東大と各施設測定値の相関

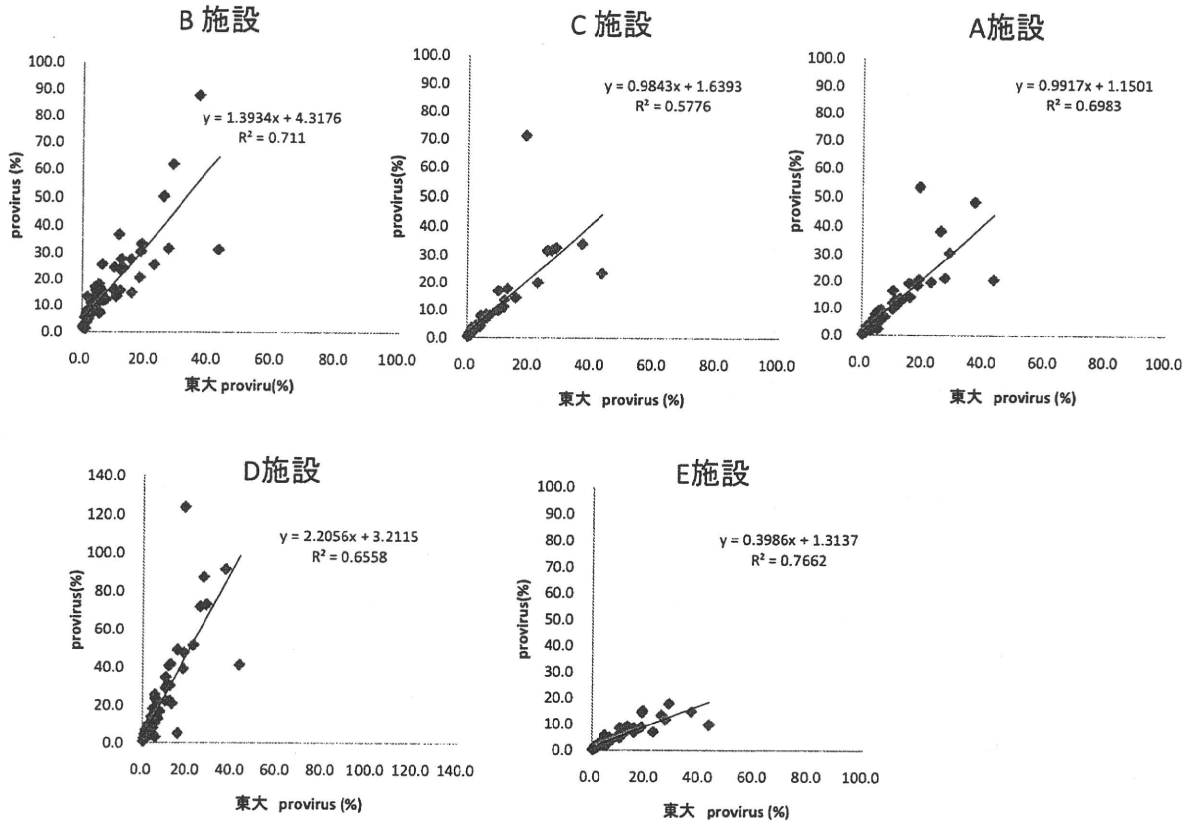
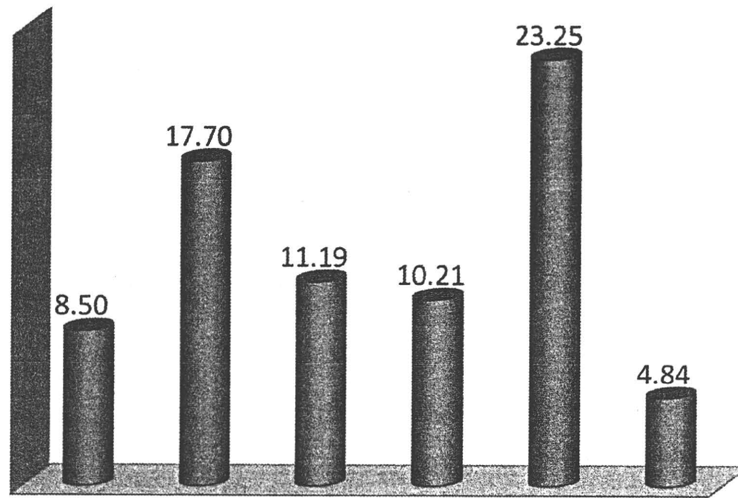


図 20

各施設の平均値の比較と補正係数
-施設間で最大約 5 倍の差-



	Tokyo Univ	B施設	C施設	A施設	D施設	E施設
Average	8.50	17.70	11.19	10.21	23.25	4.84
slope	1.000	1.393	0.984	0.992	2.206	0.399
Ajusted factor	1.000	0.718	1.016	1.008	0.453	2.506

図 21

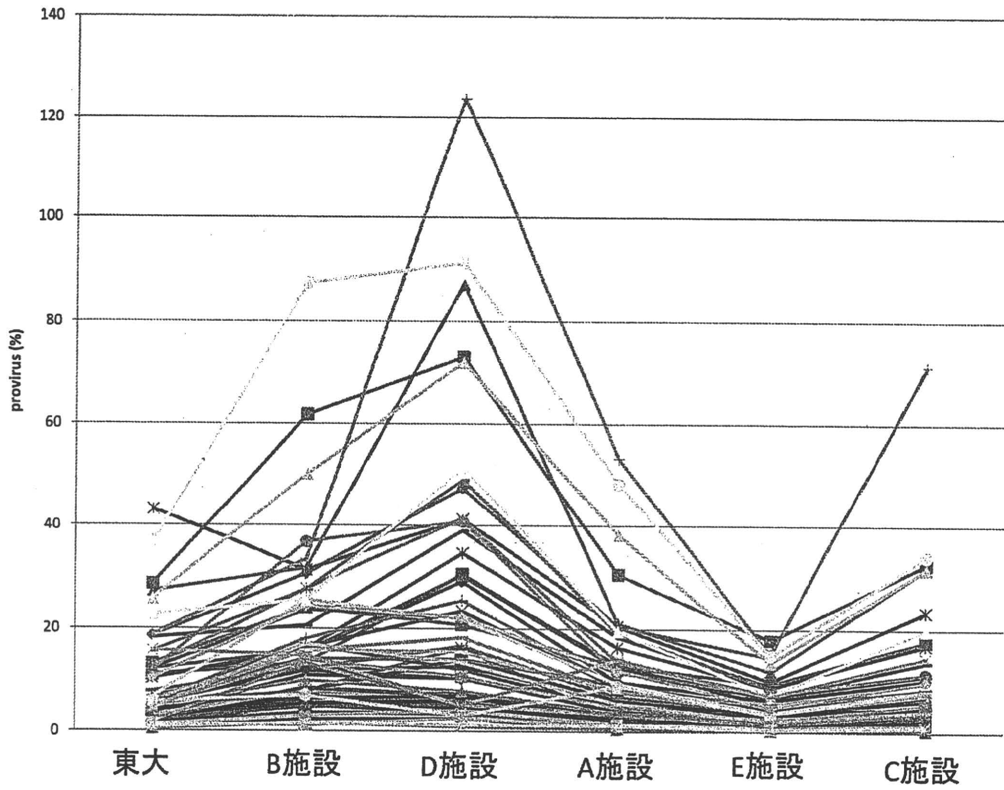


図 22

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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齋藤　滋	ヒト白血病ウイルス-I型(HTLV-I)の母子感染に関する情報の提供について	月刊母子保健	第617号	10	2010
齋藤　滋	妊娠中のHTLV-1検査と母子感染予防	日産婦医会報	平成22年11月号	10-11	2010
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III. 研究成果の刊行物・印刷

Cancer Sci. 2011 Jan 18. doi: 10.1111/j.1349-7006.2011.01873.x. [Epub ahead of print]

c-Maf Suppresses HTLV-1 Tax by Competing for CREB-binding Protein.

Hieshima K, Nagakubo D, Shigeta A, Tanaka Y, Hoshino H, Tsukasaki K, Yamada Y, Yoshie O.

Department of Microbiology, Kinki University Faculty of Medicine, Osaka-Sayama, Osaka 589-8511, Japan Department of Immunology, G Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan Department of Virology and Preventive Medicine, Gunma Univ Medicine, Maebashi 371-8511, Japan. Present address: Department of Developmental Immunology, Max-Planck Institute of Immunobiolo Freiburg, Germany Department of Molecular Medicine and Hematology, Molecular Medicine Unit, Atomic Bomb Disease Institute, Nagasal School of Biomedical Science, Nagasaki, Japan Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical 8501, Japan.

Abstract

The latent infection of HTLV-1 is considered to be preferentially associated with CCR4(+) CD4(+) T cells. Here we report that c-Maf, one c factors for Th2 differentiation, suppresses the transcriptional activity of HTLV-1 Tax by competing for CREB-binding protein. Notably, c-ma induced in a fraction of CCR4(+) CD4(+) T cells upon activation. Furthermore, c-Maf significantly decreases Tax-induced HTLV-1 envelop from an infectious HTLV-1 molecular clone and tax expression in a cell-free HTLV-1 infection system. Collectively, c-Maf may play a role HTLV-1 in CCR4(+) CD4(+) T cells by negatively regulating Tax activity.

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Haematologica. 2011 Jan 12. [Epub ahead of print]

Overexpression of enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in a leukemia/lymphoma as a target for epigenetic therapy.

Sasaki D, Imaizumi Y, Hasegawa H, Osaka A, Tsukasaki K, Choi YL, Mano H, Marquez V, Hayashi T, Yanagihara K, Moriwaki Y, Miyaz
Yamada Y.

Nagasaki, Japan;

Abstract

ABSTRACT Background. Enhancer of zeste homolog 2 is a component of the Polycomb repressive complex 2 that mediates chromatin-b through trimethylation of lysine 27 on histone H3. This complex plays vital roles in the regulation of development-specific gene expression this study, a comparative microarray analysis of gene expression in primary adult T-cell leukemia/lymphoma samples was performed, and evaluated for their oncogenic and clinical significance. Results. Significantly higher levels of Enhancr of zeste homolog 2 and RING1 and transcripts with enhanced levels of trimethylation of lysine 27 on histone H3 were found in adult T-cell leukemia/lymphoma cells comparec CD4+ T-cells. Furthermore, there was an inverse correlation between the expression level of Enhancer of zeste homolog 2 and that of mi suggesting that the altered expression of the latter miRNAs accounts for the overexpression of the former. Patients with high Enhancer of RING1 and YY1 binding protein transcripts had a significantly worse prognosis than those without it, indicating a possible role of these ge and progression of this disease. Indeed, adult T-cell leukemia/lymphoma cells were sensitive to a histone methylation inhibitor, 3-deazane 3-deazaneplanocin A and histone deacetylase inhibitor panobinostat showed a synergistic effect in killing the cells. Conclusions. These fir cell leukemia/lymphoma cells have deregulated Polycomb repressive complex 2 with overexpressed Enhancer of zeste homolog 2, and th of a new therapeutic strategy targeting histone methylation in this disease.

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Defective human T-lymphotropic virus Type 1 provirus in asymptomatic carriers

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Few studies have specifically examined defective provirus in asymptomatic human T-lymphotropic virus Type 1 (HTLV-1) carriers and its relation to proviral DNA loads (PVLs). To assess the significance of defective provirus in asymptomatic carriers, we examined PVLs in peripheral blood mononuclear cells of 208 asymptomatic HTLV-1 carriers. The mean PVLs determined using primers for the *pol* region were less than that for the *pX* region in these carriers. Analysis of seven carriers with high PVLs for the *pX* region but lower PVLs for the *pol* region showed that four had single nucleotide polymorphisms of proviral genomes for the *pol* region and three had HTLV-1-infected cells with defective provirus. Three carriers with defective provirus showed high PVLs at their initial screens, and PVLs increased after a 10- to 12-year interval in two carriers. Southern blot assay showed clonal expansion of HTLV-1-infected cells, and the predominant clones changed during the observation period. These data suggest that although HTLV-1-infected cells with defective provirus may have a growth advantage, the predominant clones of HTLV-1-infected cells do not always survive for many years in asymptomatic carriers.

Infectious Causes of Cancer

Human T-lymphotropic virus Type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL) and a progressive neurological disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] When an individual is infected by HTLV-1, the virus randomly integrates into the genome of affected T-cells in the form of a provirus.[5] The majority of HTLV-1 carriers are asymptomatic, and only a fraction of the number of carriers develops ATL after a long latent period.[6,7] It is thought that HTLV-1 infection drives the proliferation of T-cells, leading to the clonal expansion of HTLV-1-infected cells.[8–11] A high level of HTLV-1-infected cells is considered a risk factor for developing ATL.[12]

Key words: HTLV-1, asymptomatic carrier, proviral DNA loads
Abbreviations: ATL: adult T-cell leukemia/lymphoma; CTL: cytotoxic T-lymphocytes; HBZ: HTLV-1 basic leucine zipper factor; HTLV-1: human T-lymphotropic virus Type 1; LTR: long-terminal repeat; PBMCs: peripheral mononuclear cells; PCR: polymerase chain reaction; PVLs: proviral DNA loads
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The complete HTLV-1 provirus is ~9 kb and contains the coding regions for core protein (*gag*), protease (*pro*), polymerase (*pol*), envelope protein (*env*), regulatory proteins, such as Tax and Rex, and some accessory molecules between 5′ and 3′ long-terminal repeats (LTRs).[5,13] It has been reported that defective provirus was detectable in approximately half of patients with ATL.[14–18] Tamiya *et al.* reported two types of genome deletion in defective provirus.[16] One form (*i.e.*, Type 1) retains both LTRs and lacks internal sequences, such as the *gag* and *pol* regions. The other form (*i.e.*, Type 2) has only the 3′ LTR, and the 5′ LTR and its flanking internal sequences are preferentially deleted. HTLV-1-infected cells harboring Type 2 defective virus were frequently found in patients with ATL.[18] Defective provirus has also been reported to be detectable in asymptomatic HTLV-1 carriers. Morozov *et al.* reported that defective provirus, which lacked large internal sequences, was detectable in 18 of 20 HTLV-1 carriers.[19] However, it has not yet been determined whether the HTLV-1-infected cells with defective provirus are maintained for a long time in asymptomatic carriers and whether the defective provirus is associated with the development of ATL.

In our study, to clarify the significance of defective provirus in asymptomatic carriers, the peripheral mononuclear cells (PBMCs) of 208 HTLV-1 carriers were screened for the presence of defective provirus. Long polymerase chain reaction (PCR) and Southern blot analysis were performed to determine the changes in clonality of HTLV-1-infected cells

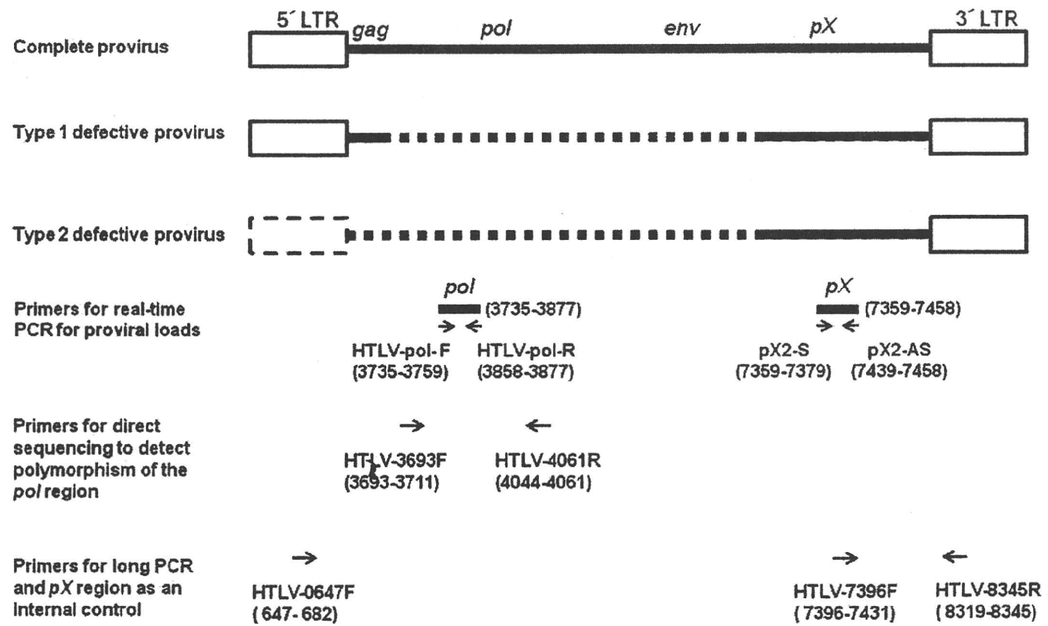

Figure 1. Schemas of structure of complete, Type 1 defective and Type 2 defective HTLV-1 (human T-lymphotropic virus Type 1) provirus. Dotted lines represent the defective regions of HTLV-1 provirus. Locations of primers for polymerase chain reactions in our study are revealed.

harboring defective provirus. Time-sequential samples of greater than 10 years obtained from asymptomatic carriers with large numbers of HTLV-1-positive cells with defective provirus were analyzed.

Material and Methods

Samples

Samples of PBMCs were obtained from 208 asymptomatic HTLV-1 carriers in the Miyazaki Cohort Study.[20] Informed consent was obtained from the study participants, and the study protocol was approved by the institutional review board at the University of Miyazaki. Genomic DNA was isolated from the PBMCs of HTLV-1 carriers by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform extraction and ethanol precipitation.

Quantification of HTLV-1 provirus in PBMCs

Schemas of the structure of complete, Type 1 defective and Type 2 defective HTLV-1 provirus are shown in Figure 1.[16] The nucleotide position number of HTLV-1 provirus was according to Seiki *et al.* (accession no. J02029).[21]

Proviral DNA loads (PVLs) for the *pol* (positions 3735–3877) and *pX* (positions 7359–7458) regions were measured by real-time PCR using a Light Cycler DX 400 (Roche Diagnostics, Mannheim, Germany). When multiple time-sequential samples were available from one subject, the most recent sample was used for the first screening. The primers and the probe for the *pol* region of HTLV-1 provirus were as follows: the forward primer (HTLV-pol-F 5′-AACCAATT CATTCAAACATCTGACC-3′: positions 3735–3759), the reverse primer (HTLV-pol-R 5′-GCTTTCACAGGAGCCAA TGG-3′: positions 3877–3858) and the FAM-labeled probe (5′-FAM-TGTTCCTATCTTACTCCACCACAGTCACCGA-TA MRA-3′: positions 3767–3797).[22] The primers and the probe for the *pX* region of HTLV-1 provirus were as follows: the forward primer (pX2-S 5′-CGGATACCCAGTCTACGTGTT-3′: positions 7359–7379), the reverse primer (pX2-AS 5′-CAGTAGGG CGTGACGATGTA-3′: positions 7458–7439) and the FAM-labeled probe (5′-FAM-CTGTGTACAAGGCGACTGGTGCC-TAM RA-3′).[11] *RNase* P control Reagent (Applied Biosystems, Foster City, CA) was used for the primers and the probe for human *RNase* P DNA as internal control. PVLs were shown by the copy number of HTLV-1 provirus in 100 PBMCs.

Determination of DNA polymorphism in the *pol* primer region

To determine whether the lower PVLs for the *pol* region compared to that for the *pX* region in a same subject was due to the polymorphism of the DNA sequence of primers for the *pol* region, DNA sequence of PCR products of the *pol* region was identified in the cases described below. Primers used for PCR for this purpose were as follows: the forward primer (HTLV-3693F 5′-CTCTGCCAAACCATAC-3′: positions 3693–3711) and the reverse primer (HTLV-4061R 5′-ATGCAAAAGTCCGAGAAG-3′: positions 4061–4044). PCR products were supplied for direct sequencing using an

ABI PRISM Genetic Analyzer 310 (Applied Biosystems). To verify whether the polymorphism found affected the amplification efficiency of real-time PCR for measuring PVLs for the *pol* region, PCR products were subcloned by pGEM-T Easy vector system (Promega, Madison, WI). The amplification efficiency of real-time PCR for the *pol* region was compared between the DNA sequences with and without this polymorphism.

Detection of Type 1 defective provirus by long PCR

To assess whether the Type 1 defective provirus exists in the HTLV-1 carriers with lower PVLs for the *pol* region compared to those for the *pX* region, long PCR, which amplifies the complete provirus and the Type 1 defective provirus with 5′ LTR conserved, was performed. The primers were as follows: 5′LTR(HTLV-0647F 5′-GTTCCACCCCTTTCCCTTTC ATTCACGACTGACTGC-3′: positions 647–682) and 3′LTR (HTLV-8345R 5′-GGCTCTAAGCCCCCGGGGGATATTTG GGGCTCATGG-3′: positions 8345–8319).[18] Long PCR was performed using LA Taq DNA polymerase (Takara Bio, Shiga, Japan). Cycles for long PCR were as follows: one cycle of 98°C for 20 sec, 35 cycles of denaturation at 98°C for 10 sec, annealing at 65°C for 20 sec and extension at 72°C for 7 min. Genomic DNA containing 100 copies of HTLV-1 provirus for the *pX* region from each subject was used. To ensure that same amount of provirus was used for each reaction, PCR for the *pX* region was performed as an internal control. Primers used for this PCR were as follows: the forward primer (HTLV-7396F 5′-GGCGACTGGTGCCCCATCT CTGGGGGACTATGTTCG-3′: positions 7396–7431) and the reverse primer described above (HTLV-8345R).

DNA sequence analysis for Type 1 defective provirus

Long PCR products from subjects suspected of having defective provirus were subcloned by pGEM-T Easy vector system (Promega). The resulting plasmid DNA was purified by GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO). The DNA sequence of long PCR product was identified using an ABI PRISM Genetic Analyzer 310 (Applied Biosystems).

Southern blot hybridization analysis

To analyze the clonality of HTLV-1-infected cells, Southern blot analysis for HTLV-1 provirus was performed based on the method previously described by Kamihira *et al.* with slight modification.[17] Genomic DNA samples (10 μg) from cases were digested with restriction enzyme *Eco*RI (Fermentas, Barlington, Canada), electrophoresed on 0.7% agarose gel and transferred to nylon membrane (Roche). The filter was hybridized with DIG-PCR-labeled HTLV-1 DNA probe mix, which was prepared by a mixture of PCR products to cover the genome of 5′LTR-*gag* (positions 655–1624), *pro* (positions 2109–2619), *pol* (positions 3410–4059), *env* (positions 5464–6114) and *pX* (positions 7461–8646) and by incorporating DIG-11-dUTP (Roche). Finally, the band patterns were visualized with a CDP-star (Roche).

Results

PVLs of 208 asymptomatic HTLV-1 carriers based on the real-time PCR for the *pol* and *pX* regions

PVLs of 208 asymptomatic HTLV-1 carriers were determined by real-time PCR using primers for the *pol* and *pX* regions. The mean PVLs determined using primers for the *pol* region (2.3 copies per 100 PBMCs) were lower than that for the *pX* region (3.6 copies per 100 PBMCs). Because the *pX* region has been reported to be conserved in the HTLV-1 provirus,[14,16] the carriers, whose PVLs for the *pol* region were much lower than those for the *pX* region, were assumed to have many PBMCs harboring defective HTLV-1 provirus. Therefore, to characterize the carriers with defective HTLV-1 provirus, the subjects with relatively high PVLs for the *pX* region, which were equal to or greater than 1.0 copy per 100 PBMCs, and with PVLs for the *pol* region, which were less than half of those for the *pX* region, were supplied for further analysis. Seven carriers (Cases A–G) among 111 carriers with PVLs for the *pX* region, which were equal to or greater than 1.0 copy per 100 PBMCs, met this condition (Table 1).

DNA polymorphism analysis for the *pol* primer region

Although these seven carriers were potential carriers with relatively high PVLs and defective provirus, there was a possibility that the low PVLs for the *pol* region were due to the polymorphism of the DNA sequence of primers and probe for the *pol* region. Therefore, DNA sequences of the *pol* regions for PCR in Cases A–G were determined by the direct sequencing of PCR products. In Cases A–G, the polymorphism was not detected in the forward primer and probe annealing sequences. However, as shown in Table 1, the polymorphism of the DNA sequence was identified in two positions (3860 A>C and 3876 G>A) of the genome of provirus for the reverse primer for the *pol* region in four of seven cases (Cases D–G, Table 1). This DNA sequence was cloned into the plasmid, and the amplification efficacy of real-time PCR for the *pol* region was assessed. As expected, the amplification efficacy of real-time PCR in the plasmid with two nucleotide substitutions was ~3–4% of that in the plasmid without nucleotide substitutions (data not shown). These results accounted for the low PVLs for the *pol* region in Cases D–G shown in Table 1. Therefore, only Cases A–C were thought to potentially have many PBMCs with defective HTLV-1 provirus.

Sequential change of PVLs determined by real-time PCR for the *pol* and *pX* regions

All of three cases (Cases A, B and C) were followed-up for 10 or more years, and the samples from several screens were available (Fig. 2). None of these cases showed any signs or

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Table 1. Proviral DNA loads for the *pol* and *pX* regions and polymorphism found in the *pol* region

Cases	Sex	Age (years)	PVLs (copies/100 PBMCs)			Polymorphism of *pol* region	
			pX	*pol*	*pX/pol*	3860[1]	3876[1]
A	Male	61	57.5	2.8	21.7	A	G
B	Female	73	31.7	0.5	59.2	A	G
C	Female	84	17.3	3.8	4.6	A	G
D	Male	82	12.6	0.4	5.5	C	A
E	Male	45	3.7	0.1	30.6	C	A
F	Male	75	2.5	0.1	27.2	C	A
G	Male	83	1.9	0.1	24.5	C	A

PVLs: proviral DNA loads; PBMCs: peripheral blood mononuclear cells.
[1]Position of proviral genome sequence.

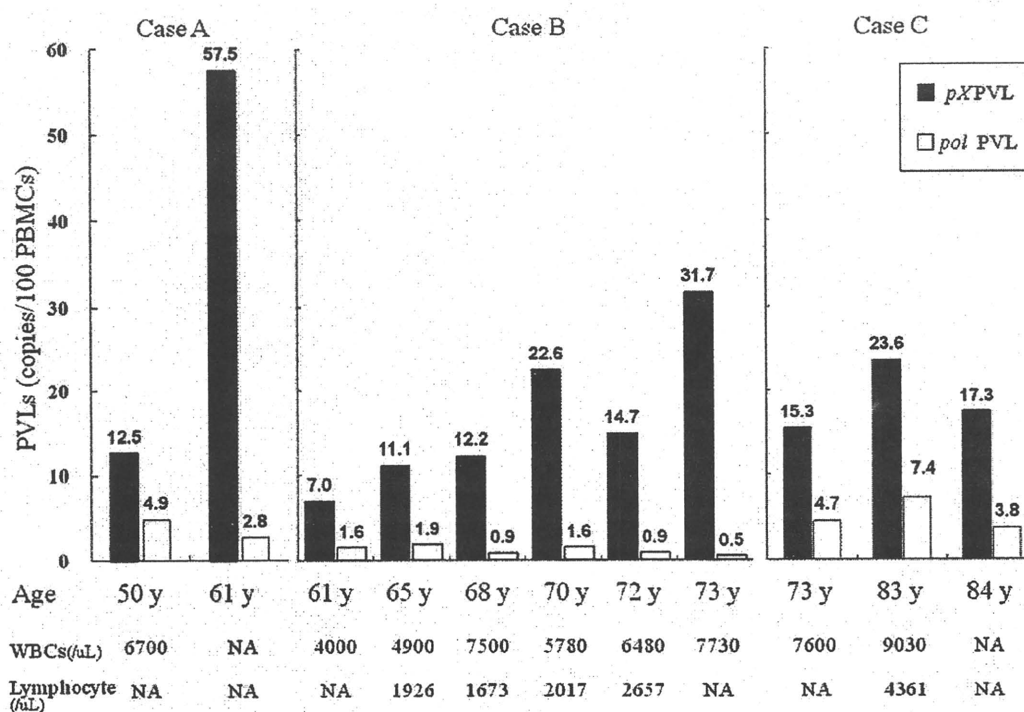

Figure 2. Proviral DNA loads for *pol* and *pX* regions at different ages of Cases A, B and C. WBCs: white blood cells; NA: not available.

symptoms suggesting ATL- and HTLV-1-associated diseases. The numbers of white blood cells and lymphocytes in their peripheral blood were within normal limits with no abnormal cells observed during the follow-up period. Cases A, B and C had high PVLs for the *pX* region, which were greater than 15 copies per 100 PBMCs at the most recent screens, when the cases were 61, 73 and 84 years old, respectively. PVLs for both the *pol* and *pX* regions were measured in previous time-sequential samples from these cases (Fig. 2). PVLs for the *pX* region in Cases A and B showed a marked increase during the 11- and 12-year follow-up, and those for the *pol* region showed either no change or decreased.

Sequencing and analysis for defective provirus in three cases

Long PCR to amplify the HTLV-1 provirus using primers for 5′ LTR and for the *pX* region was performed in the time-sequential samples from Cases A, B and C (Fig. 3). This long PCR amplifies the complete provirus and the Type 1 defective provirus with 5′ LTR conserved. In other words, Type 2 defective provirus, which does not conserve 5′LTR, is not amplified by this long PCR. If the subject had a complete proviral genome, the size of PCR product would be expected to be 7.7 kb. If the PCR products were smaller than 7.7 kb, they were judged to be derived from Type 1 defective provirus.

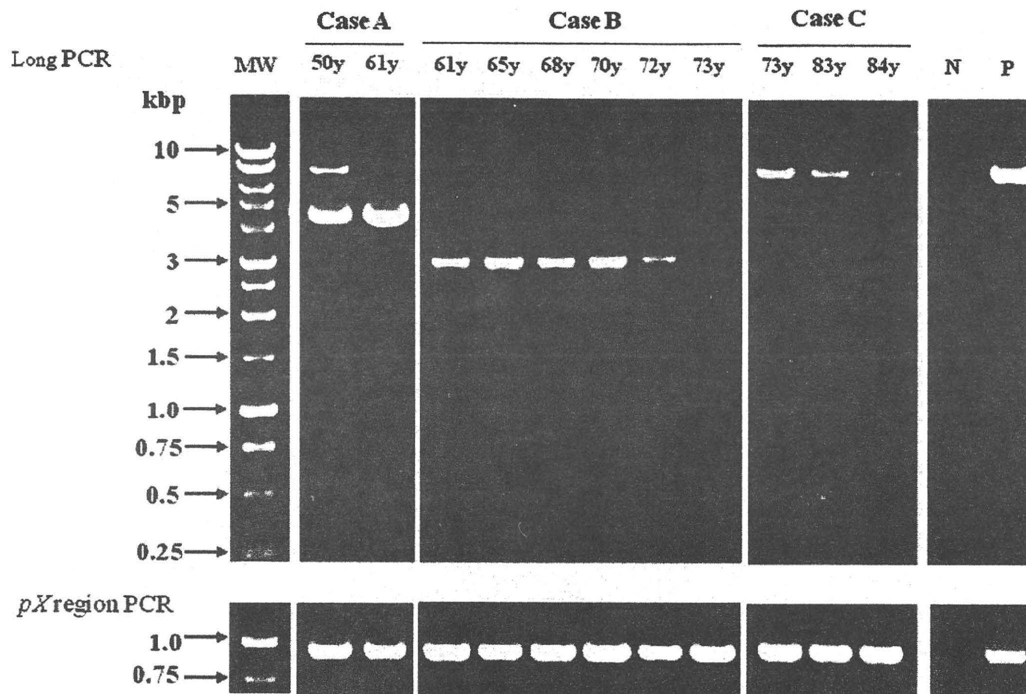

Figure 3. Detection of defective provirus by long polymerase chain reaction at different ages of Cases A, B and C. MW: molecular weight marker; N: human T-lymphotropic virus Type 1 (HTLV-1) negative subject; P: HTLV-1-positive cell line, ED-40515.

In Case A, a strong band of 4.5 kb and a weak band of 2.7 kb were detected in addition to a band of 7.7 kb at age of 50 years. When Case A was 61 years old, the strong 4.5 kb band increased its intensity. In contrast, the weak band of 2.7 kb was not detectable, and the band for the complete proviral genome decreased its intensity. The DNA sequence of the strong band of 4.5 kb showed that this band represented a Type 1 defective provirus with a 3.2-kb deficiency (positions 1203–4368, Fig. 4).

In Case B, a strong band of 2.9 kb was detected in addition to a weak band of 7.7 kb at age of 61 years. The DNA sequence of this 2.9 kb band showed that this band also represented a Type 1 defective provirus with 4.8 kb deficiency (positions 1173–5958, Fig. 4). When Case B was 73 years old, the intensity of the 2.9 kb band decreased markedly (Fig. 3). However, PVLs in Case B gradually increased as time passed (Fig. 2). Therefore, HTLV-1-infected cells harboring 2.9-kb Type 1 defective provirus were assumed not to be responsible for the increase of PVLs in Case B. In other words, HTLV-1-infected cells harboring provirus, which was not detected by long PCR used in our study, increased in number.

In Case C, several bands smaller in size than 7.7 kb, which might represent different Type 1 defective provirus, were detected. However, they were not consistently detectable at the ages of 73, 83 and 84. The 7.7-kb band of the complete proviral genome also decreased its intensity at the age of 84 years. The PCR product at age of 83 years was subcloned, and the DNA sequence was identified (Fig. 4). Thirteen Type 1 defective proviruses were detected in the 33 colonies derived from PCR products except for provirus with complete genome. Four of these were found to have insertions of nonviral sequences (clone cc-1,-3,-4 and -6, in Fig. 4).

Analysis of clonality of HTLV-1-infected cells by Southern blotting

To examine the clonal expansion of HTLV-1-infected cells, samples of genomic DNA (10 μg) from Cases A, B and C were analyzed by Southern blotting (Fig. 5). In Case A, a 17-kb band (a-1) was detected both at 50 and 61 years of age. The intensity of a-1 increased markedly at age 61. The increased intensity of clone a-1was consistent with the finding of increased PVLs for the *pX* region (Fig. 2) and with the increased intensity of the 4.5-kb band of Type 1 defective provirus by long PCR (Fig. 3). In addition, another weak band (a-2) was detected at age 61. Because the size of a-2 was ~7 kb, which was smaller than the size of complete HTLV-1 provirus (9 kb), a-2 was considered to be a clone with defective provirus, which was not detected by long PCR. In Case B, two clones (b-1 and b-2) were detected both at

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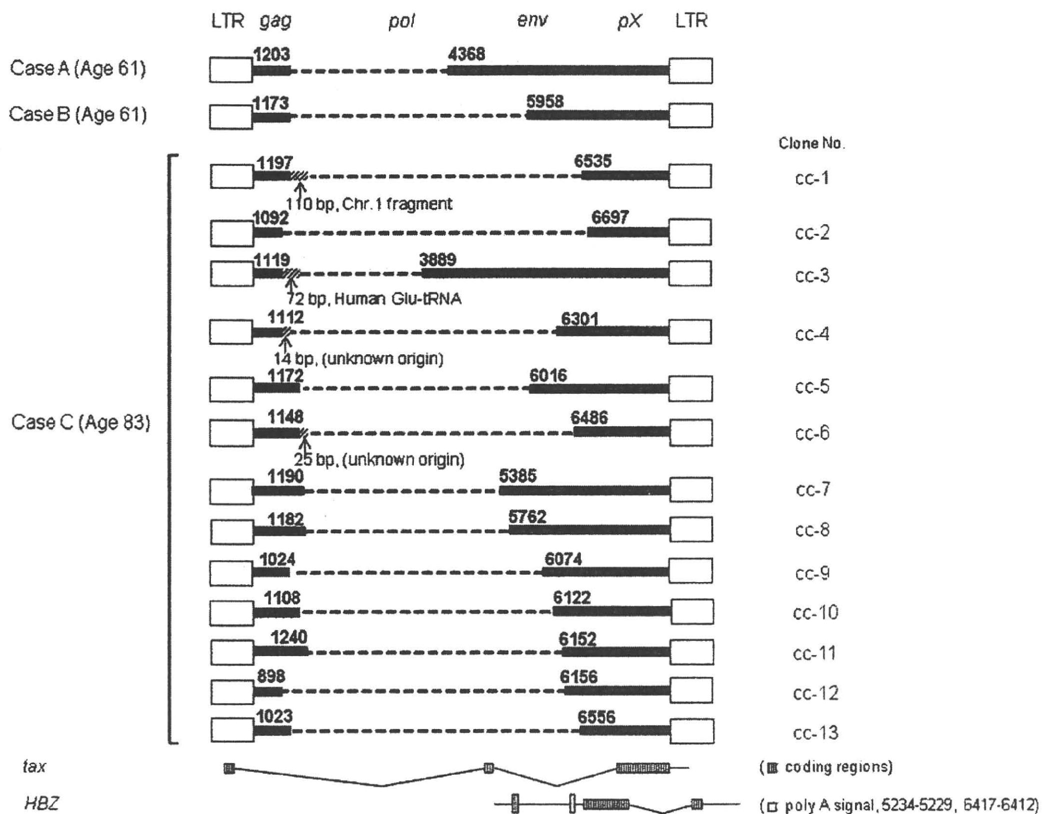

Figure 4. The schema of Type 1 defective provirus in Cases A, B and C. Dotted lines represent the defective regions of provirus. Splicing patterns of *tax* and *HBZ* genes are revealed. The nucleotide position numbers of human T-lymphotropic virus Type 1 (HTLV-1) provirus are same as those of Figure 1.

ages 61 and 70. The intensity of b-2 did not change during 9 years; however, that of b-1 increased at the age of 70. PVLs for the *pX* region increased (Fig. 2); however, the intensity of 2.9-kb Type 1 defective provirus by long PCR showed no change or decreased at age 70 (Fig. 3). Therefore, it was possible that clone b-2 represented the HTLV-1-infected cells with 2.9-kb Type 1 defective provirus detected by long PCR, and that another clone (b-1) of HTLV-1-infected cells with provirus, which was not detectable by long PCR, contributed to the increase of the PVLs in Case B. In Case C, one clone (c-1) was detected both at ages 73 and 84, and the other clone (c-2) was detected only at age 84. The intensity of c-1 was somewhat increased at the age of 84. The size of c-2 was ~7 kb and was considered to be a clone with defective provirus. However, clones c-1 and c-2 were not considered to be harboring Type 1 defective provirus because no band was observed to be increased in intensity at age 84 by long PCR (Fig. 3).

Discussion

To identify asymptomatic carriers, who have PBMCs harboring defective provirus with large deletions, PVLs of 208 asymptomatic HTLV-1 carriers were determined by real-time PCR using primers for the *pol* and *pX* regions. HTLV-1 *pX* region has been reported to be well conserved in the proviral genome.[14,16] Therefore, as expected, PVLs for the *pol* region were lower than those for the *pX* region. The carriers showing PVLs for the *pol* region, which were lower than those for the *pX* region, were considered to be candidates who have many PBMCs harboring defective provirus with large deletions of internal sequences. One hundred and eleven asymptomatic carriers showed relatively high PVLs (equal to or greater than 1.0 copy per 100 PBMCs). Seven showed low PVLs for the *pol* region (less than half of those for the *pX* region) among these 111 carriers. Four cases were excluded from further analysis because their low PVLs for the *pol* region were due to polymorphism of the proviral genome at the site of primer annealing. Three (Cases A, B and C) were considered as candidates for asymptomatic carriers, who have many HTLV-1-infected cells harboring defective provirus with large deletions. PVLs for the *pX* region increased in Cases A and B during follow-up for equal to or greater than 10 years. In contrast, PVLs for the *pol* region showed no change or decreased. These data suggested that the number

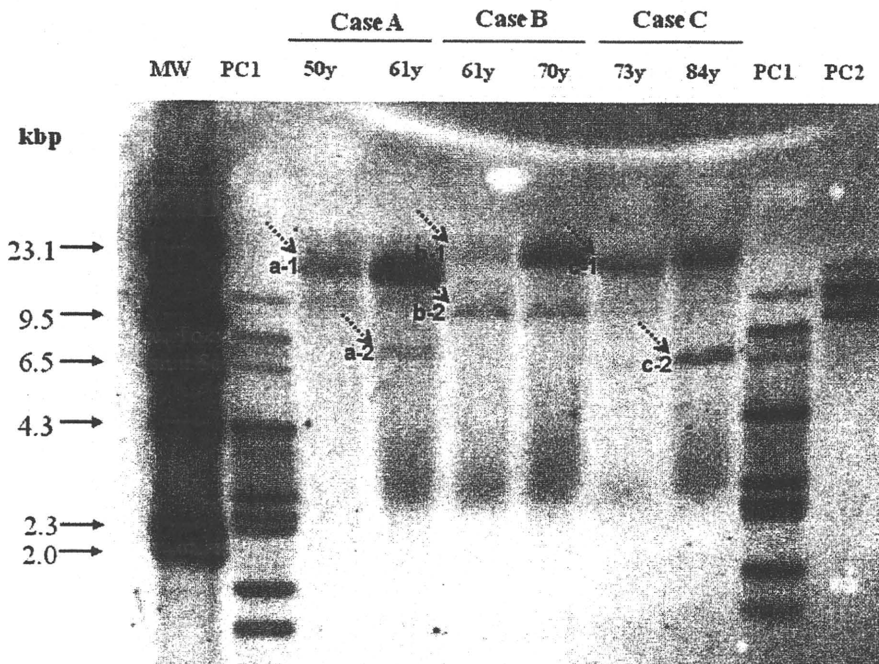

Figure 5. Southern blot analysis for human T-lymphotropic virus Type 1 (HTLV-1) provirus at different ages of Cases A, B and C. Arrows indicate predominant clones of HTLV-1-infected cells. PC1: DNA sample from HTLV-1-positive cell line, ST-1, which was digested with *Pst* I; PC2: DNA sample from HTLV-1-positive cell line, SO_4, which was digested with *Eco* RI; MW: molecular weight marker.

of HTLV-1-infected cells harboring defective provirus increased in Cases A and B.

Then, the defective provirus and the clonality of HTLV-1-infected cells were analyzed for the time-sequential samples from each subject. In Case A, a Type 1 defective provirus with a deletion of internal sequence of 3.2 kb was evident at the age of 50, and its intensity increased at age 61 (Fig. 3). Therefore, the increase of PVLs for the *pX* region, not for the *pol* region, was considered because of the clonal expansion of the HTLV-1-infected cells with this Type 1 defective provirus. Southern blot analysis showing the increased intensity of clone a-1 at age 61 supported this hypothesis. In Case B, a Type 1 defective provirus with the deletion of internal sequence of 4.8 kb was evident at age 61 (Fig. 3). The increase of PVLs for the *pX* region was not explained by the expansion of the HTLV-1-infected cells with this Type 1 defective provirus because the intensity of the PCR product for this defective provirus decreased at ages 72 and 73. Southern blot analysis showed two clones at the age of 61. Clone b-2 showed the same intensity at ages 61 and 70; however, clone b-1 increased in intensity at age 70. Therefore, the HTLV-1-infected cells harboring the Type 1 defective provirus in this case were more likely to belong to clone b-2. The increased PVLs at ages 70–73 were considered to be due to an increased number of HTLV-1-infected cells, which belonged to clone b-1. Clone b-1 was assumed to harbor the defective provirus because only a very weak band for complete provirus was detectable by long PCR in Case B. However, the defective provirus accounting for clone b-1 was not detectable by long PCR and could be a Type 2 defective provirus. Alternatively, a polymorphism of the DNA sequence in the site of primers of long PCR in 5′-LTR for clone b-1 may explain the absence of the band for defective provirus by long PCR. Therefore, it was considered that two major clones with defective provirus existed in Case B at age 61, and only clone b-1 survived as time passed. In Case C, several bands, which may have represented different Type 1 defective provirus, were detected by long PCR in addition to the band for the complete provirus at age 73. These defective proviruses were not consistently detectable at ages 73, 83 and 84. The intensity of the band for the HTLV-1-infected cells with complete provirus was also decreased as time passed. Therefore, maintenance of high PVLs at the age of 84 was not explained by these Type 1 defective proviruses alone. HTLV-1-infected cells with defective provirus, which was not detectable by long PCR in our study, might exist in Case C. In fact, Southern blot analysis showed increased intensity of clone c-1 and the appearance of new clone c-2 at age 84 (Fig. 5). The data from Cases A, B and C in our study suggested that HTLV-1-infected cells with certain types of defective provirus could be the predominant clones and persist for several years; however, the clones of HTLV-1-infected cells do not always survive for a long time in asymptomatic carriers.

Furukawa *et al.* reported that clonally proliferated cells infected with HTLV-1 were detected and were stable for from 4 months to 3 years in patients with HAM/TSP and their seropositive family members without showing any significant indication of ATL.[23] None of Cases A, B and C in our study showed any symptoms and data suggesting ATL- or HTLV-1-associated diseases, even at the end of the follow-up. Therefore, these carriers were judged not to have developed clinical ATL although they had high PVLs and clonal expansion of HTLV-1-infected cells with defective provirus. HTLV-1 Tax protein has been shown to promote the proliferation of infected cells.[13,24] On the other hand, Tax is also reported to be a good target for the host cellular immune response to HTLV-1.[25] HBZ protein was also reported to be important for the proliferation of HTLV-1-infected cells.[26–28] The proviral genome for *HBZ* gene, which is transcribed from 3′LTR, can be conserved even in the Type 2 defective provirus.[16,18] The Type 1 defective provirus found in Case A possessed internal deletion (positions 1203–4368). Theoretically, the expression of Tax and HBZ protein is not prevented by this internal deletion. Therefore, Tax and HBZ may have promoted the proliferation of HTLV-1-infected cells harboring this defective provirus although this proliferation might have been controlled by cytotoxic T-lymphocytes (CTL) through the recognition of Tax. At the same time, this defective provirus cannot express envelope and core proteins, which were also reported as the targets for CTL in HTLV-1 carriers.[29] Therefore, HTLV-1-infected cells harboring this defective provirus may be able to avoid attack from CTL more efficiently. In Case B, Type 1 defective provirus detected by long PCR possessed larger internal deletion (positions 1173–5958). Theoretically, the expression of Tax was prevented because of the deletion of the second exon of the *tax* gene in this defective provirus. It is not clear whether the loss of the expression of Tax protein was related to the decreased intensity of this clone at age 73. Theoretically, this Type 1 defective provirus was able to express HBZ protein because the provirus genome of *HBZ* gene was conserved. In Case C, 13 Type 1 defective proviruses were found at age 83. Twelve among these 13 clones (except clone cc-3 in Fig. 4) had large internal deletions, which theoretically prevent the expression of Tax. Moreover, 4 of 12 clones had the deletions, which theoretically prevent the expression of HBZ protein because of either the deficiencies of the coding regions of *HBZ* and/or deficiencies of two poly A signals (clone cc-1, −2, −6 and −13 in Fig. 4). These large deletions of defective provirus might account for clones not being consistently detectable during a long period in Case C.

In Cases B and C, the increase of PVLs in the time-sequential samples could not be explained by the existence of HTLV-1-infected cells with Type 1 defective provirus. The different clones of HTLV-1-infected cells with defective provirus, which was not detectable by the long-PCR used in our study, might exist in these cases. Clonal expansion of HTLV-1-infected cells with defective provirus, which does not express Tax protein and may not be recognized by the CTL, but which does promote the proliferation of HTLV-1-infected cells under the expression of HBZ protein, was possible. Indeed, HTLV-1-infected cells harboring Type 2 defective provirus were found more frequently in patients with ATL, suggesting a greater potential for leukemogenesis.[17,18]

In Case C, 4 of 13 Type 1 defective proviruses were found to have insertions of nonviral sequences (clone cc-1, −3, −4 and −6 in Fig. 4). Tamiya *et al.* also reported that insertion of a nonviral sequence (35 bp), which was derived from human proline transfer RNA, between the primer binding site and *env* region of HTLV-1 provirus in a patient with ATL.[16] They assumed that this nonviral sequence was inserted into the defective provirus during reverse transcription because human proline transfer RNA had the 16-bp homologous sequence with the 5′-region of HTLV-1. In our study, the DNA sequences of the inserted nonviral sequences in clone cc-1 and −3 were compared to the sequence of the 5′-region of HTLV-1. However, the homologous sequence was not found, and we could not clarify the mechanism of insertions of nonviral sequences in the defective provirus in Case C.

A major limitation of our study is that we were unable to identify genome sequences of Type 2 defective provirus, which possibly existed in Cases B and C, because of technical limitations. Further study to identify Type 2 defective provirus in asymptomatic carriers through improved methodology is necessary. In addition, the number of cases in which defective provirus was analyzed was small in our study. The analysis of more cases may clarify whether the HTLV-1-infected cells harboring the defective provirus have a growth advantage.

In our study, PVLs measured using primers for the *pol* region were less than those for the *pX* region in 208 asymptomatic HTLV-1 carriers. Analysis of seven carriers, who had relatively high PVLs for the *pX* region but much lower PVLs for the *pol* region, showed that they had HTLV-1-infected cells with polymorphism of proviral genome for the *pol* region or with defective provirus. All three asymptomatic HTLV-1 carriers, who had many HTLV-1-infected cells with defective provirus, showed high PVLs. The PVLs in two of the carriers increased markedly after a 10- to 12-year interval. This increase was considered to be due to the expansion of HTLV-1-infected cells with defective provirus. Accordingly, it is suggested that HTLV-1-infected cells with certain types of defective provirus can be predominant clones; however, not all predominant clones of HTLV-1-infected cells survive for a long time. Therefore, the detection of major clones of HTLV-1-infected cells may not always predict the development of ATL. Further study is necessary to clarify whether certain types of defective provirus are related to disease outcome such as ATL.

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