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Kyoto University

1 **APOBEC3G Generates Nonsense Mutations in HTLV-1 Proviral Genomes *In Vivo***

2 **Running title: Nonsense mutations by hA3G in HTLV-1**

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19

1	**ABSTRACT**

2	 Human T-cell leukemia virus type 1 (HTLV-1) induces cell proliferation after

3	infection, leading to efficient transmission by cell-to-cell contact. After a long latent

4	period, a fraction of carriers develop adult T-cell leukemia (ATL). In ATL cells, genetic

5	changes in the *tax* gene were reported in about 10% of ATL cases. To determine genetic

6	changes that may occur throughout the provirus, we determined the whole sequences of

7	HTLV-1 provirus in 60 ATL cases. Abortive genetic changes including deletion,

8	insertion and nonsense mutations were frequent in all viral genes except in the *HBZ*

9	gene, which is transcribed from the minus strand of the virus. G-to-A base substitutions

10	were the most frequent mutations in ATL cells. Sequence context of G-to-A mutations

11	was in accordance with the preferred target sequence of human APOBEC3G (hA3G).

12	Target sequences of hA3G were less frequent in the plus strand of the *HBZ* coding

13	region than in other coding regions of HTLV-1 provirus. Nonsense mutations in viral

14	genes including *tax* were also observed in proviruses from asymptomatic carriers,

15	indicating that these mutations were generated during reverse transcription and prior to

16	oncogenesis. That hA3G targets the minus strand during reverse transcription explains

17	why the *HBZ* gene, which is encoded by the minus strand of provirus, is not susceptible

18	to such nonsense mutations. HTLV-1 infected cells likely take advantage of hA3G to

19	escape from the host immune system by losing expression of viral proteins.

2

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of both adult

T-cell leukemia (ATL) and inflammatory HTLV-1 associated myelopathy/tropical

spastic paraparesis (HAM/TSP) (9, 40). HTLV-1 is a complex retrovirus that encodes

regulatory genes, *tax* and *rex*, and accessory genes, *p12*, *p13*, and *p30* (6, 29). An

accessory gene, *HTLV-1 bZIP factor* (*HBZ*), is transcribed from the 3' long terminal

repeat (LTR) as an anti-sense transcript (10, 47). Previous studies have shown that Tax

expression can immortalize T-lymphocytes *in vitro* (1, 11), and *in vivo* the expression of

Tax in transgenic mice causes various tumors depending on the tissue-specific promoter

that expresses Tax (12, 14, 20). In more than half of human ATLs, the *tax* gene is not

transcribed (41). Three mechanisms have been identified to inactivate Tax expression

(29): 1) abortive genetic changes of the *tax* gene (7, 41), 2) silencing by DNA

methylation in the 5'LTR (18, 43), and 3) deletion of the 5'LTR (31, 42). Since Tax is a

major target of cytotoxic T-lymphocytes (CTLs) *in vivo* (17), the tumor cells might

escape from the host immune system by suppressing Tax expression. However, the

actual mechanism used to create genetic changes in the *tax* gene remains to be

elucidated. Nonsense mutations in the *tax* gene have also been observed in HTLV-1

carriers and the mechanism to generate these mutations is similarly unknown (8).

As a host defense against retroviruses, mammalian cells employ the APOBEC3

family, which causes deamination during reverse transcription, resulting in nucleotide

mutations (3, 26). Human APOBEC3G (hA3G) deaminates cytosine residues of

single-stranded DNA during reverse transcription, resulting in high levels of plus-strand

1 G-to-A mutations. The human immunodeficiency virus (HIV) nucleocapsid is critical

2 for incorporation of hA3G into virions (2, 24), while its accessory protein, Vif,

3 counteracts hA3G. Vif inhibits hA3G packaging into HIV-1 virion through

4 ubiquitination and proteasomal degradation of hA3G (39, 49). HTLV-1 does not encode

5 a protein analogous to Vif that inactivates hA3G. Instead, it has been demonstrated that

6 a domain of the HTLV-1 nucleocapsid suppresses the incorporation of hA3G into the

7 virion (4). Consistent with this finding, it has been reported that G-to-A mutations are

8 rare in HTLV-1 carriers (25).

9   In this study, we analyzed the sequences of whole proviruses in ATL and carrier cells,

10 and found that most nonsense mutations in the proviruses were caused by deamination.

11 The sites of nonsense mutation coincided with the preferred target sequences of hA3G.

12 In ATL cases, non-sense mutations, deletions and insertions were detected in most of

13 the viral genes except the *HBZ* gene, supporting the critical role of the *HBZ* gene in

14 ATL.

15

MATERIALS AND METHODS

2   **Cell lines and clinical samples.** MT-2 and MT-4 are HTLV-1-transformed cell lines.

3   MT-1 and TL-Om1 were derived from leukemia cells. Jurkat cells were negative for

4   HTLV-1. These cells were cultured in RPMI-1640 medium supplemented with 10%

5   fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM

6   L-glutamine at 37°C under a 5% CO_2 atmosphere. Clinical samples were collected from

7   60 ATL patients and 10 HTLV-1 carriers. Genomic DNA was extracted from peripheral

8   blood mononuclear cells using standard phenol-chloroform methods. This study was

9   conducted according to the principles expressed in the Declaration of Helsinki. The

10   study was approved by the Institutional Review Board of Kyoto University (G204). All

11   patients provided written informed consent for the collection of samples and subsequent

12   analysis.

13

14   **Sequencing of complete provirus genomes in ATL cells.** The copy number of

15   HTLV-1 provirus in ATL cases was determined by inverse PCR (5) and ATL samples

16   with one complete provirus were selected for direct sequencing. The complete provirus

17   genome was amplified as two halves (fragment I and II) from genomic DNA samples

18   derived from ATL patients. Fragment I was amplified using primers

19   5'-TGACAATGACCATGAGCCCCAAATATCC-3' and

20   5'-CGGCTATTAAGACCAGGAAG-3'. PCR conditions were as follows: 1 cycle of

21   94°C for 5 min, 30 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 5 min, 1 cycle of

22   72°C for 10 min. Fragment II was amplified using primers 5'-

1   AGAAACAAGCTCAGAAGCTA -3' and 5'-

2   TGTACTAAATTTCTCTCCTGAGAGTGC-3'. PCR conditions were as follows: 1

3   cycle of 94°C for 5 min, 30 cycles of 94°C for 30 s, 60 ^{o}C for 30 s and 72 ^{o}C for 5 min, 1

4   cycle of 72 ^{o}C for 10 min. PCR products were then subjected to nested PCR to amplify

5   I-1 and I-1 from fragment I, and II-1 and II-2 from fragment II. Primers for I-1 were

6   5'-TGACAATGACCATGAGCCCCAAATATCC-3' and

7   5'-GAGCTTAAAGTGATCTTGG-3'. Primers for I-2 were

8   5'-TTCCGATAGCCTTGTTCTCA-3' and 5'-CGGCTATTAAGACCAGGAAG-3'.

9   Primers for II-1 were 5'-TGGTATTATTTCAAGCTTCC-3' and

10   5'-AAATGCAGGAGTTGGGGATT-3'. Primers for II-2 were

11   5'-AACTGTCTAGTATAGCCATC-3' and

12   5'-TGTACTAAATTTCTCTCCTGAGAGTGC-3'. PCR conditions for the nested PCR

13   were as follows: 1 cycle of 94°C for 5 min, 28 cycles of 94°C for 30 s, X ^{o}C for 30 s and

14   72 ^{o}C for 2.5 min, 1 cycle of 72 ^{o}C for 10 min. X was 60 ^{o}C for I-1 and II-2, 58 ^{o}C for

15   I-2 and II-1. The final PCR products were gel purified and sequence was determined by

16   direct sequencing.

17

18   **Sequencing of the *pol* and *tax* regions in HTLV-1 carriers.** Proviral load was

19   determined as previously reported (32). Herculase II Fusion DNA Polymerase

20   (Stratagene, La Jolla, CA), a Pfu-based DNA polymerase, was used to amplify *pol* and

21   *tax* regions from genomic DNA derived from HTLV-1 carriers. Primers to amplify the

22   *pol* region were 5'- TACACCTTGCAATCCTATGG-3' and 5'-

1   GCTAGGCTTGCCTAGATGGG-3'. PCR conditions were as follow: 1 cycle of $95^{\circ}C$

2   for 5 min, 30 cycles of $95^{\circ}C$ for 20 s, $60^{\circ}C$ for 20 s and $72^{\circ}C$ for 50 sec, 1 cycle of 72

3   $^{\circ}C$ for 5 min. Primers to amplify the *tax* region were 5'-

4   GGTCCTCCGGGCATGACACA-3' and 5'- TCTCCACGCTTTTATAGACT-3'. PCR

5   condition were as follow: 1 cycle of $95^{\circ}C$ for 5 min, 28 cycles of $95^{\circ}C$ for 20 s, $68^{\circ}C$

6   for 20 s and $72^{\circ}C$ for 50 sec, 1 cycle of $72^{\circ}C$ for 5 min. PCR products were purified,

7   cloned and at least 20 clones sequenced. DNA polymerase fidelity was studied by using

8   a clone carrying an amplified *tax* region as template instead of genomic DNA. The *tax*

9   region was amplified using the same PCR conditions described above, cloned and 25

10 clones were sequenced.

11

12 **Sequencing of HTLV-1 genome in non-tumor cells derived from ATL samples.**

13 ATL patient-derived tumor cells are considered a mixture of a major population of

14 monoclonal expanded tumor cells and a minor population of HTLV-1 infected

15 non-tumor cells. To amplify the HTLV-1 genome from non-tumor infected cells, ATL

16 samples carrying defective HTLV-1 genome based on direct sequencing were selected.

17 Target regions were amplified by setting one of the primers at the deleted region.

18 Sequences were determined by direct sequencing.

19

20 **Mutation analysis.** Nucleotide sequences of all ATL samples were aligned using

21 GENETYX-MAC Ver.13.0 software. Mutations were determined after filtering for i)

22 polymorphisms, defined as a nucleotide substitution occurring in >5 cases; ii) linkers,

7

1   defined as >7 common nucleotide substitutions occurring in >2 cases. With these

2   criteria, the sequence of an ATL case, ATL-25, showed the fewest mutations (3 single

3   nucleotide substitutions with no deletions or insertions) and was selected as a standard

4   sequence. The nucleotide positions of identified mutations were numbered relative to

5   this standard sequence, which has been submitted to the DDBJ (DNA Data Bank of

6   Japan) database under the accession number AB513134.

7   For carrier samples, the sequence of a given region from all clones amplified from a

8   carrier was aligned and compared with the standard sequence described above with

9   mutated bases corrected. When a nucleotide substitution occurred in all clones, it was

10   considered a polymorphism and excluded from the mutation repertoire.

11

12   **cDNA synthesis and quantitative real-time RT-PCR.** Total RNA was extracted

13   from transfectants using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was treated

14   with DNase I (Invitrogen) to eliminate the genomic DNA. cDNA was synthesized from

15   1 μg of total RNA with the Superscript preamplification system (Invitrogen) according

16   to the manufacturer's protocol. cDNA product was quantified by real-time RT-PCR

17   with Power SYBR Green PCR Master Mix and 7900HT Fast Real Time PCR System

18   (Applied Biosystems) according to the manufacturer's instructions. Specific primers for

19   *hA3G* gene were 5'-CGCGTGCCACCATGAAGATC-3' (forward) and

20   5'-TGTGGGTGGATCCATCGAGT-3' (reverse). Specific primers for the human *AID*

21   gene were 5'-TTCACCGCGCGCCTCTACTT-3' (forward) and

22   5'-GCTGTCTGGAGAGACGAACT-3' (reverse). Target cDNA was normalized to the

1   amount of endogenous mRNA of *ACTB*. Primers used for *ACTB* were

2   5'-AGGCCAACCGCGAGAAGATG-3' (forward) and

3   5'-CCAGAGGCGTACAGGGATAG-3' (reverse). PCRs were carried out in triplicate.

4   Data was analyzed by the comparative C_t method according to the manufacturer's

5   protocols.

6

7   ***In vitro* editing of HTLV-1 genome by hA3G.** QT6 quail cells were transfected with

8   an infectious molecular clone of HTLV-1 (pX1MT-M) (30) in the presence or absence

9   of hA3G-expression plasmid. Twenty-four hours post-transfection, HTLV-1-producing

10   QT6 cells were treated with 200 μg/ml of mitomycin C at 37˚C for 30 min, well-washed

11   and then co-cultivated 1:1 with fresh QT6 cells (13). 72 hours later, cells were collected

12   and genomic DNA was extracted. hA3G-mediated DNA hypermutation were detected

13   using 3DPCR that allows differential amplification of G-to-A hypermutants using a Taq

14   DNA polymerase as previously described (25). The first round of PCR was performed

15   with a denaturation temperature of 94°C using the primer pair

16   5'-CTGCAGATACAAAGTTAACC-3' (forward) and

17   5'-TGGAGGAAGGAGGGTGGAAT-3' (reverse). PCR conditions were 94˚C for 5 min,

18   followed by 35 cycles of 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec, with a

19   final extension for 10 min at 72˚C. Then, 0.5 μl of the first-round reaction was used as

20   template for the second round of PCR using the following primers:

21   5'-CCATGCTTATTATCAGCCCA-3' (forward) and

22   5'-GTTGGGGGTTGTATGAGTGA-3' (reverse). PCR program was the same as the

1   first round of reaction except that the denaturation gradient ranged from 94-82˚C. PCR

2   products were purified, cloned and up to 20 clones were sequenced.

3

1   **RESULTS**

2   **HTLV-1 proviral sequence changes in ATL**. Previous studies showed that Tax

3   expression was frequently lost by genetic changes in the *tax* gene, by DNA methylation

4   of the 5'LTR, and by deletion of the 5'LTR (29). Similarly, although not well studied to

5   date, the expression of other viral proteins might also be lost through genetic changes in

6   the provirus sequence. To analyze the genetic changes of the HTLV-1 provirus in ATL

7   cells, we determined the whole sequence of proviruses (9034bp) in 60 ATL cases. For

8   this study, we chose ATL cases with HTLV-1 proviruses containing both LTRs. As

9   shown in Table 1, we detected genetic changes (nonsense mutations, deletions and

10   insertions) in the coding region of viral genes in 28 of 60 ATL cases (46.7%). Deletions

11   in the proviruses were detected in 27 cases (45%) while insertions were found in 10

12   cases (16.7%) (See Table S1 in the supplemental material). These deletions and

13   insertions resulted in the loss or the truncation of protein(s) in 18 cases (Table 1 and see

14   Table S1 in the supplemental material).

15   Nonsense mutations, deletions, and insertions in the viral genes were found in all

16   viral genes except the *HBZ* gene (Table 1). Since *p30* and *p13* use an identical coding

17   frame, the same mutations generate nonsense changes in both *p30* and *p13* (Fig. 1). The

18   frequency of genetic changes in the *tax* gene was 16.7% (10/60) (Table 1), which was

19   higher than in previous studies (7, 41). 27 of a total of 29 nonsense mutations

20   preferentially accumulated in tryptophan codons (27/29: 89.7%) (See Table S2 in the

21   supplemental material). Since the tryptophan codon is TGG, a G-to-A mutation

22   generates either a TGA or a TAG stop codon. In one nonsense mutation, the codon for

11

1 arginine (CGA) was converted to the TGA stop codon by a C-to-T mutation.

2 Interestingly, nonsense mutations tended to accumulate in the same cases (Table 1).

3   It was difficult to discriminate between mutations and polymorphisms in provirus

4 from ATL patients since the proviral sequences in non-leukemic cells could not be

5 analyzed in most ATL cases without an internal deletion of provirus. Some mutations

6 might be characteristic of a subgroup of proviruses (polymorphism). Since nonsense

7 mutations at the *tax* gene (7469) were detected in 5 ATL cases, we tentatively judged

8 base substitutions as polymorphisms when more than 5 identical base substitutions were

9 observed in different cases. Based on this criterion, we observed 591 mutations in all

10 cases (See Table S3 in the supplemental material). Among them, the G-to-A mutation

11 was the most frequent, contributing to 28.8% of the total mutations. Other frequently

12 detected mutations were C-to-T (23.7%), A-to-G (17.1%) and T-to-C (12.9%). Frequent

13 G-to-A and C-to-T mutations strongly suggested the activity of deamination enzymes in

14 the generation of these mutations.

15

16 **Mutations in leukemic and non-leukemic cells in ATL cases.** In ATL patients,

17 non-leukemic HTLV-1 infected cells co-exist with leukemic cells. To analyze the

18 proviral sequences of non-leukemic cells, we amplified proviral sequences using

19 primers within regions deleted in leukemic cells to avoid amplification of provirus from

20 leukemic cells. Nonsense mutations that were observed in leukemic cells were not

21 detected in non-leukemic cells (Fig. 2), indicating that nonsense mutations generated in

22 the provirus of leukemic cells were selected for. We compared the whole sequences of

12

1   HTLV-1 provirus in both leukemic cells and non-leukemic cells from five ATL cases.

2   As shown in Table 2, only 37% of base substitutions were considered to be real

3   mutations since the nucleotide differed between leukemic cells and non-leukemic cells.

4   However, 71% of G-to-A transitions were actually mutations while 73% of other base

5   substitutions were polymorphisms (Table 2), indicating that G-to-A mutations are

6   predominant in ATL cells.

7   **Association of G-to-A mutations with human APOBEC3G**. A high frequency of

8   G-to-A mutations suggests the role of deaminase(s) in generating these mutations.

9   Deamination enzymes are known to have individually preferred target sequences for

10   deamination (44). Sequences surrounding the nonsense mutations generated by the

11   G-to-A mutation in HTLV-1 proviruses showed a predominance of the 5'-GG

12   dinucleotide context (target underlined) (Fig. 3A). Among trinucleotides containing GG

13   dinucleotides, CGG, TGG and GGG were preferred (Fig. 3A); these are consistent with

14   the target sequences of hA3G (48). The AGG sequence was also targeted in HTLV-1,

15   unlike in HIV-1. When we checked tetrameric sequences that contain a central GG

16   dinucleotide, CGGG and TGGG were the preferred targets in the HTLV-1 provirus,

17   similar to those reported for HIV-1 (Fig. 3B). As in HIV-1, a C at the +2 position

18   (NGGC) was disfavored in HTLV-1. These findings suggest that the observed G-to-A

19   mutations were generated by hA3G. Another deaminase, APOBEC3F (hA3F), which is

20   largely coexpressed with hA3G, is also reported to target single-stranded minus DNA

21   (46). Unlike hA3G, which favors the 5'-GG dinucleotide, the consensus target sequence

22   of hA3F is 5'-GA (21). G-to-A mutations at GA sites contributed 13.4% of all G-to-A

13

1 mutations (Fig. 3A), suggesting that hA3F might also play a role in these mutations in

2 the HTLV-1 provirus.

3   Since hA3G targets the minus strand during reverse transcription, nonsense mutations

4 generated by G-to-A mutations are not found in the *HBZ* gene**.** A G-to-A mutation in

5 the proviral sense strand caused a nonsense mutation in the *p12* gene (TGG to TGA),

6 whereas the same mutation generated a missense mutation in the *HBZ* gene (CCA to

7 TCA) (Fig. 1). G-to-A mutations occurred most frequently at GG sites with a T or C at

8 their 5' terminus (Fig. 3A). We therefore counted the TGG or CGG sites within

9 individual HTLV-1 regions (Table 3). As NGGC (N indicates T or C) was disfavored

10 by hA3G (Fig. 3B), it was not included. Among HTLV-1 regions, the plus-strand

11 sequence of the *HBZ* gene had the fewest TGG and CGG sites, even when the number

12 of sites was normalized by size of the region. This indicates that mutations in the *HBZ*

13 gene-coding region caused by hA3G are also rare.

14

15   **Mutations in the HTLV-1 proviral sequences from carriers**. Frequent detection of

16 G-to-A mutations in HTLV-1 proviral sequences in ATL cases suggests that these

17 nonsense mutations potentially occur in both HTLV-1 carriers and ATL patients. To

18 explore this possibility, we next studied the proviral sequences (*pol, env, p12, p13/p30,*

19 *p27, tax,* and *HBZ*) in asymptomatic HTLV-1 carriers. The proviral sequences in

20 asymptomatic carriers were amplified using a high fidelity DNA polymerase with

21 proofreading activity, and after subcloning, the sequences were determined for at least

22 20 subclones. As seen in ATL cases, the mutation frequency differed drastically among

14

1   individual carriers (representative data are shown in Table S4 and S5 in the

2   supplemental material), and the distribution of base substitutions among clones varied

3   within individual carriers. G-to-A mutations accounted for 81% and 72 % of all

4   mutations in the *pol* and the *tax* genes, respectively (Table 4 and 5). High frequency of

5   G-to-A mutations was also observed in other viral genes (data not shown). Analyses of

6   the sequence context of G-to-A mutations showed a predominance of GG sequences,

7   which is consistent with the finding that hA3G deaminates the viral genome during

8   reverse transcription (48). In order to compare the frequencies of nonsense mutations in

9   carriers versus ATL cases, the occurrence of nonsense mutations was further analyzed.

10   Consistent with findings in ATL cases, nonsense mutations were detected in the *pol*,

11   *env, p30/p13, p27* and *tax* genes, but not in the *p12* or *HBZ* genes (Table 6). As in ATL

12   cases, nonsense mutations in carriers were most frequently observed in the *tax* and *pol*

13   genes. This result suggests that there is no bias for mutations in specific viral genes in

14   ATL cases compared with carriers. These results suggest that nonsense mutations in

15   provirus are not generated during oncogenesis, but are present in the carrier state.

16   In addition to G-to-A mutation, C-to-T mutations were detected in the *pol* and *tax*

17   genes in carrier cells as in ATL cells (See Table S2 in the supplemental material) as

18   well as other viral regions (data not shown). Comparative analyses of leukemic cell and

19   non-leukemic cells showed that 79% of C-to-T base substitutions were polymorphisms

20   (Table 2). However, after G-to-T mutations, C-to-T mutations were still the most

21   frequent in ATL patients and HTLV-1 carriers.

22

Expression of the *hA3G* gene and activation-induced deaminase gene

In order to analyze the correlation between G-to-A mutation and *hA3G* expression, we

first studied the mRNA level of *hA3G* using real-time PCR assay. The *hA3G* gene was

expressed in normal T cells and the expression level of *hA3G* moderately decreased in

ATL cells. In ATL samples, there was no obvious correlation between the expression

level of *hA3G* and number of G-to-A mutations in the proviral genome.

Although only 21% of C-to-T base substitutions were mutations (Table 2), C-to-T was

the second most frequent mutation (See Table S2 in the supplemental material).

Activation-induced deaminase (AID) was found to be a key factor for the switch

recombination of immunoglobulin (Ig), and is also implicated in hypermutations of Ig

genes (15). AID deaminates cytosine to uracil. Aberrant expression of AID driven by

NF-κB activation is found in gastric cancers and hepatomas, and AID expression has

been invoked to explain induced mutations observed in cancer cells (28). Therefore, we

studied *AID* expression in 7 ATL cases with different proviral mutation frequencies. As

shown in Fig.4, the *AID* gene transcription increased in ATL-15. In this case, we found

six C-to-T mutations in the provirus, and the sequence context showed more AID

preferred sequences in C-to-T mutated sites compared to other cases without AID

activation (data not shown). Thus, AID might also play a role in the deamination of the

HTLV-1 genome in at least some ATL cases. Since AID is thought to target double

strand DNA of provirus, it likely generates C-to-T mutations in the plus strand of

provirus. However, C-to-T base substitutions contained many polymorphism, as shown

in Table 2. Therefore, correlation between C-to-T mutations and AID expression

16

1   remains to be studied.

2

3   *In vitro* **editing of the HTLV-1 genome by hA3G**

4   Previous reports have suggested that HTLV-1 is relatively resistant to the antiviral

5   effect of hA3G (4), consistent with our finding that the frequency of G-to-A mutations

6   throughout the HTLV-1 proviral genome was low. In order to provide direct evidence

7   that G-to-A mutations observed in the proviruses are the result of hA3G-mediated

8   genome editing, we studied *in vitro* the editing effect of hA3G on the HTLV-1 genome.

9   HTLV-1 viruses were generated in the presence of exogenous hA3G to allow packaging

10 into budding viral particles, a step that is required for exerting editing activity of the

11 cytidine deaminase. After a round of virus infection, the HTLV-1 DNA was analyzed

12 using a highly sensitive PCR-based protocol, referred to as 3D PCR, that is capable of

13 amplifying G-to-A hypermutated genomes as described in the Materials & Methods.

14 Differential amplification of a tax region from cells infected with viral particles

15 produced in the presence of hA3G was achieved when the denaturation PCR

16 temperature was lowered to between 84-86˚C (data not shown). All sequences of

17 differentially amplified PCR clones exhibited extensive G-to-A hypermutation, and the

18 number (percentage) of G-to-A transitions ranged from 8 (13%) to 29 (48%) per clone

19 (Fig. 5). In ATL cases in the same region, G-to-A mutations at four nucleotide positions

20 resulted in premature stop codons in the *p30* and *tax* genes (Fig. 1). The occurrence of

21 such mutations was observed *in vitro* when viruses were produced together with hA3G.

22 G-to-A mutations at two sites, in which nonsense mutations were more frequently

1 detected in ATL cases, were also frequent among 3D PCR clones. As expected, a

2 preference for GG dinucleotide context in hA3G-mediated G-to-A editing was observed

3 (Fig. 5C). Our data suggested that hA3G is indeed able to actively deaminate HTLV-1

4 viral genome during reverse transcription although with low editing frequency, leading

5 to G-to-A mutations in the plus-strand of the provirus.

6

DISCUSSION

1

2   In this study, in order to acquire a full mutation spectrum of HTLV-1 virus,

3   sequences of proviral genomes were determined in 60 ATL cases as well as 10

4   asymptomatic carriers. In addition to deletion and insertion, we identified base

5   substitutions, and found that G-to-A mutations were most prevalent. In addition to the

6   observation that G-to-A mutations in ATL and carriers occurred at the preferred target

7   sequence of hA3G, we also experimentally confirmed that hA3G actively introduced

8   G-to-A mutations to the HTLV-1 viral genome. It has been reported that hypermutation

9   in the HTLV-1 genome was a rare event (35), and HTLV-1 was relatively resistant to

10   the antiviral activity of hA3G (33). Unlike HIV, which counteracts hA3G by producing

11   Vif, HTLV-1 does not express an accessory protein that interferes with hA3G. A

12   peptide motif in the C-terminus of the HTLV-1 nucleocapsid has been shown to inhibit

13   hA3G packaging into nascent virions (4). However, inhibition of hA3G induced G-to-A

14   mutations by HTLV-1 was partial (37). Previous studies analyzed small regions of

15   provirus amplified by PCR in carriers or HAM/TSP patients, while proviral sequences

16   of ATL cells were determined by direct sequencing. Since previous studies used

17   error-prone Taq polymerase, it was very difficult to identify mutations after cloning (36).

18   The number of ATL cases was so limited in the previous studies that mutations could

19   not be discriminated from polymorphisms (34). This study first analyzed the whole

20   sequence of 60 ATL cases by direct sequencing, and also determined the proviral

21   sequences of clones derived from HTLV-1 carriers using a precise DNA polymerase,

22   which enabled us to identify mutations in HTLV-1 genomes. Consistent with previous

19

1 studies (34, 35), our results showed that mutations were relatively rare. However,

2 among the rare mutations in HTLV-1 genomes, G-to-A mutations in the target

3 sequences of hA3G were predominant. In addition to G-to-A mutations, other mutations

4 were also observed in ATL and carriers, indicating the role of other deaminases

5 including AID and error-prone reverse transcriptase (27).

6   This study demonstrated important roles for cytidine deaminase(s) in generating

7 mutations in HTLV-1 proviruses. So far, at least 12 cytidine deaminases have been

8 identified, many of which share significant sequence homology. They are considered to

9 have evolved through gene transpositions and duplications (45). Although some

10 members, including APOBEC1 and AID, target cellular genes, most human APOBEC

11 proteins are thought to defend the host against retroviruses. Our study suggests that

12 hA3G is responsible for G-to-A mutations in HTLV-1. hA3G binds to single-stranded

13 DNA, and preferentially deaminates CCCA or CCCG sequences during reverse

14 transcription (48). This strategy of hA3G can induce nonsense mutations in the

15 plus-strand coding sequence since TGG is a target of hA3G, resulting in nonsense

16 mutations like TAG or TGA. However, the *HBZ* gene is encoded by the minus strand of

17 the provirus. The *HBZ* gene is therefore much less susceptible to nonsense mutations

18 generated by hA3G. Furthermore, there are few target sequences for hA3G in the plus

19 strand of the *HBZ* coding RNA. In addition, the coding sequence of the *HBZ* gene

20 overlaps that of *p12* gene. Thus, HBZ further avoids missense mutation in the minus

21 strand.

22   The frequencies of G-to-A changes in the HTLV-1 provirus (0.21% for the *pol* gene

1   and 0.11% for the *tax* gene) in the carriers are slightly lower than that for vif positive

2   HIV-1 (0.57%) (48), indicating that HTLV-1 is resistant to hA3G during reverse

3   transcription. Why are the few nonsense mutations that do occur retained in the

4   HTLV-1 provirus? The difference between HTLV-1 and HIV-1 is related to their

5   distinct strategies of propagation. HIV-1 replicates vigorously *in vivo*, producing

6   tremendous numbers of viral particles. Viruses with nonsense mutations cannot

7   replicate, and thereby disappear *in vivo*. On the other hand, HTLV-1 promotes the

8   proliferation of the infected cells themselves by the action of its regulatory and

9   accessory genes. Therefore, HTLV-1 infected cells can proliferate despite nonsense

10   mutations occurring in most of the viral genes, provided the cells retain the minimum

11   set of viral genes that relate to proliferation. This might be a reason why so many

12   nonsense mutations in various viral genes remain in the provirus. The only gene without

13   nonsense mutation is *HBZ*. It is likely that the *HBZ* gene is indispensable for

14   proliferation of ATL cells, as our previous studies reported (38).

15   Previous studies suggest that Tax is critical for proliferation of HTLV-1 infected cells

16   and oncogenesis. However, we reported that Tax expression is frequently disrupted by

17   three mechanisms (29). It has been speculated that Tax expression is not necessary in

18   the late stages of ATL. Rather, since Tax is a major target of CTLs, ATL cells without

19   Tax expression are selected during leukemogenesis. However, as shown in this study,

20   nonsense mutations were likely generated by hA3G during reverse transcription ,

21   indicating that Tax expression was not necessary even in the carrier state. In one carrier,

22   9 of 21 clones shared a nonsense mutation in the *tax* gene, demonstrating clonal

1   expansion of HTLV-1 infected cells with this mutation (Table S5). Furthermore, 7 of 60

2   ATL cases contained nonsense mutations in the *tax* gene. HTLV-1 infected cells with

3   nonsense mutations could proliferate *in vivo* and be transformed to ATL cells. A

4   nonsense mutation in the *tax* gene has been detected in asymptomatic carriers (8). This

5   study suggests that this nonsense mutation was generated by hA3G during reverse

6   transcription. Another explanation is that loss of the expression of some viral gene(s) by

7   nonsense mutations benefits the cells. Tax generates DNA damage that activates

8   checkpoints (16, 22). In addition, it has been reported that HTLV-1-infected cells

9   become cell cycle-arrested due to the activity of Tax (19, 23). Therefore, HTLV-1

10   infected cells with nonsense mutations in the tax gene might have growth-advantage by

11   loosing Tax expression. We previously reported that HTLV-1 provirus lacking the

12   5'LTR and the second exon of the *tax* gene was detected in ATL cases. By sequencing

13   the integration sites in such defective provirus, we found 6 bp short repeats generated by

14   integrase. This finding showed that defective proviruses that could not produce Tax

15   were generated before integration (31). Taken together, these findings indicate that Tax

16   is not necessary for oncogenesis at least in some ATL cases.

17   This study suggests that nonsense mutations in the HTLV-1 provirus are generated by

18   hA3G in both ATL cases and HTLV-1 carriers. That hA3G targets the minus strand

19   during reverse transcription explains why the *HBZ* gene is not susceptible to such

20   nonsense mutations. On the contrary, HTLV-1 infected cells take advantage of hA3G to

21   escape from the host immune system by losing expression of other viral proteins, while

22   the *HBZ* gene remains intact.

10   **REFERENCES**

11

12 1. **Akagi, T., H. Ono, and K. Shimotohno.** 1995. Characterization of T cells
13   immortalized by Tax1 of human T-cell leukemia virus type 1. Blood **86:**4243-9.
14 2. **Cen, S., F. Guo, M. Niu, J. Saadatmand, J. Deflassieux, and L. Kleiman.**
15   2004. The interaction between HIV-1 Gag and APOBEC3G. J Biol Chem
16   **279:**33177-84.
17 3. **Chiu, Y. L., and W. C. Greene.** 2008. The APOBEC3 cytidine deaminases: an
18   innate defensive network opposing exogenous retroviruses and endogenous
19   retroelements. Annu Rev Immunol **26:**317-53.
20 4. **Derse, D., S. A. Hill, G. Princler, P. Lloyd, and G. Heidecker.** 2007.
21   Resistance of human T cell leukemia virus type 1 to APOBEC3G restriction is
22   mediated by elements in nucleocapsid. Proc Natl Acad Sci U S A **104:**2915-20.
23 5. **Etoh, K., S. Tamiya, K. Yamaguchi, A. Okayama, H. Tsubouchi, T. Ideta, N.**
24   **Mueller, K. Takatsuki, and M. Matsuoka.** 1997. Persistent clonal
25   proliferation of human T-lymphotropic virus type I-infected cells in vivo.
26   Cancer Res **57:**4862-7.
27 6. **Franchini, G., R. Fukumoto, and J. R. Fullen.** 2003. T-cell control by human

1   T-cell leukemia/lymphoma virus type 1. Int J Hematol **78:**280-96.

2   7.   **Furukawa, Y., R. Kubota, M. Tara, S. Izumo, and M. Osame.** 2001.

3   Existence of escape mutant in HTLV-I tax during the development of adult

4   T-cell leukemia. Blood **97:**987-93.

5   8.   **Furukawa, Y., M. Tara, S. Izumo, K. Arimura, and M. Osame.** 2006.

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

7   leukemia. Int J Cancer **118:**381-7.

8   9.   **Gallo, R. C.** 2005. The discovery of the first human retrovirus: HTLV-1 and

9   HTLV-2. Retrovirology **2:**17.

10   10.   **Gaudray, G., F. Gachon, J. Basbous, M. Biard-Piechaczyk, C. Devaux, and**

11   **J. M. Mesnard.** 2002. The complementary strand of the human T-cell leukemia

12   virus type 1 RNA genome encodes a bZIP transcription factor that

13   down-regulates viral transcription. J Virol **76:**12813-22.

14   11.   **Grassmann, R., C. Dengler, I. Muller-Fleckenstein, B. Fleckenstein, K.**

15   **McGuire, M. C. Dokhelar, J. G. Sodroski, and W. A. Haseltine.** 1989.

16   Transformation to continuous growth of primary human T lymphocytes by

17   human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus

18   saimiri vector. Proc Natl Acad Sci U S A **86:**3351-5.

19   12.   **Grossman, W. J., J. T. Kimata, F. H. Wong, M. Zutter, T. J. Ley, and L.**

20   **Ratner.** 1995. Development of leukemia in mice transgenic for the tax gene of

21   human T-cell leukemia virus type I. Proc Natl Acad Sci U S A **92:**1057-61.

22   13.   **Harris, R. S., and M. T. Liddament.** 2004. Retroviral restriction by APOBEC

23   proteins. Nat Rev Immunol **4:**868-77.

24   14.   **Hasegawa, H., H. Sawa, M. J. Lewis, Y. Orba, N. Sheehy, Y. Yamamoto, T.**

25   **Ichinohe, Y. Tsunetsugu-Yokota, H. Katano, H. Takahashi, J. Matsuda, T.**

26   **Sata, T. Kurata, K. Nagashima, and W. W. Hall.** 2006. Thymus-derived

27   leukemia-lymphoma in mice transgenic for the Tax gene of human

28   T-lymphotropic virus type I. Nat Med **12:**466-72.

29   15.   **Honjo, T., H. Nagaoka, R. Shinkura, and M. Muramatsu.** 2005. AID to

30   overcome the limitations of genomic information. Nat Immunol **6:**655-61.

31   16.   **Jin, D. Y., F. Spencer, and K. T. Jeang.** 1998. Human T cell leukemia virus

32   type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1.

33   Cell **93:**81-91.

1   17.   **Kannagi, M., S. Harada, I. Maruyama, H. Inoko, H. Igarashi, G.**

2   **Kuwashima, S. Sato, M. Morita, M. Kidokoro, M. Sugimoto, and et al.** 1991.

3   Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX

4   gene products by human CD8+ cytotoxic T cells directed against HTLV-

5   I-infected cells. Int Immunol **3:**761-7.

6   18.   **Koiwa, T., A. Hamano-Usami, T. Ishida, A. Okayama, K. Yamaguchi, S.**

7   **Kamihira, and T. Watanabe.** 2002. 5'-long terminal repeat-selective CpG

8   methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in

9   vivo. J Virol **76:**9389-97.

10   19.   **Kuo, Y. L., and C. Z. Giam.** 2006. Activation of the anaphase promoting

11   complex by HTLV-1 tax leads to senescence. EMBO J **25:**1741-52.

12   20.   **Lairmore, M. D., L. Silverman, and L. Ratner.** 2005. Animal models for

13   human T-lymphotropic virus type 1 (HTLV-1) infection and transformation.

14   Oncogene **24:**6005-15.

15   21.   **Liddament, M. T., W. L. Brown, A. J. Schumacher, and R. S. Harris.** 2004.

16   APOBEC3F properties and hypermutation preferences indicate activity against

17   HIV-1 in vivo. Curr Biol **14:**1385-91.

18   22.   **Liu, B., S. Hong, Z. Tang, H. Yu, and C. Z. Giam.** 2005. HTLV-I Tax directly

19   binds the Cdc20-associated anaphase-promoting complex and activates it ahead

20   of schedule. Proc Natl Acad Sci U S A **102:**63-8.

21   23.   **Liu, M., L. Yang, L. Zhang, B. Liu, R. Merling, Z. Xia, and C. Z. Giam.**

22   2008. Human T-cell leukemia virus type 1 infection leads to arrest in the G1

23   phase of the cell cycle. J Virol **82:**8442-55.

24   24.   **Luo, K., B. Liu, Z. Xiao, Y. Yu, X. Yu, R. Gorelick, and X. F. Yu.** 2004.

25   Amino-terminal region of the human immunodeficiency virus type 1

26   nucleocapsid is required for human APOBEC3G packaging. J Virol

27   **78:**11841-52.

28   25.   **Mahieux, R., R. Suspene, F. Delebecque, M. Henry, O. Schwartz, S.**

29   **Wain-Hobson, and J. P. Vartanian.** 2005. Extensive editing of a small fraction

30   of human T-cell leukemia virus type 1 genomes by four APOBEC3 cytidine

31   deaminases. J Gen Virol **86:**2489-94.

32   26.   **Malim, M. H., and M. Emerman.** 2008. HIV-1 accessory proteins--ensuring

33   viral survival in a hostile environment. Cell Host Microbe **3:**388-98.

1   27.   **Mansky, L. M.** 2000. In vivo analysis of human T-cell leukemia virus type 1
2   reverse transcription accuracy. J Virol **74:**9525-31.

3   28.   **Matsumoto, Y., H. Marusawa, K. Kinoshita, Y. Endo, T. Kou, T. Morisawa,**
4   **T. Azuma, I. M. Okazaki, T. Honjo, and T. Chiba.** 2007. Helicobacter pylori
5   infection triggers aberrant expression of activation-induced cytidine deaminase
6   in gastric epithelium. Nat Med **13:**470-6.

7   29.   **Matsuoka, M., and K. T. Jeang.** 2007. Human T-cell leukaemia virus type 1
8   (HTLV-1) infectivity and cellular transformation. Nat Rev Cancer **7:**270-80.

9   30.   **Mitchell, M. S., E. T. Bodine, S. Hill, G. Princler, P. Lloyd, H. Mitsuya, M.**
10   **Matsuoka, and D. Derse.** 2007. Phenotypic and genotypic comparisons of
11   human T-cell leukemia virus type 1 reverse transcriptases from infected T-cell
12   lines and patient samples. J Virol **81:**4422-8.

13   31.   **Miyazaki, M., J. Yasunaga, Y. Taniguchi, S. Tamiya, T. Nakahata, and M.**
14   **Matsuoka.** 2007. Preferential Selection of Human T-Cell Leukemia Virus Type
15   1 Provirus Lacking the 5' Long Terminal Repeat during Oncogenesis. J Virol
16   **81:**5714-23.

17   32.   **Miyazato, P., J. Yasunaga, Y. Taniguchi, Y. Koyanagi, H. Mitsuya, and M.**
18   **Matsuoka.** 2006. De novo human T-cell leukemia virus type 1 infection of
19   human lymphocytes in NOD-SCID, common gamma-chain knockout mice. J
20   Virol **80:**10683-91.

21   33.   **Navarro, F., B. Bollman, H. Chen, R. Konig, Q. Yu, K. Chiles, and N. R.**
22   **Landau.** 2005. Complementary function of the two catalytic domains of
23   APOBEC3G. Virology **333:**374-86.

24   34.   **Okazaki, S., R. Moriuchi, N. Yosizuka, K. Sugahara, T. Maeda, I. Jinnai, M.**
25   **Tomonaga, S. Kamihira, and S. Katamine.** 2001. HTLV-1 proviruses
26   encoding non-functional TAX in adult T-cell leukemia. Virus Genes **23:**123-35.

27   35.   **Renjifo, B., I. Borrero, and M. Essex.** 1995. Tax mutation associated with
28   tropical spastic paraparesis/human T-cell leukemia virus type I-associated
29   myelopathy. J Virol **69:**2611-6.

30   36.   **Saito, M., Y. Furukawa, R. Kubota, K. Usuku, S. Sonoda, S. Izumo, M.**
31   **Osame, and M. Yoshida.** 1995. Frequent mutation in pX region of HTLV-1 is
32   observed in HAM/TSP patients, but is not specifically associated with the
33   central nervous system lesions. J Neurovirol **1:**286-94.

1   37.   **Sasada, A., A. Takaori-Kondo, K. Shirakawa, M. Kobayashi, A. Abudu, M.**
2   **Hishizawa, K. Imada, Y. Tanaka, and T. Uchiyama.** 2005. APOBEC3G
3   targets human T-cell leukemia virus type 1. Retrovirology **2:**32.

4   38.   **Satou, Y., J. Yasunaga, M. Yoshida, and M. Matsuoka.** 2006. HTLV-I basic
5   leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia
6   cells. Proc Natl Acad Sci U S A **103:**720-5.

7   39.   **Takatsuki, K.** 2005. Discovery of adult T-cell leukemia. Retrovirology **2:**16.

8   40.   **Takeda, S., M. Maeda, S. Morikawa, Y. Taniguchi, J. Yasunaga, K. Nosaka,**
9   **Y. Tanaka, and M. Matsuoka.** 2004. Genetic and epigenetic inactivation of tax
10   gene in adult T-cell leukemia cells. Int J Cancer **109:**559-67.

11   41.   **Tamiya, S., M. Matsuoka, K. Etoh, T. Watanabe, S. Kamihira, K.**
12   **Yamaguchi, and K. Takatsuki.** 1996. Two types of defective human
13   T-lymphotropic virus type I provirus in adult T-cell leukemia. Blood
14   **88:**3065-73.

15   42.   **Taniguchi, Y., K. Nosaka, J. Yasunaga, M. Maeda, N. Mueller, A. Okayama,**
16   **and M. Matsuoka.** 2005. Silencing of human T-cell leukemia virus type I gene
17   transcription by epigenetic mechanisms. Retrovirology **2:**64.

18   43.   **Trono, D.** 2004. Retroviruses under editing crossfire: a second member of the
19   human APOBEC3 family is a Vif-blockable innate antiretroviral factor. EMBO
20   Rep **5:**679-80.

21   44.   **Turelli, P., and D. Trono.** 2005. Editing at the crossroad of innate and adaptive
22   immunity. Science **307:**1061-5.

23   45.   **Wiegand, H. L., B. P. Doehle, H. P. Bogerd, and B. R. Cullen.** 2004. A
24   second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and
25   HIV-2 Vif proteins. EMBO J **23:**2451-8.

26   46.   **Yoshida, M., Y. Satou, J. Yasunaga, J. Fujisawa, and M. Matsuoka.** 2008.
27   Transcriptional control of spliced and unspliced human T-cell leukemia virus
28   type 1 bZIP factor (HBZ) gene. J Virol **82:**9359-68.

29   47.   **Yu, Q., R. Konig, S. Pillai, K. Chiles, M. Kearney, S. Palmer, D. Richman, J.**
30   **M. Coffin, and N. R. Landau.** 2004. Single-strand specificity of APOBEC3G
31   accounts for minus-strand deamination of the HIV genome. Nat Struct Mol Biol
32   **11:**435-42.

33

1   **FIGURE LEGENDS**

2   **Fig. 1**. **Schema of the distribution of nonsense mutations in HTLV-1 provirus.** The

3   *p13* gene has the same reading frame as the *p30* gene. A star indicates a nonsense

4   mutation observed in an ATL case.

5

6   **Fig. 2. Mutations in non-tumor cell-derived proviral sequence in ATL patients.**

7   HTLV-1 genomes from two ATL samples with internal deleted regions were

8   schematically demonstrated. As indicated by horizontal arrows, one of the PCR primers

9   was set in the deleted region in order to specifically amplify a proviral region originated

10   from non-tumor cells (See Materials & Methods). Nested PCR products, represented by

11   short lines, were subject to direct sequencing. Nucleotide bases mutated in tumor cells

12   according to the criteria mentioned in Materials & Methods were displayed. A base

13   substitution is considered as a polymorphism if the same base was found also in the

14   non-tumor cells derived from the same patient sample. Positions underlined are those at

15   which a G-to-A mutation will result in a nonsense mutation.

16

17   **Fig. 3. Target sequence preference of G-to-A mutations. (A)** Sequence context of

18   G-to-A mutations. In all G-to-A mutations, the percentage of ones at the indicated

19   dinucleotide sequence context is shown (left panel). In GG context, the influence of 5'

20   (middle panel) and 3' (right panel) nucleotides neighboring the GG dinucleotide on

21   G-to-A mutations is displayed. **(B)** Frequency of G-to-A mutations on sites of

22   tetranucleotides containing a central GG. HTLV-1 genome sequence from ATL-25 was

1   used as a standard sequence with mutated bases corrected to calculate the total number

2   of GG sites that exist in the provirus genome (See Materials & Methods). The

3   percentage of GG sites that underwent G-to-A mutation for at least once in 60 ATL

4   cases was plotted against each sequence context. Mutated bases are underlined. CGGG

5   and TGGG were the preferred targets for G-to-A mutation in the HTLV-1 provirus. A C

6   at the +2 position (NGGC) was disfavored.

7

8   **Fig. 4. Expression of the *hA3G* and *AID* gene in ATL cases and in HTLV-1**

9   **associated cell lines.** Expression of the *hA3G* (A) and *AID* gene (B) was investigated

10   by real-time PCR. Normal resting T cells isolated from 3 healthy blood donors were

11   used as a control. *ACTN* was used as an internal control. Relative quantification was

12   performed using a comparative Ct method (Applied Biosystems). Expression of *hA3G*

13   and *AID* in resting T cells was artificially set as 1. Number of nucleotide mutations in

14   the 7 ATL cases was shown below. Among mutated sites, number of AID preferred

15   sites was demonstrated in parenthesis. (C) Number of nucleotide mutations in the 7

16   ATL cases. Among mutated sites, the number of AID preferred sites was demonstrated

17   in parenthesis.

18

19

20   **Fig. 5. In vitro editing of HTLV-1 genome by hA3G.** (A) Mutation matrix for

21   hyperedited *tax* gene sequences derived from cloned 86˚C 3DPCR products. n indicates

22   the number of bases sequenced. (B) A selection of hypermutated HTLV-1 *tax* sequences

1 from tissue culture. The sequence is given with respect to the viral plus strand. Only

2 differences are shown. # and % denotes respectively the number of mutations and the

3 percentage of G-to-A transitions per sequence. The base composition of the sequence is

4 75 T (24 %), 113 C (36.2 %), 60 G (19.2 %) and 64 A (20.5 %). Position number of the

5 first base is indicated above the base. Arrows indicate positions where G-to-A-mediated

6 nonsense mutations were detected in ATL cases. (C) Bulk dinucleotide context of

7 HTLV tax region DNA strand by hA3G. The ordinate represents the substitution

8 frequency as a function of the 3' nucleotide. Mutated G residues are underlined. A χ^2

9 analysis showed that the observed frequencies by hA3G deviated significantly from the

10 expected values (p < 0.001).

Table 1. Abortive genetic changes of HTLV-1 viral genes in 60 ATL cases[a]

	gag	pol	env	p12	p30	p13	p27	tax	HBZ
ATL-2		DEL							
ATL-3								W248*	
ATL-7			DEL						
ATL-8			DEL				W139*		
ATL-10	DEL	DEL	DEL						
ATL-11		W680*	W427*						
ATL-12	DEL		W387*		W165*	W11*	W139*	W56*	
ATL-13	W59*	W680*	DEL		W165*	W11*		W56*	
					W190*	W36*			
ATL-14							DEL	DEL	
ATL-15		W680*							
ATL-27		DEL							
ATL-29			W431*		W165*	W11*		W56*	
ATL-32		W271*							
ATL-36								DEL	
ATL-37		W485*	W50*					W56*	
		W680*							
ATL-39				W87*					
ATL-40					R184*	R30*			
ATL-41		W485*						W56*	
ATL-42			DEL						
ATL-43		DEL							
ATL-44	DEL	DEL		W87*					
ATL-45	DEL							Y286*	
ATL-46	DEL	DEL							
ATL-47	DEL	DEL							
ATL-48	DEL							IN	
ATL-49	DEL	DEL							
ATL-51					W178*	W24*			
ATL-53	DEL								

[a]Only cases with deletion, insertion or non-sense mutations are shown. DEL, deletion.

IN, insertion. W, Tryptophan. *, stop codon. Number indicates position of non-sense

mutation.

Table 2. Concordance rate of mutations between leukemic cells and non-leukemic cells in five ATL cases.

	Total[a]	G-A	C-T	A-G	T-C	A-C	A-T	C-G	C-A	T-G	G-C	G-T	T-A
ATL-8	10 (4)	2 (1)	3 (1)	2 (0)	1 (0)	-	-	1 (1)	-	-	1 (1)	-	-
ATL-12	25 (8)	8 (6)	7 (2)	5 (0)	3 (0)	-	-	2 (0)	-	-	-	-	-
ATL-13	36 (20)	10 (8)	8 (3)	7 (2)	2 (1)	2 (0)	-	-	1 (0)	1 (0)	3 (3)	1 (0)	1 (0)
ATL-14	12 (2)	1 (1)	7 (0)	1 (0)	2 (0)	-	-	-	1 (1)	-	-	-	-
ATL-45	16 (3)	3 (1)	3 (0)	4 (0)	2 (1)	-	3 (0)	-	1 (1)	-	-	-	-
Total	99 (37)	24 (17)	28 (6)	19 (2)	10 (2)	2 (0)	3 (0)	3 (1)	3 (2)	1 (0)	4 (4)	1 (0)	1 (0)
Concordance rate (%)	37%	71%	21%	11%	20%	0%	0%	33%	67%	0%	100%	0%	0%

Each entry *A(B)* shows the number *A* of each type of mutation in ATL judged by our criteria and the number *B* of confirmed mutations. Concordance rate of mutations was calculated by dividing *B* by *A*.

[a]Number of mutations of all types in each ATL case.

Table 3. Distribution of hA3G target sequence in the plus strand of HTLV-1 genome.

Genome Regions	5'LTR	*gag*	*pol*	*env*	*p27*	*p30*	*tax*	*HBZ*	3'LTR
Size (bp)	755	1290	2691	1467	570	726	1062	621	755
CGGX[a]	8	12	16	8	3	2	5	1	7
CGGX/1 kb	10.60	9.3	5.95	5.45	5.26	2.75	4.71	1.61	9.27
TGGX	3	10	25	17	10	7	11	1	3
TGGX/1 kb	3.97	7.75	9.29	11.59	17.54	9.64	10.36	1.61	3.97

[a]X indicates A, T or G.

Table 4. Mutations in the *pol* region of 10 HTLV-1 carriers[a]

	Number and type of single nucleotide substitution									Sequence context of G-to-A mutations			
	G-A	C-T	A-G	T-C	A-C	C-G	G-C	G-T	T-A	GA	GC	GT	GG
C72 (25)	5	1					1						5
C79 (20)	8		1		1			1		1	1		6
C63 (28)	1							1				1	
C40 (26)	13		1										13
C32 (22)		2											
C62 (22)	1	2											1
C87 (22)	14									1	1		13
C5 (23)	19	1						1	2				19
C29 (25)	9					1		1				1	8
C82 (27)	7			1								1	6
Total	77	6	2	1	1	1	1	4	2	2	4	1	71

[a]Number of clones studied for each samples was indicated in the brackets. Mutated

bases are underlined.

Table 5. Mutations in the *tax* region of 10 HTLV-1 carriers[a]

	Number and type of single nucleotide substitution								Sequence context of G-to-A mutations			
	G-A	C-T	A-G	A-C	C-A	G-C	G-T	T-A	GA	GC	GT	GG
C72 (24)	3								1			2
C79 (24)	2	4		2	1	1					2	
C63 (21)	19											19
C40 (21)	2											2
C32 (25)	4	1										4
C62 (27)	5	1									1	4
C87 (24)	1	1							1			
C5 (26)	2											2
C29 (25)	3		3				1					3
C82 (22)	5							1				5
Total	46	7	3	2	1	1	1	1	2	1	2	41

[a]Number of clones studied for each samples was indicated in the brackets. Mutated

bases are underlined.

Table 6. Nonsense mutations in ten HTLV-1 carriers.

Genes	pol		env						p12	p30/p13	p27[a]		tax		HBZ
Position	3955	4540	5465	6158	6342	6362	6482	6494	-	7361	7652	7660	7469	8045	-
C72	**1/25**	-/25	-/23	-/23	-/23	-/23	-/23	-/23	-/31	-/31	-/31	-/31	**1/24**	-/24	-/31
C79	**2/20**	**2/20**	-/20	-/20	-/20	**1/20**	-/20	-/20	-/24	-/24	-/24	-/24	**1/24**	-/24	-/24
C63	-/28	-/28	-/20	-/20	-/20	-/20	-/20	-/20	-/24	**2/24**	-/24	-/24	**9/21**	-/21	-/24
C40	-/26	**13/26**	-/22	-/22	-/22	-/22	-/22	-/22	-/30	-/30	-/30	-/30	**2/21**	-/21	-/30
C32	-/22	-/22	-/22	-/22	-/22	-/22	-/22	-/22	-/32	-/32	-/32	**1/32**	-/25	-/25	-/32
C62	**1/22**	-/22	-/25	-/25	**2/25**	-/25	-/25	-/25	-/28	**1/28**	**1/28**		**1/27**	-/27	-/28
C87	**1/22**	**3/22**	-/20	-/20	-/20	-/20	-/20	-/20	-/21	-/21	**1/21**	-/21	-/24	-/24	-/21
C5	-/22	**19/22**	-/20	-/20	-/20	**1/20**	-/20	-/20	-/24	-/24	-/24	**1/24**	**1/26**	-/26	-/24
C29	-/25	**3/25**	-/20	**1/20**	-/20	-/20	-/20	**1/20**	-/24	**2/24**	**5/24**	-/24	**1/25**	-/25	-/24
C82	**3/27**	**2/27**	**1/20**	-/20	-/20	-/20	**1/20**	-/20	-/24	1/24	-/24	-/24	**1/22**	**1/22**	-/24

Each entry *A/B* in the table shows the number *A* of clones harboring nonsense mutations at a given nucleotide position, from a given carrier, along with the number of clones *B* analyzed in that region from that carrier.

[a]Sequences of exon 2 were determined.

Figure 1

Figure 2

A

% of G-to-A in dinucleotide

GA GC GT GG

% of G-to-A in GG site

AGG CGG TGG GGG

% of G-to-A in GG site

GGA GGC GGT GGG

B

Percentage of GG sites that are mutated

AGGA AGGC AGGT AGGG CGGA CGGC CGGT CGGG TGGA TGGC TGGT TGGG GGGA GGGC GGGT GGGG

Site	AGGA	AGGC	AGGT	AGGG	CGGA	CGGC	CGGT	CGGG	TGGA	TGGC	TGGT	TGGG	GGGA	GGGC	GGGT	GGGG
Mutated	3	2	5	3	1	2	5	13	10	3	0	9	1	1	0	2
All	29	51	17	19	16	38	15	28	37	44	10	24	22	42	8	32
%	10.3	3.9	29.4	15.8	6.3	5.3	33.3	46.4	27.0	6.8	0.0	37.5	4.5	2.4	0.0	6.3

Figure 3

A

B

C

	G-A	C-T	A-G	T-C	A-C	A-T	C-A	C-G	T-G	Total
ATL-4	3(0)	4(1)	1			2				10
ATL-11	6(1)	9(1)	3	8		1				27
ATL-12	8(2)	8(1)	4	3				2		25
ATL-15	10(3)	6(2)	2	2	1	1			1	23
ATL-30	2(0)	1(0)				1				4
ATL-39	2(0)	8(0)	2	5	3	1				21
ATL-45	3(0)	3(0)	4	2		3	1			16

Figure 4

A

→	T	C	G	A
T		2	0	0
C	0		0	1
G	0	0		231
A	0	0	1	

HTLV+A3G
n=3432 bp

C

%

☐ Expected
■ hA3G
* P < 0.001

GA GG GC GT

B

```
7291
CCATGCTTATTATCAGCCCACTTCCCAGGGTTTGGACAGAGTCTTCTTTTCGGATACCCAGTCTACGTGTTTGGAGACTGTGTACAAGGCGATTGGTGCCCCAT    #    %
.......................................A.....A....A...................A....................A................................    8   13
...................................A................A..........................A........................A......A.........   12   20
...................................A................A........................A............A........   19   32
...........................AA...A....A...............A.......A........A......AA.......   26   43
...........................AAA...A....A.............AA..........A.....AA.A...A.A.....A..A...A.......   29   48

CTCTGGGGGACTATGTTCGGCCCGCCTACATCGTCACGCCCTACTGGCCACCTGTCCAGAGCATCAGATCACCTGGGACCCCATCGATGGACGCGTTATCAGCT
....A..................................A........A....................A.................
....AAAA...................A........A....A.............A..............
....AAAA.........A..............A.......A...A.A......A.....AA..........A...............
....AAAAA.....A...A.........A.................AA.........A...A...A.A........
....AAAAA....A..........................AA.........A...AA..A...........

CAGCTCTACAGTTCCTTATCCCTCGACTCCCCTCCTTCCCCACCCAGAGAACCTCTAAGACCCTCAAGGTCCTTACCCCGCCAATCACTCATACAACCCCCAAC
............................A..............G.........................
..............................................................
..................................A........A....................
.....................A...........A........A.....................
.........................A.....................
```

Figure 5