Human Cell (2017) 30:117–123
DOI 10.1007/s13577-016-0156-4

RESEARCH ARTICLE

Infection of defective human T-lymphotropic virus type 1

Yuuki Hashikura[1] · Kazumi Umeki[1] · Kunihiko Umekita[1] · Hajime Nomura[1] · Akiteru Yamada[1] · Ikuo Yamamoto[1] · Hiroo Hasegawa[2] · Katsunori Yanagihara[2] · Akihiko Okayama[1]

Received: 16 November 2016 / Accepted: 23 December 2016 / Published online: 9 January 2017
© Japan Human Cell Society and Springer Japan 2017

Abstract In a previous study, we reported that an identical defective provirus had integrated into multiple sites of the genome of a representative human T-lymphotropic virus type 1 (HTLV-1) cell line, MT-2. A possible explanation for this may be the repeated infection of this defective provirus to a cell. Therefore, we attempted to determine whether a defective provirus could transmit during the co-culture of HTLV-1 uninfected human T-cell line, Jurkat, with MT-2 cells treated with mitomycin C. As a result, we established not only a cell line with the integration of one complete provirus, but also a cell line with the integration of one defective provirus. The rearrangement of the T-cell receptor -γ gene of these cell lines showed them to be derived from Jurkat cells. Both HTLV-1 *Tax/Rex* and *HBZ* RNA were detected in the cell line, which harbors a complete provirus. On the other hand, *HBZ* RNA and transcriptional product specific for the defective provirus were detected in the cell line, which harbors a defective HTLV-1 provirus only. These results suggested that a defective HTLV-1 provirus with large depletion of internal sequence could transmit to other cells. Moreover, the defective provirus can be transcriptionally active. This suggested the possibility that the defective HTLV-1 provirus found in the lymphocytes of HTLV-1 carriers and patients with adult T-cell leukemia may transmit to other T-cells in vivo. The results also suggested that defective provirus in HTLV-1 carriers could be functional and may play a role in leukemogenesis.

Keywords HTLV-1 · Defective virus · MT-2 cell line

Introduction

We analyzed human T-lymphotropic virus type 1 (HTLV-1) provirus integrated into a reference MT-2 cell line, and reported that five types of HTLV-1 proviral sequences were detected in 11 different sites of the genome [1]. The five types of HTLV-1 proviral sequences were one complete proviral genome, two types of proviruses with deletions of large internal viral sequences (5.3 and 3.9 kB), one provirus with a large deletion (6.2 kB) from 5′LTR to position 6257, and one provirus of LTR only. The provirus with identical deletion of large internal viral sequence (5.3 kB) was found to be integrated into six different sites (chromosomes). Efficient infection of HTLV-1 requires cell-to-cell contact of HTLV-1 infected cells and uninfected cells [2–4], followed by the reverse transcription of HTLV-1 RNA and integration of proviral DNA into the host genome. One explanation for the integration of a provirus with identical deletion into multiple sites of the MT-2 genome may be the repeated infection of the defective provirus to cells. To test this hypothesis, we investigated whether a cell line with the integration of a defective provirus could be established after the co-culture of HTLV-1 uninfected human T-cell line, Jurkat, with MT-2 cells treated with mitomycin C, which prevents cell proliferation of MT-2 [5].

✉ Akihiko Okayama
okayama@med.miyazaki-u.ac.jp

[1] Department of Rheumatology, Infectious Diseases and Laboratory Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan

[2] Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8102, Japan

Materials and methods

Cell lines

Human T-cell line Jurkat (Cat No. RCB3052) was purchased from RIKEN BioResource Center (Tsukuba, Japan). An HTLV-1 positive cell line, MT-2, has been passaged at University of Nagasaki. Integration of complete and defective proviruses in this cell line was identified previously [1].

Establishment of the HTLV-1 positive Jurkat cell line

MT-2 cells (1×10^5/ml) processed with mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) in a concentration of 100 μg/ml for 1 h were washed twice with PBS and were co-cultured with Jurkat cells (1×10^5/ml). In the preliminary experiment before the co-culture, it was confirmed that MT-2 cells processed with mitomycin C did not proliferate and were not alive on day 20 (data not shown). Co-cultured cells were passaged twice per week. In each passage, the cells were reduced to the number to 1/10. These passages were repeated 8 times and limiting dilution of the cultured cells was performed on day 30 of culture. Mouse Embryonic Fibroblasts (MEF, COSMO BIO, Tokyo, Japan), which was pre-treated with mitomycin C, were cultured for 24 h in 96 well-Falcon Tissue Culture Plate (Corning, NY, USA) as the concentration of 7500 cells per well. This was for the purpose of supporting single cell culture [6]. Before this experiment, it was confirmed that single cell of Jurkat can proliferate, when MEF cells were used as feeder cells (data not shown). Co-cultured cells were diluted as 7.5 cells/ml and 0.1 ml of them was added in each 96 wells. Culture medium consisted of RPMI1640 (Nacalai Tesque, Kyoto, Japan), 10% fetal bovine serum (Sigma-Aldrich Japan, Tokyo, Japan) and PSN (Life Technologies Japan, Tokyo, Japan). All 96 wells were checked by the microscope for cell proliferation every day. On day 14, the cell proliferation from a single colony was observed in 45 wells and 90% of cells in each well were harvested. Genomic DNA was isolated from the harvested cells using a QIAamp® DNA Mini kit (QIAGEN, Hilden, Germany) according to the instructions provided in the kit. Genomic DNA was used as the template for polymerase chain reaction (PCR). The nucleotide position number of HTLV-1 provirus was according to Seiki et al. (accession No. J02029) [7]. To detect new HTLV-1 provirus integration to Jurkat cells, the *pX* region was amplified by nested PCR as reported by Umeki et al. [8]. The primers for HTLV-1 *pX* region for primary PCR of nested PCR were as follows: the forward primer HTLV-7180F (5′-CTTCCTCCTCCTCCTTGTCC-3′), the reverse primer HTLV-7518 (5′-TGAGCCGATAACGCGTCCATC-3′). The primers for secondary PCR were as follows: the forward primer HTLV-7220F (5′-AATAGCCCGTCCAC CAATTCC-3′), the reverse primer HTLV-7491R (5′-GT CCCAGGTGATCTGATGCTC-3′). The PCR products were electrophoresed on 0.8% agarose gel, and visualized by ethidium bromide staining.

The copy number of HTLV-1 provirus (5′LTR-*gag*, *gag*, and *pX* coding regions) in the cultured cells was then measured by the real-time PCR based on the method described by Ueno et al. [9]. The gene for albumin was used as an internal control. In addition, the copy number of HTLV-1 proviral LTR was measured. The primers of PCR for HTLV-1 LTR region were as follows: forward primer HTLV-421F (5′-CCATCCACGCCGGTTGAG-3′), reverse primer HTLV-561R (5′-CTGAGTCTAGGTAGGCTCCA AG-3′), The FAM-labeled probe HTLV-456P (5′-CCGC CTGTGGTGCCTCCTGAACTG-3′). The copy numbers of HTLV-1 provirus for 5′LTR-*gag*, *gag*, *pX* coding regions and LTR per one cell (two albumin genomes) were measured [10].

Analysis of the rearranged T-cell receptor-γ gene

To confirm the newly HTLV-1 infected cell lines derived from the Jurkat cells, the rearrangement of T-cell receptor-γ (TCR-γ) gene of the cell lines was amplified by PCR based on the report by Benhattar et al. [11], using forward primer (TVG 5′-AGGGTTGTGTTGGAATCAGG-3′) and reverse primer (TJG 5′-CGTCGACAACAAGTGTTGTTC CAC-3′). DNA sequences of the PCR products were analyzed using Big Dye Terminator ver3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 3500/3500xL Genetic Analyzer (Thermo Fisher Scientific), according to the instructions provided with the assay kits.

Confirmation of defective HTLV-1 provirus integrated into Jurkat cells by long PCR

To identify the large defective provirus in the newly HTLV-1 infected cells, long PCR was performed based on the report by Takenouchi et al. [12], using primers for forward primer HTLV-647F (5′-GTTCCACCCCTTTCCC TTTCATTCACGACTGACTGC- 3′) and reverse primer HTLV-8345R (5′-GGCTCTAAGCCCCCGGGGGATATT TGGGGCTCATGG-3′).

Identification of the structure and integration site of the HTLV-1 provirus in the newly HTLV-1 infected cell lines using inverse-long-PCR

To detect the sites of HTLV-1 provirus integration, inverse-long-PCR (IL-PCR) was first performed using a method described previously [13]. Briefly, genomic DNA was digested with *Eco* RI and self-ligated by T4 ligase. This was followed by digestion with *Mlu* I. The primers used in this analysis were a forward primer in the U5 region of the LTR (HTLV-8856F 5′-TGCCTGACCCTGC TTGCTCAACTCTACGTCTTTG-3′) and a reverse primer in the U3 region of the LTR, (HTLV-123R 5′-AGTCTGG GCCCTGACC TTTTCAGACTTCTGTTTC-3′). PCR was performed using LA Taq DNA polymerase (TAKARA BIO, Ozu, Japan). Yeast RNA (Thermo Fisher Scientific) was added to PCR reaction mixture at a final concentration of 100 ng/μL to increase amplification efficiency. PCR products were subjected to sequencing assay as described above. The DNA sequence of the provirus and adjacent human genome was referenced using BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat). Based on the genome DNA sequence adjacent to each provirus, we then performed integration-site-specific PCR to identify the whole structure of the HTLV-1 provirus. The primer, which was specific to the new cell line genome adjacent to the integration site of each provirus, was synthesized. Two primers for the HTLV-1 proviral sequence were used for the integration-site-specific PCR (HTLV-7002R: 5′-AGTATTTG AAAAGGAAGGAAGAGGAGAAGGCA-3′ and HTLV-1206TF: 5′-AA GTCCTTCCAGTCATGCATCCACATG GTG-3′). The resultant PCR products were subjected to sequencing assay as described above.

Detection of transcriptional products derived from HTLV-1 provirus

RNAs were purified from the newly HTLV-1 infected cell lines, Jurkat cells, and MT-2 cells using TRIzol® Reagent (Life Technology Japan, Tokyo, Japan) according to the instructions provided. Resultant RNA was reverse transcribed to complementary DNA (cDNA) using an M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, USA) with random primer (TAKARA BIO) according to the instructions. The primers of PCR for *Tax/Rex* cDNA were as follows: forward primer (Tax-F: 5′-CCCGCCGATCCCA AAGAAA-3′), reverse primer (Tax-R: 5′-GGGTATCC GAAAAGAAGACTCTG-3′). The primers of PCR for *HBZ* cDNA were as follows: forward primer (HBZ-F: 5′-G GCAGAACGCGACTCAACC-3′), reverse primer (HBZ -R: 5′-CGGGCATGACACAGGCAAG-3′). Specific primers for both adjunctive sites of the deficient provirus (positions 1333–6658) were prepared as follows: forward primer (MT-2 def-F 5′-AGCAAGAAGTCTCCCAAGCA G-3′), reverse primer (MT-2 def-R 5′-GGAGGCGATGT GGTTGCA-3′). *GAPDH* RNA was used as an internal control. The primers for the PCR of *GAPDH* cDNA were as follows: forward primer (GAPDH-F 5′-GATGCTGGC GCTGAGTACG-3′), reverse primer (GAPDH-R 5′-GCAG AGATGATGACCCTTTTGG-3′). Based on the amount of PCR products, the cycles of PCR were adjusted to 37, 37, 25 and 25 for *Tax/Rex, HBZ*, a defective provirus and *GAPDH* cDNA, respectively [1].

Results

Establishment of Jurkat cell lines harboring a complete and a defective provirus

Twelve of 45 clones obtained after the co-culture of Jurkat and MT-2 cells processed with mitomycin C tested positive for HTLV-1 genome of the *pX* region by PCR. Then, the copy numbers of HTLV-1 provirus for 5′LTR-*gag*, *gag*, *pX* coding regions and LTR per one cell (two albumin genomes) in these 12 clones were measured. Finally, one clone (MBJ24) showed the copy number of HTLV-1 provirus for 5′LTR-*gag*, *gag*, *pX* coding regions and LTR to be 0.9, 1, 0.9, and 1.8, respectively. Therefore, MBJ24 was assumed to harbor only one complete HTLV-1 genome per cell. Another clone (MBJ46) showed the copy number of HTLV-1 provirus for 5′LTR-*gag*, *gag*, *pX* coding regions and LTR to be 1, 0, 0.9, and 2.1, respectively. These data suggested MBJ46 harbors only one HTLV-1 genome with deficiency of *gag* region per cell.

To confirm that MBJ24 and MBJ46 were derived from Jurkat and not MT-2 cells, the rearrangement of TCR-γ gene of these cell lines was identified by PCR followed by DNA sequencing. Twenty-three of 181 nucleic acids sequenced differed between Jurkat and MT-2. The DNA sequences of MBJ24 and MBJ46 matched those of Jurkat (Fig. 1), suggesting that MBJ24 and MBJ46 cells were derived from Jurkat cells.

Analysis of HTLV-1 provirus in MBJ24 and MBJ46 cells

HTLV-1 provirus in MBJ24 and MBJ46 cells amplified by long PCR showed single bands measuring approximately 8 and 2.4 kB, respectively (Fig. 2). The sizes of these PCR products were the same as the complete HTLV-1 provirus and defective HTLV-1 provirus with 5.3 kB deletion detected in MT-2 cells. To identify the sites of HTLV-1 provirus integration and the DNA sequence of MBJ24 and MBJ46, IL-PCR using *Eco*RI digestion followed by DNA sequencing was performed. The HTLV-1 provirus in

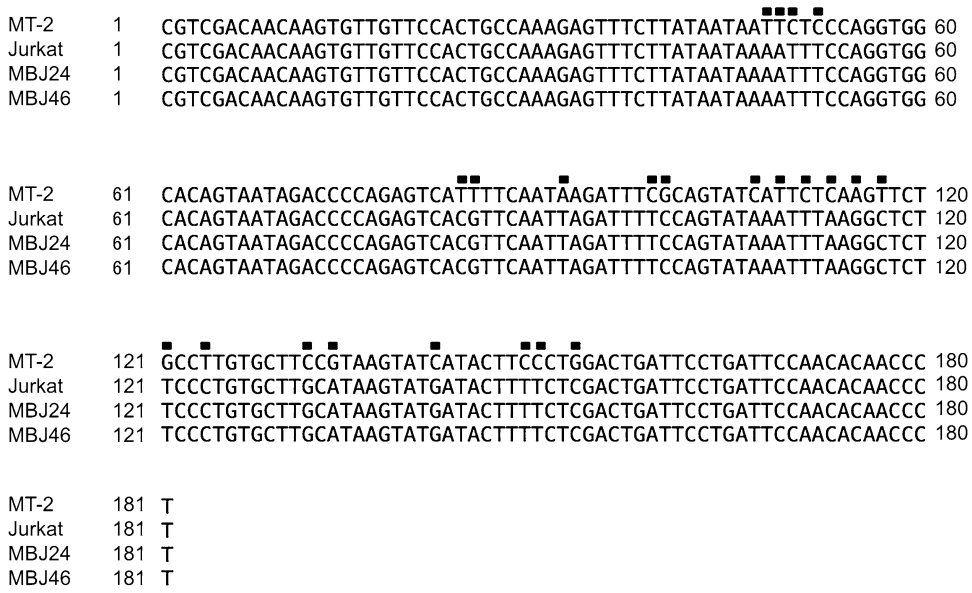

Fig. 1 The sequence alignment of the PCR product for TCR-γ gene derived from the newly established human T-lymphotropic virus type 1 (HTLV-1) infected cell lines (MBJ24 and MBJ46), Jurkat cell line, and MT-2 cell line. *Closed squares* indicate the differences between MT-2 cell and 3 cell lines (Jurkat, MBJ24, MBJ46)

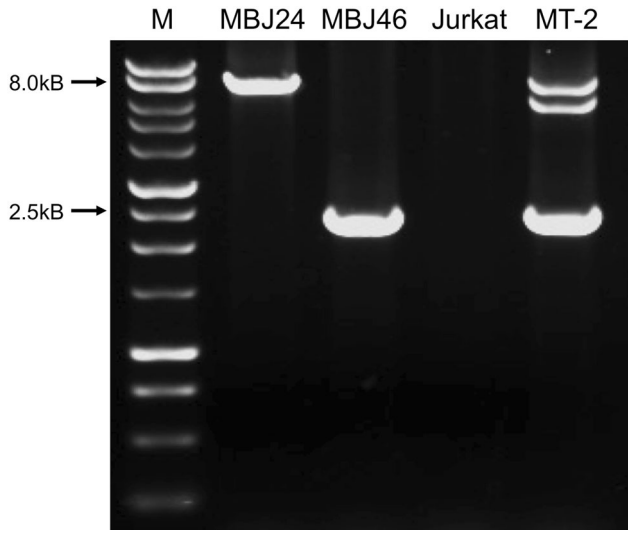

Fig. 2 Long PCR for human T-lymphotropic virus type 1 (HTLV-1) provirus of newly established HTLV-1 infected cell lines (MBJ24 and MBJ46), Jurkat, and MT-2. PCR products were electrophoresed on 0.8% agarose gel. *M* DNA marker

MBJ24 was found to be integrated into the satellite region; however, the chromosome could not be confirmed. We confirmed that the HTLV-1 provirus in MBJ46 was integrated into chromosome 8 (p12). Then, to identify the whole structure of provirus at each integration site, integration-site-specific PCR was performed. Based on the information from the MBJ24 and MBJ46 genomic DNA sequence adjacent to each HTLV-1 provirus, primers that anneal to the expected genomic DNAs on the upstream or the downstream of the HTLV-1 provirus of each integration site were synthesized. PCR was then performed using these primers in combination with primers that anneal to the HTLV-1 proviral genome. The DNA sequence of integration-site-specific PCR products was analyzed, and the whole structure of HTLV-1 provirus was identified. The structure and integration sites of HTLV-1 proviruses in newly established cell lines are summarized in Fig. 3. Only one complete HTLV-1 provirus and a defective provirus with 5.3 kB deficiency from the *gag* to *env* region were integrated into MBJ24 and MBJ46, respectively. The deficiency seen in MBJ46 was the same as that most frequently found in MT-2 cells [1].

Detection of transcriptional products of HTLV-1 *Tax/Rex*,*HBZ* and a defective provirus in MBJ24 and MBJ46

To determine whether RNA specific for proviruses is transcribed from provirus in MBJ24 and MBJ46 cells, we performed RT-PCR to detect HTLV-1 *Tax/Rex* and *HBZ* RNA. In addition, we also investigated whether the specific RNA for defective HTLV-1 provirus (with 5.3 kB deletion) was transcribed. As shown in Fig. 4, *Tax/Rex* and *HBZ* RNA was detected in MBJ24 cell line, which harbors a complete provirus. A higher level of *HBZ* RNA than *Tax/Rex* RNA was observed in MBJ24. *HBZ* RNA, but not *Tax/Rex* RNA, was detected in MBJ46 cells, which harbors a defective HTLV-1 provirus (with 5.3 kB deletion). Transcriptional product specific for the defective provirus was detectable only in MBJ46 cells.

Discussion

Miyoshi et al. established an HTLV-1 positive cell line, MT-2, from human cord leukocytes co-cultured with ATL cells [14–16]. MT-2 cells have been utilized in numerous studies to investigate HTLV-1 infection and the

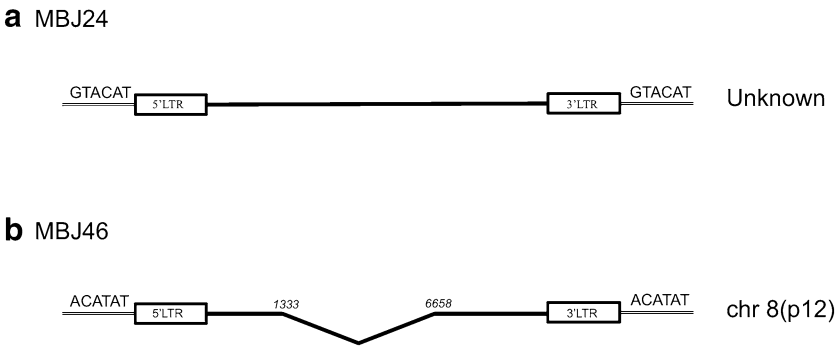

Fig. 3 Structure of human T-lymphotropic virus type 1 (HTLV-1) provirus and their integration sites (chromosomes) in MBJ24 and MBJ46 cells was shown. Positions of deletion or truncated provirus were indicated as HTLV-1 provirus base numbers. Six bases repeat sequences derived from host genome DNA were shown at the both end of provirus

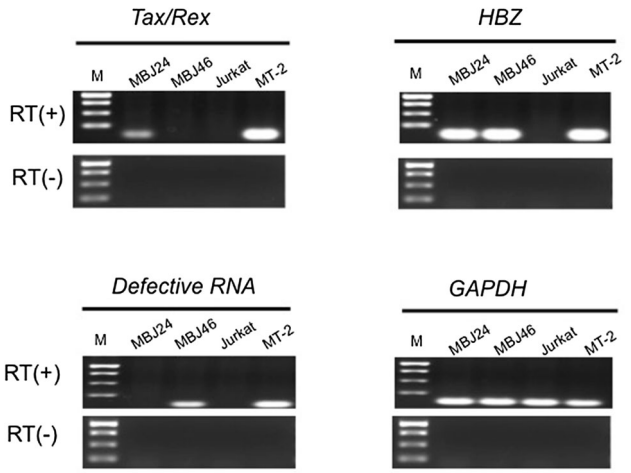

Fig. 4 Identification of RNA transcribed from genes of HTLV-1 *Tax/Rex, HBZ,* a defective provirus and *GAPDH* in MBJ24 and MBJ46 cells with or without reverse transcription was shown. PCR products were electrophoresed on 0.8% agarose gel and visualized by ethidium bromide staining. *M* 100 bp ladder marker

mechanism of leukemogenesis. In a previous report, we demonstrated that MT-2 cell harbors five types of HTLV-1 proviral sequences, including one complete proviral genome and several types of defective provirus [1]. A provirus of identical 5.3 kB deletion (positions 1333–6658) of internal sequence was found to be integrated into 6 different sites. Repeated sequences consisting of six bases derived from the host genome, which is characteristic of the integrated form of retrovirus [17], were found at both ends of this provirus. In general, it is thought that a complete virus integrates into the host genome and that the internal sequence is deleted by a mechanism that results in a defective provirus [18]. However, the identical 5.3 kB deletion (positions 1333–6658) was found in the defective provirus at 6 different sites of MT-2. It is unlikely that this identical 5.3 kB deletion occurred accidentally in the complete proviruses, which were already integrated into 6 different sites of the MT-2 genome.

One possible explanation is that a virus with identical 5.3 kB deletion of internal sequence integrated into several sites of the MT-2 genome [1]. Therefore, we investigated whether this virus could integrate into the HTLV-1 negative cells when MT-2 cells were co-cultured. As a result, a cell line, MBJ46, with the integration of provirus with identical 5.3 kB deletion of internal sequence into the Jurkat genome was established. No other complete or defective provirus was detected in MBJ46. The defective provirus integrated into MBJ46 could not produce proteins for core, envelope, reverse transcriptase and integrase due to the 5.3 kB deletion of internal sequence. Therefore, it was assumed that infection of this defective virus occurred with the assistance of proteins derived from complete provirus in MT-2 cells. In fact, it has been reported that HTLV-1 particles including defective viral RNA are produced by MT-2 cells [19, 20]. Morozov et al. reported that light particles released from MT-2 cells are composed primarily of p28 protein corded by 5.3 kB deleted HTLV-1 provirus DNA, but also includes a smaller amount of p19 (MA), $Pr53^{gag}$, gp21(TM), and gp46 (SU). The viral products (transcriptional products and functional proteins) provided by a complete provirus may help the infection of defective provirus.

Next, we wondered whether transcriptional product of HTLV-1 provirus was detectable in the MBJ46 cell line. We tested to determine whether *Tax/Rex* RNA and *HBZ* RNA were detectable. As a control, we used another cell line, MBJ24, which was also established by the co-culture of MT-2 and Jurkat cells. MBJ24 was shown to harbor only one complete HTLV-1 provirus without defective provirus. Both *Tax/Rex* RNA and *HBZ* RNA were detected in MBJ24, although the signal of *Tax/Rex* RNA appeared weaker than that of *HBZ* RNA. Testing of the MBJ46 cell line showed *HBZ* RNA, but no *Tax/Rex* RNA. *Tax/Rex* RNA signal was not expected because *Tax/Rex* RNA cannot be transcribed due to the 5.3 kB deletion of internal sequence. It was important to show that *HBZ* RNA could

be transcribed even from this defective provirus, which was infected to Jurkat cells. In addition, a transcriptional product specific for the defective provirus with deletion of internal viral sequence (5.3 kB) was also detectable in MBJ46 cells. This RNA transcript was supposed to be the same as the 24S (3.4 kB) RNA transcript, which was previously reported to translate into p28 and to work as a protein kinase [21]. Taken together, these results suggested the possibility that the defective provirus in MBJ46 is transcriptionally active.

The leukemic cells in patients with ATL have been reported to frequently harbor defective provirus [22–26]. Both *Tax/Rex* RNA and *HBZ* RNA have been thought to play an important role in leukemogenesis; however, while *HBZ* RNA is always detected in ATL cells, *Tax/Rex* RNA is not [18, 27]. Because Tax protein is a good target for immune surveillance, pre-leukemic cells, which lose the expression of Tax, are considered more likely to survive [28]. Therefore, HTLV-1 infected cells such as MBJ46, which harbor a defective provirus to express *HBZ* RNA, but not *Tax/Rex* RNA, may serve as a good model for further experiments to investigate the role of defective provirus in leukemogenesis.

Moreover, in the current study, it was shown that a defective viral sequence with deletion of internal viral sequence (5.3 kB) can transmit alone to T-cells such as Jurkat cells. It was shown that HTLV-1 asymptomatic carriers frequently have HTLV-1 infected cells with defective provirus of the *gag* region [9]. One mechanism for the deficiency of internal sequence in provirus involves non-homologous recombination between two sites, which was reported in human immunodeficiency virus type 1 genetic recombination [29]. Alternatively, the results of the current study suggest the possibility of a direct transmission of defective provirus in cell-to-cell contact. It is assumed that products from a complete HTLV-1 genome are essential to this process because HTLV-1 infected cells with the integration of defective provirus cannot produce the components necessary for the transmission of a virus. It is still unclear whether this can occur in vivo (in HTLV-1 carriers).

There are several limitations to the current study. We attempted to test whether translation to viral products from proviruses existed in MBJ24 and MBJ46 cell lines; however, our attempt was unsuccessful. It was not clear whether this was due to the lack of translation from provirus or because of technical problems. It has also not yet been confirmed whether the change of Jurkat cell phenotype occurred after the infection of complete or defective provirus. It is also important to show that MBJ 46 cells, which harbor only a defective provirus, alone cannot transmit HTLV-1 virus to the other T-cells. Further study is necessary.

In conclusion, we demonstrated that a defective HTLV-1 provirus with large depletion of internal sequence derived from MT-2 could transmit to a T-cell line, Jurkat. Moreover, transcription of HTLV-1 *HBZ*, not *Tax*, was detectable in the established MBJ46 cell line harboring only one copy of defective provirus per cell. These results suggested the possibility that the defective HTLV-1 provirus found in the lymphocytes of HTLV-1 carriers can transmit to other T-cells in vivo. It also suggested that defective provirus in the HTLV-1 carriers can be functional and may play a role in leukemogenesis. Further study to clarify the mechanism of the transmission of defective HTLV-1 provirus is necessary.

Acknowledgements The authors would like to thank Ms. Y. Kaseda and Ms. K. Takatsuka (Miyazaki University) for their technical support and assistance.

Compliance with ethical standards

Conflict of interest We have no disclosure.

References

1. Hashikura Y, Umeki K, Umekita K, Nomura H, Yamamoto I, Hasegawa H, et al. The diversity of the structure and genomic integration sites of HTLV-1 provirus in MT-2 cell lines. Hum Cell. 2016;29:122–9.
2. Igakura T, Stinchcombe JC, Goon PK, Taylor GP, Weber JN, Griffiths GM, Tanaka Y, Osame M, Bangham CR. Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. Science. 2003;299:1713–6.
3. Thoulouze MI, Alcover A. Can viruses form biofilms? Trends Microbiol. 2011;19:257–62.
4. Van Prooyen N, Gold H, Andresen V, Schwartz O, Jones K, Ruscetti F, et al. Human T-cell leukemia virus type 1 p8 protein increases cellular conduits and virus transmission. Proc Natl Acad Sci USA. 2010;107:20738–43.
5. Grassmann R, Dengler C, Müller-Fleckenstein I, Fleckenstein B, McGuire K, Dokhelar MC, et al. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a *Herpesvirus saimiri* vector. Proc Natl Acad Sci USA. 1989;86:3351–5.
6. Fisher HW, Puck TT. On the functions of X-irradiated "feeder" cells in supporting growth of single mammalian cells. Proc Natl Acad Sci USA. 1956;42:900–6.
7. Seiki M, Hattori S, Hirayama Y, Yoshida M. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc Natl Acad Sci USA. 1983;80:3618–22.
8. Umeki K, Umekita K, Hashikura Y, Yamamoto I, Kubo K, Nagatomo Y, Okayama A (2017) Evaluation of line immunoassay to detect HTLV-1 infection in an endemic area, Southwest of Japan; comparison with polymerase chain reaction and Western blot. Clin Lab 63.doi:10.7754/Clin.Lab.2016.160501
9. Ueno S, Umeki K, Takajo I, Nagatomo Y, Kusumoto N, Umekita K, et al. Proviral loads of human T-lymphotropic virus Type 1 in asymptomatic carriers with different infection routes. Int J Cancer. 2011;130:2318–26.

10. Kuramitsu M, Okuma K, Yamagishi M, Yamochi T, Firouzi S, Momose H, et al. Identification of TL-Om1, an adult T-cell leukemia (ATL) cell line, as reference material for quantitative PCR for human T-lymphotropic virus 1. J Clin Microbiol. 2015;53:587–96.
11. Benhattar J, Delacretaz F, Martin P, Chau-bert P, Costa J. Improved polymerase chain reaction detection of clonal T-cell lymphoid neoplasms. Diagn Mol Pathol. 1995;4:108–12.
12. Takenouchi H, Umeki K, Sasaki D, Yamamoto I, Nomura H, Takajo I, et al. Defective human T-lymphotropic virus type 1 provirus in asymptomatic carriers. Int J Cancer. 2011;128:1335–43.
13. Etoh K, Tamiya S, Yamaguchi K, Okayama A, Tsubouchi H, Ideta T, et al. Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells in vivo. Cancer Res. 1997;57:4862–7.
14. Miyoshi I, Kubonishi I, Yoshimoto S, Shiraishi Y. A T-cell line derived from normal human cord leukocytes by co-culturing with human leukemic T-cells. Gann. 1981;72:978–81.
15. Miyoshi I, Kubonishi I, Yoshimoto S, Shiraishi Y. Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. Nature. 1981;294:770–1.
16. Yamamoto N, Okada M, Koyanagi Y, Kannagi M, Hinuma Y. Transformation of human leukocytes by cocultivation with an adult T cell leukemia virus producer cell line. Science. 1982;217:737–9.
17. Miyazaki M, Yasunaga J, Taniguchi Y, Tamiya S, Nakahata T, Matsuoka M. Preferential selection of human T-cell leukemia virus type 1 provirus lacking the 5′ long terminal repeat during oncogenesis. J Virol. 2007;81:5714–23.
18. Tamiya S, Matsuoka M, Etho K, Watanabe T, Kamihira S, Yamaguchi K, et al. Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia. Blood. 1996;88:3065–73.
19. Morozov VA, Weiss RA. Two types of HTLV-1 particles are released from MT-2 cells. Virology. 1999;255:279–84.
20. Benovic S, Kok T, Stephenson A, McInnes J, Burrell C, Li P. De novo reverse transcription of HTLV-1 following cell-to-cell transmission of infection. Virology. 1998;244:294–301.
21. Iino T, Takeuchi K, Nam SH, Siomi H, Sabe H, Kobayashi N, et al. Structural analysis of p28 adult T-cell leukaemia-associated antigen. J Gen Virol. 1986;67:1373–9.
22. Takeda S, Maeda M, Morikawa S, Taniguchi Y, Yasunaga J, Nosaka K, et al. Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. Int J Cancer. 2004;109:559–67.
23. Tsukasaki K, Tsushima H, Yamamura M, Hata T, Murata K, Maeda T, et al. Integration patterns of HTLV-I provirus in relation to the clinical course of ATL: frequent clonal change at crisis from indolent disease. Blood. 1997;89:948–56.
24. Kamihira S, Sugahara K, Tsuruda K, Minami S, Uemura A, Akamatsu N, et al. Proviral status of HTLV-1 integrated into the host genomic DNA of adult T-cell leukemia cells. Clin Lab Haematol. 2005;27:235–41.
25. Ohshima K, Kikuchi M, Masuda Y, Kobari S, Sumiyoshi Y, Eguchi F, et al. Defective provirus form of human T-cell leukemia virus type I in adult T-cell leukemia/lymphoma: clinicopathological features. Cancer Res. 1991;51:4639–42.
26. Korber B, Okayama A, Donnelly R, Tachibana N, Essex M. Polymerase chain reaction analysis of defective human T-cell leukemia virus type I proviral genomes in leukemic cells of patients with adult T-cell leukemia. J Virol. 1991;65:5471–6.
27. Satou Y, Yasunaga J, Yoshida M, Matsuoka M. HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. Proc Natl Acad Sci USA. 2006;103:720–5.
28. Bangham CR. Human T-lymphotropic virus type 1 (HTLV-1): persistence and immune control. Int J Hematol. 2003;78:297–303.
29. Onafuwa-Nuga A, Telesnitsky A. The remarkable frequency of human immunodeficiency virus type 1 genetic recombination. Microbiol Mol Biol Rev. 2009;73:451–80.