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Research Article

DNA methylation analysis of the tumor suppressor gene *CDKN2B* in Brazilian leukemia patients

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Abstract

The aim of this work was to evaluate the methylation profile of the *p15 (CDKN2B)* gene in Brazilian patients with leukemia and to correlate the *CDKN2B* gene expression with the percentage of methylated CpG dinucleotides in its promoter region. Thirty-one samples from six patients with acute lymphocytic leukemia (ALL), four with chronic myeloid leukemia (CML), and 21 with acute myeloid leukemia (AML) were evaluated by MSP (Methylation-Specific PCR). The *CDKN2B* gene was found to be methylated in four (67%) of the six ALL samples and in 16 (76%) of the 21 AML samples, but in none of the four CML samples analyzed. We observed a correlation between the *CDKN2B* mRNA expression (RT-PCR) and the percentage of methylated CpG dinucleotides. Therefore, this study in Brazilian patients confirms that the *CDKN2B* gene is methylated in the majority of leukemia patients.

Key words: CpG island, DNA methylation, *CDKN2B*.

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Introduction

DNA methylation is a covalent modification and results from the activity of a family of DNA methyltransferase (DNMT) enzymes which catalyze the transfer of a methyl group (CH3) from S-adenosylmethionine (SAM) to the cytosine residues at CpG dinucleotides (Strathdee and Brown, 2002). The distribution of CpG dinucleotides in the human genome is not uniform, but there are small stretches (0.5 kb to several kb) of CpG-rich DNA regions termed CpG islands (Galm *et al.*, 2006). It has been estimated that the human genome contains about 29,000 CpG islands (Nephew and Huang, 2003). These CpG islands are usually located in the vicinity of genes, are often found near the promoters of widely expressed genes, and typically extend into the first exon (Jones, 2003). In contrast to CpG dinucleotides, which are dispersed throughout the genome, the cytosines within CpG islands, especially those associated with promoter regions, are normally unmethylated, allowing the expression of a gene. The exception to this unmethylated state of CpG islands involves the imprinted genes and X-chromosome inactivation, and this indicates the tight association of promoter DNA methylation with transcriptional silencing during normal mammalian development (Galm *et al.*, 2006).

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The methylation pattern of normal cells is kept through successive cellular divisions in adult tissues and the heritage of this information is called epigenetic inheritance (Laird, 2003). Therefore, epigenetics is the study of modifications in gene expression that are not caused by alterations of DNA sequence (Verma and Srivastava, 2002; Galm *et al.*, 2006).

In tumorigenesis, the balance of the methylation state of normal cells is lost, and some of the possible alterations are: (I) transcriptional silencing of tumor suppressor genes by CpG island promoter hypermethylation; and (II) histone deacetylation and global genomic hypomethylation. Hypo-

methylation contributes to carcinogenesis and is responsible for the chromosomal instability, reactivation of transposable elements, and loss of imprinting (Esteller and Herman, 2002). Therefore, the profile of hypermethylation promoters differs according to each cancer type, because for each tumor type specific genes are methylated. In addition to that, the epigenetic inactivation may affect all molecular mechanisms involved in cell immortalization and transformation. Last but not least, it seems that epigenetic changes are among the several early steps in carcinogenesis (Mukai and Sekiguchi, 2002).

According to the estimates of cancer incidence for 2008 by INCA (Instituto Nacional do Câncer – Brazilian National Cancer Institute), leukemias will afflict 5220 men and 4320 women. Hematologic neoplasias can jeopardize the lymphoid or myeloid lineages. Lymphoid neoplasias start from lymphoid lineage cells in different stages of maturation. Myeloid neoplasias result from a pluripotential progenitor cell mutation which maintain the capacity, albeit in an imperfect manner, of differentiation and maturation for each one of the myeloid lineages (Zago *et al.*, 2001). Most acute leukemias appear to be the consequence of the collaboration between one class of mutations or gene rearrangements that confer a proliferative and/or survival advantage to hematopoietic progenitors and a second class of mutations that serve primarily to impair hematopoietic differentiation and subsequent apoptosis of cells (Kelly and Gilliland, 2002).

In hematological neoplasms, hypermethylation genes were identified in multiple fundamental pathways related to cancer, including cell cycle control (*p15*, *p16*, *Rb1*, *p27*, *p73*), DNA repair (*O^6MGMT*), apoptosis inhibition (*DAPK*), tumoral metastasis (*E-cadherin*), and growth factors (*ER*, *EphA3*) (French *et al.*, 2002). One of the most frequent methylated genes in leukemia, mainly in ALM, is *CDKN2B* (Herman *et al.*, 1996; Issa *et al.*, 1997; Wong *et al.*, 2000; Toyota *et al.*, 2001; Claus and Lubbert, 2003).

In this work, we determined the methylation profile of the *CDKN2B* gene in samples from Brazilian patients with acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), and chronic myeloid leukemia (CML). We also evaluated the *CDKN2B* mRNA expression and the percentage of methylated CpG dinucleotides by sequencing of sodium bisulfite-treated DNA.

Material and Methods

Samples

Genomic DNA was extracted from bone marrow cells of six ALL patients, four CML patients, 21 AML patients and one sample of normal bone marrow, using a Super Quik Gene Kit (AGTC). The AML samples were subdivided based on French-American-British (FAB) into: M0 (1), M1 (2), M2 (10), M4 (2), M5 (4) and M6 (2). All patients signed a consent form approved by the Ethics Committee of the Institution.

Bisulfite DNA modification

Bisulfite DNA modification was accomplished in agreement with the technique described in *Current Protocols In Human Genetics* (Dracopoli *et al.*, 1994). DNA (2 μg) was denatured with NaOH (2 M) for 10 min at 37 °C. Ten millimoles of hydroquinone and 3 M of sodium bisulfite at pH 5, both freshly prepared, were added and mixed, and then samples were incubated at 50 °C for 16 h. The modified DNA was purified using a *Wizard DNA clean-up* purification kit (Promega) according to the manufacturer's instructions and eluted into water. Modification was completed by treatment with NaOH 3 M for 5 min at room temperature, followed by ethanol precipitation. The DNA was resuspended in water and used immediately or stored at -80 °C.

Methylation-Specific PCR (MSP)

A modified DNA was used for two MSP reactions, both for the *CDKN2B* gene: one reaction specific for methylated DNA and other specific for unmethylated DNA. The primer sequences used were: *CDKN2B*-F(M) 5'-CGTTCG TATTTTGCGGTT-3'; *CDKN2B*-R(M) 5'-CGTACAATA ACCGAACGACCGA-3'; *CDKN2B*-F(U) 5' -TGTGATG TGTTTGTATTTTGTGGTT-3'; and *CDKN2B*-R(U) 5'-CCATACAATAACCAAACAACCAA-3' (Herman *et al.*, 1996). For all reactions the amplification conditions were: bisulfite-modified DNA; 10X PCR buffer (Invitrogen); 50 mM $MgCl_2$; 1.25 mM dNTP, and 300 ng/μL of each primer. Reactions were hot-started at 95 °C for 5 min before addition of 1.25 units of *Taq* polymerase (Invitrogen), followed by 35 cycles (30 s at 95 °C, 30 s at 61 °C for primer methylated or 60 °C for primer unmethylated, 30 s at 72 °C), and a final extension step of 4 min at 72 °C. Each PCR product was analyzed using 3% agarose gel, stained with ethidium bromide and directly visualized under UV illumination.

Sequencing of sodium bisulfite-treated DNA

Bisulfite-treated DNA was amplified by a nested-PCR protocol, using the following primers: *CDKN2B*-F1 5'-GGTTGAAGGAATAGAAATTT-3' and *CDKN2B*-R1 5'-ACACTCTTCCCTTCTTTCCC-3' for the first reaction; and *CDKN2B*-F2 5'-TTAGTTTTGGTTTTATTGG A-3' and *CDKN2B*-R2 5'-TCTCTCCTTCCTAAAAAAC C-3' for the second reaction. PCR was performed in a solution containing 10 X buffer (Biotools); 1.25 mM dNTP; 2.5 μM of each primer, and 2U of *Taq* DNA polymerase (Biotools). The PCR conditions were: 94 °C for 5 min and 55 °C for 2 min, followed by 35 cycles (72 °C for 1 min, 94 °C for 1 min, and 55 °C for 1 min) and 72 °C for 10 min for the first reaction. For the nested reaction the conditions were similar, only the annealing temperature being chan-

ged to 51 °C. The amplified products were sequenced directly in a MegaBace 1000 sequencer.

RNA isolation and reverse transcriptase PCR (RT-PCR)

Total RNA was isolated using TRIZOL (Invitrogen) and the cDNA was synthesized with the use of a High Capacity kit (Applied Biosystems). Amplification of *CDKN2B* mRNA was performed with specific primers: *CDKN2B*-RTF 5'-AACGGAGTCAACCGTTTCGG-3' and *CDKN2B*-RTR 5'-TGTGCGCAGGTACCCTGCA A-3' (Hoshino *et al.*, 2002). The PCR conditions were as follows: 10 X buffer (Biotools); 1.25 mM dNTP; 20 pmol of each primer, and 2 U of *Taq* DNA polymerase (Biotools). PCR was initiated with one cycle at 95 °C for 1 min, followed by 30 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The internal control was the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The amplified products were analyzed on 1.5% agarose gel, stained with ethidium bromide and directly visualized under UV illumination.

Results

Methylation analysis

Different methylation patterns of the *CDKN2B* gene were detected among leukemia types analyzed by MSP. The gene was methylated in four (67%) of the six ALL samples and in 16 (76%) of the 21 AML samples, but in none of the four CML samples analyzed. Regarding AML subtypes, the frequency distribution of *CDKN2B* gene methylation was: M0 1/1, M1 2/2, M2 9/10, M4 0/2, M5 3/4, and M6 1/2. In 12 AML samples, amplification was achieved with both primers, specific for methylated DNA and for unmethylated DNA, and therefore these samples were classified as hemimethylated (Figure 1). For frequency estimates, these hemimethylated samples were considered as methylated.

Expression analysis

The expression of *CDKN2B* mRNA was evaluated in 19 samples with available RNA (six methylated, five unmethylated, and eight hemimethylated) and in one normal bone marrow sample. In the methylated samples, no *CDKN2B* mRNA expression was detected (Figure 2A). On the other hand, *CDKN2B* expression was detected in the unmethylated and in some hemimethylated samples (Figures 2B and C). However, the level of expression was higher in the unmethylated than in the hemimethylated samples. The highest level of *CDKN2B* gene expression was detected in the normal bone marrow sample.

Sequencing analysis

The region analyzed (498 bp) after bisulfite treatment of the DNA is located approximately -234 to +264 bp from the transcription start site, comprising two CpG islands according to the MethPrimer program (Li and Dahiya, 2002). However, the CpG dinucleotides analyzed by sequencing contained only the region located -15 to +208 bp (223 bp) from the transcription start site that shows 26 CpG dinucleotides. The sequence amplified by MSP (147 bp), which shows 19 CpG dinucleotides, is within the sequenced region (Figure 3).

Thirteen out of the 31 samples analyzed by MSP were sequenced (one normal bone marrow, four methylated, four hemimethylated and four unmethylated). The methylation percentage for each sample was calculated as the number of methylated CpG dinucleotides in the total number of analyzed CpGs (Figure 4). The normal sample did not show any methylated CpG (data not shown). Seven out of eight samples, which were amplified with the primer set specific for the methylated DNA, presented a methylated CpG frequency of 46% to 100%. The exception was sample XIX (Figure 4), considered to be hemimethylated by MSP, which showed only 26% of methylated CpG dinucleotides. In the unmethylated samples, the percentage of methylated CpGs ranged from 15% to 46%.

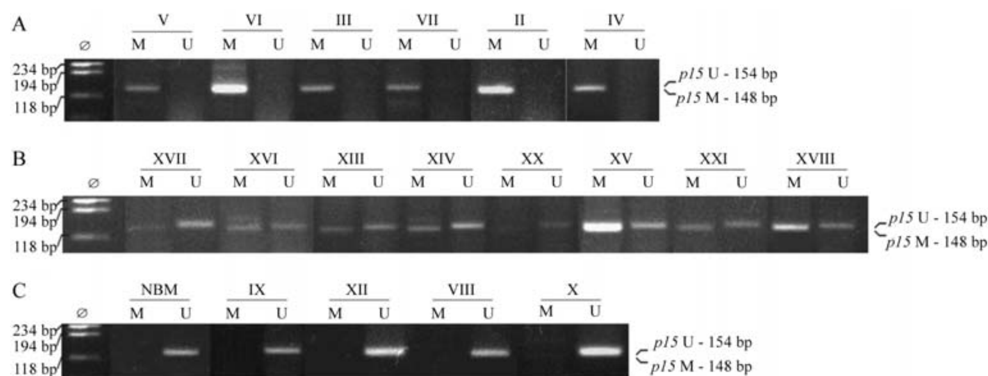

Figure 1 - MSP of *CDKN2B*. Primer sets used for amplification are designated as methylated (M) or unmethylated (U). The leukemia samples are represented in Roman numbers. NBM = normal bone marrow. (A) Samples amplified with methylated primer set. (B) Samples amplified with methylated and unmethylated primer sets. (C) Samples amplified with unmethylated primer set.

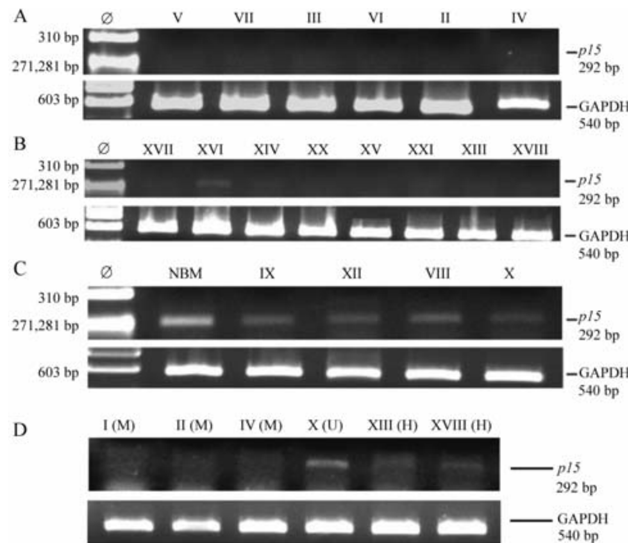

Figure 2 - *CDKN2B* mRNA expression evaluated by RT-PCR. NBM = normal bone marrow. The samples are represented in roman numbers. (A) methylated samples (B) hemimethylated samples. (C) unmethylated samples. (D) Comparative RT-PCR. *GAPDH* was used to control the integrity of the RNA samples.

Discussion

The *CDKN2B* gene encodes a 15-kDa protein that is a cyclin-dependent kinase inhibitor. The role of *CDKN2B* as a tumor suppressor in acute myelogenous leukemia (AML) was established in several previous studies (Herman *et al.*, 1996; Issa *et al.*, 1997; Wong *et al.*, 2000; Toyota *et al.*, 2001; Claus and Lubbert, 2003). Hypermethylation of *CDKN2B* CpG islands has been shown to occur in up to 80% of human AML, and this epigenetic state is associated with reduced expression (Markus *et al.*, 2007).

In this study, analysis results of the methylated state of the *CDKN2B* gene, performed by MSP, were confirmed by direct sequencing and expression analysis. Our MSP results showed that *CDKN2B* was methylated in 76% of the AML samples. These results are in agreement with previous studies that found the *CDKN2B* gene to be methylated in more than 50% of the cases (Herman *et al.*, 1996; Issa *et al.*, 1997; Toyota *et al.*, 2001; Claus and Lubbert, 2003).

Among patients with AML, Wong *et al.* (2000) reported *CDKN2B* methylation frequencies to be higher in subtypes M2, M3 or M4 than in M1, M5, M6 or M7. In nine of our 10 subtype M2 samples, the gene was methylated; for the other subtypes, the number of samples was too small to allow a correlation analysis.

In ALL, previous studies indicated different methylation frequencies for the *CDKN2B* gene, depending on the methodology used (Issa *et al.*, 1997; Melki *et al.*, 1999; Garcia-Manero *et al.*, 2002; Melki and Clark, 2002; Chim *et al.*, 2003). We found a higher frequency (67%) than that (40%) described by Chim and colleagues (2003), using the same methodology (MSP). This might be due to our smaller sample size (six samples) compared to theirs (25 samples). Even with a small number of LMC samples (four), our data showed the unmethylated state of the *CDKN2B* gene, which is in agreement with previous reports (Issa *et al.*, 1997; Toyota *et al.*, 2001).

A sample of genomic DNA usually consists of a large pool of molecules that may display methylation heterogeneity. This heterogeneity can be due to the fact that two alleles of any given genomic locus in a cell may differ in their methylation patterns, and that the DNA sample was derived from multiple cells with potentially different methylation patterns (Siegmund and Laird, 2002). This could explain the simultaneous amplification by MSP using primer sets for both methylated and unmethylated DNA. This methylation heterogeneity of samples can also be difficult to analyze by direct sequencing of sodium bisulfite-treated DNA, once it does not allow the methylation state of individual alleles to be determined. Hence, in some chromatograms the

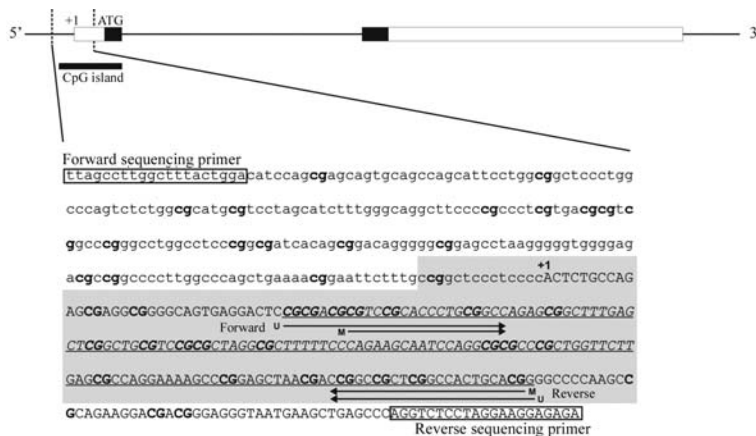

Figure 3 - *CDKN2B* gene sequence amplified by nested-PCR. The sequence analyzed by direct sequencing is highlighted in gray. The region amplified by MSP is underlined. Intron sequences, in short letters, exon sequences, in capital letters, and CpG dinucleotides in boldface. RefSeq accession NM_004936.

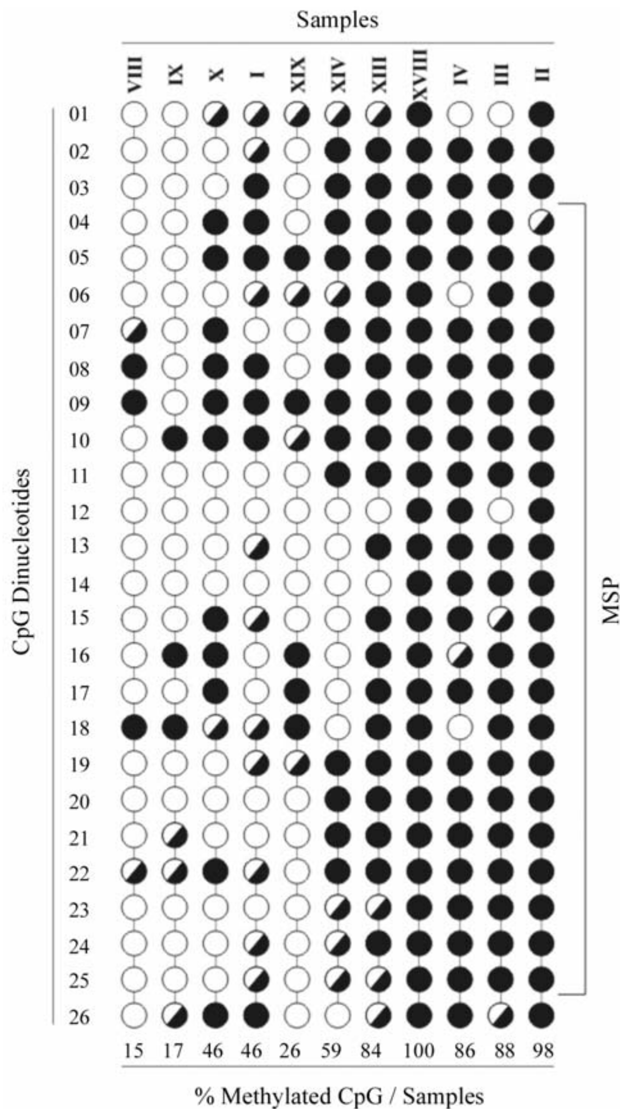

Figure 4 - Scheme of direct bisulfite sequencing of region containing 26 CpG dinucleotides. Each circle represents a CpG dinucleotide. Spacing between circles is relative. Open circle, unmethylated; black circle, methylated; partly open circle, hemimethylated. M, methylated; U, unmethylated and H, hemimethylated. The numbers at the top indicate the samples: VIII, IX and X are unmethylated; XIII, XIV, XVIII and XIX, hemimethylated; I, II, III and IV, methylated. The numbers to the left indicate the CpG dinucleotides, and those below, the percentage of methylation in each sample. The CpG dinucleotides amplified by MSP are shown on the right.

TCpG sequence was observed instead of TpG or CpG. We expected to find TpG in the case of an unmethylated dinucleotide and CpG in the case of a methylated dinucleotide. The term hemimethylated was also used to designate those CpG nucleotides for which the methylation state could not be defined by sequencing, as it occurred in CpG 04, 06 and 10 (Figure 5C).

In general, the amount of methylated CpG sites within a locus, as well as the number of methylated loci, increase in more advanced stages of cancer (Nephew and Huang, 2003). The consequence of this dynamic epigenetic gene silencing is that the degree of loss of protein production is not uniform throughout the tumor-cell population, unlike the one that is produced by the genetic changes (Jones and Baylin, 2002). We analyzed the expression of *CDKN2B* mRNA, which in general agreed both with the MSP results and with the percentage of methylated CpG dinucleotides, as determined by direct PCR sequencing. In the methylated samples that showed more than 80% of methylated CpG dinucleotides, no *CDKN2B* mRNA expression was observed by RT-PCR (Figures 2A and 4). In the unmethylated samples, a relationship between the expression level and the percentage of methylated CpG was observed, as in sample X (46% methylated CpG) and samples VIII and IX (15% and 17% methylated CpG, respectively) (Figures 2C and 4). The *CDKN2B* gene expression was very low when the gene was found to be hemimethylated by MSP (Figure 2B). *CDKN2B* mRNA expression differed between the normal bone marrow and the unmethylated samples (Figure 2C). These unmethylated samples showed a low percentage of methylated CpG, which seems to correlate with the levels of mRNA transcription.

The percentage of methylated CpGs was similar (46%) in samples X and I (unmethylated and methylated, respectively). However, their level of gene expression and the number of hemimethylated CpGs (Figures 2D and 4) were different. There were 10 hemimethylated CpGs in sample I, while in sample X only 2 hemimethylated CpGs were identified. Hence, the methylation density of the region analyzed was higher in sample I than in sample X, and this might have caused the apparent difference in *CDKN2B* gene expression observed between these samples.

Another difference between the samples was the state of the methylated CpG 3 dinucleotide at the transcription factor Sp1 site. Sp1 is associated with the transcription of the *CDKN2B* gene and is a regulator of cell cycle progression (Pagliuca *et al.*, 2000). Many factors are known to bind CpG-containing sequences, and some of them fail to bind when the CpG is methylated, therefore inhibiting transcription (Bird, 2002). CpG 3 and CpG 25 are part of two Sp1 sites (Figure 4), and in seven of the eight sequenced samples which were amplified by the specific primer set for methylated DNA at least one of these dinucleotide CpGs was methylated (Figure 4).

Methylation of specific targets may explain the observation that different hematopoietic malignancies harbor distinct methylation signatures (Rush and Plass, 2002). The cell type-specific pattern of hypermethylation suggests that the methylation of certain CpG islands may be used as disease marker and is also useful in the detection of minimal residual disease after chemotherapy. This pattern may therefore influence any adjuvant treatments (Melki and Clark, 2002) or be used as a marker for disease progression (Laird, 2003). Agrawal *et al.* (2007) showed increased *CDKN2B* methylation levels in the bone marrow of patients

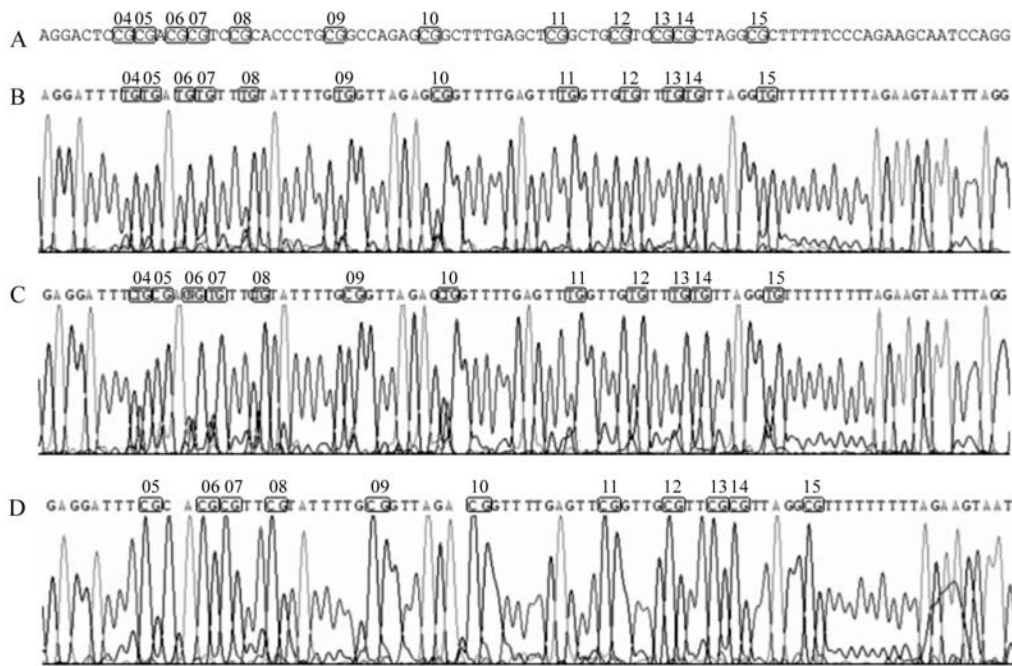

Figure 5 - Chromatogram of the direct bisulfite sequencing from CpG 4 to CpG 15 of the region: (A) Original sequence. (B) Sample IX, unmethylated. (C) Sample XIX, hemimethylated. (D) Sample II, methylated. The CpG dinucleotides are highlighted. After bisulfite treatment, the CpG dinucleotides remained as such in the methylated samples, but were converted to TpG in the unmethylated samples. In the hemimethylated samples, some CpG dinucleotides were converted but others not.

with acute leukemias in clinical remission and, according to these authors, the presence of aberrant DNA methylation in remission is a powerful indicator of a high risk of leukemia relapse.

In this first report on the methylation status of the *CDKN2B* gene in Brazilian leukemia patients, *CDKN2B* was found to be methylated, in agreement with data obtained in similar analyses performed in leukemia patients of other countries.

Acknowledgments

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Internet Resources

INCA (Instituto Nacional do Câncer), http://www.inca.gov.br (March 5, 2008).

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