Manuscript #IJC-04-2515.R2

HTLV-I viral escape and host genetic changes in the development of adult T-cell leukemia

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(1) Presented in part: 46th Conference Annual Meeting of the Japanese Society of Hematology, Kyoto, Japan, 19 September 2004.

(2) Informed consent was obtained from all HTLV-I carriers and patients. This research was approved by the institutional review boards of the author's institutions, and human experimentation guidelines of the US Development of Health and Human Services and those of the author's institutions were followed in the conduct of clinical research.

(3) The authors have no commercial or other associations that might pose a conflict of interest.

(4) This study was supported by in part from JAPAN LEUKEMIA RESEARCH FUND.

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ABSTRACT

In the pathogenesis of Adult T-cell leukemia (ATL), an oncogenetic role of the human T cell lymphotropic virus type I (HTLV-I) Tax protein, viral escape from the host immune system, and host genetic changes have each been proposed as contributory factors.

In this study, we examined the premature stop codons in *tax* gene as one of the mutations that may lead to escape of HTLV-I from the cytotoxic T lymphocyte (CTL) response in HTLV-I carriers, to test whether a putative CTL escape mutant can emerge in the early stage of ATL development and whether HTLV-I infected cells with such a mutation can proliferate subsequently. We also examined deletion of cyclin-dependent kinase inhibitor 4 (*INK4*) genes and mutation of *p53* gene in combination with changes in the HTLV-I genome in acute type ATL to test whether host genetic changes promoted the malignant transformation of ATL cells that carry putative CTL escape mutations.

The premature stop codon in *tax* gene existed in many non-ATL HTLV-I carriers as a minor population but not in the commonest HTLV-I sequence of the individual. This minor population with a premature stop codon did not expand subsequently in three asymptomatic carriers tested. There were cases who had a mutation or deletion in HTLV-I who also have deletion of *INK4* genes or mutation in *p53* gene. These findings suggest that CTL escape mutation can occur at an early stage of ATL development, and that certain host genetic changes favor the development of the aggressive form of ATL.

INTRODUCTION

Adult T-cell leukemia (ATL) is a T cell malignancy with clonal proliferation of human T-cell leukemia virus type I (HTLV-I) infected cells (1, 2). HTLV-I is also an etiologic agent for HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (3, 4). ATL is subdivided into four types (smoldering, chronic, lymphoma and acute) (5). ATL has a long incubation period and smoldering and chronic type ATL sometimes transform into a more aggressive acute/lymphoma type of ATL, suggesting a multistep leukemogenesis model (6) for the development of ATL.

HTLV-I Tax protein is a key regulator for immortalization, transformation, and oncogenesis of the HTLV-I infected lymphocytes through its interaction with many cellular proteins. For example, Tax binds to CBP/p300 and determines the accessibility of CBP/p300 to protein complexes on specific DNA elements (7), resulting in Tax mediated trans-activation of viral genes (8) and growth factors (9), or trans-repression of p18 (10), DNA polymerase β and bax genes (11). Tax also modifies the cell cycle through binding $p16^{INK4A}$ (12), hDLG (13) and MAD1 (14) and contributes to the development of ATL.

Tax also plays a role as an immunodominant target antigen for the cytotoxic T lymphocyte response (CTL) (15, 16) to HTLV-I and Tax-expressing cells will be rejected by the host immune response. Therefore it is possible that immortalized cells that elicit weaker CTL responses are selected during the development of ATL. We have previously reported several mutations and deletions in the *tax* gene in ATL that can escape from the host immune system (17). A premature stop codon in the *tax* gene (substitution at nt. position 7464 from G to A) that was frequently observed in ATL, is one such escape mutation, because the resulting truncated Tax protein loses its

transactivational activity (18) and thus expression of HTLV-I related proteins is diminished. This premature stop codon was also observed in the chronic type of ATL as a consensus sequence of the patient (17), suggesting that Tax is dispensable at least in some chronic ATL cases.

These findings suggest that viral escape from CTL recognition is one of the important steps for the development of ATL; however host genetic changes such as cyclin-dependent kinase inhibitor genes and *p53* gene have also been observed in ATL. The signaling pathway governed by G1 cyclins, cyclin-dependent kinases (CDKs), pRb and E2F plays a major regulatory role during G1 to S transition in the cell cycle (19, 20). The complex formed by CDK4 and D-type cyclins controls the passage of cells through G1 phase, and the function of CDK4/CDK6 complexes is inhibited by a number of inhibitor of CDK4 (INK4) , i.e., $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$ (21-25). The human $p16^{INK4A}$ and $p15^{INK4B}$ genes are situated within 30kb on chromosome 9p21 (26). $p18^{INK4C}$ and $p19^{INK4D}$ proteins also inhibit the activities of D-type CDKs (23-25). However, among these INK4s, $p16^{INK4A}$ is most frequently impaired in tumor cells (27). There is another tumor suppressor gene named $p14^{ARF}$ (28), encoded in an alternative reading frame (ARF) of the $p16^{INK4A}$ gene. The *p53* gene is another tumor suppressor gene (29). Mutations of the *p53* gene have been found in several malignancies including ATL (30).

We wondered when a premature stop codon in the tax gene, a putative CTL escape mutant, emerged during the development of ATL, and whether HTLV-I with this stop codon would subsequently proliferate. To this end, we examined the occurrence of a premature stop codon in the tax gene in 219 asymptomatic carriers and 143 HAM/TSP patients. We also examined the proportion of HTLV-I infected cells with this stop

codon in three asymptomatic carriers at different time points to test whether such HTLV-I infected cells continuously proliferate in asymptomatic HTLV-I carriers without ATL. We also examined the deletion of cyclin-dependent kinase (CDK) 4 inhibitor genes (*p15*^{INK4B}, *p16*^{INK4A}, *p18*^{INK4C}, *p19*^{INK4D}) in 23 acute ATL patients and mutations of *p53* gene in 22 ATL patients to investigate whether additional host genetic changes favor the development of the aggressive form of ATL.

MATERIALS AND METHODS

Study population. Two hundred and nineteen HTLV-I seropositive asymptomatic blood donors (ACs) and 143 cases of HAM/TSP whose *tax* gene had not been sequenced in our previous study (17) were examined for the stop codon in the *tax* gene. Twenty-three cases of acute type ATL whose tax genes were sequenced in the previous study (17) were tested for the deletion of cyclin-dependent kinase inhibitor 4 genes and twenty two of these cases were tested for a mutation in the *p53* gene. All cases were of Japanese ethnic origin and resided in Kagoshima prefecture, Japan. The diagnosis and clinical subtyping of ATL were made according to Shimoyama's criteria (5). The diagnosis of HAM/TSP was made according to the World Health Organization diagnostic criteria (31).

Proviral load measurement. The HTLV-I provirus load in peripheral blood mononuclear cells (PBMC) was measured in HAM/TSP patients and HCs as described (32). A quantitative PCR reaction was performed using the ABI PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems). The amount of HTLV-I proviral DNA was calculated as follows: copy number of HTLV-1 (*tax*) per 10^4 PBMC = [copy number of *tax* / (copy number of β-actin/2)] x 10^4. The lower limit of

detection was 1 copy per 10^4 PBMC.

Statistical Analysis. The Mann-Whitney-*U* test was used for statistical analysis of HTLV-I provirus load and the variables were treated as continuous.

Restriction fragment length polymorphism (RFLP) analysis of the HTLV-I tax gene

Substitution at nt. position 7464 from G to A on the tax gene created a premature stop codon (17) and also created a Bln I restriction site (CCTGGG to CCTAGG). This stop codon was observed frequently in ATL in the commonest sequence of the individual ATL patients. RFLP analysis using Bln I was done on 219 ACs and in 143 HAM/TSP cases. Nested polymerase chain reaction (PCR) was performed on the extracted DNA to amplify proviral DNA and the amplified product was digested with Bln I. One hundred nanograms of DNA were amplified by 35 cycles of PCR using expand high fidelity PCR system (Boehringer Mannheim, Tokyo, Japan) and 1μM primers (PXO1+: 5'- TCGAAACAGCCCTGCAGATA-3' (7257--7276) and PXO2-: 5'- TGAGCTTATGATTTGTCTTCA -3' (8447--8467)). After the first PCR reaction, 1μl aliquots of the amplified products were subjected to further 20 cycles of the second PCR using internal primers (PXI1+: 5'- ATACAAAGTTAACCATGCTT -3' (7274--7293) and PXI1-: 5'- GGGTTCCATGTATCCATTTC -3' (7644-7663)). Each PCR cycle consisted of denaturation at 94°C for 60 s, annealing at 58°C for 75 s, extension at 72°C for 90 s and extension of the final cycle at 72°C for 10 min. Two μl of the nested PCR product was digested with 5 U of Bln I (Takara, Japan) in 10μl volume at 37°C for 18 hours and was then electrophoresed on 1 % agarose gel.

Proportion of HTLV-I infected cells with stop codon in the tax gene in asymptomatic carriers at different time points

RFLP analysis revealed that there are ACs and HAM/TSP patients that have a stop

codon in the *tax* gene as a minor subpopulation of HTLV-I infected cells. To test whether such HTLV-I infected cells with a premature stop codon in the *tax* gene that can escape from the host immune response to HTLV-I can subsequently expand as a major population, we carried out RFLP analysis at different time points in three asymptomatic carriers. Case 1 was examined with samples taken on June 25 1999 and June 26 2000. Case 2 was examined with samples taken on Jan 14 2000 and Jan 26 2001. Case 3 was examined with samples taken on May 26 2000, Jan 25 2002 and Oct 22 2004. RFLP analysis suggested that in Case1, the proportion of HTLV-I infected cells with the premature stop codon decreased after one year. In this case, to quantify the ratio of HTLV-I infected cells with the premature stop codon to HTLV-I infected cells without this premature stop codon, the nested PCR product was cloned into pCR-Blunt II-TOPO vector (Zero Blunt TOPO PCR cloning kit: Invitrogen), transformed into competent E.coli cells and spread on LB plates containing 50 μg/ml kanamycin. Colonies from the plate were cultured overnight in LB medium containing 50 μg/ml kanamycin, and plasmids containing subcloned tax genes were extracted. Purified plasmids containing the subcloned tax gene were digested with Eco RI and Bln I and then electrophoresed on 1 % agarose gel. When the subcloned tax gene was cleaved by Bln I, the subclone was judged as having the stop codon, and if uncleaved, the subclone was judged as not having the stop codon. The proportion of HTLV-I cells that carry the stop codon was then calculated.

Southern blot analysis of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ and HTLV-I

Southern blot analysis of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$ was performed in 23 cases with acute type ATL. Southern blot analysis of HTLV-I was also performed. High molecular weight DNA was extracted by a standard method using phenol

extraction. In southern blot analysis for cyclin-dependent kinase inhibitor genes ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$), 10μg of genomic DNA was digested with Hind III, separated on a 1% agarose gel, and transferred to a nylon membrane. Probes used in hybridization were a EcoRI-XhoI fragment of $p16^{INK4A}$ cDNA, EcoRI-XhoI fragment of $p15^{INK4B}$ cDNA, BamIII-BamIII fragment of $p18^{INK4C}$ cDNA, and EcoRI-EcoRI fragment of $p19^{INK4D}$cDNA, all of these probes were provided from Dr Hirai (Banyu Tsukuba Research Institute). The same filters were rehybridized successively with the respective probes. Nylon membranes were also hybridized with β-globin probe. Probe DNA fragments were labeled with α-^{32}P-dCTP by random priming. Blots were hybridized at 65°C for 12 hours in a mixture containing 4×SSC (1×SSC; 0.15MNaCl, 0.015M sodium citrate) and 50μg of sonicated and denatured salmon sperm DNA and then washed in 0.1% sodium dodecylsulfate (SDS) and 1× SSC at 65°C for 30min, and autoradiographed, then exposed to a imaging plate and analyzed by a laser image analyzer (MAC-BAS-1000). Southern blot analysis of HTLV-I was also done in our previous study (17) with 10μg of genomic DNA digested with Pst I and hybridized with total sequence of HTLV-I as a probe. The same filters were rehybridized with a ^{32}P-labelled HTLV-1 long terminal repeat (LTR) probe.

Sequence of p53 gene

The sequence of *p53* was examined in 22 ATL cases. Three p53 fragments were amplified using nested PCR: one was 371 bp encompassing the entire exon 4; the second was 499 bp encompassing the entire exons 5 and 6; the other was 692 bp encompassing the entire exons 7 and 8. The primers used for PCR encompassing exon 4 were sense 5'-AACGTTCTGGTAAGGACAAGGG-3' (p53_41) and antisense 5'-AAGGGTGAAGAGGAATCCCAAA-3' (p53_42) for the first PCR and sense

5'-AGGACCTGGTCCTCTGACTG-3' (p53_43) and antisense 5'-ATACGGCCAGGCATTGAAGT-3' (p53_44) for the second PCR. The primers used for PCR encompassing exons 5 and 6 were sense 5'-TAGTGGGTTGCAGGAGGTGCTT-3' (p53_51) and antisense 5'-GCAGGAGAAAGCCCCCCTACTG-3' (p53_62) for the first PCR and sense 5'-TATCTGTTCACTTGTGCCCT-3' (p53_53) and antisense 5'-GGCCACTGACAACCACCCTT-3' (p53_64) for the second PCR. The primers used for PCR encompassing exons 7 and 8 were sense 5'-GACAGAGCGAGATTCCATCTCA-3' (p53_71) and antisense 5'-GCTGGTGTTGTTGGGCAGTGCT-3' (p53_82) for the first PCR and sense 5'-AGGTCTCCCCAAGGCGCACTGG-3' (p53_73) and antisense 5'-GGCATAACTGCACCCTTGGTCT-3' (p53_84) for the second PCR. One hundred nanogram DNA was amplified by 35 cycles for the first PCR using the Expand high-fidelity PCR system (Boehringer Mannheim, Japan) and 1uM of each primers. After the first PCR , 1 μl of aliquots of the amplified products were subjected to an additional 20 cycles of the second PCR using internal primers. Each PCR cycle consisted of denaturation at 95°C for 60 s, annealing at 60°C for 75 s, extension at 72°C for 120 s and extension of the final cycle at 72°C for 10 min. Amplified DNA products were purified using QIA quick purification kit (Qiagen, Japan) and 0.1μg of PCR products were sequenced using dye terminator DNA sequencing kit (Applied Biosystems, Japan) with 3.2pmol of each primers (p53_43 and p53_44 for exon 4, p53_53 and p53_64 for exons 5 and 6, p53_73 and p53_84 for exons 7 and 8) in an automatic sequencer (377 DNA Sequencer, Applied Biosystems).

Result

Premature stop codon in the tax gene in ACs and in HAM/TSP patients

In 219 asymptomatic carriers and in 143 HAM/TSP patients, there was no case that had a premature stop codon in the *tax* gene in the commonest sequence of the individual. However, there are cases who had HTLV-I infected cells with this premature stop codon in the *tax* gene as a minor population of the HTLV-I infected cells. Figure 1 shows representative results. In the ATL case with a premature stop codon in the HTLV-I *tax* gene, all of the nested PCR product was cut by Bln I (Fig. 1 lane1). Under the same experimental conditions, there were no ACs or HAM/TSP patients whose nested PCR products were completely cut by Bln I, but there was partial cleavage in some cases (Fig 1. lane 3 and lane 5). There were 79 cases out of 219 ACs (36.1%) and 78 cases out of 143 HAM/TSP patients (54.5%) that had HTLV-I infected cells with the premature stop codon in the *tax* gene as a minor population of the individuals (Table 1).

The median provirus load in ACs who had the premature stop codon in the *tax* gene as a minor population of HTLV-I infected cells was 166 and the median provirus load in ACs who did not have this premature stop codon in the HTLV-I infected cells was 34.5, and this difference was significant ($p<0.001$). The median provirus load in HAM patients who had the premature stop codon in *tax* gene as a minor population of HTLV-I infected cells was 523 and the median provirus load in HAM patients who did not have this premature stop codon in HTLV-I infected cells was 420, and this difference was not significant ($p=0.305$) (Table 1).

Proportion of HTLV-I infected cells with a stop codon in the tax gene as a minor population in asymptomatic carriers at different time points

In three asymptomatic carriers having the premature stop codon in the *tax* gene as a

minor population of HTLV-I infected cells, we examined whether this minor population expanded subsequently. Fig 2 shows that this minor population with the premature stop codon in the *tax* gene in these three asymptomatic carriers did not expand subsequently (Case lane 1 and 2, Case 2 lane 3 and 4, Case lane 5,6 and 7) . In Case1, the proportion of the population with premature stop codon decreased subsequently when analyzed by RFLP (Fig 2 lane 1 and 2). To quantify this, we subcloned the PCR product and counted the number of clones that had a stop codon at different time points. The number of subclones that had a premature stop codon in the *tax* gene were 9 out of 59 (15.3%) on June 25 1999, and was 2 out of 63 (3.2%) on June 26 2000.

Deletion of cyclin-dependent kinase 4 inhibitor genes and mutations in HTLV-I provirus in acute type ATL patients

Judged from the density of the $p16^{INK4A}$ gene band in southern blot hybridization compared to the density of the band of β-globin using an image analyzer, 9 of 23 (39.1%) acute-type ATL patients deleted the $p16^{INK4A}$ gene in leukemic cells (Fig. 3). Similarly, an absent or weak hybridization band indicating deletion of the $p15^{INK4B}$ gene was observed in 8 of 23 acute type ATL patients and a shorter size of the $p15^{INK4B}$ gene band indicating partial deletion or rearrangement of this gene was observed in one patient. No genetic alteration was detected by southern blot analysis in $p18^{INK4C}$ and in $p19^{INK4D}$.

Case 9 had the stop codon in the *tax* gene but did not have deletion in the HTLV-I provirus genome by southern blotting (Fig 4 lane 1A, 1B), and $p15^{INK4B}$ and $p16^{INK4A}$ were both deleted (Table 2). Case 11 had only one LTR band by southern blotting (data not shown) and $p16^{INK4A}$ was deleted. Case 22 had a large deletion in HTLV-I genome suggested by the southern blotting (Fig 4 lane2A, lane2B), and $p15^{INK4B}$ and

p16^{INK4A} were deleted.

Mutations in p53 gene

Sequencing of p53 was performed from codons 33 to 307 (exons 4,5,6,7 and 8) in 22 acute ATL cases. Mutations were found in 3 cases. All of them were homozygous missense mutations. Case 13 had a mutation at codon 285 (Glu to Lys) in exon 8. This case had only one LTR band by southern blot analysis of HTLV-I (Fig 4 lane 3A, lane 3B). In this case (Case13), 1.8kb band was absent when hybridized with a total HTLV-I probe, suggesting a large deletion encompassing the 5' LTR through the first Pst I restriction site. Case 17 had a mutation at codon 266 (Gly to Arg) in exon 8 and had a deletion in HTLV-I genome by southern blot analysis of HTLV-I. Case 19 had a mutation at codon 193 (His to Leu) in exon 6. There were also ATL cases that had deletion in HTLV-I provirus genome but did not have deletion in INK4 genes and did not have mutation in *p53* gene. Case 15 represent one such ATL case. Case 15 had only one LTR band when hybridized with a LTR probe (Fig 4 lane 4B) and there was a larger size of band instead of 1.8kb band when hybridized with a total HLTV-I probe (Fig 4 lane 4A), suggesting a deletion encompassing the 5' LTR through the first Pst I restriction site. The deletions and mutations observed in the HTLV-I provirus genome, INK genes and p53 gene are summarized in Table 2. There were 9 cases of acute ATL who had a putative CTL escape mutation in the HTLV-I provirus genome and 4 of these 9 patients had either a deletion in INK4A and/or INK4B gene or *p53* gene.

Discussion

The purpose of the present study was to examine when the premature stop codon in the

tax gene, a putative escape mutation from the anti-HTLV-I CTL response can emerge in the proviral population, and to examine whether such HTLV-I infected cells with escape mutation will subsequently proliferate even in an asymptomatic carrier. We found that the premature stop codon in the *tax* gene exist frequently in HTLV-I carriers as a minor population of the individual carriers: however, such a minor population did not expand subsequently, and deletions in certain host genes still favored the development of ATL.

In this study, we focused on the mutation in *tax* gene especially the premature stop codon of *tax*. Although we could not detect this premature stop codon in the *tax* gene in the majority of ATL patients, there were 4 cases among 55 ATL patients as we previously reported (17) and 4 cases among 47 ATL patients were reported in other paper (33). In addition to this premature stop codon, deletions in the *tax* gene (17), deletion of 5'-LTR (33) which is a promoter of viral genes, and silencing of the *tax* gene (34) have previously been reported in ATL, suggesting that ATL cells that do not express HTLV-I-encoded proteins are selected by the cytotoxic T cell (CTL) response to HTLV-I, during the development of ATL. A premature stop codon in the *tax* gene is one such escape mutation that is observed in some ATL patient as the commonest sequence in the individual (17). It was clear that this premature stop codon in the ATL cell emerged after the viral transmission, because the HTLV-I *tax* sequence in the family member was identical with other nucleotide alterations specific for this family except this premature stop codon (17). However, it was not clear if this premature stop codon emerged in the early stage of ATL development, or whether the mutation emerged after ATL had developed. To infer at which stage the premature stop codon could have emerged, and to investigate whether there is any HTLV-I carrier with this premature stop codon in the *tax* gene in the commonest proviral sequence of the

individual, we examined this premature stop codon in the tax gene in 219 ACs and in 143 HAM/TSP patients. Although the premature stop codon in the *tax* gene did not exist as the commonest sequence in any ACs or HAM/TSP patients, a stop codon in the *tax* gene was observed as a minor population of the HTLV-I infected cells in many ACs and in HAM/TSP patients. This observation was consistent with our previous study by direct sequencing in which we found no ACs or HAM/TSP patients that had this premature stop codon as the commonest sequence of the individual (17). The observation is also consistent with our previous study of a small number of ACs and HAM/TSP patients that the premature stop codon in the *tax* gene is present in a minor population in some of the HTLV-I carriers (35). In the present study, we found that many ACs and HAM/TSP patients carried the premature stop codon in the *tax* gene in a minor population of the infected cells. We also previously reported a chronic type of ATL with this premature stop codon as the commonest sequence of the individual (17). These findings suggest that this premature stop codon emerged in the early stages of ATL development rather than as a consequence of genetic instability after the progression to an aggressive form of ATL. The median HTLV-I provirus load was significantly higher in ACs who had a minor population of HTLV-I infected cells with this premature stop codon in the *tax* gene compared to ACs who did not have the premature stop codon in the *tax* gene as a minor population, but was not different in HAM patients regardless of the presence of premature stop codon in the *tax* gene in HTLV-I infected cells. We do not know why this difference happens, but it is possible to speculate that the proportion of the role of viral transcription versus cell division in maintaining the provirus load is different among ACs and HAM because, the HTLV-I sequence mutation are frequently caused by the reverse transcriptase, but rarely caused

by the host DNA polymerase.

Although the RFLP analysis of the premature stop codon in the *tax* gene was not quantitative, there were HTLV-I carriers with a significant proportion of HTLV-I infected cells with this premature stop codon, as judged from the density of the band cut by the Bln I restriction enzyme. To investigate whether such HTLV-I infected cells were already in the process of developing ATL, we followed three asymptomatic carriers who had this premature stop codon in the tax gene as minor population (Fig 2). In these three carriers, the population with the premature stop codon in the *tax* gene did not expand, although the observation time was one year in each of two cases and 4 year in one case. Interestingly, in one case, in which we quantified the proportion of HTLV-I infected cells with the premature stop codon in the *tax* gene, the proportion of HTLV-I infected cells with this premature stop codon decreased after a year. The provirus load was 64 when the proportion of HTLV-I infected cells had the premature stop codon in tax gene was 15.3%, but the provirus load was 5 after a year when the proportion of HTLV-I infected cells with the premature stop codon in tax gene decreased to 3.2%. These findings suggest that, although HTLV-I infected cells with escape mutation can accumulate in non-ATL HTLV-I carriers, HTLV-I infected cells with a mutation that abolishes the function of Tax lose their proliferative advantage and progressively disappear. It is likely that an escape mutation that abolishes the function of Tax should appear after the immortalization of HTLV-I infected cells for the subsequent development of ATL.

Secondly, we examined whether host genetic changes promote the malignant transformation of HTLV-I infected cells even in ATL cells that can escape from the host immune response to HTLV-I. To this end, we examined four known cyclin dependent

kinase inhibitors (INKs) and *p53* gene, in combination with the alterations in the HTLV-I genome. In the case of $p16^{INK4A}$ and $p15^{INK4B}$ genes, there is a report that these genes are deleted in many ATL patients (36), while 18^{INK4C} and $p19^{INK4D}$ are not deleted (37, 38). This is consistent with our finding that 9 of 23 (39.1%) acute-phase ATL samples had a deletion in $p16^{INK4A}$ gene and 8 of these patients (36%) who deleted $p16^{INK4A}$ also had deletion or rearrangement of $p15^{INK4B}$, while $p18^{INK4C}$ and $p19^{INK4D}$ were not deleted. In our ATL cases that deleted $p16^{INK4A}$ and/or $p15^{INK4B}$, there were three cases that could potentially escape from the host CTL response to HTLV-I. One had a premature stop codon in the tax gene, one other case had only one LTR region, and the other had a large deletion in the HTLV-I genome. Each of these genomic alterations could lead to escape from the host immune response to HTLV-I. In the case of *p53* gene, we sequenced the entire exons 4, 5, 6, 7 and 8, where are highly conserved regions of the *p53* gene (40), and are also identified as hot spots for mutations in several malignancies including ATL (30). The missense mutations of *p53* gene observed in our study were at codons 193, 266 and 285 where mutation had been reported in other malignancies (anonymous ftp to ftp.ebi.ac.uk, in the directory/pub/databases/p53) and two of them had alterations in the HTLV-I provirus genome. These deletions in the HTLV-I genome could also lead to escape from the host immune response to HTLV-I. Regarding the host genetic changes in the development of ATL, Yamada et al. reported that three chronic ATL cases who progressed to acute type lost the $p16^{INK4A}$ gene alone or both the $p15^{INK4B}$ and $p16^{INK4A}$ genes at their exacerbation phase (39). Hatta et al. reported an ATL patient with a homozygously deleted $p16^{INK4A}$ gene in the chronic phase who rapidly progressed to acute ATL and died within 6 months of the initial diagnosis. There was also a case

reported in whom *p53* gene was intact at chronic stage but was mutated when the disease progressed to acute type ATL (30). These reports suggest that deletion/ mutation of tumor suppressor genes such as $p16^{INK4A}$ gene and *p53* in ATL are not a result of genetic instability after the exacerbation of ATL, but are factors that predict poor prognosis. In summary, we speculate that in the early stage of HTLV-I infection, Tax gives advantage for proliferation of HTLV-I infected cells, but those cells that continuously express HTLV-I viral proteins are likely to be rejected by host immune system. If an escape mutation emerges after the immortalization of the HTLV-I infected cells, cells that carry such mutations are selected by the host immune system and when additional host genetic changes have accumulated, such as in the $p16^{INK4A}$ or p53 gene, HTLV-I infected cells will progress further to an aggressive form of ATL.

Acknowledgments

This study was supported by in part from JAPAN LEUKEMIA RESEARCH FUND.

We thank Ms. T. Muramoto and Ms. Y. Nishino (Department of Neurology and Geriatrics, Kagoshima University, Japan) for their excellent technical assistance. We also thank Professor Charles R. M. Bangham (Immunology Department, Imperial College Faculty of Medicine, United Kingdom) for critical reading of the manuscript.

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FIGURE LEGENDS

Fig 1. RFLP analysis of *tax* gene. Nested PCR products of *tax* was digested by restriction enzyme Bln I. PCR product in ATL case with a premature stop codon at nt. 7464 in HTLV-I genome was completely cut by Bln I (lane1), while in ATL case without the stop codon at nt. 7464 was not cut by Bln I (lane2). PCR products in HAM case (lane3) and in AC case (lane5) was partially cut by Bln I, suggesting existence of a minor population of HTLV-I infected cells with the premature stop codon at nt. 7464 in HTLV-I *tax* genome, while in other HAM case (lane4) and in AC case (lane6), PCR products were not cut by Bln I. Long arrow indicates the nested PCR product and short arrow indicates the band cut by Bln I. M: 100 base marker.

Fig. 2. RFLP analysis of *tax* gene at different time points. RFLP analysis of nested PCR product of *tax* gene digested by Bln I was done in three asymptomatic carriers at different time points. Case 1 (lane 1 at June 25 1999 and lane 2 at June 26 2000). Case 2 (lane 3 at Jan 14 2000 and lane 4 at Jan 26 2001). Case 3 (lane 5 at May 26 2000, lane 6 at Jan 25 2002 and lane 7 at Oct 22 2004). Long arrow indicates the nested PCR product and short arrow indicates the band cut by Bln I. M: 100 base marker.

Fig. 3. Southern blot analysis of $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$ and β-globin in 23 acute ATL. Ten μg of genomic DNA was digested with Hind III and hybridized with each probe. Case 1, 7, 8, 9, 10, 11, 12, 14 and 22 has weak or decreased $p16^{INK4A}$ band when compared to the density of β-globin band. Case 1, 8, 9, 10, 12, 14, 18 and 22 has weak or decreased $p15^{INK4B}$ band and case 7 has a shorter $p15^{INK4B}$ band. Abbreviations: p15; $p15^{INK4B}$, p16; $p16^{INK4A}$, p18; $p18^{INK4C}$, p19; $p19^{INK4D}$.

Fig. 4. Southern blot analysis of the HTLV-I provirus genome.

Ten micrograms cellular DNA was digested with Pst I and subjected to standard Southern blot analysis. The filter was hybridized with a total HTLV-I probe (A) and then with a LTR probe (B). Arrowheads show the viral-cellular junction bands with LTR probe, in addition to 3 internal bands (2.5kb, 1.8kb, 1.2kb). Schematic illustration of the HTLV-I genome, restriction map, and probes are shown on the upper column. (↓)Pst I site. Lane 1 (Case 9 in Table 2): Typical patient with ATL showing 3 major internal bands (2.5kb, 1.8kb, 1.2kb) with additional 2 viral-cellular junction bands (arrow heads). Lane 2 (Case 22 in Table 2): Three major internal bands are absent (A), but two LTR bands are observed, suggesting a large deletion in HTLV-I proviral genome. Lane 3 (Case 13 in Table 2): 1.8kb band is absent when hybridized with a total HTLV-I probe (A) and there is only one LTR band hybridized with a LTR probe (B), suggesting a large deletion encompassing the 5' LTR through the first Pst I restriction site. Lane 4 (Case 15 in Table 2): Another example of ATL who have only one LTR band hybridized with a LTR probe (B) and 1.8kb band is absent hybridized with a total HTLV-I probe (A).

Table 1. HTLV-I provirus load in ACs and in HAM/TSP in association with or without the premature stop codon in tax gene as minor population of HTLV-I infected cells

	ACs (n=219)		HAM(n=143)	
	+	–	+	–
n	79	140	78	65
Median	166	34	523	420
	*			

HTLV-I copy number per 10^4 PBMC was represented. n=number of subjects.

+ represent subjects with the premature stop codon in tax gene as minor population of HTLV-I infected cells detected by RFLP analysis. – represent subjects without premature stop codon in tax gene. HAM=patients with HAM. ACs=asymptomatic carriers. * P value <0.001 by Mann-Whitney-*U* test.

Table 2. Mutation/Deletion in HTLV-I genome, p15, p16 and p53 genes in acute ATL

	Southern Blot/ Mutation Analysis	Southern Blot Analysis		Mutation Analysis		
Case No.	HTLV-I	p15	p16	p53 codon	Nucleotide Substitution	Amino Acid
1		Del	Del			
2						
3						
4						
5	G7464A[a]					
6						
7		Del	Del			
8		Del	Del			
9	G7464A[a]	Del	Del			
10		Del	Del			
11	1 LTR		Del			
12		Del	Del			
13	1 LTR			285	GAG-AAG	Glu-Lys
14		Del	Del			
15	1 LTR					
16						
17	P.D.			266	GGA-AGA	Gly-Arg
18		Del				
19				193	CAT-CTT	His-Leu
20						
21	A7337G[b]			not examined		
22	P.D.	Del	Del			
23	P.D.					

a: G7464A: substitution at nt. position 7464 from G to A that creates a premature stop codon in tax.

b: A7337G: substitution at nt. position 7337 from A to G that causes an amino acid change from Gly to Arg which is an putative escape mutation (Ref 17).

1LTR: Cases that show only one LTR band by Southern blot analysis using HTLV-I LTR as a probe.

Abbreviations: P.D., Partial Deletion; Del. Deletion