Increasing Methylation of the CDKN2A Gene Is Associated with the Progression of Adult T-Cell Leukemia

Kisato Nosaka, Michiyuki Maeda, Sadahiro Tamiya, Tatsunori Sakai, Hiroaki Mitsuya, and Masao Matsuoka

Department of Internal Medicine II, Kumamoto University School of Medicine, Kumamoto 860-8556 [K. N., S. T., S. T., H. M., M. M.]; Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507 [M. M.], Japan

ABSTRACT

In this study, we examined the methylation status of the CDKN2A gene in patients with different forms of adult T-cell leukemia (ATL) using Southern blot analysis, methylation-specific PCR (MSPCR), and nucleotide sequencing. We found that the CDKN2A gene was more frequently methylated in fresh tumor cells isolated from patients with acute ATL (47%) or lymphoma-type ATL (73%) than in those with less malignant chronic (17%) and smoldering (17%) ATL. In addition, deletions of the CDKN2A gene were found in 24% of acute ATL patients; thus, abnormalities of the CDKN2A gene totaled 71% in acute ATL patients. In contrast, no CDKN2A gene methylation was found in asymptomatic carriers or uninfected individuals. Methylation of the p15 gene was not found in any samples from 36 ATL patients. Direct sequencing of the CDKN2A gene after sodium bisulfite treatment of genomic DNA revealed that the methylation of CpG sites had occurred in 24 of 32 ATL cases (75%) including chronic and smoldering ATL, even when MSPCR and the Southern blot had failed to detect CDKN2A gene methylation. Among fresh ATL samples with methylation, methylation was detected in the promoter region and exon in 17 of 24 cases, and methylation in the exon without promoter region was detected in 7 of 24 cases. In one case, the pattern of methylation proved to be different between peripheral blood cells and lymph node cells, suggesting the presence of multiple subclones with regard to methylation patterns, despite the same HTLV-I integration site. Quantitative PCR showed a marked decrease in CDKN2A mRNA expression in the cells with a methylated CDKN2A gene, especially if the promoter region was methylated. These findings suggest that CpG methylation decreases CDKN2A expression and represents a critical factor in the disease progression of ATL.

INTRODUCTION

ATL is a highly aggressive neoplasm of helper T lymphocytes and is etiologically associated with HTLV-I (1–6). HTLV-I has a unique structure, named the pX region, between env and the 3' long terminal repeat. The pX region encodes at least three proteins, Tax, Rex, and p21. Among them, Tax protein is thought to play a central role in the development of ATL. The pX region encodes a region of unknown function, named the pX region, between env and the 3' long terminal repeat. This region encodes at least three proteins, Tax, Rex, and p21. Among them, Tax protein is thought to play a central role in the development of ATL.

METHODS AND PROJECTS

ATL Patients. PBMCs or lymph node cells were obtained from patients with various subtypes of ATL [smoldering (6 cases), chronic (16 cases), acute (38 cases), and lymphoma-type (8 cases)], three asymptomatic HTLV-I carriers, and three uninfected healthy individuals. Diagnosis was made based on the criteria proposed previously (25), and monoclonal integrations of the HTLV-I provirus were confirmed by Southern blot analyses using a whole HTLV-I probe.

Cell Lines. HTLV-I transformed cell lines and ATL-derived cell lines were used in this study (26, 27). ED, ATL-43T, STK1B, ATL-55T, TL-Om1, and ATL-48T were cell lines derived from leukemic clones, as confirmed by Southern blot analysis with an HTLV-I probe or a T-cell receptor gene probe.

The abbreviations used are: ATL, adult T-cell leukemia; HTLV-I, human T-cell leukemia virus type I; CDK, cyclin-dependent kinase; PBMC, peripheral blood mononuclear cell; 5-Aza-CdR, 5-aza-2'-deoxycytidine; MSPCR, methylation-specific PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
control. To demethylate the CDKN2A gene, cell lines were grown in media supplemented with 10 μM 5-Aza-CdR (Sigma) for 3 days, and then RNAs were isolated using Trizol (Life Technologies, Inc., Paisley, United Kingdom). Reverse transcription-PCR using primers specific for the CDKN2A gene amplified transcripts of the CDKN2A gene.

**Southern Blot Analysis.** Genomic DNA isolated from PBMCs or lymph node cells was first digested with a methylation-sensitive enzyme, SacII, then digested with EcoRI, and electrophoresed in a 1.0% agarose gel, transferred to a nylon membrane, and subjected to hybridization with a probe corresponding to the CDKN2A gene.

**MSPCR.** MSPCR was performed as described previously by Herman et al. (28). In brief, 2 μg of genomic DNA (10 μl) were denatured by the addition of an equal volume of 0.6 M NaOH for 10 min, and then 208 μl of 3.6 M sodium bisulfite and 12 μl of 10 M hydroxyquinone were added. This mixture was incubated at 55°C for 16 h to convert cytosine to uracil. Treated genomic DNA was subsequently purified using the Wizard clean up system (Promega), precipitated with ethanol, and resuspended in 100 μl of distilled H2O. The genomic DNA obtained was used in MSPCR using primers as follows: CDKN2A M (methylated): sense primer 5′-TTATAGGATTGGGGCGG-3′, antisense primer 5′-CCACCAATACCCCTCCACCG-3′; CDKN2A U (unmethylated): sense primer 5′-TTATAGGATTGGGGCGG-3′, antisense primer 5′-CCACCAATACCCCTCCACCG-3′, and p15 U (methylated): sense primer 5′-CGTGGTTATTTTGTGT-3′, antisense primer 5′-CGTACAAATAACGGACACCGA-3′, and p15 U (unmethylated): sense primer 5′-CCATACAAATAACGGACACCGA-3′, antisense primer 5′-CCATACAAATAACGGACACCGA-3′. The PCR mixture contained 1× buffer with 1.5 mM MgCl2, 0.2 μl of each primer, 0.1 mM deoxynucleotide triphosphates, 2 units of Taq DNA polymerase (Promega), and 100 ng of sodium bisulfite-olean treated genomic DNAs (100 ng). Conditions of amplification were as follows: 95°C for 3 min and 35 cycles of 94°C for 30 s, 65°C for 30 s (60°C for CDKN2A U, p15 M, and p15 U primers), 72°C for 30 s, and finally 4 min at 72°C.

**Direct Sequencing after Sodium Bisulfite Treatment.** Sodium bisulfite-treated genomic DNAs (100 ng) were amplified with the CDKN2A gene-specific primers as follows (29). Primers for amplification of the promoter region of the CDKN2A gene (from −378 to −89) were 5′-TGGTGGTTGCTGGGCTGCT-3′ (sense) and 5′-TACCTATATACACACCTC-3′ (antisense). To obtain products for sequencing, a second round of PCR was performed with 20 pmol of nested primers: the sense primer, 5′-GATTTAGGATTGGGGCGG-3′, and the antisense primer 5′-CCACCAATACCCCTCCACCG-3′. Amplification was carried out for 35 cycles (30 s at 95°C, 1 min at 58°C, and 1 min at 72°C) followed by a final 4 min extension at 72°C. Primers used for amplification of the promoter and exon 1 (from −115 to 119) were as follows: for the PCR round, 5′-GAGGAGGATGGAGGATTATAT-3′ (sense) and 5′-CCACCTACTTTTAACACTT-3′ (antisense), and for the nested PCR, 5′-GGATG-3′ (sense) and 5′-CTACCTTTTAACACTT-3′ (antisense). Conditions of amplification were as follows: 35 cycles (30 s at 95°C, 1 min at 52°C, and 1 min at 72°C) and then 4 min at 72°C. Sequencing was performed using Big Dye Terminator (Applied BioSystems) with an ABI 377 autoscaler. Six HTLV-I-transformed cell lines were analyzed [ED (CL-1), 43T (CL-2), SKT-1B (CL-3), ATL-55T (CL-5), and M 5-Aza-CdR (Sigma), 43T (CL-2), SKT-1B (CL-3), ATL-55T (CL-5), and M 5-Aza-CdR (Sigma), and ATTO densitograph 4.0 (ATTO, Tokyo, Japan).

**RESULTS**

**Methylation of the CDKN2A Gene Detected by Southern Blot and MSPCR.** We analyzed the methylation of the CDKN2A gene of HTLV-I-transformed cell lines and fresh ATL cells using Southern blot analysis and MSPCR. Digestion of the CDKN2A gene with a methylation-sensitive restriction enzyme, SacII, produced a 3.3-kb band when detected with the exon 1 probe; however, when methylation occurred in the SacII site (within exon 1) of the CDKN2A gene, a 4.3-kb band was detected (data not shown). In addition to methylation, Southern blot analysis also revealed deletions of the CDKN2A gene. MSPCR was performed after sodium bisulfite treatment of the genomic DNA (Fig. 1). MSPCR relies on the fact that methylated cytosines are resistant to sodium bisulfite treatment, and thus primers can be made to distinguish between unmethylated and methylated templates. In samples with highly methylated CpG sites, only the methylated band was detected (Fig. 1, no. 3). When the CDKN2A gene was methylated partially, both methylated and unmethylated bands were found (Fig. 1, no. 6). A total of 71 samples of primary cells (38 cases with acute ATL, 8 cases with lymphoma-type ATL, 16 cases with chronic ATL, 6 cases with smoldering ATL, and 3 asymptomatic carriers) and 19 HTLV-I-associated cell lines were analyzed with Southern blot and MSPCR. This experiment detected a high incidence of methylation in both cell lines and fresh samples, and methylation was more prevalent in the more malignant forms of ATL, although MSPCR can detect methylation of CpG sites only in the exon. In contrast, no methylation was detected in asymptomatic HTLV-I carriers and uninfected healthy individuals. In acute ATL, methylation and deletion of CDKN2A was found in 47 and 24% of cases, respectively, resulting in either genetic or epigenetic changes of CDKN2A in 71% of acute ATL patients. In lymphoma-type ATL, methylation of the CDKN2A gene was found in 73% of cases without deletion. Methylation was less frequent in chronic (17%) and smoldering type (17%), as assessed by MSPCR and Southern blot analysis. Moreover, MSPCR with primers specific to the p15 gene failed to detect the methylation in 36 ATL samples (20 cases with acute ATL, 11 cases with chronic ATL, 3 cases with lymphoma-type ATL, and 2 cases with smoldering ATL). Because the CDKN2A gene is adjacent to the p15 gene, these data support the idea that the CDKN2A gene is preferentially methylated in lymphoid cells in contrast to the p15 gene.

Nineteen cell lines transformed with HTLV-I were analyzed in this study. There were two types of HTLV-I-transformed cell lines: those derived from leukemic clones (identified by comparison of leukemic and cell lines with the Southern blot method using the HTLV-I provirus or T-cell receptor gene as a probe), and those derived from nonleukemic clones. All six cell lines derived from leukemic clones...
had methylation of the CDKN2A gene; however, there were 3 methylation-negative cell lines of 13 cell lines derived from nonleukemic clones, suggesting that methylation of the CDKN2A gene is more common in leukemic cells.

**Direct Sequencing of Sodium Bisulfite-modified Genomic DNA.** A major limitation of Southern blot analysis and MSPCR is that these methods detect methylation in only a few CpG sites in the CDKN2A gene. In contrast, direct sequencing after treatment with sodium bisulfite enables us to identify the methylation state of all of the CpG sites in the amplified sequences. Therefore, we amplified and sequenced the promoter region and exon 1 of the CDKN2A gene using sodium bisulfite-treated genomic DNAs obtained from PBMCs of patients at various clinical stages (13 cases with acute ATL, 4 cases with lymphoma-type ATL, 11 cases with chronic ATL, and 4 cases with smoldering ATL) and three carriers. The level of methylation of the CpG sites (42 sites) was judged to be a methylated one. Representative data from this analysis are shown in Fig. 2. Most of the CpG sites were heavily methylated in a case of acute ATL, as shown in Fig. 2A. Only partial methylation was observed in a case of chronic ATL and another case with lymphoma type ATL (Fig. 2, B and C). A summary of this analysis is shown in Fig. 3, in which each column represents one CpG site of the CDKN2A gene and the extent of its methylation. We frequently observed complete methylation in HTLV-I-transformed cell lines, although the extent of methylation differed among cell lines. Cell lines derived from leukemic clones, like ATL-43T (CL-2), had heavily methylated CpG sites; however, MT-2 cells (CL-4), which were derived from a nonleukemic cell (30), had partially methylated CpG sites (Fig. 3). The 5' region of the promoter was particularly less methylated.

Direct sequencing after treatment with sodium bisulfite detected the methylation of the CDKN2A gene, even in cases (five cases: A-8, L-3, C-7, C-8, and C-9) in which methylation of the CDKN2A gene could not be found by Southern blot or MSPCR. Because the single methylation of a single site is unlikely to suppress transcription, only cases with more than two methylated CpG sites were judged as methylation positive. Methylation of the CDKN2A gene was detected in 24 of 32 ATL cases (75%), including chronic and smoldering ATL, and on the other hand, no methylation was detected in asymptomatic carriers (data not shown). CpG sites in exon 1 tend to be methylated in all cases. Methylation of the CDKN2A gene was observed in the exon region without upstream methylation in 7 of 32 cases. In 17 of 32 fresh ATL cases and all cell lines, methylation was also detected in the promoter region in addition to exon. In the promoter region, the −17 CpG site was preferentially methylated among fresh ATL samples (10 cases of 32; 31%).

Although none of methylation-negative cases (C-10, C-11, S-3, and S-4) among chronic and smoldering type ATL showed progression to acute ATL, the clinical progression was frequently observed in methylation-positive ATL cases (8 of 11 cases), which showed that methylation-negative ATL cases had more indolent clinical courses than methylation-positive cases. Two different subgroups among patients with chronic ATL have been reported; patients in one group progress to acute crisis within a few years, and patients in the other group have indolent clinical courses (31). Chronic ATL patients with risk factors identified previously (high WBC counts and high lactate dehydrogenase) had more methylation of the CDKN2A gene (C-1–6) when compared with those without risk factors (C-7–11).

In one case, partial methylation (~50%) of the CDKN2A gene was found in PBMCs, whereas no methylation was identified in lymph node cells (Fig. 2D). It was confirmed that both ATL cell samples had the same HTLV-I provirus integration site, showing that multiple subclones with different methylation patterns were present in the patient.

**Semiquantitative Reverse Transcription-PCR of the CDKN2A Gene.** Methylation of the CDKN2A gene may result in the suppression of its transcription, leading to decreased expression. Therefore, we analyzed the expression of the CDKN2A gene using semiquantitative PCR (30 cycles) using primers specific for the CDKN2A gene. As shown in Fig. 4A, 293 cells (Lane 1) and normal T lymphocytes stimulated in the presence of interleukin 2 (Lane 6) contain CDKN2A gene transcripts (330 bp) and were used as positive controls. The relative levels of expression of the CDKN2A gene was shown by the histogram after normalization with the GAPDH transcript. Transcripts of the CDKN2A gene could not be detected in any cell line, such as ED (Lane 3) or ATL 43T (Lane 4), which had a heavily methylated CDKN2A gene (Fig. 3), and the MT-2 cell line, which contains a partially methylated gene, showed a markedly suppressed expression of the CDKN2A gene (Lane 5). Thus, expression of CDKN2A gene was inversely correlated with its methylation. For example, ATL cells with extensive methylation, which ranged to the promoter region, were found to express low levels of CDKN2A gene transcripts (Lanes 7; A-5 in Fig. 3 and Lane 8; C-1 in Fig. 3) when compared with controls. On the other hand, ATL cells with no methylation had equivalent amounts of CDKN2A gene transcripts (Lane 9, A-13; Lane 1045).
These results suggest that the decreased expression of the CDKN2A gene is caused by the methylation of the gene. To confirm this possibility, we tested whether a demethylating agent, 5-Aza-CdR, reversed the suppressive effects of methylation. As shown in Fig. 4
B, an exposure of ATL cell lines (ED, ATL-43T, and MT-2) to 5-Aza-CdR for 3 days reactivated or potentiated the expression of CDKN2A mRNA, corroborating our observation that the methylation of the CDKN2A gene was associated with CDKN2A gene silencing in ATL cells.

DISCUSSION

Inactivation of the CDKN2A gene by deletions has been reported in cancer cells of various types, indicating that CDKN2A is a tumor suppressor gene (17–19). The methylation of CpG sites in the CDKN2A gene has been recognized as another mechanism of suppressing its transcription in lymphoma, myeloma, melanoma, and bladder cancer (23, 24, 32). The INK4 family includes the p15, p18, and p19 genes in addition to the CDKN2A gene, and the methylation of p15 was also reported in gliomas and leukemia cells (33). In contrast to the methylation of the p15 gene observed predominantly in myeloid cells, the CDKN2A gene has been reported to be preferentially methylated in lymphoid malignant cells (34), which is consistent with our findings in this study. Such preferential silencing specific for cell lineages suggests that each gene in the INK4 family is functionally predominant in a lymphoid lineage.

Deletion of the CDKN2A gene was reported in 27% (21) and 11.4% (22) of ATL cases, whereas this study revealed that methylation of this gene was a more common phenomenon in ATL, suggesting that methylation is the predominant mechanism of suppressing the expression of the CDKN2A gene in ATL cells. In this work, we reported an
increasing methylation as the disease progressed. Increasing methylation is also associated with progressive suppression of CDKN2A expression, as shown by analysis of CDKN2A transcripts. Examination of ATL cells at each clinical stage showed that methylation first occurs in the exon region and progresses to the promoter region with clinical progression of ATL. It has been reported that methylation in the exon region does not have any effect on the expression of the CDKN2A gene (29, 35). This study supports that finding because the ED and ATL-43T cell lines, which had completely methylated CDKN2A genes, did not express CDKN2A gene transcripts, whereas the MT-2 cell line with a less methylated promoter region expressed low levels of CDKN2A gene transcripts. Methylation of the promoter region is thought to be related to the silencing of CDKN2A gene transcription because 5-Aza-CdR could restore the transcription of the CDKN2A gene.

Analysis of bladder cancer cell lines with reactivated CDKN2A gene expression after 5-Aza-CdR treatment revealed that a small number of CpG sites can significantly down-regulate CDKN2A pro-moter activity (29). In this study, methylation of a specific CpG site (~17) was observed frequently in ATL samples; this site may influence the expression of the CDKN2A gene. Transcriptional repression by DNA methylation has also been reported for other tumor suppressor genes such as E-cadherin (36) and the VHL tumor suppressor gene (37). These reports suggest that methylation is a common mechanism of inhibiting tumor suppressor genes.

Among chronic ATL cases, there are variations in clinical course; some patients progress to acute or lymphoma-type ATL shortly, and other patients show no progression for a long time. Thus, the identifi- cation of the subgroup of chronic ATL cases who will progress to acute crisis is clinically important. Chronic ATL cases without CDKN2A gene methylation had more indolent clinical courses than those with methylation. Thus, analysis of the methylation status of the CDKN2A gene may be useful in predicting when the patients will progress to acute ATL.

In this study, we found different methylation patterns of the CDKN2A gene within a single patient, demonstrating the presence of subclones within leukemic cells carrying a single integration site of HTLV-I provirus. Cells with different methylation pattern of the CDKN2A gene in the same patient may have different tissue affinities, perhaps reflecting different patterns of expression of adhesion molecules.

Of various proteins associated with Tax, p16 has been shown to be functionally inactivated, resulting in cell cycle perturbation (12). However, at the late stage of leukemogenesis, such as acute or lymphoma-type ATL, the expression of tax is significantly lower than in asymptomatic carriers (38). Fresh leukemic cells in some acute ATL patients do not produce Tax in vitro because of deletion of the long terminal repeat and other mechanisms (39, 40). Thus, whether Tax expression is required in ATL cells remains unclear. On the other hand, such deletions in the HTLV-I provirus are rare in low-grade malignant ATL cases (smoldering or chronic ATL), suggesting that Tax may play an important role at such stages but may not be essential for late stages of leukemia. The increasing methylation of the CDKN2A gene shown in this study suggests that at an early stage of leukemogenesis, Tax is responsible for inactivating the p16 protein, whereas at a late stage, methylation or even deletion of the CDKN2A gene abolishes the expression of p16. Methylation might be a way to transform HTLV-I infected T cells in the absence of Tax expression. ATL cells express activation antigens like CD25 and secrete various cytokines; their phenotype resembles that of cells expressing Tax in vitro. Modification of methylation in ATL cells might fix a phenotype initially induced by Tax and replace the functions of Tax.

The progressive methylation of the CDKN2A gene in ATL cells observed in this study suggests that methylation suppresses the expression of the CDKN2A gene, which initially is functionally inac- tivated by Tax. Therefore, cells with methylated CDKN2A gene may not depend on the expression of viral proteins for their growth and thus may escape host immune surveillance.

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REFERENCES


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