from June 2001 to January 2004. After transplantation, samples were collected over a period ranging from 22 to 380 days. Peripheral blood samples from 30 healthy volunteers were used as controls. The average ages were 38.8 and 36.2 for patients and controls, respectively. At the time of transplantation, 27 patients were in the chronic phase, one patient in the accelerated phase and two patients in the blastic crisis of the disease. All patients were found to be t(9;22) positive by cytogenetics and BCR/ABL positive by molecular methods at the time of diagnosis.

The local Ethical Committee approved this study.

DNA extraction

DNA was extracted from peripheral blood and bone marrow samples using Wizard® Genomic DNA purification Kit (PROMEGA-USA), according to manufacturer's instruction and its quality was evaluated by polymerase chain reaction (PCR) amplification of the GAPDH housekeeping gene.

Methylation-specific PCR analysis (MSP) and direct sequencing of bisulfite

The methylation status of the promoter region of SOCS-1 was analyzed by methylation specific polymerase chain reaction (PCR) as previously described. Primers for MSP were designed to amplify both methylated (M) or unmethylated (U) alleles. SOCS-1 gene (GenBank accession number U20734) was amplified using specific primers for the promoter region and exon2. (Table 1) JUNB gene (GenBank accession number U20734) was amplified using primers specific for the promoter region of the JUNB gene. (Table 1).

DNA from healthy donors was used as negative controls. Normal DNA from peripheral blood was treated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA) in order to generate positive controls for methylated alleles of SOCS-1. Two μg of genomic DNA diluted to 17μL with water and 1μg of salmon sperm DNA (Invitrogen) were denatured by treatment with 2μL of 3M NaOH. The mixture was heated at 50°C for 20 minutes. Five hundred μL of bisulfite (0.24 mol/L) (Sigma-Aldrich) and hydroquinone (0.02 mol/L) mixture were added to the denatured DNA solution and heated at 70°C for 3 hours. The DNA was purified using Wizard SV Gel and PCR Clean-Up System (Promega) and treated with 3M NaOH, followed by ethanol precipitation. DNA was resuspended in 50μL of water and either used immediately or stored at -80°C. For MSP analysis, approximately 1μg of DNA was sodium bisulfite-modified, and amplified with either a methylation-
specific or unmethylation-specific primer as described previously (Table 01). MSP cycling conditions, JUNB and SOCS-1 promoters were: 95°C for 7 min, 35 cycles of 95°C for 1 min 30 s, annealing for 1 min (SOCS-1 at 60°C and JunB at 55°C), 72°C for 2 min and a final extension at 72°C for 7 min. For exon 2 of SOCS-1, cycling conditions were: 95°C for 05 min followed by 40 cycles of 95°C for 30 s, annealing at 60°C for 45 s, 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were electrophoresed on 6 % non-denaturing polyacrylamide gels, silver-stained. Results from duplicate experiments were used to determine methylation status. To confirm the efficiency of DNA modification, direct sequencing of the PCR products was done using primers methylated SOCS1-M/JUNB-M and unmethylated SOCS1-U/JUNB-U PCR in both forward and reverse ways. PCR products were sequenced on an ABI Prism 377 DNA Sequencer with DNA Sequencing kit (Applied Biosystem, UK) according to the manufacturer's instructions.

Statistical analysis
The Chi-square and Fischer tests were used for statistical analysis. Probability values of ≤ 0.05 were regarded as statistically significant.

Results
The analysis of the methylation status of the SOCS-1 gene and the JUNB gene was carried out by MSP (Methylation-Specific PCR) (Figure 1). The results were confirmed by direct DNA sequencing (data not shown).

Table 1. PCR primer sequences used for methylation in CML patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’-3’) forward</th>
<th>Primer (5’-3’) reverse</th>
<th>Size (bp)</th>
<th>T (°C)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS1</td>
<td>M TTGTTCGGAGGTGACATT</td>
<td>ACTAAAAACGGCTACGAAAACC</td>
<td>218</td>
<td>60</td>
<td>Liu et al. (2003)</td>
</tr>
<tr>
<td>promoter</td>
<td>U TTATTTTCTCTTGGAGTTTGC</td>
<td>AAAACAAAAAACAATGAACTACACTACAAAACCA</td>
<td>243</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>SOCS2</td>
<td>M GGTGAGGCGGAGAGTTTGC</td>
<td>ACAGCGAGCTACGCAACG</td>
<td>104</td>
<td>60</td>
<td>Brakensiek et al. (2005)</td>
</tr>
<tr>
<td>exon2</td>
<td>U TTTGAGTGGGTGTTGAGAGTTTGC</td>
<td>CCACACACACACACTACAAAACA</td>
<td>124</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>JUNB</td>
<td>M GAGCTTAGGAAATGTGAGTTTGC</td>
<td>CGAATACATACCTACGCA</td>
<td>136</td>
<td>55</td>
<td>Yang et al.</td>
</tr>
<tr>
<td>promoter</td>
<td>U TTGGGAAATGATGTTAGGAAATGTGAGTTTGC</td>
<td>ACTACACACACACACACTACAAAACCA</td>
<td>192</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

M = methylated primer; U = unmethylated DNA, MW indicates molecular weight control (100pb ladder); C+, positive control of methylated DNA U-MSP, M-MSP; B water blank. (A) SOCS-1 promoter was methylated in CML samples, 1, 2 and 3 samples after allogeneic haematopoietic stem cell transplantation (HSCT). (B) exon2 of SOCS-1 was methylated in samples 1 (CML) and 2 after HSCT. (C) JunB promoter was methylated in 1 (CML), 2 and 3 samples after HSCT.
Table 2. SOCS-1, JUNB methylation status in CML patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>SOCS-1 promoter</th>
<th>SOCS-1 exon 2</th>
<th>JUNB promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U</td>
<td>M</td>
<td>P</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 35 anos</td>
<td>30</td>
<td>0</td>
<td>0.246</td>
</tr>
<tr>
<td>&gt; 35 anos</td>
<td>28</td>
<td>2</td>
<td>0.485</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>0</td>
<td>0.246</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>2</td>
<td>0.485</td>
</tr>
<tr>
<td><strong>Clinical phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cronic</td>
<td>25</td>
<td>2</td>
<td>0.246</td>
</tr>
<tr>
<td>Accelerated/Blastic</td>
<td>1/2</td>
<td>0/0</td>
<td>0.246</td>
</tr>
<tr>
<td><strong>Normal controls</strong></td>
<td>30</td>
<td>0</td>
<td>27</td>
</tr>
</tbody>
</table>

U ( unmethylated); M (methylated); P (probability value)

Table 3. Alterations in the methylation status of the promoter region of the SOCS-1 gene, exon2 of SOCS-1 and the promoter region of the JUNB gene in CML patients after HSCT

<table>
<thead>
<tr>
<th>Gene</th>
<th>U → M (n = 30)</th>
<th>M → U (n = 30)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS1 promoter</td>
<td>1</td>
<td>1</td>
<td>6.6%</td>
</tr>
<tr>
<td>SOCS exon2</td>
<td>12</td>
<td>2</td>
<td>46%</td>
</tr>
<tr>
<td>JUNB promoter</td>
<td>5</td>
<td>0</td>
<td>16.6%</td>
</tr>
</tbody>
</table>

U = unmethylated; M = methylated

Methylation was not detected in the promoter region of SOCS-1 and JUNB genes in hematopoietic stem cell donors.

After HSCT, 36.6% (11/30) of the patients developed acute graft-versus-host disease (GVHD), and 70% (21/30) of the patients developed chronic graft-versus-host disease.

Discussion and Conclusions

The methylation status in the promoter region and exon 2 of the SOCS-1 gene, and in the promoter region of the JUNB gene was analyzed in CML patients.

The methylation status of these genes was not influenced by the age of the patients or their gender, although several studies have suggested that DNA methylation is related to patients’ age.31,32

In this study, the two patients who showed methylation in the promoter region of the SOCS-1 gene were in the chronic phase of the disease. No methylation was detected in the three patients who were in the accelerated phase or blast crisis. Hartirnaz et al.33 analyzed 56 blood samples of patients with CML and no methylation was detected in the promoter region of the SOCS-1 gene. In contrast, Liu et al.14 found methylation levels of 52% (52/100) in the same region, and they were more frequent in patients who were in the blast crisis (67%) than in those who were in the chronic phase of the disease (46%). In the
present study, no methylation was detected in the control group, which is in agreement with Liu et al. 14 Johan et al. 25 and Hartirnaz et al. 33

Regarding the analysis of the methylation status in exon 2 of the SOCS-1 gene in patients with CML, our findings (46.6%) are similar to those found by Hartirnaz et al. 33 which detected methylation levels of 58.9% in this region. The greatest difference was the frequency of the methylation status in exon2 of the SOCS-1 gene in the control group: in our study we observed low rate of methylation similar to those observed by Johan et al. 25 and Chin et al. 20 while Hartirnaz et al. 33 found methylation level of 93.8% in the exon2 of the SOCS-1 gene.

It's important to point out that Hartirnaz et al. 33 used primers directed against regions slightly different from those used by the other authors.

Methylation was detected in the promoter region of the JUNB gene in only one patient who was in the chronic phase of the disease (3.3%). These results were not in agreement with those of Yang et al. 13 which observed methylation of 100% (32/32) in samples of CML patients, in which 21 patients were in the chronic phase and 11 in the blastic crises. Findings in the literature suggest that lower levels of methylation in the chronic phase are explained by a higher proportion of normal circulating cells in the peripheral blood. 14

In this study, 18 gains of methylation were observed in the genes studied after HSCT. The probability of transference of such alterations to the recipients was discarded once no methylation was detected in the hematopoietic stem cell donors.

The three patients who were in the accelerated phase or blastic crises had some gain of methylation after HSCT. A higher number of patients would be necessary to evaluate the real significance of our finding.

When a possible correlation between methylation status and graft-versus-host disease was investigated, no statistically significant result was found. However, patients who acquired methylation in exon2 of the SOCS-1 gene were more prone to develop chronic GVHD after HSCT.

Whether the immunosuppressants (specially metotrexate - MTX) used had any influence on alterations of the methylation status could not be determined because 29 out of 30 patients received the same kind of treatment containing MTX. These results showed that methylation in the promoter regions of SOCS-1 and JUNB are not a frequent event in CML patients. In contrast, methylation in exon2 of the SOCS-1 gene is a frequent event, susceptible to alterations of status after HSCT. Lack of negative regulation of cytokine signaling pathways due to methylation can lead to alterations of cell proliferation. Further studies are needed to determine the mechanisms involved in alterations of the methylation status in chronic myeloid leukemia.

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Resumo

Alteração no padrão de metilação gênica pode contribuir para a progressão da leucemia mieloide crônica (LMC). Neste estudo, o padrão de metilação no exon 2 do gene SOCS-1 e região promotora de ambos SOCS-1 e JUNB foram avaliadas em pacientes com LMC. O padrão de metilação desses genes foi analisado usando a técnica "methylation-specific polymerase chain reaction (MSP)" em 30 amostras de pacientes com LMC, 30 amostras desses mesmos pacientes após transplante de medula óssea (TMO) e 30 amostras controle de indivíduos saudáveis. As amostras de pacientes com LMC apresentaram o seguinte padrão de metilação: gene JUNB (3.3%), região promotora do gene SOCS-1 (6.6%) e exon2 do gene SOCS-1 (46.6%). As amostras dos indivíduos saudáveis apresentaram metilação somente no exon 2 do gene SOCS-1 (10%, P = 0.002). Após o transplante, os pacientes apresentaram alterações no padrão de metilação da região promotora do gene SOCS-1 (6.6%), no exon2 do gene SOCS-1 (46.6%) e na região promotora do gene JUNB (16.6%). Metilação das regiões promotoras dos genes SOCS-1 e JUNB não é um evento frequente em LMC. Em contraste, metilação no exon 2 do gene SOCS-1 apresenta-se como um evento frequente, suscetível a alterações no padrão de metilação após TMO.

Palavras-chave: Leucemia mieloide crônica; metilação; MSP-PCR SOCS-1; JUNB.

References


Avaliação: Editor e dois revisores externos
Conflito de interesse: sem conflito de interesse

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