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Artigo / Article

Methylation status of the *SOCS 1* and *JUNB* genes in chronic myeloid leukemia patients

Padrão de metilação dos genes SOCS 1 e JUNB em pacientes com leucemia mieloide crônica

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*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.*

Key words: *Chronic myeloid leukemia; methylation; MSP-PCR, SOCS-1, JUNB.*

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 *ABL* oncogene with the *BCR* gene resulting in the *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

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see the antigens of the recipient as foreign.[5] The preventive treatment for GVHD is performed by using immunosuppressants.[6]

DNA methylation in the promoter region is an event which may alter the expression of a specific gene.[7] 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').[8]

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 hypometylation of protooncogenes and deacetylation of histones.[7] Many genes in CML, including *p53*, *p16*, *calcitonin, cadherin 13, ABL , hPER3, JUNB* and *SOCS-1* have been reported to be inactivated by methylation.[9-14]

Proliferation and differentiation of hematopoietic precursor cells are regulated by many cytokines.[15] 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.[16,17] Loss of this regulation may be associated with leukemogenesis and the progression of CML.[14] *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.[16,18]

Suppression of the *SOCS-1* gene by aberrant hypermethylation has been observed in several diseases such as hepatocellular carcinoma,[17] multiple myeloma,[19] acute myeloid leukemia,[20,21,22] ovarian and breast carcinomas,[23] myelodysplastic syndrome,[24,25,26] and chronic myeloid leukemia.[14,33]

JUNB gene is a member of the AP-1 transcription factor complex (activating protein-1). This gene, is constitutively expressed in human mature granulocytes and its expression promotes myeloid differentiation.[27] JUNB protein can regulate transcription of several genes either in a positive or negative way.[28] Mice lacking JunB protein expression in the myeloid lineage develop a myeloproliferative disease, progressing to blast crisis that resembles human chronic myeloid leukemia.[13]

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)[29] 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.[29] 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.[14,24] (Table 1) *JUNB* gene (GenBank accession number U20734) was amplified using primers specific for the promoter region of the *JUNB* gene.[13] (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*.[30] 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 50ºC 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 70ºC 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 ressuspended in 50µL of water and either used immediately or stored at -80ºC. For MSP analysis, approximately 1µg of DNA was sodium bisulfite-modified, and amplified with either a methylation-

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

Gene		Primer (5' - 3') foward	Primer (5' - 3') reverse	Size (bp)	T (ºC)	Ref
SOCS1 promoter	M	TTGTTCGGAGGTGGATTT	ACTAAAACGCTACGAAACCG	218	60	Liu *et al.* (2003)
	U	TTTTTTCCTCTTGTTTGGAGGTTGGATTTT	AAAACAAAACAATAAACTAAAACACTACAAAACCA	243	60	
SOCS1 exon2	M	GGATGGTAGTCGCGAGAGTTTC	ACGCGACGCTAACGCAACG	104	60	Brakensiek *et al.* (2005)
	U	TTTGGATGGTAGTTGTGAGAGTTTT	CCACACACAACACTAACACAACA	124	61	
JUNB promoter	M	GACGTTAGGAAAGTTATCGC	CGAACTAAATACCTAATCGCG	136	55	Yang *et al.* (2003)
	U	TTGGGGAAATGATGTTAGGAAAGTTATTGT	ACTACAACAAACAACAAACTCTCCACTACA	192	63	

M = methylated primer; U = unmethylated

specific or unmethylation-specific primer as described previously (Table 01). MSP cycling conditions, *JUNB* and *SOCS-1* promoters were: 95ºC for 7 min, 35 cycles of 95°C for 1min 30 s, annealing for 1min (*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).

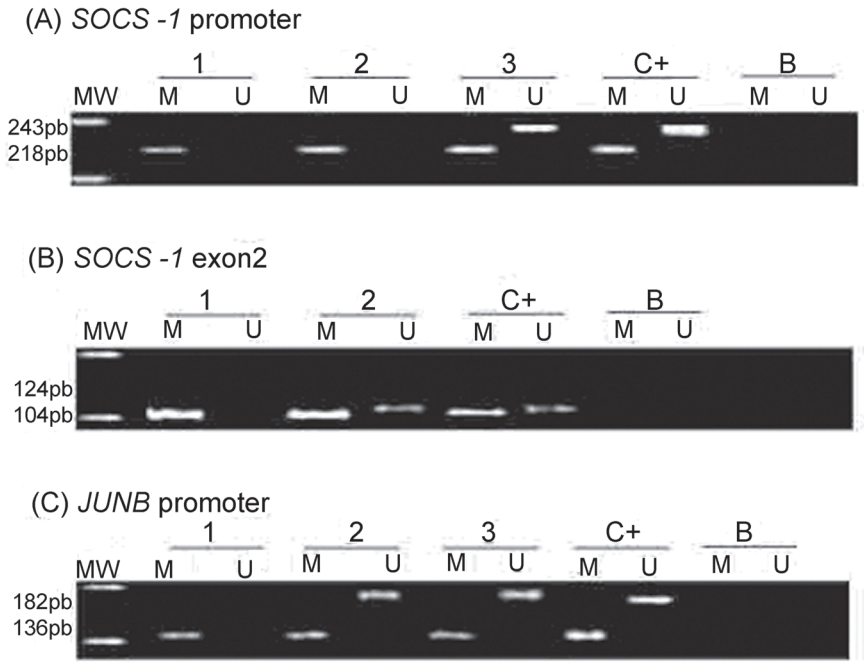

Figure 1. Representative data showing the methylation status of *SOCS-1* promoter, exon2 of *SOCS-1* and *JunB* promoter. U unmethylated DNA, M methylated DNA, MW indicates molecular weight control (100pb ladder); C+, positive control of methylated DNA U-MSP, M-MSP; B water blank. (A) *SOCS-1* promoter 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

	SOCS-1 promoter			*SOCS-1* exon 2			*JUNB* promoter		
	U	M	*P*	U	M	*P*	U	M	*P*
Age									
≤ 35 anos	30	0	0.246	5	7	0.501	30	0	1.000
> 35 anos	28	2		11	7		29	1	
Sex									
Male	14	0	0.485	8	6	0.980	14	0	1.000
Female	14	2		8	8		15	1	
Clinical phase									
Cronic	25	2	0.246	13	14	0.002*	26	1	1.000
Accelerated/Blastic	1/2	0/0		1/2	0/0		1/2	0/0	
Normal controls	30	0		27	3		30	0	

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

Alterations in the methylation status after HSCT			
Gene	U → M (*n = 30*)	M → U (*n = 30*)	Total
SOCS1 promoter	1	1	6.6%
SOCS exon2	12	2	46%
JUNB promoter	5	0	16.6%

U = ummethylated; M = methylated

The resulting *BCR-ABL* mRNA research was identified in 10% (3/30) of patients after HSCT, and 90% (27/30) had shown molecular remission. There was no significant difference between patients who had molecular remission after HSCT with the methylation of genes studied. Twenty-seven out of the 30 patients were in the chronic phase of the disease and only two patients showed methylation in the promoter region of the *SOCS-1*gene. No methylation was found in the three patients who were in the accelerated phase or blastic crises.

Before the hematopoietic stem cell transplantation (HSCT), methylation levels of 6.6% (2/30) in the promoter region of the *SOCS-1* gene; 46.6% (14/30) (P = 0.002) in exon2 of the *SOCS-1* gene and 3.3% (1/30) in the promoter region of the *JUNB* gene were observed (Table 2).

In the control samples without the disease, methylation of 10% (3/30) in exon2 of the *SOCS-1* gene was found, and no methylation was found in the promoter regions of *SOCS-1* and JUNB genes (Table 2).

After HSCT, two patients (6.6%) showed alteration of methylation status in the promoter region of the *SOCS-1*gene (one gained methylation and one lost methylation); 14 patientes (46.6%) showed alterations of methylation status in exon2 of the *SOCS-1* gene (12 gained and 2 lost methylation) (Table 3), in 16 patients (53,3%) the methylation status did not change, while 11 patients continued to show the methylation status of *SOCS-1* gene exon 2 after HSCT and 5 patients was not detected the methylation; 5 patients (16.6%) showed alteration of methylation status in the *JUNB* gene (they gained methylation) (Table 3).

In two patients was observed the disease recurrence. One patient showed methylation in all genes studied after HSCT, had a survival of 1020 days and died of non-Hodgkins lymphoma. The other patient showed only methylation in gene *SOCS-1* exon2 after HSCT and is still alive.

The patients studied before and after the HSCT, showed a low frequency of methylation in the promoter region of genes *SOCS-1* and *JUNB*, no statistically significant result was found. In contrast, there is a significant difference in methylation of *SOCS-1* gene exon2 found in patients with CML before and after the HSCT.

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 blastic 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 mielóide 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%). 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. Rev. Bras. Hematol. Hemoter. 2009;31(3):147-152.

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

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