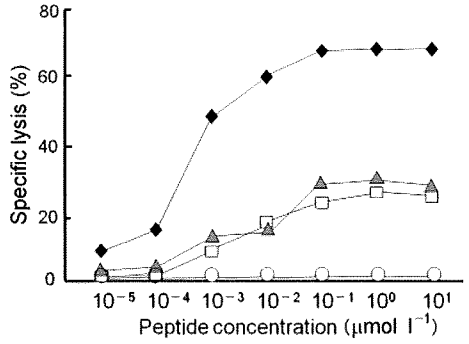


**Fig. 3.** Effect of HBZ peptide concentration on cytotoxicity of HBZ-1 against B-LCL and HTLV-1-transformed T-cell lines. The cytotoxicity of HBZ-1 against autologous B-LCL (◆), autologous HTLV-1-transformed $CD4^+$ T-cell line (□), HLA-A*0201-positive allogeneic HTLV-1-transformed $CD4^+$ T-cell line (▲), and HLA-A*0201-negative allogeneic HTLV-1-transformed $CD4^+$ T-cell line (○), loaded with various concentrations of HBZ peptide for 1 h was determined by 4 h $^{51}Cr$ release assays at an E:T ratio of 5:1. Experiments were performed three times and representative data are shown. The cytotoxic activities of HBZ-1 against HBZ peptide-loaded autologous and HLA-A*0201-positive allogeneic HTLV-1-transformed $CD4^+$ T-cell lines are significantly lower than that against HBZ peptide-loaded autologous B-LCL ($P<0.01$ by paired sample *t*-test).

are still obscure. Notably, we also found that the expression levels of *HBZ* mRNA and HBZ protein in HTLV-1-infected cells were not parallel. This suggests that the machinery for translation of *HBZ* mRNA and/or the degradation pathway of HBZ protein may differ, and that the degree of this difference may be determined by cell type.

Another interesting finding of this study was that HTLV-1-infected T lymphocytes were relatively resistant to CTL-mediated cytotoxicity, compared with B-LCLs. We have previously reported that myeloma cells are more sensitive to the perforin-mediated granule exocytosis pathway of CTLs than lymphoma cells, and that susceptibility of membranes to perforin is an important factor determining the sensitivity of target cells to CTL-mediated cytotoxicity (Azuma *et al.*, 2004). Resistance to perforin-mediated cytotoxicity, possibly induced by membrane-stabilizing mechanisms, has been demonstrated in human cytomegalovirus-infected fibroblasts (Odeberg *et al.*, 2003). In addition, it has been reported that the human leukaemia cell line ML-2 can be recognized by natural killer (NK) cells but is resistant to NK cell-mediated cytotoxicity because of a defect in perforin binding (Lehmann *et al.*, 2000). Some molecules, including cathepsin B (Balaji *et al.*, 2002) and PI-9 (Bird *et al.*, 1998), have been proposed to play an important role in protection of target cells from CTL-mediated cytotoxicity. Although there has been no obvious evidence in the relationship between the resistance of HTLV-1-infected T lymphocytes to CTL-mediated cytotoxicity and cathepsin B or PI-9, further studies focusing on these molecules seem to be needed to clarify the mechanism underlying the resistance of HTLV-1-infected T lymphocytes to CTL-mediated cytotoxicity.

In summary, we conclude that HBZ is certainly immunogenic for CTLs, but that ATL cells cannot be lysed by HBZ-specific CTLs. Although further clarification of the mechanism underlying the resistance of HTLV-1-infected T lymphocytes to CTLs is needed, our present data strongly suggest the presence of a novel mechanism that allows HTLV-1 to evade immune recognition.

The authors declare no competing financial interests.

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*Tumorigenesis and Neoplastic Progression*

# Multi-Step Aberrant CpG Island Hyper-Methylation Is Associated with the Progression of Adult T–Cell Leukemia/Lymphoma

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**Aberrant CpG island methylation contributes to the pathogenesis of various malignancies. However, little is known about the association of epigenetic abnormalities with multistep tumorigenic events in adult T cell leukemia/lymphoma (ATLL). To determine whether epigenetic abnormalities induce the progression of ATLL, we analyzed the methylation profiles of the *SHP1, p15, p16, p73, HCAD, DAPK, hMLH-1,* and *MGMT* genes by methylation specific PCR assay in 65 cases with ATLL patients. The number of CpG island methylated genes increased with disease progression and aberrant hypermethylation in specific genes was detected even in HTLV-1 carriers and correlated with progression to ATLL. The CpG island methylator phenotype (CIMP) was observed most frequently in lymphoma type ATLL and was also closely associated with the progression and crisis of ATLL. The high number of methylated genes and increase of CIMP incidence were shown to be unfavorable prognostic factors and correlated with a shorter overall survival by Kaplan-Meyer analysis. The present findings strongly suggest that the multistep accumulation of aberrant CpG methylation in specific target genes and the presence of CIMP are deeply involved in the crisis, progression, and prognosis of ATLL, as well as indicate the value of CpG methylation and CIMP for new diagnostic and prognostic biomarkers. *(Am J Pathol 2010, 176:402–415; DOI: 10.2353/ajpath.2010.090236)***

Adult T cell leukemia/lymphoma (ATLL) is an aggressive malignant disease of CD4-positive T lymphocytes caused by infection with human T-lymphotropic virus type I (HTLV-1).[1–3] HTLV-1 causes ATLL in 3% to 5% of infected individuals after a long latent period of 40 to 60 years.[4] Advanced acute ATLL has a poor prognosis. ATLL is divided into four stages: smoldering, chronic, lymphoma, and acute types.[5] The smoldering and chronic types are indolent, but the acute and lymphoma types are aggressive ATLL characterized by resistance to chemotherapy and a poor prognosis.[5,6] Such a long latent period suggests that a multistep leukemogenic/lymphomagenic mechanism is involved in the development of ATLL, although the critical events in the progression have not been characterized. The pathogenesis of HTLV-1 has been investigated intensively in terms of the viral regulatory protein HTLV-1 Tax or Rex, which is supposed to play key roles in the HTLV-1 leukemogenesis/lymphomagenesis, as well as the recently discovered HTLV-1 basic leucine zipper factor.[6–8] We and others have reported the progression mechanism of ATLL from various genetic aspects, including specific chromosome abnormalities,[9–14] changes of characteristic HTLV-1 Tax and Rex protein expression pattern,[14] and aberrant expression of the *SHP1*,[10,15] *p53*,[16,17] *MEL1S*,[17] *DRS*,[18] and ASY/Nogo[19]

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genes, although, the detailed mechanism triggering the onset and progression of ATLL remains to be elucidated.

On the other hand, epigenetic aberration processes have been recognized to play another important role in carcinogenesis.[20,21] The aberrant hypermethylation of CpG islands within the promoter and 5′-regions of genes is the most widely studied epigenetic abnormality in cancer and is associated with loss of gene function. Target genes of aberrant hypermethylation of CpG islands seem to be tumor type-specific[22,23] and current efforts are concentrated on finding ways to exploit the diagnostic and therapeutic implications of these abnormalities.[24,25] A comprehensive knowledge of the methylation profile of a given tumor may provide important information for risk assessment, diagnosis, monitoring, and treatments.[20,26]

Recently, we have reported that a frequent epigenetic aberration of DNA hypermethylation associated with the *SHP1* gene silencing has been identified in a wide range of hematopoietic malignancies.[15,27] SHP1 is a nonreceptor type protein-tyrosine phosphatase, which acts as a negative regulator in hematopoietic cells. A decrease or loss of the *SHP1* gene expression may be related to the malignant transformation in lymphoma and leukemia cells.[15,27]

Multiple genes have recently been shown to be methylated simultaneously (a condition termed CpG island methylator phenotype: CIMP)[28,29] in various types of human malignancies. This mechanism is a fundamental process involved in the development of many tumors. However, analysis of CIMP in leukemia/lymphoma is limited,[30] and CIMP status has not yet been elucidated in ATLL.

Our goal in the current study was to clarify the contribution of epigenetic abnormalities to disease development and progression. We comparatively evaluated the methylation status of eight genes in four stages of ATLL: smoldering, chronic, lymphoma, and acute types, HTLV-1 carriers and healthy donor peripheral blood mononuclear cells (PBMCs). In addition, we addressed the questions of whether CIMP is associated with ATLL development and/or progression and if HTLV-1 infection induces aberrant DNA hypermethylation in HTLV-1 carriers.

## Materials and Methods

### Patients

PBMCs or lymph node tissues were collected from 16 healthy volunteers and 65 patients with HTLV-1 carrier ($n$ = 10), smoldering type ($n$ = 15), chronic type ($n$ = 5), acute type ($n$ = 15), and lymphoma type ATLL ($n$ = 20). These samples were collected at the Department of Pathology & Oncology, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University as the collaboration with Okayama University Hospital, Fukuoka University Hospital and Imamura Bun-in Hospital. Informed consent was obtained from all of the patients. This study was approved by the Institutional Review Board at Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University and related hospitals, in accordance with the Declaration of Helsinki. The mean and SD of age (years) in each group was as follows; healthy volunteers, 40.1 ± 11.3; HTLV-1 carrier, 48.2 ± 11.4; smoldering-, 57.1 ± 9.7; chronic-, 54.4 ± 13.9; acute-, 60.8 ± 9.0; and lymphoma-type ATLL, 65.5 ± 13.3. The mean age of healthy volunteers was matched with that of HTLV-1 carriers to eliminate the possibility of DNA methylation by aging. The diagnosis of ATLL was based on the clinical features, hematological characteristics, and monoclonal integrations of the HTLV-1 provirus by Southern blot analyses.[5] HTLV-1 proviral DNA load was determined by real time PCR as described.[31] The mononuclear cell fraction was isolated by Ficoll/Hypaque according to the manufacturer's protocol. Genomic DNA was isolated using QIAamp DNA Mini Kit (QIAGEN GmbH., Germany).

### Methylation-Specific PCR

The methylation status of the *SHP1, p15, p16, p73, HCAD, DAPK, hMLH-1,* and *MGMT* genes were analyzed using a methylation-specific polymerase chain reaction (MSP) assay. These genes include negative regulators of the Jak/STAT signaling pathway (*SHP1*), tumor suppressor genes associated with the cell cycle (*p15, p16, p73*), cell adhesion and metastasis processes (*HCAD*), apoptosis (*DAPK*), and DNA repair (*hMLH-1, MGMT*) related genes. After bisulfite-treatment, genomic DNA was amplified by PCR using unmethylated (U) or methylated (M) DNA specific primer sets (Table 1).[15,32] Nested PCR was performed for the *SHP-1, p16*, and *HCAD* genes.

PCR was performed using the following cycling conditions: 95°C for 10 minutes, at 94°C for 30 s, an annealing step for 1 minute, and an extension step at 72°C for 1 minute followed by a final extension step of 72°C for 7 minutes, with 20 μl of reaction mixture including 0.4 μmol/L each primer, 1 μl of bisulfite-treated DNA, 1× PCR gold buffer, 200 μmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, and 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster, CA).

To determine the methylation status, DNA methylation was standardized using a DNA specimen from PBMCs of healthy volunteers as the negative control in each experiment. CpGenome Universal Methylated DNA (Chemicon International Inc., Temecula, CA) or DNA from healthy PBMCs treated with SssI methylase (New England BioLabs Inc., Beverly, MA) were used as methylation-positive control. The SssI methylase reaction was performed in a total volume of 400 μl with 2 μg of genomic DNA, 40 μl NEB buffer 2, 2 μl SAM (32 mmol/L SAM), and 8 units SssI methylase at 37°C for 24 hours.

### DNA Bisulfite Sequencing

The PCR products were cloned to pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Independent plasmid clones were purified from several bacterial colonies, and were subjected to a sequencing reaction with BigDye Terminator Cycle Sequencing Ready Reaction kit V2.0 (Applied Bio-

**Table 1.** Primer Sequences and PCR Conditions for MSP Analysis

| Genes | Primer sequences forward | Primer sequences reverse | Product size (bp) | AT °C | No. of cycles |
|---|---|---|---|---|---|
| *p15* | | | | | |
| U | 5′-TGTGATGTGTTTGTATTTTGTGGTT-3′ | 5′-CCATACAATAACCAAACAACCAA-3′ | 148 | 60 | 40 |
| M | 5′-GCGTTCGTATTTTGCGGTT-3′ | 5′-CGTACAATAACCGAACGACCGA-3′ | 154 | 64 | 40 |
| *p73* | | | | | |
| U | 5′-AGGGGATGTAGTGAAATTGGGGTTT-3′ | 5′-ATCACAACCCCAAACATCAACATCCA-3′ | 69 | 66 | 35 |
| M | 5′-GGACGTAGCGAAATCGGGGTTC-3′ | 5′-ACCCCGAACATCGACGTCCG-3′ | 60 | 63 | 35 |
| *DAPK* | | | | | |
| U | 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′ | 5′-CAAATCCCTCCCAAACACCAA-3′ | 106 | 64 | 35 |
| M | 5′-GGATAGTCGGATCGAGTTAACGTC-3′ | 5′-CCCTCCCAAACGCCGA-3′ | 98 | 64 | 35 |
| *hMLH1* | | | | | |
| U | 5′-TTTTGATGTAGATGTTTTATTAGGGTTGT-3′ | 5′-ACCACCTCATCATAACTACCCACA-3′ | 124 | 60 | 35 |
| M | 5′-ACGTAGACGTTTTATTAGGGTCGC-3′ | 5′-CCTCATCGTAACTACCCGCG-3′ | 153 | 60 | 35 |
| *MGMT* | | | | | |
| U | 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ | 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ | 91 | 66 | 35 |
| M | 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ | 5′-GCACTCTTCCGAAAACGAAACG-3′ | 81 | 66 | 35 |
| *SHP1* | | | | | |
| 1st | 5′-GGGTTGTGGTGAGAAATTAATTAG-3′ | 5′-CCTCAAATACAACTCCCAATACC-3′ | | 64 | 35 |
| U | 5′-GGATTGTTTGGGTTTGTATGTGT-3′ | 5′-AACAAAAAAAAAACACAACCAACCC-3′ | 250 | 64 | 25 |
| M | 5′-GGATCGTTTGGGTTCGTATGC-3′ | 5′-ACAAAAAAAAAACGCGACCGACC-3′ | 251 | 64 | 25 |
| *p16* | | | | | |
| 1st | 5′-CAGAGGGTGGGGCGGACCGC-3′ | 5′-CGGGCCGCGGCCGTGG-3′ | | 54 | 35 |
| M | 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ | 5′-CAACCCCAAACCACAACCATAA-3′ | 151 | 69 | 25 |
| U | 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ | 5′-GACCCCGAACCGCGACCGTAA-3′ | 150 | 69 | 21 |
| *HCAD* | | | | | |
| 1st | 5′-TTGGAAAAGTGGAATTAGTTGG-3′ | 5′-CCTCTTCCCTACCTAAAACA-3′ | | 54 | 35 |
| U | 5′-GTAAAATGAGGGAGTGTTAGG-3′ | 5′-AAACACACCCAACAACCCCTCT-3′ | 260 | 50 | 25 |
| M | 5′-TCGCGGGGTTCGTTTTTCGC-3′ | 5′-GACGTTTTCATTCATACACGCG-3′ | 243 | 69 | 25 |

*U, unmethylated sequences; M, methylated sequences; AT, annealing temperatures.

systems, Foster City, CA) and applied on ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

## *Flow Cytometry*

PBMCs obtained from patients with HTLV-1 carrier and acute type ATLL were analyzed using antibodies of mouse phycoerythrin-conjugated anti-CD4 (clone RPA-74; eBiosciences, San Diego, CA), allophycocyanin-conjugated anti-CD25 (eBiosciences, San Diego, CA). Flow cytometry was performed with FACSAria (BD Biosciences, NJ) using FlowJo (Tree Star, Inc. Ashland, OR) software.

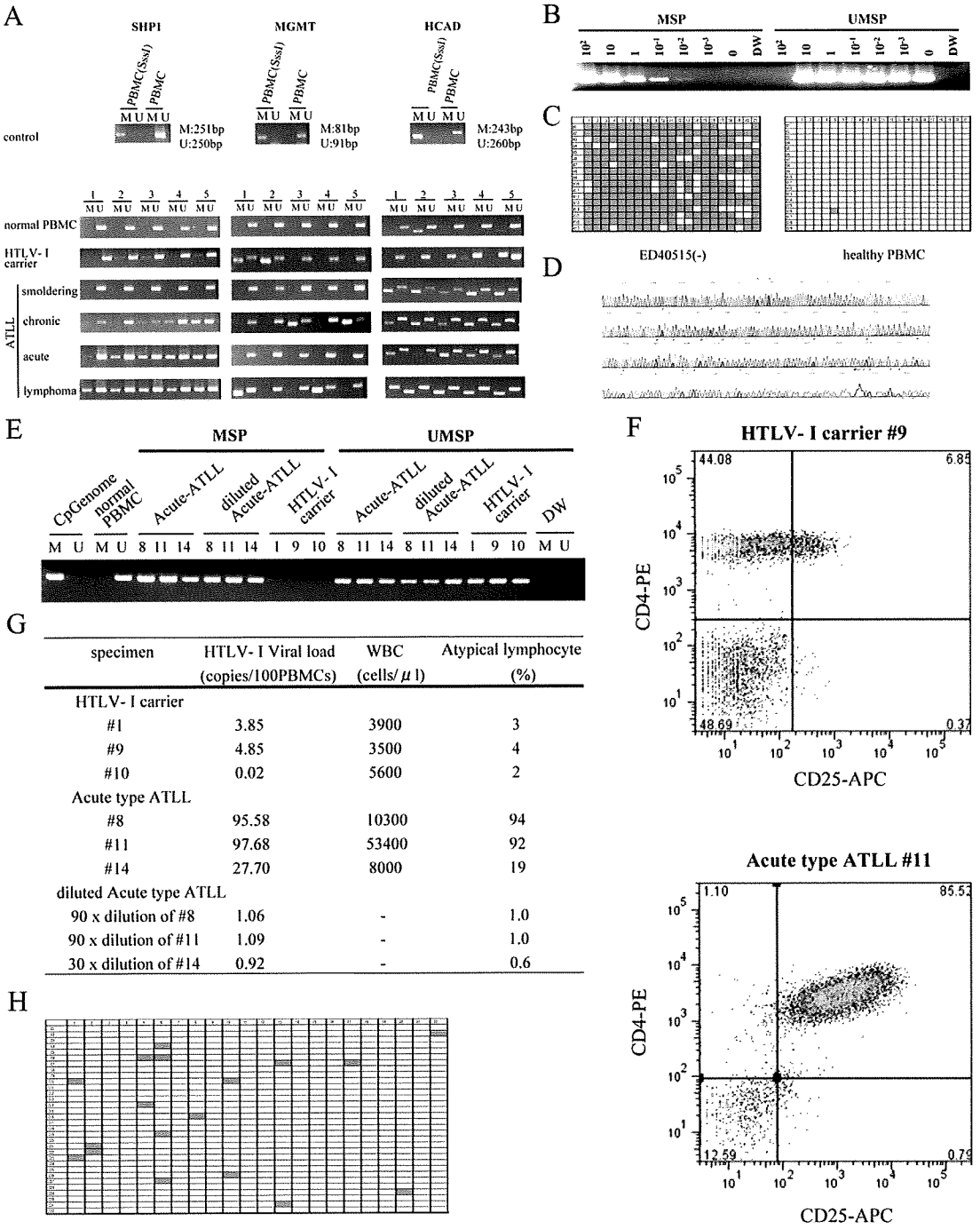
## *Immunohistochemistry*

Specimens obtained from patients with ATLL were processed routinely by fixation in 10% formaldehyde and embedding in paraffin. Immunohistological examination used a panel of antibodies specific for SHP1 (C19; affinity purified rabbit polyclonal antibody against the c-terminus of human SHP1; Santa Cruz, CA), p16 (Ab-4 clone 16P04; mouse monoclonal antibody against full length human p16, Thermo Scientific, CA), and CD4 (clone 4B12; mouse monoclonal antibody, MBL, Japan), CD25 (clone 4C9; mouse monoclonal antibody, Novocastra, UK). Immunohistochemical staining was performed as described previously.[27] We used the normal reactive lymph node as a positive control for *SHP1*, CD4, and CD25, and endocervical squamous cell carcinoma as a positive control for *p16*.

Triple-staining of fluorescent immunohistochemistry for SHP1, CD25, and Haexist33258 were performed to skin and lymph node specimens obtained from smoldering or chronic type ATLL, comparing with lymph node specimens from lymphoma type ATLL. The following secondary antibodies were used: goat Alexa fluor 488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, CA) for detection of rabbit anti-SHP1 IgG, goat Alexa fluor 555-conjugated anti-mouse IgG (Molecular Probes, Eugene, CA) for mouse anti-human CD25 IgG. Haexist33258 (Sigma-Aldrich, Japan) was used to counterstain the cell nuclei.

## *Statistical Analyses*

The methylation frequencies between the two phases were analyzed using Fisher's exact tests. The methylated genes of each phase were compared using the two-sample Mann-Whitney's *U*-tests. The overall survival was analyzed by Kaplan–Meyer method and then it was compared using the log-rank test. For all tests, probability values of $P < 0.05$ were regarded as statistically significant. The program SPSS for Windows, Release 11.5 was used for the statistical analysis.




| specimen | HTLV- I Viral load (copies/100PBMCs) | WBC (cells/μl) | Atypical lymphocyte (%) |
|---|---|---|---|
| HTLV- I carrier | | | |
| #1 | 3.85 | 3900 | 3 |
| #9 | 4.85 | 3500 | 4 |
| #10 | 0.02 | 5600 | 2 |
| Acute type ATLL | | | |
| #8 | 95.58 | 10300 | 94 |
| #11 | 97.68 | 53400 | 92 |
| #14 | 27.70 | 8000 | 19 |
| diluted Acute type ATLL | | | |
| 90 x dilution of #8 | 1.06 | - | 1.0 |
| 90 x dilution of #11 | 1.09 | - | 1.0 |
| 30 x dilution of #14 | 0.92 | - | 0.6 |

**Figure 1.** MSP and UMSP analysis of normal PBMC, HTLV-1 carriers, and ATLL patients. **A:** Representative results of MSP/UMSP of ATLL specimens; PBMC (SssI) was used as a positive control; healthy volunteer PBMC used as negative control; U, unmethylated DNA; and M, methylated DNA. PCR products' sizes are shown on the right. The number on each lane indicates patients' specimen number. **B:** To evaluate the sensitivity of MSP/UMSP in the present condition, various template DNA ratio of ATLL tumor cell line ED40515(−), showing hypermethylation of the *SHP1* gene, to normal PBMC without methylation of that gene from 100, 10, 1, 0.1, 0.01, 0.001, to 0%, analyzed by MSP/UMSP. **C:** The results of a bisulfite sequencing analysis of ED40515(−) and healthy PBMC: filled box is methylated and open box is unmethylated. Horizontal axis shows the CpG sites and vertical axis shows the patient specimen. **D:** Representative data of bisulfite sequencing. **E:** To evaluate the effects of HTLV-1 infected cells and/or neoplastic ATLL cells content on MSP/UMSP, specimens of acute type ATLL were diluted to about 1% content of neoplastic ATLL cells with normal PBMCs, which was less than the content of HTLV-1 infected cells in carrier specimens. HTLV-1 viral load, white blood cell counts, and content of atypical lymphocytes (%) of each carrier and acute type ATLL specimen were summarized. **F:** The typical results of flow cytometry showed that $CD4^+CD25^+$ T cells, which was enriched with HTLV-1 infected cells and/or neoplastic ATLL cells, were contained in acute type ATLL patient #11 (85.52%) and in HTLV-1 carrier #9 (6.85%) specimens. **G:** Summary of the clinical data including HTLV-1 viral load, white blood cell counts, and content of atypical lymphocytes in HTLV-1 carriers and acute type ATLL specimen. **H:** Results of a bisulfite sequencing analysis of the *SHP1* gene with HTLV-1 carrier specimen, which showed faint signal in MSP analysis. Filled box, methylated; open box, unmethylated. Horizontal axis shows the CpG sites and vertical axis shows the patient specimen.

**Table 2.** Methylation Profile of Eight Genes in Normal PBMC, HTLV- I Carrier, and Various Types of ATLL

| Age | Sex | *SHP1* | *p15* | *p16* | *p73* | *hMLH1* | *MGMT* | *DAPK* | *HCAD* | *CIMP* |
|---|---|---|---|---|---|---|---|---|---|---|
| **Normal PBMC ($n$ = 16)** | | | | | | | | | | |
| 49 | M | | | | | | | | | |
| 46 | M | | | | | | | | | |
| 31 | M | | | | | | | | | |
| 29 | F | | | | | | | | | |
| 26 | F | | + | | | | | | | |
| 29 | F | | + | | | | | | + | |
| 27 | F | | | | | | | | | |
| 54 | M | | | | | | | | + | |
| 43 | M | | | | | | | | + | |
| 30 | F | | | | | | | | | |
| 29 | F | | | | | | | | + | |
| 52 | M | | + | | | | | | | |
| 43 | M | | | | | | | | | |
| 56 | F | | | | | | | | | |
| 57 | F | | | | | | | | + | |
| 40 | M | | | | | | | | + | |
| % methylation | | 0% (0/16) | 19% (3/16) | 0% (0/16) | 0% (0/16) | 0% (0/16) | 0% (0/16) | 0% (0/16) | 38% (6/16) | 0% (0/16) |
| **HTLV-1 carrier ($n$ = 10)** | | | | | | | | | | |
| 50 | F | | | | | | | | | |
| 52 | F | | | | | | + | | | |
| 38 | M | | | | + | | | | | |
| 49 | M | + | | | | | | | | |
| 57 | M | | | | | | + | | | |
| 42 | M | | | | | | | | + | |
| 72 | F | + | | | | | | | | |
| 34 | F | + | + | | + | | | | | + |
| 36 | M | | + | | | | | + | + | + |
| 52 | F | | | | + | + | | + | + | + |
| % methylation | | 30% (3/10) | 20% (2/10) | 0% (0/10) | 30% (3/10) | 10% (1/10) | 20% (2/10) | 20% (2/10) | 30% (3/10) | 30% (3/10) |
| **Smoldering type ($n$ = 15)** | | | | | | | | | | |
| 66 | M | | | | | | | | + | |
| 56 | F | | | | | | | | + | |
| 62 | F | | | | | | | | + | |
| 53 | M | | | | | | | | + | |
| 48 | F | + | | | | | | | + | |
| 43 | F | | | + | | | | | + | |
| 63 | M | | | | | | | + | + | |
| 54 | M | | | | | | | + | + | |
| 48 | F | | | | | | | + | + | |
| 51 | F | | + | | | | | | + | |
| 58 | M | + | + | | | | | | + | + |
| 55 | F | | | | + | | | + | + | + |
| 79 | M | | + | | | | | + | + | + |
| 49 | F | | | | | | + | + | + | + |
| 71 | M | + | + | + | | | | + | + | + |
| % methylation | | 20% (3/15) | 27% (4/15) | 13% (2/15) | 7% (1/15) | 0% (0/15) | 7% (1/15) | 47% (7/15) | 100% (15/15) | 33% (5/15) |
| **Chronic type ($n$ = 5)** | | | | | | | | | | |
| 64 | M | | | | | | | | + | |
| 68 | M | + | | | | | | + | + | + |
| 34 | F | | | | + | | + | + | + | + |
| 47 | M | + | + | | | | + | | + | + |
| 59 | F | + | + | | | | | + | + | + |
| % methylation | | 60% (3/5) | 40% (2/5) | 0% (0/5) | 40% (1/5) | 0% (0/5) | 40% (2/5) | 60% (3/5) | 100% (5/5) | 80% (4/5) |

(*table continues*)

**Table 2.** *Continued*

| Age | Sex | *SHP1* | *p15* | *p16* | *p73* | *hMLH1* | *MGMT* | *DAPK* | *HCAD* | *CIMP* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Acute type ($n$ = 15) | | | | | |
| 60 | F | | | | | | | | | |
| 64 | M | | | | | + | | | + | |
| 60 | M | + | | | | | | | | |
| 58 | M | | | | | | | + | + | |
| 73 | M | + | | | | | | | + | |
| 45 | F | + | | | | | | | + | |
| 65 | F | + | | | | | | | + | |
| 47 | F | + | | | | + | | | + | + |
| 67 | F | + | | | | | | + | + | + |
| 67 | F | + | | | | | | + | + | + |
| 53 | F | + | | | | | | + | + | + |
| 53 | F | + | | | | | | + | + | + |
| 55 | F | + | + | | | | | + | + | + |
| 72 | M | + | + | | | | | + | + | + |
| 73 | M | + | + | | | + | | + | + | + |
| % methylation | | 80% (12/15) | 20% (3/15) | 0% (0/15) | 0% (0/15) | 20% (3/15) | 0% (0/15) | 53% (8/15) | 87% (13/15) | 53% (8/15) |
| | | | | | Lymphoma type ($n$ = 20) | | | | | |
| 47 | M | | | | | | | | + | |
| 70 | M | | | | | | + | + | | |
| 47 | M | + | + | | | | | | + | + |
| 74 | M | + | | | | | + | + | | + |
| 70 | M | + | | + | | | | | + | + |
| 59 | F | + | | | | | | + | + | + |
| 41 | M | + | | | | | | + | + | + |
| 64 | F | + | | | | | | + | + | + |
| 77 | M | + | | | | | | + | + | + |
| 57 | M | + | | + | | + | | | + | + |
| 36 | F | + | | + | | | | + | + | + |
| 74 | M | + | | | | | + | + | + | + |
| 47 | M | + | | | | | + | + | + | + |
| 67 | M | + | | + | | | | + | + | + |
| 69 | M | + | | + | | | + | + | + | + |
| 82 | F | + | | + | + | | | + | + | + |
| 48 | F | + | + | | | | + | + | + | + |
| 67 | F | + | + | | + | | + | + | + | + |
| 58 | M | + | | + | + | | + | + | + | + |
| 75 | M | + | + | + | | | + | + | + | + |
| % methylation | | 90% (18/20) | 20% (4/20) | 40% (8/20) | 15% (3/20) | 5% (1/20) | 45% (9/20) | 80% (16/20) | 90% (18/20) | 90% (18/20) |
| Total % methylation | | *SHP1* 60% (39/65) | *p15* 23% (15/65) | *p16* 15% (10/65) | *p73* 12% (8/65) | *hMLH1* 8% (5/65) | *MGMT* 22% (14/65) | *DAPK* 55% (36/65) | *HCAD* 63% (41/65) | *CIMP* 58% (38/65) |

The methylation profile, showing the + represents methylated specimens, while the blank columns represent unmethylated specimens. M, male; F, female.

## Results

### Sensitivity and Accuracy of MSP/UMSP Analyses

The methylation status of the *SHP1, p15, p16, p73, HCAD, DAPK, hMLH-1,* and *MGMT* genes were analyzed by MSP assay in normal PBMCs ($n$ = 16), HTLV-1 carriers ($n$ = 10), smoldering type ($n$ = 15), chronic type ($n$ = 5), acute type ($n$ = 15), and lymphoma type ATLL ($n$ = 20). Representative results of the MSP assay are shown in Figure 1A. To assess the sensitivity of MSP (Methylation Specific PCR)/UMSP (Un-methylation Specific PCR) in the present experimental condition, various template DNA ratio of ATLL neoplastic cell line ED40515 (−), showing strong CpG island hypermethylation of the *SHP1* gene to normal PBMCs without methylation of that gene from 100, 10, 1, 0.1, 0.01, 0.001 to 0% was analyzed by bisulfite sequencing analysis, thus showing that present system of MSP/UMSP has the sensitivity to detect a 0.01% presence of ATLL neoplastic cell ED40515 (−) among normal PBMCs (Figure 1, B–D). Next, we investigated the possibility whether different proportions of HTLV-1 infected cells and/or neoplastic cells among carrier stage, smoldering type, chronic type, acute type, and lymphoma type ATLL may affect on the difference of MSP/UMSP, HTLV-1 viral load and atypical lymphocytes content analyses were performed as well as flow cytometry analysis (Figure 1, E–G). Representative HTLV-1 carriers ($n$ = 3) and acute type ATLL ($n$ = 3) specimens were selected, showing HTLV-1 viral load to 3.85, 4.85, and 0.02 copies/100

PBMCs in HTLV-1 carriers and 95.58, 97.68, and 27.70 copies/100 PBMCs in acute type ATLL specimens respectively. Atypical lymphocyte contents were 3%, 4%, and 2% in HTLV-1 carriers and 94%, 92%, and 19% in acute type ATLL in each specimen. Typical flow cytometric analyses showed that $CD4^+CD25^+$ T cells fraction, which was enriched with HTLV-1 infected cells and/or neoplastic ATLL cells, contained 12.5 times higher number of cells in acute type ATLL patient #11 (85.52%) than in HTLV-1 carrier #9 (6.85%). These data indicate that acute type ATLL specimens contain HTLV-1 infected cells and/or neoplastic ATLL cells about 10 to 50 times more than in HTLV-1 carrier specimens. To estimate the effect of content of HTLV-1 infected cells and/or neoplastic ATLL cells on MSP/UMSP, specimens of acute type ATLL were diluted to about 1% content of neoplastic ATLL cells with normal PBMCs, which was less than the content of HTLV-1 infected cells in carrier specimens (Figure 1, E–G). Results of MSP/UMSP showed that MSP signals in diluted acute type ATLL specimens were almost as strong as those in original acute type ATLL specimens in contrast to no signals in HTLV-1 carrier specimens. This clearly indicated that the different content of HTLV-1 infected cells and/or neoplastic ATLL cells among HTLV-1 carriers and various types of ATLL specimens has no prominent effect on MSP/UMSP signal strength in the present conditions. Some cases of HTLV-1 carriers and indolent type ATLL specimen showed faint or intermediate signals with MSP/UMSP analyses. One of these specimens of HTLV-1 carriers was analyzed with bisulfite sequencing, showing that many clones contained several methylated CpG sites. It is quite different methylation profile compared with that of normal PBMCs (Figure 1H).

## *Aberrant CpG Methylation during the Progression of ATLL*

The methylation profile of the *SHP1, p15, p16, p73, HCAD, DAPK, hMLH-1,* and *MGMT* genes was summarized in Table 2. Normal PBMC from 16 healthy volunteers showed negative signals of DNA methylation in every gene except for faint methylation-positive signals of the *p15* (15.4% [2/13]) and *HCAD* genes (30.8% [4/13]). An overview of the methylation profile presented the characteristic pattern, showing that the total number of methylated genes gradually increased from healthy donors to HTLV-1 carrier, smoldering type, chronic type, acute type, and lymphoma type ATLL (Table 2). This tendency was more prominent in the distribution of the number of CpG island methylated genes among these categories, indicating that distribution profile shifted from a small number of methylated genes to larger numbers according to the progression from healthy volunteer to HTLV-1 carrier, indolent (smoldering and chronic) type and aggressive (acute and lymphoma) type of ATLL (Figure 2). Moreover, the average number of methylated genes significantly increased according to the progression of the ATLL stages: from 0.5 genes in normal PBMC to1.6 genes in HTLV-1 carrier, 2.2 in smoldering type, 3.2 in chronic type, 2.6 in acute type, and 3.9 genes in lymphoma type- ATLL ($P < 0.05$, Mann-Whitney's *U*-test.). Specifically, the CpG island methylation frequency of the *SHP1* gene clearly increased from 0% (0/16) in normal PBMC to 30% (3/10) in HTLV-1 carrier, 20% (3/15) in smoldering type, 60% (3/5) in chronic type, 80% (12/15) in acute type, and 90% (18/20) in lymphoma type-ATLL (Table 2).

## *Specific Gene Methylation During Progression of ATLL*

Next, statistical significance of the differences in methylation frequencies in each gene between two clinical stages of ATLL was analyzed in detail using Fisher's exact test (Table 3). This demonstrated that the methylation frequency of several specific genes showed statistically significant differences between two clinical stages. The *SHP1* and *p73* genes were methylated at the stage of infection to become a HTLV-1 carrier from healthy individual ($P < 0.046$). The *SHP1* gene was significantly highly methylated at the crisis of ATLL from the carrier state to acute type or lymphoma type ATLL and also during the disease progression from the smoldering to aggressive type: acute type or lymphoma type ATLL ($P < 0.05$). The *HCAD* gene was statistically significantly hypermethylated at the crisis of indolent and aggressive type of ATLL from the carrier state ($P < 0.05$). For the *DAPK, p16,* and *MGMT* genes, these genes were hypermethylated during the progression to lymphoma type ATLL. It is noteworthy that the methylation profile of lymphoma type ATLL was quite different from that of acute type; the *p16* (40%, 8/20) and *MGMT* (45%, 9/20) genes were specifically hypermethylated in lymphoma type ATLL, on the other hand, none of them (0%, 0/15) were methylated in acute type ATLL (Tables 2 and 3). CIMP was defined as the presence of more than or equal to three methylated genes among the eight genes investigated, according to the distribution of the number of methylated genes (Figure 2). CIMP was detected in 0% (0/16) of normal PBMC, 30% (3/10) of HTLV-1 carrier, 33% (5/15) of smoldering type ATLL, 80% (4/5) of chronic type ATLL, 53% (8/15) of acute type ATLL, and 90% (18/20) of lymphoma type ATLL (Table 2). The incidence of CIMP in lymphoma type ATLL was significantly higher than that in HTLV-1 carrier, smoldering type- and acute type-ATLL ($P < 0.05$, Fisher's exact test), thus indicating that CIMP was associated with the progression to lymphoma type ATLL (Table 2, Table 4). In addition, the correlation between presence of CIMP and specific gene methylation was investigated, thus revealing that the methylation of the *SHP1, p16, DAPK, p15,* and *HCAD* genes closely correlated with the incidence of CIMP ($P < 0.05$, Fisher's exact test; Table 4). This indicates that the methylation of these genes strongly contributes to the presence of CIMP.

## *Gene Silencing Associated with CpG Island Methylation*

To make sure the gene silencing associated with CpG hypermethylation, protein expression was examined with

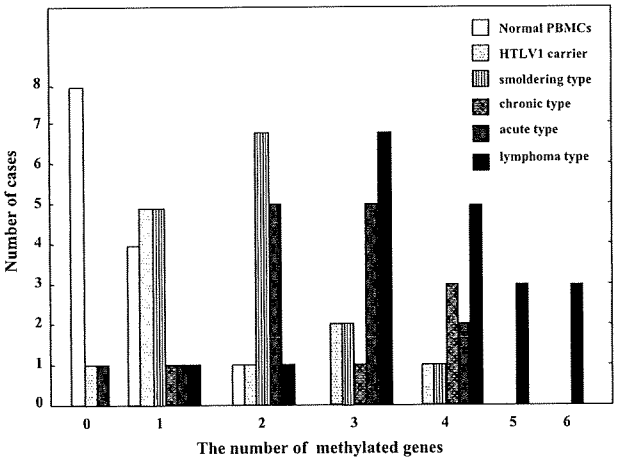



**Figure 2.** Distribution of the number of methylated genes in normal PBMC, HTLV-1 carrier, smoldering-, chronic-, acute-, and lymphoma-type ATLL.

smoldering type, chronic type, and lymphoma type ATLL specimens by immunohistochemistry, thus showing a typical ATLL cell phenotype to be clearly positive for CD4 (Figure 3A-B) and CD25 (Figure 3C). SHP1 protein expression was detected in the cytoplasm of the quiescent small lymphocytes of the mantle zone and interfollicular lymphocytes in the reactive lymphoid hyperplasia as a positive control (Figure 3D). In contrast, all of the lymphoma type ATLL specimens showed no expression of SHP1 ($n$ = 2) (Figure 3F) in addition to our previous results of entirely no expression of SHP1 protein in lymphoma type ATLL specimens ($n$ = 9) with tissue microarray analysis.[10,27] The lymphoma type ATLL specimen showed no signal of p16 expression with immunohistochemistry (Figure 3G) in contrast to the strong positive staining in endocervical squamous cell carcinoma, which was positive control (Figure 3E). These observations were consistent with the CpG hypermethylation of the *p16* gene.

In addition, expression pattern of SHP1 protein in skin and/or lymph node specimens from smoldering type (skin; $n$ = 2, lymph node; $n$ = 1) and chronic type ATLL (skin; $n$ = 1, lymph node; $n$ = 2) was examined with triple-staining of fluorescent immunohistochemistry for SHP1, CD25, and Haexist33258, comparing with lymphoma type ATLL. Expression of SHP1 protein was completely abolished in $CD25^+$ lymphoma type ATLL specimen (Figure 3N, O, P), which was clearly contrast to the strong expression of SHP1 protein in interfollicular lymphocytes of reactive lymphoid hyperplasia (Figure 3H, I, J). Expression strength of SHP1 protein in the skin and/or lymph node of smoldering type and chronic type ATLL was intermediate, which was consistent with the faint or intermediate MSP signals of the *SHP1* gene (Figure 3K, L, M).

## *Clinical Significance of CpG Island Methylation*

To clarify the clinical significance of the number of methylated genes and CIMP, overall survival was analyzed with Kaplan–Meyer method and was compared using Log-Rank test (Figure 4, A and B). The overall survival significantly decreased according to the increase of the number of methylated genes ($P$ = 0.008). The overall survival of CIMP(+) patients was quickly decreased, thus showing a poor prognosis in comparison with CIMP(−) cases ($P$ = 0.002).

Finally, the simultaneous methylation between two specific genes was investigated. A positive correlation was detected between methylation of specific genes (such as the *SHP1* and *DAPK*, $P$ = 0.001; *SHP1* and *p16*, $P$ = 0.038; *SHP1* and *HCAD*, $P$ = 0.038; *DAPK* and *MGMT*, $P$ = 0.009; and *DAPK* and *HCAD* genes, $P$ = 0.000), indicating that the *SHP1*, *p16*, *DAPK*, and *HCAD* genes were methylated simultaneously with statistical significance ($P < 0.05$, Fisher's exact test; Table 5).

## *Discussion*

ATLL is a T cell malignancy that occurs after a 40- to 60-year period of clinical latency in about 3% to 5% of all HTLV-1-infected individuals. ATLL is an interesting model for the investigation of multistep lymphomagenesis/leukemogenesis. The presence of age-dependent accumulation of leukemogenic events within HTLV-1-infected T cells have been suggested before the development of ATLL, and the approximate number of independent leukemogenic events in ATLL is estimated to be five.[9] The progression mechanism of ATLL has been studied from various genetic aspects,[9–19] although, the detailed mechanism triggering the onset and progression of ATLL remains to be elucidated. An epigenetic mechanism may be another possibility to induce the progression and onset of ATLL.[33] Epigenetic regulatory mechanisms are finely controlled by chromatin modification and various key epigenetic regulators, including the DNA methyltransferases,[34,35] histone deacetylases,[36] histone acetyltransferase,[37] trithorax group proteins,[38] polycomb group proteins,[38,39] methylated DNA binding protein,[40] heterochromatin protein 1,[41] and non-coding RNA.[42] One important candidate for an epigenetic mechanism associated with malignant transformation is gene silencing associated with aberrant CpG island DNA hypermethylation. Generally, CpG island DNA methylation is strictly regulated in the course of development and tissue specific differentiation responding to the changes in the microenvironment.

Assessment of the sensitivity of present MSP/UMSP analyses showed that MSP/UMSP has the high sensitivity to detect a 0.01% presence of ATLL neoplastic cell ED40515 (−), having hypermethylated CpG island in the specific genes, among normal PBMCs (Figure 1, B–D), suggesting the present system of MSP/UMSP is an extremely sensitive method to detect neoplastic cells in the crisis of HTLV-1 carriers to ATLL. The analysis of MSP/UMSP with specimens of acute type ATLL were diluted to about 1% content of neoplastic ATLL cells with normal PBMCs, which was less than the content of HTLV-1 infected cells in carrier specimens (Figure 1, E–G), clearly showed that the different proportion of HTLV-1 infected cells and/or neoplastic ATLL cells from range of HTLV-1 carriers (usually 1% to 5% content of HTLV-1 infected

**Table 3.** Correlation between Methylation of Eight Genes and Progression of ATLL

| | *SHP1* | | *p16* | | *DAPK* | | *hMLH1* | | *MGMT* | | *p73* | | *p15* | | *HCAD* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | U | M | U | M | U | M | U | M | U | M | U | M | U | M | U |
| Normal PBMC | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 3 | 13 | 6 | 10 |
| HTLV-I carrier | 3 | 7 | 0 | 10 | 2 | 8 | 1 | 9 | 2 | 8 | 3 | 7 | 2 | 8 | 3 | 7 |
| *P* value | **0.046** | | – | | 0.138 | | 0.385 | | 0.138 | | **0.046** | | 1.000 | | 1.000 | |
| Normal PBMC | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 3 | 13 | 6 | 10 |
| Smoldering type | 3 | 12 | 2 | 13 | 7 | 8 | 0 | 15 | 1 | 14 | 1 | 14 | 4 | 11 | 15 | 0 |
| *P* value | 0.101 | | 0.226 | | **0.002** | | – | | 0.484 | | 0.484 | | 0.685 | | **0.000** | |
| Normal PBMC | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 3 | 13 | 6 | 10 |
| Chronic type | 3 | 2 | 0 | 5 | 3 | 2 | 0 | 5 | 2 | 3 | 1 | 4 | 2 | 3 | 5 | 0 |
| *P* value | **0.006** | | – | | **0.006** | | – | | **0.048** | | 0.238 | | 0.553 | | **0.035** | |
| Normal PBMC | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 3 | 13 | 6 | 10 |
| Acute type | 12 | 3 | 0 | 17 | 8 | 7 | 3 | 12 | 0 | 15 | 0 | 15 | 3 | 12 | 13 | 2 |
| *P* value | **0.000** | | – | | **0.001** | | 0.101 | | – | | – | | 1.000 | | **0.009** | |
| Normal PBMC | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 0 | 16 | 3 | 13 | 6 | 10 |
| Lymphoma type | 18 | 2 | 8 | 12 | 16 | 4 | 1 | 19 | 9 | 11 | 3 | 17 | 4 | 16 | 18 | 2 |
| *P* value | **0.001** | | **0.005** | | **0.000** | | 1.000 | | **0.002** | | 0.557 | | 0.675 | | **0.045** | |
| HTLV-I carrier | 3 | 7 | 0 | 10 | 2 | 8 | 1 | 9 | 2 | 8 | 3 | 7 | 2 | 8 | 3 | 7 |
| Smoldering type | 3 | 12 | 2 | 13 | 7 | 8 | 0 | 15 | 1 | 14 | 1 | 14 | 4 | 11 | 15 | 0 |
| *P* value | 0.653 | | 0.350 | | 0.176 | | 0.400 | | 0.346 | | 0.267 | | 0.545 | | **0.000** | |
| HTLV-I carrier | 3 | 7 | 0 | 10 | 2 | 8 | 1 | 9 | 2 | 8 | 3 | 7 | 2 | 8 | 3 | 7 |
| chronic type | 3 | 2 | 0 | 5 | 3 | 2 | 0 | 5 | 2 | 3 | 1 | 4 | 2 | 3 | 5 | 0 |
| *P* value | 0.287 | | – | | 0.167 | | 0.667 | | 0.407 | | 0.593 | | 0.407 | | **0.019** | |
| HTLV-I carrier | 3 | 7 | 0 | 10 | 2 | 8 | 1 | 9 | 2 | 8 | 3 | 7 | 2 | 8 | 3 | 7 |
| Acute type | 12 | 3 | 0 | 15 | 8 | 7 | 3 | 12 | 0 | 15 | 0 | 15 | 3 | 12 | 13 | 2 |
| *P* value | **0.018** | | – | | 0.105 | | 0.468 | | 0.150 | | 0.052 | | 0.687 | | **0.007** | |
| HTLV-I carrier | 3 | 7 | 0 | 10 | 2 | 8 | 1 | 9 | 2 | 8 | 3 | 7 | 2 | 8 | 3 | 7 |
| Lymphoma type | 18 | 2 | 8 | 12 | 16 | 4 | 1 | 19 | 9 | 11 | 3 | 17 | 4 | 16 | 18 | 2 |
| *P* value | **0.002** | | **0.022** | | **0.003** | | 0.563 | | 0.175 | | 0.306 | | 0.694 | | **0.002** | |
| Smoldering type | 3 | 12 | 2 | 13 | 7 | 8 | 0 | 15 | 1 | 14 | 1 | 14 | 4 | 11 | 15 | 0 |
| Chronic type | 3 | 2 | 3 | 2 | 3 | 2 | 0 | 5 | 2 | 3 | 1 | 4 | 2 | 3 | 5 | 0 |
| *P* value | 0.131 | | 0.553 | | 0.500 | | – | | 0.140 | | 0.447 | | 0.483 | | – | |
| Smoldering type | 3 | 12 | 2 | 13 | 7 | 8 | 0 | 15 | 1 | 14 | 1 | 14 | 4 | 11 | 15 | 0 |
| Acute type | 12 | 3 | 0 | 15 | 8 | 7 | 3 | 12 | 0 | 15 | 0 | 15 | 3 | 12 | 13 | 2 |
| *P* value | **0.003** | | 0.241 | | 0.500 | | 0.112 | | 0.500 | | 1.000 | | 0.500 | | 0.241 | |
| Smoldering type | 3 | 12 | 2 | 13 | 7 | 8 | 0 | 15 | 1 | 14 | 1 | 14 | 4 | 11 | 15 | 0 |
| Lymphoma type | 18 | 2 | 8 | 12 | 16 | 4 | 1 | 19 | 9 | 11 | 3 | 17 | 4 | 16 | 18 | 2 |
| *P* value | **0.000** | | **0.087** | | **0.045** | | 0.571 | | **0.015** | | 0.619 | | 0.473 | | 0.319 | |
| Chronic type | 3 | 2 | 0 | 5 | 3 | 2 | 0 | 5 | 2 | 3 | 1 | 4 | 2 | 3 | 5 | 0 |
| Acute type | 12 | 3 | 0 | 15 | 8 | 7 | 3 | 12 | 0 | 15 | 0 | 15 | 3 | 12 | 13 | 2 |
| *P* value | 0.366 | | – | | 0.604 | | 0.399 | | 0.053 | | 0.250 | | 0.366 | | 0.553 | |
| Chronic type | 3 | 2 | 0 | 5 | 3 | 2 | 0 | 5 | 2 | 3 | 1 | 4 | 2 | 3 | 5 | 0 |
| Lymphoma type | 18 | 2 | 8 | 12 | 16 | 4 | 1 | 19 | 9 | 11 | 3 | 17 | 4 | 16 | 18 | 2 |
| *P* value | 0.166 | | 0.116 | | 0.343 | | 0.800 | | 0.622 | | 0.617 | | 0.343 | | 0.633 | |
| Acute type | 12 | 3 | 0 | 15 | 8 | 7 | 3 | 12 | 0 | 15 | 0 | 15 | 3 | 12 | 13 | 2 |
| Lymphoma type | 18 | 2 | 8 | 12 | 16 | 4 | 1 | 19 | 9 | 11 | 3 | 17 | 4 | 16 | 18 | 2 |
| *P* value | 0.360 | | **0.005** | | **0.095** | | 0.200 | | **0.002** | | 0.174 | | 0.668 | | 0.581 | |

Bold numbers indicate significant differences of methylation frequency ($P < 0.05$).

cells) to various types of ATLL specimens (30% to 98% content of neoplastic ATLL cells) does not strongly affect on MSP/UMSP signal strength.

Although weak and strong MSP-positive signals related to positive DNA methylation, the MSP-positive signals in healthy volunteers, HTLV-1 carrier, and smoldering type ATLL tended be rather faint in general, in comparison with the strong signals in acute and lymphoma type ATLL. For the case of faint or intermediate signals of MSP, several possible situations could be expected. First, extremely small populations of highly methylated cells can be detected, such as the presence of highly methylated neoplastic ATLL cells among 1000 to 20,000 or more of normal cells (Figure 1B). Second, there are a little or some populations having intermediate frequency or density of methylation in the specific CpG islands. Third, wide populations of cells contain the low or intermediate methylation density at the specific locus. Bisulfite sequencing analysis of a HTLV-1 carrier specimen, which showed a faint band with MSP, revealed that lots of clones showed methylation in several CpG sites. This is a quite different methylation profile compared with that of normal PBMCs (Figure 1, C and H), suggesting many clones are accumulating the ongoing methylation at the specific locus in some population of HTLV-1 carriers. It would be quite interesting question whether

**Table 4.** Correlation between the Presence of CIMP and Progression of ATLL

| | CIMP | | |
|---|---|---|---|
| | Positive | Negative | *P* value |
| HTLV-1 carrier | 3 | 7 | 1.000 |
| Smoldering type | 5 | 10 | |
| HTLV-1 carrier | 3 | 7 | 0.119 |
| Chronic type | 4 | 1 | |
| HTLV-1 carrier | 3 | 7 | 0.414 |
| Acute type | 8 | 7 | |
| HTLV-1 carrier | 3 | 7 | **0.002** |
| Lymphoma type | 18 | 2 | |
| Smoldering type | 5 | 10 | 0.127 |
| Chronic type | 4 | 1 | |
| Smoldering type | 5 | 10 | 0.462 |
| Acute type | 8 | 7 | |
| Smoldering type | 5 | 10 | **0.001** |
| Lymphoma type | 18 | 2 | |
| Chronic type | 4 | 1 | 0.603 |
| Acute type | 8 | 7 | |
| Chronic type | 4 | 1 | 0.504 |
| Lymphoma type | 18 | 2 | |
| Acute type | 8 | 7 | **0.022** |
| Lymphoma type | 18 | 2 | |

Fisher's exact test.
Bold numbers indicate significant differences of CIMP incidence ($P < 0.05$).

Correlation between the Presence of CIMP and Methylation of Each Gene

| | CIMP | | |
|---|---|---|---|
| | Positive | Negative | *P* value |
| *SHP1* | | | |
| Methylated | 32 | 7 | **0.000** |
| Unmethylated | 6 | 20 | |
| *p16* | | | |
| Methylated | 9 | 1 | **0.037** |
| Unmethylated | 29 | 26 | |
| *DAPK* | | | |
| Methylated | 31 | 5 | **0.000** |
| Unmethylated | 7 | 22 | |
| *hMLH1* | | | |
| Methylated | 4 | 1 | 0.393 |
| Unmethylated | 33 | 26 | |
| *MGMT* | | | |
| Methylated | 11 | 3 | 0.127 |
| Unmethylated | 27 | 24 | |
| *p73* | | | |
| Methylated | 7 | 1 | 0.126 |
| Unmethylated | 31 | 26 | |
| *p15* | | | |
| Methylated | 14 | 1 | **0.002** |
| Unmethylated | 24 | 26 | |
| *HCAD* | | | |
| Methylated | 36 | 18 | **0.005** |
| Unmethylated | 2 | 9 | |

Fisher's exact test.
Bold numbers indicate significant differences of CIMP incidence ($P < 0.05$).

HTLV-1 carriers having faint signal in MSP are at high risk to progress to ATLL, whether these persons have multiple locus of ongoing methylation, and what kinds of factors induce this kind of progressive methylation. The slight methylation of some genes in HTLV-1 carriers and/or indolent ATLL as well as healthy donors may reflect fluctuations of genome-wide methylation in response to environmental changes, inflammation, nutrition, aging, gender, lifestyle, and so on.[28]

To determine whether aberrant CpG island methylation of the specific genes and CIMP contribute to the progression of ATLL, the methylation status of eight genes was analyzed by MSP. Methylation profiles of PBMCs from healthy volunteers, HTLV-1 carriers and various stages of ATLL were shown to be quite different and characteristic (Figure 1 and Table 2). The total number of methylated genes and the incidence of CIMP significantly increased from healthy PBMC or HTLV-1 carriers to various types of ATLL. In the present investigation, some specific genes were stage-dependently hypermethylated. The frequency of methylation of the *SHP1* and *p73* genes increased at the stage of HTLV-1 infection from healthy volunteers to HTLV-1 carrier ($P < 0.046$; Table 3 and Figure 5), thereby indicating that HTLV-1 infection induces epigenetic changes in the cells even in HTLV-1 carrier state. Such epigenetic changes, triggered by HTLV-1 infection, could abrogate the normal cellular regulatory mechanism.

The *HCAD* genes were found to be hypermethylated with statistical significance at the crisis of every type of ATLL from carrier state on ($P < 0.05$). The hypermethylation of the *SHP1* gene was significantly associated with the progression to aggressive type (acute and lymphoma type) ATLL from carrier and smoldering type ATLL ($P < 0.05$). The progression from the carrier state or smoldering type of ATLL to the lymphoma type ATLL was associated with the specific hypermethylation of another type of genes such as the *DAPK, MGMT,* or *p16* genes, thus indicating that epigenetic changes of these specific genes contribute to the stage-specific progression of ATLL. Significant differences were also found between the methylation profiles of the acute type and lymphoma type ATLL. The lymphoma type showed more frequent methylation in the *p16, p73,* and *MGMT* genes, also a higher number of simultaneously methylated genes and higher incidence of CIMP than acute type (Figure 2), suggesting that epigenetically different abnormalities in target genes and also regulatory machinery may contribute to the distinct molecular pathogenesis of the lymphoma type ATLL different from acute type ATLL. This is consistent with the findings of a previous investigation, which showed the profiles of CGH (comparative genomic hybridization) microarray to be quite different between acute and lymphoma type of ATLL.[29] This indicates that structural abnormalities in the whole genome also contribute to the different tumorigenesis between the two subtypes of ATLL as well as distinct type of epigenetic abnormalities.

The *SHP1* gene showed the most significant correlation between the methylation levels and disease progression. This indicated that the *SHP1* gene methylation may be the most useful marker to distinguish between normal and ATLL. The *SHP1* gene methylation is more prevalent in ATLL, and silencing of the *SHP1* gene expression by CpG methylation may contribute to the transformation

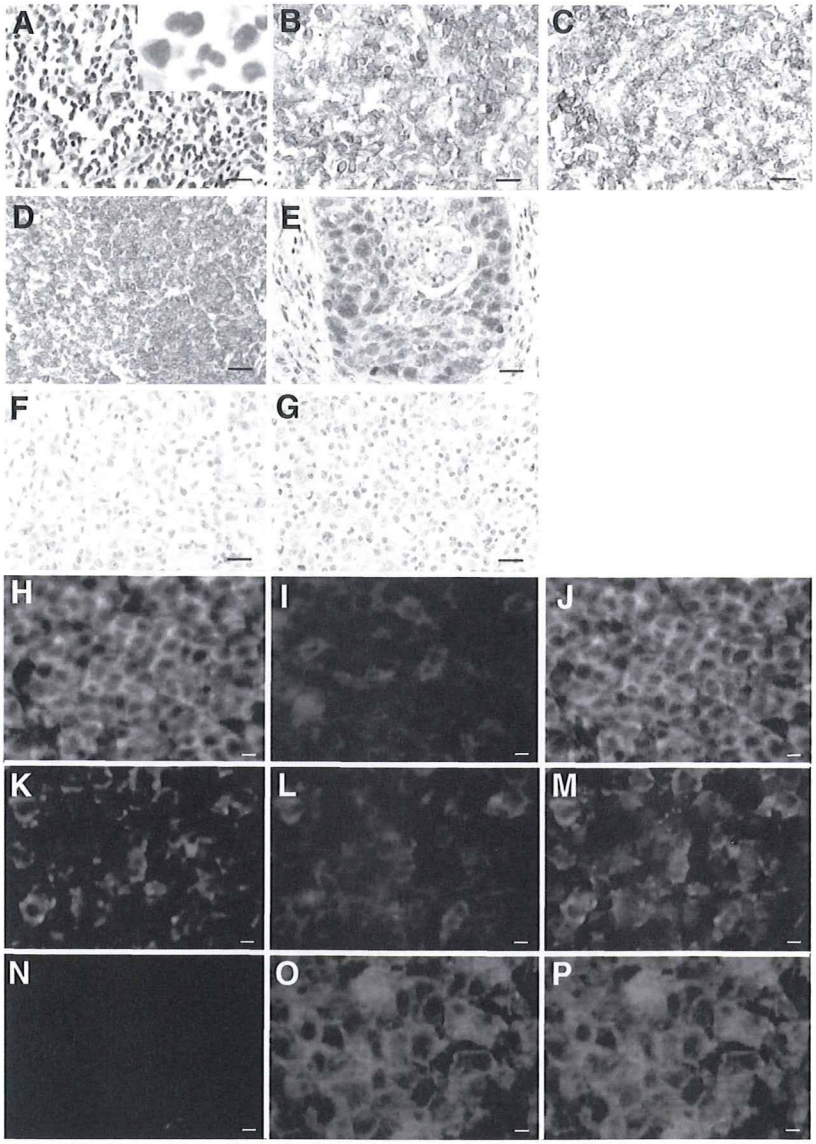



**Figure 3.** SHP1 and p16 protein expression in smoldering, chronic, and lymphoma type ATLL specimens. **Top three rows**: A typical case with lymph node involvement of ATLL cells in lymphoma type ATLL (**A**, H&E staining, ×400). Immunohistochemical staining showed that atypical ATLL lymphocytes were positive for CD4 (**B**, ×400), CD25 (**C**; ×400), and negative for SHP1 (**F**, ×400) and p16 (**G**, ×400) in lymphoma type ATLL specimen. The normal reactive lymph node was used for SHP1 positive control (**D**, ×400). The endocervical squamous cell carcinoma specimen was used as positive control for *p16* (**E**, ×400). Scale bars = 50 μm. **Bottom three rows**: Triple-staining of fluorescent immunohistochemistry showed that the expression of SHP1 protein was completely abolished in $CD25^+$ lymphoma type ATLL specimen (**N, O, P** ×400), which was clearly contrast to the strong expression of SHP1 protein in interfollicular lymphocytes of reactive lymphoid hyperplasia (**H, I, J** ×400). Expression of SHP1 protein in the skin of smoldering type ATLL was intermediate (**K, L, M** ×400). Alexa fluor 488 (green): anti-SHP1 Ab (**H, K** and **N**); Alexa fluor 555 (red): anti-CD25 Ab (**I, L** and **O**) Hoechst33258 (blue). Merged pictures were shown in (**J, M** and **P**). Scale bar = 20 μm.

from normal to various stage of ATLL.[15,27] The HTLV-1-encoded multifunctional protein Tax is thought to play an important role in the early stages of tumorigenesis in ATLL.[43,44] Tax stimulates various signaling pathway, deregulation of the cell cycle arrest, DNA repair and apoptosis by binding to CDK inhibitors and inhibiting some tumor-suppressor proteins.[43,45–47] Tax expression also has been reported to be regulated by the SUV39H1 histone methyltransferase[48] and histone deacetylases,[49] which negatively regulate the viral gene expression.

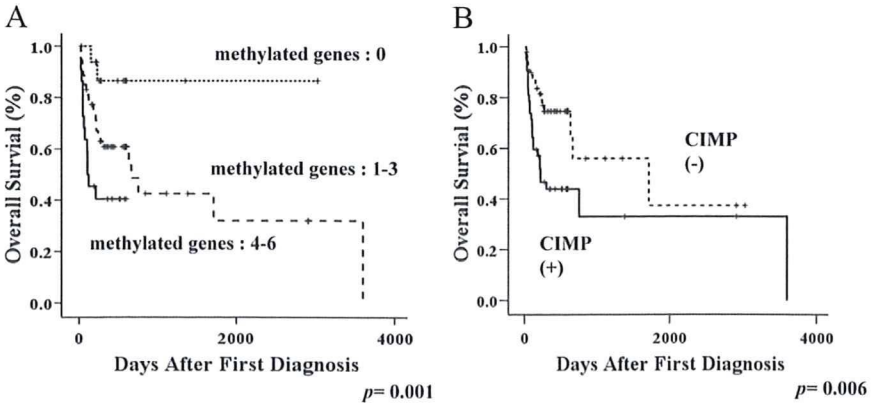



**Figure 4.** The overall survival analyzed by the Kaplan-Meyer method. **A:** The overall survival of ATLL patients according to the number of methylated genes with Kaplan–Meyer analysis. The overall survival of the ATLL patients significantly decreased as the number of methylated genes increased. **B:** The overall survival of ATLL patients in terms of the CIMP presence. The CIMP-positive ATLL patients showed a significantly poor prognosis.

**Table 5.** Simultaneous Methylation of Two Genes during Crisis or Progression of ATLL

| | *p16* | | *DAPK* | | *hMLH1* | | *MGMT* | | *p73* | | *p15* | | *HCAD* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | U | M | U | M | U | M | U | M | U | M | U | M | U |
| *SHP1* | | | | | | | | | | | | | | |
| M | 8 | 28 | 24 | 12 | 3 | 33 | 9 | 27 | 5 | 31 | 10 | 26 | 31 | 5 |
| U | 2 | 40 | 12 | 30 | 2 | 40 | 5 | 37 | 5 | 37 | 7 | 35 | 27 | 15 |
| *P* value | **0.038** | | **0.001** | | 0.657 | | 0.152 | | 1.000 | | 0.279 | | **0.038** | |
| *p16* | | | | | | | | | | | | | | |
| M | | | 7 | 3 | 1 | 9 | 3 | 7 | 2 | 8 | 2 | 8 | 10 | 0 |
| U | | | 29 | 39 | 4 | 64 | 11 | 57 | 8 | 60 | 15 | 53 | 48 | 20 |
| *P* value | | | 0.173 | | 0.506 | | 0.373 | | 0.608 | | 1.000 | | 0.057 | |
| *DAPK* | | | | | | | | | | | | | | |
| M | | | | | 2 | 34 | 11 | 25 | 6 | 30 | 10 | 26 | 34 | 2 |
| U | | | | | 3 | 39 | 3 | 39 | 4 | 38 | 7 | 35 | 24 | 18 |
| *P* value | | | | | 1.000 | | **0.009** | | 0.500 | | 0.279 | | **0.000** | |
| *hMLH1* | | | | | | | | | | | | | | |
| M | | | | | | | 0 | 5 | 1 | 4 | 1 | 4 | 5 | 0 |
| U | | | | | | | 14 | 59 | 9 | 64 | 16 | 57 | 53 | 20 |
| *P* value | | | | | | | 90.579 | | 0.506 | | 1.000 | | 0.320 | |
| *MGMT* | | | | | | | | | | | | | | |
| M | | | | | | | | | 4 | 10 | 4 | 10 | 10 | 4 |
| U | | | | | | | | | 6 | 58 | 13 | 51 | 48 | 16 |
| *P* value | | | | | | | | | 0.073 | | 0.491 | | 0.746 | |
| *p73* | | | | | | | | | | | | | | |
| M | | | | | | | | | | | 3 | 7 | 7 | 3 |
| U | | | | | | | | | | | 14 | 54 | 51 | 17 |
| *P* value | | | | | | | | | | | 0.682 | | 0.711 | |
| *p15* | | | | | | | | | | | | | | |
| M | | | | | | | | | | | | | 15 | 2 |
| U | | | | | | | | | | | | | 43 | 18 |
| *P* value | | | | | | | | | | | | | 0.211 | |

Fisher's exact test.
Bold numbers indicate significant differences of methylation frequency ($P < 0.05$).

These indicate that the epigenetic abnormalities including Tax regulation play crucial roles in the pathogenesis of ATLL. Present evidence that CpG methylation of the *SHP1* gene has started even in HTLV-1 carrier state and also accumulation of methylation frequency according to the progression, suggests the possibility that the gene silencing of the *SHP1* gene, which is a specific key negative regulator of receptor mediated signaling, is the initial step of multistep leukemogenesis/lymphomagenesis in ATLL. This hypothesis is supported by the previous evidences that the high incidence of the *SHP1* gene methylation and loss of heterozygosity, indicating the key role in initiation and progression in lymphomagenesis and/or leukemogenesis.[10,15,27] This implies that the stage-specific DNA hypermethylation of these genes could be the highly specific markers useful for the accurate early detection, diagnosis of the onset of ATLL, and also the monitoring the patient status, because present data of MSP shows the high sensitivity to detect a single ATLL tumor cell among $1 \times 10^4$ background normal PBMCs (Figure 1, B and C). Sensitive and specific epigenetic analysis would contribute to the establishment of more effective treatments and improve the prognosis of ATLL as well as early detection.

The presence of CIMP has been reported in several malignancies; however, the definition and cut-off value of

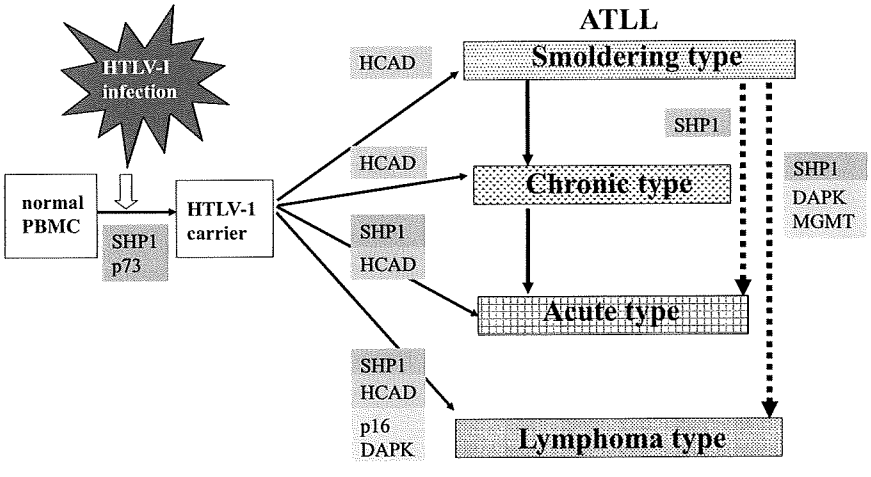



**Figure 5.** Specific genes that show statistically significant increase in the CpG island hypermethylation incidence during the progression of ATLL. Genes associated with **arrow** indicate significant increase of methylation frequency from **arrow** start-pointed status to end-point status, which does not always indicate the direction of disease progression. The *SHP1 and p73* genes were significantly methylated after HTLV-1 infection ($P < 0.05$). The hypermethylation of the *SHP1* gene was associated with the progression to aggressive type (acute and lymphoma type) ATLL from carrier and smoldering type ATLL, with statistical significance. The *HCAD* gene was significantly highly methylated at the onset of every type of ATLL, on the other hand the *DAPK, MGMT,* and *p16* genes were significantly hypermethylated at the progression to lymphoma type-ATLL. Statistical data were originated from Table 1.

CIMP remains unclear.[30,50,51] CIMP was defined in the present study as more than or equal to three genes over eight tested genes being methylated according to the distribution of the number of methylated genes among diseases. The results demonstrated that CIMP was associated with the progression and onset of ATLL, especially in lymphoma type and also significant unfavorable overall survival as shown in the Kaplan-Meyer analysis (Figure 4, Table 4). These findings may therefore imply that CIMP might be an additional valuable biomarker for an early diagnosis and progression in ATLL, as well as a prominent prognostic marker, in addition to another parameter of the number of methylated genes.

These findings indicate that treatment with a demethylation agents such as 5-aza-2′-deoxycytidine may be effective for prophylactic treatment of ATLL in HTLV-1 carriers, as well as for therapy of all types of ATLL. It is necessary to characterize the detailed methylation profiles associated with prognosis in a larger number of patient specimens. Simultaneous methylation, detected by the correlations in the methylation events of two genes (*SHP1* and *DAPK, DAPK* and *MGMT, DAPK* and *HCAD*) (Table 5) and presence of CIMP suggest that abnormalities in the epigenetic machinery, regulating target specific CpG methylation, may cause functionally or regulatory disability in the related genes during the progression and onset of ATLL. Further studies are currently underway to elucidate the mechanism of methylation in different stages of ATLL during follow-up.

In summary, present investigation provides strong evidence that the MSP assay is reliable to investigate methylation status associated with crisis and/or progression of ATLL irrespective to the different proportion of HTLV-1 infected cells and/or neoplastic ATLL cells. Present data suggested that HTLV-1 infection induces epigenetic changes in the cells, even in the HTLV-1 carrier state. Such epigenetic abnormal changes could abrogate the normal cellular regulatory mechanism and relate to the progression with the multistep accumulation of aberrant CpG hypermethylation and CIMP in ATLL. These findings suggest that the epigenetic abnormalities are deeply involved in the crisis and progression of ATLL in multistep leukemogenesis/lymphomagenesis. Furthermore, the CIMP status and stage-specific DNA hypermethylation may be potentially novel biomarkers for the early detection and prognosis of ATLL patients', and minimal residual disease, and for also determining the appropriate candidate patients for epigenetically targeted salvage therapy regimens with high sensitivity and accuracy.

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*Review Article*

# Immunogenetics and the Pathological Mechanisms of Human T-Cell Leukemia Virus Type 1- (HTLV-1-)Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

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Human T-cell leukemia virus type 1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease only in a minority of infected individuals: the malignancy known as adult T-cell leukemia (ATL) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, accumulating evidence suggests that complex virus-host interactions play an important role in determining the risk of HAM/TSP. This review focuses on the role of the immune response in controlling or limiting viral persistence in HAM/TSP patients and the reason why some HTLV-1-infected people develop HAM/TSP whereas the majority remains asymptomatic carriers of the virus.

## 1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection is of particular interest to the field of immunology as well as microbiology because HTLV-1 is never eliminated from the host in spite of vigorous cellular and humoral immune responses against the virus but causes no disease in vast majority of infected subjects (asymptomatic carriers:ACs). Although only approximately 2%-3% develop adult T cell leukemia (ATL) [1, 2] and another 0.25%–3.8% develop chronic inflammatory diseases involving the central nervous system (HTLV-1-associated myelopathy/tropical spastic paraparesis: HAM/TSP) [3, 4], evaluation of the individual risk for developing diseases in each ACs would certainly be of considerable importance especially in HTLV-1 endemic area such as southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa [5]. However, many fundamental questions are remained to be solved. First, how does HTLV-1 persist in the individual host in spite of strong host immune response? Second, why do some HTLV-1-infected people develop consequent diseases such as ATL or HAM/TSP, whereas the majority remains asymptomatic carriers of the virus? Third, how is the inflammatory lesion in HAM/TSP initiated and maintained, and why is the inflammation specifically in thoracic spinal cord? This review summarizes the past and recent works for HAM/TSP attempting to resolve each of these questions.

## 2. Clinical and Pathological Features of HAM/TSP

HTLV-1 is classified as a complex retrovirus in the genus *Deltaretrovirus* of the subfamily *Orthoretrovirinae* and infects 10–20 million people worldwide [6–8]. HTLV-1 can be transmitted through sexual contact [9], injection drug use [8], and breastfeeding from mother to child [10, 11]. Although HTLV-1 infection is associated with a range of nonmalignant chronic inflammatory diseases in the eyes, the lungs, or the skeletal muscles [7], HAM/TSP is the bestrecognized with chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [12]. To date, more

than 3,000 cases of HAM/TSP patients have been reported in HTLV-1 endemic areas. Sporadic cases have also been described in nonendemic areas such as the United States and Europe, mainly in immigrants from an HTLV-1 endemic area. Among ACs, the lifetime risk of developing HAM/TSP, which is different among different ethnic groups, ranges between 0.25% and 4%. It has been reported that the annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times higher risk for women in both populations [13–16].

Pathological analysis of HAM/TSP autopsy materials indicates that the disease affects the spinal cord, predominantly at the thoracic level [17–19]. Loss of myelin and axons in the lateral, anterior, and posterior columns is associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis. The presence of atypical lymphocytes (so-called "flower cells") in peripheral blood and cerebrospinal fluid (CSF), a moderate pleocytosis, and raised protein content in CSF are typically found in HAM/TSP patients. Oligoclonal bands, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-6 and interferon (IFN)-$\gamma$, and an increased intrathecal antibody synthesis specific for HTLV-1 antigens have also been described [20]. Clinical progression of HAM/TSP is associated with increased proviral load in individual patients, and the ratio of proviral loads in CSF cells/in peripheral blood mononuclear cells (PBMCs) is significantly associated with clinically progressive disease [21]. The major histocompatibility complex (MHC) class I tetramer analysis of lymphocytes isolated from the CSF of HAM/TSP patients showed even higher frequencies of HTLV-1 Tax11-19-specific, HLA-A*02-restricted $CD8^+$ lymphocytes compared to those of PBMCs [22]. Therefore, an increased proliferation or migration of HTLV-1-infected and/or HTLV-1-specific lymphocytes to the central nervous system (CNS) might be closely associated with HAM/TSP pathogenesis [23].

## 3. Risk Factors for HAM/TSP

*3.1. Host Genetics.* Previous population association study of 202 cases of HAM/TSP and 243 ACs in Kagoshima, HTLV-1 endemic southern Japan, revealed that one of the major risk factors is the HTLV-1 proviral load. The median proviral load was more than ten times higher in HAM/TSP patients than in ACs, and a high proviral load was also associated with an increased risk of progression to disease [24]. Higher proviral load in HAM/TSP patients than in ACs was observed in other endemic area, such as the Caribbean [25], South America [26], and the Middle East [27]. It was suggested that genetic factors such as human leukocyte antigen (HLA) are related to the high proviral load in HAM/TSP patients and genetic relatives. In southern Japan, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 proviral load and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predisposes to HAM/TSP in the same population [28, 29]. Since the function of class I HLA proteins is to present antigenic peptides to cytotoxic T lymphocytes (CTL), these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may be an important determinant of HTLV-1 proviral load and the risk of HAM/TSP. Further analysis to look at nonHLA host genetic factors revealed that nonHLA gene polymorphism also affects the risk for developing HAM/TSP. For example, the TNF-$\alpha$ promoter −863 A allele [30], and the longer CA repeat alleles of matrix metalloproteinase (MMP)-9 promoter [31] predisposed to HAM/TSP, whereas IL-10 -592 A [32], Stromal derived factor (SDF)-1 +801A [30] and IL-15 +191 C alleles [30] conferred protection against HAM/TSP. The polymorphisms of MMP-9 and IL-10 promoter each linked to the HTLV-1-encoded transactivator Tax-mediated transcriptional activity of each gene [31, 32].

*3.2. HTLV-1 Genotype.* Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 *tax* gene variation and the risk of HAM/TSP [33]. The *tax* subgroup A that belongs to cosmopolitan subtype A was more frequently observed in HAM/TSP patients and this effect was independent of protective allele HLA-A*02. HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, *tax* subgroup B that belongs to cosmopolitan subtype B but not against *tax* subgroup A in Japanese population [33]. Interestingly, HLA-A*02 appears not to give protection against infection with cosmopolitan subtype A in a population in Iran [27]. These findings suggest that both host genetic factors and HTLV-1 subgroup play a part in determining the risk of HAM/TSP.

## 4. The Immune Response to HTLV-1

*4.1. The Humoral Immune Response.* In HTLV-1 infection, anti-HTLV-1 antibody that often includes IgM is detected in all infected individuals, either ACs or patients with HAM/TSP [34]. It has been reported that HAM/TSP patients generally had higher anti-HTLV-1 antibody titer than ACs with the similar HTLV-1 proviral load [34–36]. These data suggest that there was persistent expression of HTLV-1 proteins in vivo and the existence of an augmented humoral immune response to HTLV-1 in HAM/TSP patients. Interestingly, although antibody responses to the immunodominant epitopes of the HTLV-1 Envelope (Env) proteins were similar in all of three clinical groups (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was highest in HAM/TSP (71%–93%) than ATL patients (4%–31%) or ACs (27%–37%) [37]. In 2002, Levin et al. reported that antibodies that recognize HTLV-1 Tax protein can cross-react with a heterogenous nuclear riboprotein (hnRNP)-A1, suggesting intriguing evidence for antigen mimicry in HTLV-1 infection [38]. However, since the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed [39] and is not normally accessible to antibody

attack, it is unlikely that anti-Tax antibody explains the onset or initial tissue damage of HAM/TSP. Rather, anti-Tax antibody might be associated with subsequent inflammation following initial tissue damage and disruption of blood brain barrier, which is probably caused by the antiviral immune responses to HTLV-1 and induces the release of autoantigens.

*4.2. The Natural Killer (NK) Cell Response.* Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of NK cells (especially the $CD3^+CD16^+$ subset) than ACs, although the results were not normalized with respect to the proviral load [40]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, the synthetic tetramers of HLA-E with the HLA-G signal sequence peptide were used to identify NK cells in HAM/TSP patients [41]. The results clearly showed a lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than ACs, and as in the earlier studies [40], this reduction in frequency was particularly notable in the $CD3^+$ cells whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^-$ cells between patients with HAM/TSP and ACs [41]. Recent data also suggest that the frequency of invariant NKT cells in the peripheral blood of HAM/TSP patients is significantly decreased when compared with healthy subjects and/or ACs [42, 43]. These findings indicated that the activity of the NK or NKT cell response was associated with the presence or absence of HAM/TSP. Interestingly, previous uncontrolled preliminary trial of viable *Lactobacillus casei* strain Shirota containing fermented milk for HAM/TSP patients resulted in significant increase of NK cell activity with improvements in clinical symptoms [44]. Thus, circulating NK and NKT cells might also play an important role in the disease progression and the pathogenesis of HAM/TSP.

*4.3. The Regulatory T Cells (Tregs).* It has been reported that HTLV-1 preferentially and persistently infects $CD4^+CD25^+$ lymphocytes in vivo [45], which contains the majority of the $Foxp3^+$ Tregs [46]. In HAM/TSP patients, the percentage of $Foxp3^+$ Tregs in $CD4^+CD25^+$ cells is lower than that in ACs and uninfected healthy controls [45, 47] whereas the percentage of $Foxp3^+$ cells in the $CD4^+$ population tended to be higher in the HAM/TSP patients than in the ACs [48–50]. As CD25 is induced by HTLV-1 Tax oncoprotein [51], it is most likely that the proportion of $Foxp3^+$ cells falls in the $CD4^+CD25^+$ population, which contain both Tregs and activated nonTregs, in HTLV-1-infected individuals especially HAM/TSP patients. Interestingly, the frequency of HTLV-1 negative $Foxp3^+CD4^+$ cells positively correlated with the HTLV-1 proviral load [23, 49] and the CTL activity negatively correlated with the frequency of HTLV-1 negative $Foxp3^+CD4^+$ cells [49]. These data suggest that an increase in HTLV-1 negative $FoxP3^+CD4^+$ Tregs is one of the chief determinants of the efficiency of T cell mediated immune control of HTLV-1. If such Tregs reduce CTL activity, which in turn increases the HTLV-1 proviral load, this activity increases the risk for developing HAM/TSP.

*4.4. The $CD4^+$ Helper T Cell Response.* It is well known that antiviral $CD4^+$ T cell responses are of central importance in driving B-cell and $CD8^+$ T-cell responses in vivo. The HTLV-1 antigen most commonly recognized by $CD4^+$ T cells is the Env protein [52, 53], in contrast with the immunodominance of Tax in the $CD8^+$ T cell response [54–56]. At a similar proviral load, patients with HAM/TSP had significantly increased frequency of virus-specific $CD4^+$ T-cells compared to that of ACs [53, 57]. The antiviral T helper (Th)1 phenotype is also dominant among HTLV-1-specific $CD4^+$ T cells in both ACs and patients with HAM/TSP [58], and there is a higher frequency of IFN-$\gamma$, TNF-$\alpha$, and IL-2 production by $CD4^+$ T cells in patients with HAM/TSP compared to ACs of a similar proviral load [58, 59]. A role for CD4+ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in southern Japan [28, 29] and northeastern Iran [27]. Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380–394 peptide was used to analyze Env-specific $CD4^+$ T cells directly ex vivo [57]. The results showed that the frequency of $tetramer^+CD4^+$ T cells was significantly higher in HAM/TSP patients than in ACs with similar proviral load. Moreover, direct ex vivo analysis of $tetramer^+CD4^+$ T cells from two unrelated DRB1*0101 positive HAM/TSP patients indicated that certain T cell receptor (TCR) V$\beta$s were utilized and antigen-specific amino acid motifs were identified in complementarity determining region (CDR) 3 from both patients. These data suggest that the observed increase in virus-specific $CD4^+$ T cells in HAM/TSP patients, which may contribute to $CD4^+$ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected $CD4^+$ T cells but was the result of in vivo selection by specific MHC-peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 $tetramer^+$ $CD8^+$ T cells [60] and muscle infiltrating cells from HAM/TSP patients and HTLV-1-infected polymyositis patients [61].

*4.5. The Cytotoxic T Lymphocyte (CTL) Response.* Previous reports indicated that the HTLV-1-specific $CD8^+$ CTL is typically abundant, chronically activated, and mainly targeted to the viral transactivator protein Tax [62]. Also, as already mentioned, the median proviral load in PBMCs of HAM/TSP patients was more than ten times higher than that in ACs, and a high proviral load was also associated with an increased risk of progression to disease [24]. Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower proviral load and a lower risk of HAM/TSP [28, 29], and $CD8^+$ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals [63]. These data have raised the hypothesis that the class I-restricted $CD8^+$ CTL response