

Azacitidine induces demethylation of p16INK4a and inhibits growth in adult T-cell leukemia/lymphoma

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Abstract. Adult T-cell leukemia/lymphoma (ATL) is one of the peripheral T-cell malignant neoplasms strongly associated with human T-cell leukemia virus type-I (HTLV-I). Although the viral transactivator protein Tax has been proposed to play a critical role in leukemogenesis, additional cellular events are required for the development of ATL. One of the genetic events of the disease is inactivation of tumor suppressor genes. The CDKN2A locus on chromosome 9p encodes 2 cell cycle regulatory proteins, p14ARF and p16INK4a, which share exon 2 using different reading frames. The p14ARF and p16INK4a genes have been implicated as tumor suppressor genes by their frequent mutation, deletion or promoter hypermethylation in a variety of human tumors. In this report, we describe the expression status of p14ARF and p16INK4a in 9 ATL cell lines (MT1, MT2, OKM3T, F6T, K3T, Oh13T, S1T, Su9T01 and HUT102). By reverse transcription polymerase chain reaction (RT-PCR), expression of p14ARF was not detected in one cell line (OKM3T), while expression of p16INK4a was not detected in 6 cell lines (OKM3T, MT1, MT2, Oh13T, S1T and Su9T01). In the OKM3T cell line, the shared exon 2 of the p14ARF/p16INK4a gene was deleted; however, the p16INK4a gene, was epigenetically inactivated in 5 other cells lines. In primary tumor cells obtained from ATL patients, p14ARF expression was absent in 6 of the 11 samples. We confirmed the methylation of the p16INK4a gene in MT1 and MT2 cells using the methylation-specific PCR (MSP) method. Treatment with 2.0 μ M of Azacitidine (AZA), a demethylating agent, for 72 h restored p16INK4a transcript expression and induced growth inhibition in MT2 cells. Our results demonstrate that p16INK4a is epigenetically silenced in ATL. AZA offers a potential new therapeutic approach to improve the poor outcomes associated with ATL.

Introduction

Adult T-cell leukemia/lymphoma (ATL) is an aggressive, fatal malignancy of mature CD4⁺ T lymphocytes caused by human T-cell lymphotropic virus type I (HTLV-I) infection (1). ATL has been classified into four main subtypes (2). In the relatively indolent smoldering and chronic forms, median survival is 2 years or more. In the acute and lymphoma forms, median survival time is about 13 months (3). Although a pivotal oncoprotein of HTLV-I, Tax, is strongly associated with the development of ATL (4), precise mechanisms of tumorigenesis in ATL have not been well-defined. A long period of clinical latency precedes the development of ATL (5) and only a small percentage of HTLV-I-infected individuals develop this malignancy (6), indicating that additional genetic events probably are required to develop ATL after viral infection of the target T cells. Studies to date by others and ourselves have shown deletions or mutations of several tumor suppressor genes in the pathogenesis of ATL (7-10).

p16INK4a, one of the cyclin-dependent kinase inhibitors (CDKIs), was implicated as a tumor suppressor gene. The gene harboring the CDKN2A locus on chromosome 9p21 binds to CDK4 and thereby inhibits its kinase activities (11,12), resulting in arrest of the cell cycle. The CDKN2A locus also generates another cell cycle-regulatory protein designated as alternative reading frame (ARF), which encodes a protein of 14 kDa (p14ARF) (13). The p14ARF gene has a unique exon 1 (exon 1 β), located approximately 20 kb centromeric to exon 1 of p16INK4a. Under the control of each promoter, exon 1 β and exon 1 splice into exon 2 in the CDKN2A locus, producing p14ARF and p16INK4a proteins, respectively (13,14). Therefore, the p14ARF and p16INK4a genes share the same exon 2. p14ARF interacts *in vivo* with the MDM2 protein, neutralizing MDM2-mediated degradation of p53 (15,16). Inactivation of the p14ARF and p16INK4a results from homozygous deletion or promoter methylation (17-19). We have previously reported the homozygous deletion of p16INK4a in a significant portion of patients with ATL (7). In addition, inactivation of p14ARF and p16INK4a by hypermethylation has been described in common neoplasms including T-cell lymphoma (20).

Azacitidine (AZA) has been administered as a new anti-neoplastic agent for myelodysplastic syndrome (MDS) with

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good outcome (21-24). The agent induces the demethylation of gene promoter via inhibition of DNA methyltransferase (DNMT) that methylates cytosine residues in eukaryotic DNA (22,25).

Because prognosis of ATL is extremely poor, new treatment agents need to be identified. In this study, we investigated the inactivation status of p14ARF and p16INK4a in ATL cell lines as well as primary ATL cells obtained from patients with various clinical types of ATL. We demonstrate that AZA is a potential therapeutic candidate for ATL.

Materials and methods

Cells. Nine ATL cell lines, MT1, MT2, OKM3T, F6T, K3T, Oh13T, S1T, Su9T01 and HUT102 were analyzed. OKM3T was originally established by Miyamoto *et al* (26) and obtained from the Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). F6T, K3T, Oh13T, S1T and Su9T01 (27) were established in our institute. K562 was used as a negative control. Cell lines were maintained by serial passages in RPMI containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 U/ml streptomycin, and incubated in standard tissue culture incubators with 5% CO₂-95% air at 37°C. In addition to these cell lines, mononuclear cells were isolated from either peripheral blood samples from 10 leukemia type ATL patients (6 acute, 3 chronic and 1 undetermined) or from a lymph node sample of a lymphoma type ATL patient. All patients provided written informed consent.

DNA extraction and polymerase chain reaction (PCR) analysis. DNA was isolated with the Sepagene kit (Sanko Jun-yaku, Tokyo, Japan) according to the manufacturer's instructions. All samples were screened for exon 2 of p14ARF/p16INK4a with PCR by our method (7). The primers p16-1S1 (5'-CTTCCTGGACACGCTGGTGGTGCTG-3') and p16-2A2 (5'-GTACAAATTCTCAGATCATCAGTCC-3') were used to obtain a 219-bp fragment of exon 2 of p14ARF/p16INK4a. The cycling conditions were one cycle of 15 min at 95°C, 36 cycles of 60 sec at 95°C, 30 sec at 55°C, 120 sec at 72°C, and one cycle of 10 min at 72°C. The PCR was repeated ≥ 2 times.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis. Total-RNA was extracted with a single-step method using RNeasy reagent (Sawady, Tokyo, Japan). RT reactions were performed using a First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocol. Products were then used as templates in PCR analysis. The mRNA transcription of the p14ARF and p16INK4a genes was evaluated by RT-PCR. The primer pairs specific for these genes were as follows; p14ARF sense, 5'-GGTTTTCGTGGTTCACATCCCGCG-3' and antisense, 5'-CAGGAAGCCCTCCCGGGCAGC-3'; p16INK4a sense, 5'-TTCGGCTGACTGGCTGGCCA-3' and antisense, 5'-AGCTCCTCAGCCAGGTCCAC-3'. The length of the p14ARF and p16INK4a PCR products was 254 and 330 bp, respectively. Efficacy of reverse transcription was controlled in each sample by PCR amplification of β -actin using the following primer pair: sense, 5'-TACATGGGTGGGGTGTGAA-3' and antisense, 5'-AAGAGAGGCATCCTCACCT-3'.

Methylation-specific PCR (MSP). To determine the methylation status at the 5'CpG island in the p16INK4a promoter region, we performed MSP. Bisulfite converts unmethylated cytosine residues to uracil, but methylated cytosines remain nonreactive. PCR amplifies uracil as thymine while methylated cytosines are only amplified as cytosines. MSP distinguishes unmethylated from methylated alleles in a given gene based on sequence changes after bisulfite treatment of DNA using primers designed for either methylated or unmethylated DNA (28). The CpGenome™ DNA Modification kit (Chemicon International, Inc., Temecula, CA) was used for MSP, as recommended by the supplier (29). Genomic DNA was treated with sodium bisulfite. PCR amplification was performed using p16INK4a promoter gene fragment-specific primers for either methylated or unmethylated DNA (CpG WIZ™ Amplification kit, Chemicon International, Inc.) (30). The unmethylated p16INK4a was amplified with a primer pair 5'-TTTTTGGTGTAAAGGGTGGTGTAGT-3' and 5'-CACAAAACCTCACTCACAACAA-3', which yielded a fragment of 132 bp. The primers for methylated p16INK4a were 5'-GTGTTAAAGGGCGGCGTAGC-3' and 5'-AAAACCCTCAC TCGCGACGA-3', which yielded a PCR product of 122 bp. Universal methylation DNA CpGenome™ (Chemicon International, Inc.) was used as a control for methylated DNA. PCR-amplified products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining under UV wave.

Treatment with AZA. To determine the optimal dose of AZA (Sigma, St. Louis, MO), MT2 cells were treated with increasing doses of AZA. MT2 cells at a concentration of 1×10^5 /ml were treated with 0.5, 1.0 or 2.0 μ M of AZA. After cells were cultured for 72 h with AZA, RNA was harvested. The expression of p16INK4a mRNA was detected by RT-PCR.

Growth inhibition with AZA on MT2 cells. MT2 cells at a concentration of 1×10^5 /ml were treated with 2.0 μ M of AZA. The number of viable cells was counted on days 2, 3, 4 and 5 using the trypan blue dye exclusion procedure. All experiments were repeated 3 times.

Results

Deletion of the p14ARF and p16INK4a gene. Nine cell lines were examined for deletions of the exon 2 of p14ARF/p16INK4a. The K562 cell line served as a negative control. The gene was deleted in only one cell line, OKM3T (Fig. 1).

Expression of p14ARF. Expression of p14ARF mRNA was examined in 9 cell lines by RT-PCR. Only OKM3T did not express p14ARF mRNA (Fig. 2) due to deletion of the gene. The p14ARF mRNA was also amplified for primary ATL samples. Of the 11 ATL samples analyzed, p14ARF was not expressed in 6 (4 acute, 1 chronic and 1 undetermined), while 5 samples expressed p14ARF (2 acute, 1 lymphoma and 2 chronic) (Fig. 3).

Expression of p16INK4a. Among 9 cell lines, the OKM3T cell line in which exon2 of the p16INK4a gene was deleted did not express p16INK4a. An additional 5 cell lines (MT1, MT2,

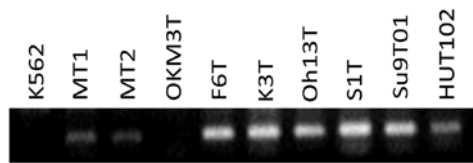


Figure 1. Deletion of the p14ARF and p16INK4a exon 2 gene in ATL cell lines. Among the 9 cell lines, the shared exon 2 of the p14ARF/p16INK4a gene was deleted in the OKM3T cell line. K562 was served as a negative control.

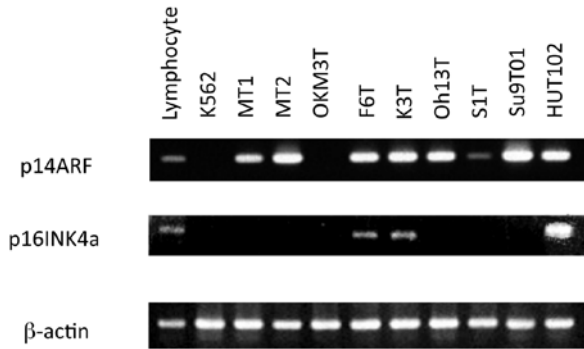


Figure 2. Expression of p14ARF and p16INK4a mRNA in ATL cell lines by RT-PCR. Among the 9 cell lines, the OKM3T cell line in which the shared p14ARF/p16INK4a exon 2 was deleted did not express either p14ARF or p16INK4a. Five other cell lines (MT, MT2, Oh13T, S1T and Su9T01) in which the p14ARF/p16INK4a exon 2 was not deleted, lacked expression of p16INK4a.

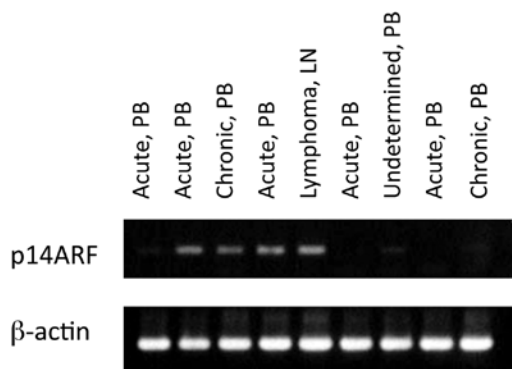


Figure 3. Expression of p14ARF in primary ATL samples. Representative cases are shown. Acute, PB, sample from peripheral blood in acute ATL; chronic, PB, sample from peripheral blood in chronic ATL; lymphoma, LN, sample from lymph node in lymphoma ATL; undetermined, PB, sample from peripheral blood in undetermined type ATL.

Oh13T, S1T and Su9T01) which had the p14ARF/p16INK4a exon 2 lacked expression of p16INK4a (Fig. 2).

Analysis of methylation of the p16INK4a gene. Because MT1 and MT2 did not express p16INK4a, the presence of methylation in the p16INK4a promoter region gene was assessed in the MT1 and MT2 cell lines using MSP. DNA obtained from lymphocytes from healthy volunteers was used as an unmethylated control. The K562 cell line served as a negative control. Both MT1 and MT2 cell lines displayed a band only when amplified with primers for methylated and not for unmethylated DNA (Fig. 4).

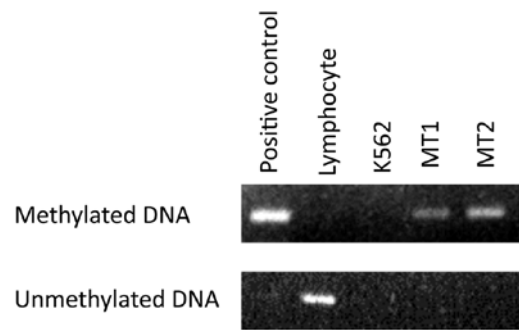


Figure 4. Methylation of the p16INK4a gene in MT1 and MT2 by methylation specific PCR (MSP). Both MT1 and MT2 cell lines displayed a band only when amplified with primers for methylated DNA but not unmethylated DNA. DNA obtained from lymphocytes from healthy volunteers was used as an unmethylated control. The K562 cell line served as a negative control.

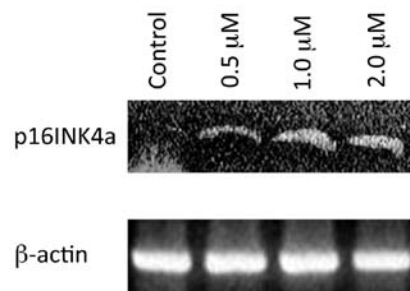


Figure 5. Restoration of p16INK4a gene expression by Azacitidine (AZA). AZA induced the demethylation of the p16INK4a gene promoter. Substantial demethylation of p16INK4a was observed after 72 h of treatment with 0.5 μ M AZA. With higher doses of AZA (1.0 or 2.0 μ M), the expression of p16INK4a mRNA was clearly up-regulated.

Restoration of p16INK4a gene expression by AZA. AZA-induced the demethylation of the p16INK4a gene promoter. Substantial demethylation of p16INK4a was observed after 72 h of treatment with 0.5 μ M AZA. With higher doses of AZA (1.0 or 2.0 μ M), the expression of p16INK4a mRNA was clearly up-regulated (Fig. 5).

AZA induces growth inhibition of MT2 cells. MT2 cells at a concentration of 1×10^5 /ml were treated with 2.0 μ M of AZA. Growth of 2.0 μ M-treated cells was inhibited compared with the control, although the difference was marginal. On Day 3, viable cell numbers in the control and 2.0 μ M AZA treated MT2 cells were 4.8 and 4.1×10^6 /ml, respectively. After obtaining maximum cell growth, viable cells decreased in both groups (Fig. 6).

Discussion

ATL is triggered by infection of the human T-cell leukemia virus type I (HTLV-I) (1), with its pivotal oncoprotein Tax being strongly associated with the development of ATL (4). However, expression of Tax disappears in most ATL cells, suggesting that alternative mechanisms may be involved in the development of ATL. A statistical analysis revealed that five independent genetic events are probably required to develop ATL after viral infection of the target T cells (31).

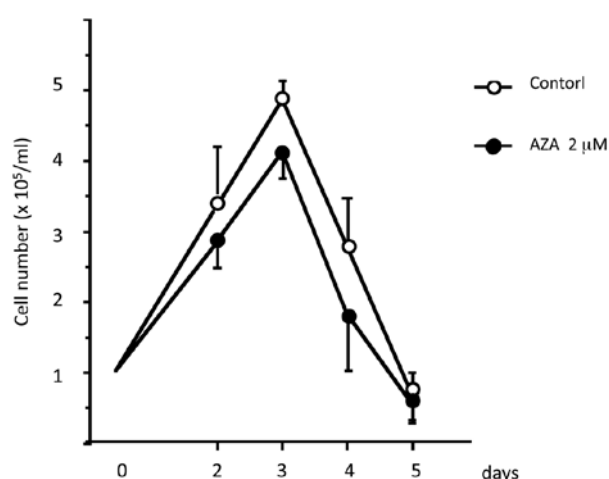


Figure 6. Azacitidine (AZA) induces growth inhibition of MT2 cells. MT2 cells at a concentration of $1 \times 10^5/\text{ml}$ were treated with $2.0 \mu\text{M}$ of AZA. Growth of $2.0 \mu\text{M}$ treated cells was inhibited compared with the control, although the difference was marginal. On Day 3, viable cell numbers in control and $2.0 \mu\text{M}$ AZA-treated MT2 cells were $4.8 \times 10^6/\text{ml}$ and $4.1 \times 10^6/\text{ml}$, respectively. After obtaining maximum cell growth, viable cells decreased in both groups.

p16INK4a, one of the CDKIs, resides in the CDKN2A locus on chromosome 9p. p16INK4a inhibits the catalytic activity of the CDK4/cyclin D complexes and blocks G1 to S transition in the cells (11,12). The CDKN2A locus also generates another cell cycle-regulatory protein, p14ARF (13,14). The human p14ARF protein causes arrest in the cell-cycle progression with an accumulation of cells in both G1 and G2/M, through binding to MDM2, which interferes with p53-MDM2 complex formation and proteasome degradation (32,33). Data suggest that both p14ARF and p16INK4A act as tumor suppressors whose inactivation contributes to the development of human tumors.

The homozygous deletion of the p16INK4a gene has been detected at a very high rate in many types of solid tumors as well as leukemias (34,35) especially in T-ALL (36,37). In ATL, we have shown that several tumor suppressor genes including p16INK4a, p18INK4c, and Rb were altered (7,10,36,37). The p16INK4a gene was frequently deleted in ATL (7). Furthermore, methylation of the 5' CpG island in the p16INK4a gene is associated with transcriptional silencing of the gene in many neoplasms including leukemias and lymphomas (38).

We screened the expression of p14ARF and p16INK4a in ATL cell lines. One (OKM3T) and 6 (MT1, MT2, OKM3T, Oh13T, SIT and Su9T01) of the 9 cell lines did not express p14ARF and p16INK4a mRNA, respectively. Absence of p16INK4a mRNA in the OKM3T cell line was due to deletion of the shared exon 2 gene of p14ARF/p16INK4a, while in the remaining 5 cell lines, it was due to gene silencing. Although we did not examine the genetic status of exon 1 of p16INK4a, deletion of the gene is unlikely, because, in our previous study, exons 1 and 2 of p16INK4a were homozygously deleted simultaneously in all ATL samples (7).

In our study, most of ATL cell lines expressed p14ARF mRNA, while 6 of 11 primary samples lacked expression of p14ARF. Esteller *et al* reported that p14ARF was epigenetically inactivated by hypermethylation in colorectal cancer cell lines

as well as primary colorectal cancer samples (30). p14ARF genomic alterations are found in T-cell acute lymphocytic leukemias (39). Inactivation, either by deletion or methylation, of p14ARF may account for developing ATL.

DNA hypermethylation is the main epigenetic modification in tumorigenesis (40). Cytosine analogues such as 5-Aza-2'-deoxycytidine and AZA can induce expression of several tumor suppressor genes with unmethylated CpGs in malignant cells by inhibiting DNMTs (41,42). In recent reports, treatment with low-dose AZA was very encouraging in older patients with MDS and AML (24). Although the target genes of AZA in MDS have not been well-defined, one of the candidates is the p15INK4b gene that shares a great deal of nucleotide sequence homology with p16INK4a and is localized 25 kb upstream of the p16INK4a locus on chromosome 9p21.

Nosaka *et al* reported the epigenetic inactivation of p16INK4a in ATL and suggested that methylation was a critical factor in disease progression (43). We demonstrate the demethylation in p16INK4a and induction of growth inhibition of the ATL cell line, MT2, with AZA. The expression of p16INK4a mRNA was up-regulated by treatment with $0.5 \mu\text{M}$ of AZA for 72 h. AZA showed inhibition of cell growth of ATL cell lines. Because no obvious toxicity of AZA was observed in phase 3 clinical trials for patients with high-risk MDS (21), our studies suggest a therapeutic approach for ATL. Although single administration of AZA is not sufficient to eradicate ATL cells, concomitant use with other anti-tumor agents such as thalidomide may be more effective as has been reported in MDS (23). This is particularly interesting because ATL cells are highly resistant to the induction of apoptosis and there is still no beneficial treatment other than allogeneic hematopoietic stem cell transplantation for this acute and fatal disease.

In conclusion, p16INK4a is epigenetically inactivated in ATL. AZA should be further investigated as a novel therapeutic agent for the management of ATL.

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