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Identification of TL-Om1, an Adult T-Cell Leukemia (ATL) Cell Line, as Reference Material for Quantitative PCR for Human T-Lymphotropic Virus 1

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Quantitative PCR (qPCR) for human T-lymphotropic virus 1 (HTLV-1) is useful for measuring the amount of integrated HTLV-1 proviral DNA in peripheral blood mononuclear cells. Many laboratories in Japan have developed different HTLV-1 qPCR methods. However, when six independent laboratories analyzed the proviral load of the same samples, there was a 5-fold difference in their results. To standardize HTLV-1 qPCR, preparation of a well-defined reference material is needed. We analyzed the integrated HTLV-1 genome and the internal control (IC) genes of TL-Om1, a cell line derived from adult T-cell leukemia, to confirm its suitability as a reference material for HTLV-1 qPCR. Fluorescent *in situ* hybridization (FISH) showed that HTLV-1 provirus was monoclonally integrated in chromosome 1 at the site of 1p13 in the TL-Om1 genome. HTLV-1 proviral genome was not transferred from TL-Om1 to an uninfected T-cell line, suggesting that the HTLV-1 proviral copy number in TL-Om1 cells is stable. To determine the copy number of HTLV-1 provirus and IC genes in TL-Om1 cells, we used FISH, digital PCR, and qPCR. HTLV-1 copy numbers obtained by these three methods were similar, suggesting that their results were accurate. Also, the ratio of the copy number of HTLV-1 provirus to one of the IC genes, RNase P, was consistent for all three methods. These findings indicate that TL-Om1 cells are an appropriate reference material for HTLV-1 qPCR.

Human T-lymphotropic virus 1 (HTLV-1) was the first retrovirus to be found in humans (1, 2). HTLV-1 is a cause of adult T-cell leukemia (ATL), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-1-associated uveitis (3). Areas where HTLV-1 is endemic are distributed across several different regions, including southern Japan, the Caribbean, South America, and tropical Africa (4, 5). A recent report has shown that the area affected by this infection has expanded from the southern part of Japan to the entire country, particularly the Tokyo metropolitan area (6). Diagnostic tests for HTLV-1 infection are performed mainly with serological assays, such as enzyme-linked immunoabsorbent assay, particle agglutination assay, and Western blotting. Recently, another diagnostic test has been developed. Quantitation of integrated proviral DNA in peripheral blood (proviral load [PVL]) can be performed by quantitative PCR (qPCR) as a risk assessment for ATL or HAM/TSP (7, 8).

A few studies reported that several samples were positive for viral DNA when tested by PCR even though those samples had been found seroindeterminate for HTLV-1 when tested by Western blotting (9, 10). Their results suggest that HTLV-1 qPCR could be used as an additional test to confirm infection in seroindeterminate samples.

Although many laboratories have developed qPCR methods for HTLV-1 detection in Japan, a wide variety of testing methods are used. For example, the target region, primers and probes, and internal control (IC) genes vary among the laboratories (8, 11–15). These variations lead to significant differences in HTLV-1 PVL when these laboratories measure the same samples (16). As a consequence of these differences, comparison of quantitative data between laboratories will continue to be difficult without standardization.

One possible solution is to establish a reference material, which is indispensable for standardizing multicenter test results. The target material for HTLV-1 qPCR is genomic DNA (gDNA) from peripheral blood mononuclear cells (PBMCs). Therefore, HTLV-1-infected cells would be an ideal source for a reference material. To date, many cell lines from ATL patients have been established, but few of them have been well characterized for the genomic features associated with reference materials for HTLV-1 qPCR.

In this study, we investigated the genomic structure of one of these ATL cell lines, TL-Om1, to establish it as a reference material for HTLV-1 nucleic acid amplification techniques (NATs), namely, HTLV-1 clonality, karyotyping, proviral sequencing, integration sites, and determination of gene copy number of HTLV-1 and cellular genes for IC.

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TABLE 1 Primers used for qPCR of HTLV-1 and IC genes

Target gene	Forward name	Forward sequence	Reverse name	Reverse sequence	Size (bp)	Primer correction factor	
						Plasmid	gDNA
HTLV-1 gene	LTR202F	ACAATGACCATGAGCCCCAAA	LTR202R	TTAGTCTGGGCCCTGACCT	101	0.9869	
	LTR215F	GCTCGCATCTCTCCTTCAC	LTR215R	AGTTCAGGAGGCACCACA	102	0.9942	
	LTR005F	CCTGACCCTGCTTGCTCAAC	LTR005R	TCAGTCGTGAATGAAAGGGAAAG	99	0.9917	
	056F	TAGTCCCACCCTGTTCGAAATG	056R	GCCAGGAGAATGTCATCCATGT	105	1.0013	
	084F	CCTGCCCCGCTTACTATCG	084R	GGCATCTGTGAGAGCGTTGA	102	0.9922	
	153F	TTGTCGCGCTACTCCTTCTTG	153R	AGGGATGACTCAGGGTTTATAAGAGA	118	0.9792	
	pX2-S[a]	CGGATACCCAGTCTACGTGTT	pX2-AS[a]	CAGTAGGGCGTGACGATGTA	100	0.9944	
RNaseP (RPPH1) gene	RPPH1-05F	TATGCACAATTATGTAATCCCCAAA	RPPH1-05R	CCAGCTCCCTATAACCTGCACTT	100	1.0025	1.0012
	RPPH1-08F	GCCGGAGCTTGGAACAGA	RPPH1-08R	AATGGGCGGAGGAGAGTAGTCT	109	0.9956	0.9937
	RPPH1-12F	AGGAAGCCCACGAAAATTCTAATT	RPPH1-12R	GTCCCCATACTCGGTGATTCTC	101	1.0019	1.0052
Albumin (ALB) gene	ALB-07F	TGCAATGAACACAGGAGAGCTACTA	ALB-07R	CCACCCAGGTAACAAAATTAGCAT	103	0.9971	0.9964
	ALB-19F	CCTGATGCTTCTCAGCCTGTT	ALB-19R	TCCATTTAAGAGTGTGTGTGGTAGGT	100	1.0019	1.0045
	ALB-26F	TGCATTGCCGAAGTGGAAA	ALB-26R	CCTCAGCATAGTTTTTGCAAACA	100	1.0038	1.0078
β-Actin (ACTB) gene	ACTB-06F	TCTGGTGTTTGTCTCTCTGACTAGGT	ACTB-06R	CCGCTTTACACCAGCCTCAT	100		0.9965
	ACTB-12F	TCCTGGGTGAGTGGAGACTGT	ACTB-12R	CCATGCCTGAGAGGGAAATG	107		1.0016
	ACTB-21F	AGCATCCCCCAAAGTTCACA	ACTB-21R	GGACTTCCTGTAACAACGCATCT	101		1.0106
CD81 gene	CD81-01F	GACACATCCCAAGGGTGCTT	CD81-01R	GGACTCAGTTCTCAATGCTTTGC	107		1.0015
	CD81-10F	ACCACGCCTTGCCCTTCT	CD81-10R	GAATCACGCCACTTCCATAACTG	111		1.0021
	CD81-21F	GGTGCACACAGCATGCATTT	CD81-21R	GTGCGCCTCTGGGTAATCAT	102		1.0009
β-Globin (HBB) gene	HBB-11F	TTGGACCCAGAGGTTCTTTGAG	HBB-11R	GGCACCGAGCACTTTCTTG	103		1.0021
	HBB-15F	AGCAGCTACAATCCAGCTACCAT	HBB-15R	GAGGTATGAACATGATTAGCAAAAGG	105		1.0033
	HBB-24F	CCCACCCCAAATGGAAGTC	HBB-24R	AGCACCATAAGGGACATGATAAGG	104		1.0111
RAG-1 gene	RAG1-03F	GCAATCCCATTTGTCCACTTTT	RAG1-03R	TCCCACTGGCCTGCATTACTA	100		1.0045
	RAG1-27F	GAAGTTTAGCAGTGCCCCATGT	RAG1-27R	ACGGGCAGTGTTGCAGATG	100		1.0006
	RAG1-32F	TCAAAGTCATGGGCAGCTATTGT	RAG1-32R	AGGGAATTCAAGACGCTCAGAA	100		0.9993

[a] Primer sequences were previously reported in reference 11.

MATERIALS AND METHODS

Cells and gDNA preparation. Jurkat clone E6-1 cells were obtained from the American Type Culture Collection. HUT102 and SLB-1 cells, which are HTLV-1-infected cell lines, were a kind gift from Masahiro Fujii (Division of Virology, Niigata University Graduate School of Medical and Dental Sciences). PBMCs were kindly provided by the Japanese Red Cross or purchased from AllCells (Alameda, CA, USA). TL-Om1 cells, an ATL-derived cell line established by Sugamura et al. (17), were maintained in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 2 mmol/liter L-glutamine, and 10 ng/ml interleukin-2 (PeproTech, London, United Kingdom). Jurkat, HUT102, and SLB-1 cells were maintained in RPMI 1640 containing 10% FBS supplemented with 100 U/ml penicillin-streptomycin and 2 mmol/liter L-glutamine. DNA was extracted using a QIAamp DNA blood mini or maxi kit (Qiagen, Valencia, CA, USA).

Southern blotting. Southern blotting was performed by SRL Inc. (Tokyo, Japan). DNA was digested with EcoRI and PstI and separated on a 0.8% agarose gel as previously reported (18, 19). DNA was transferred onto nylon membranes (Roche, Mannheim, Germany). The membrane was hybridized with digoxigenin (DIG)-labeled HTLV-1 probe at 42°C overnight. DNA fragments for HTLV-1 probes were obtained from Oncor Inc. (Gaithersburg, MD, USA). Sense and antisense HTLV-1 DNA probes were prepared by random primed labeling using a DIG-High Prime kit (Roche). After the membrane was washed, HTLV-1 probe signals were obtained using a DIG luminescent detection kit (Roche).

FISH analysis. To stop the cell cycle at M phase, Colcemid (Sigma) was added to the cell culture medium at a concentration of 0.02 μg/ml and incubated for 1 h. Cells were harvested and washed with phosphate-buffered saline (PBS). After treatment with 0.075 M KCl hypotonic solution at 37°C for 1 h, cells were fixed with a solution containing acetic acid and methanol (3:1). Cells were fixed to a glass slide and dried. The complete HTLV-1 genome inserted in pUC18 (15) was used as a probe for provirus, bacterial artificial chromosome (BAC) clone RP11-919G18 was used as a probe for the albumin (ALB) gene, and BAC clones CTD-2326H15 and RP11-203M5 were used as probes for the RNase P (RPPH1) gene. BAC clones were selected from NCBI (http://www.ncbi.nlm.nih.gov/clone/) and were purchased from Advanced Geno Techs Co. (Tsukuba, Japan). The probe for 1q44 was commercially prepared by Chromosome Science Labo Inc. (Sapporo, Japan). For the detection of ALB and RPPH1 genes, the BAC clones were labeled with cyanine 3 (Cy3) and Cy5, respectively. For the detection of provirus, the DIG-labeled probe was prepared by the nick translation method. The probe was hybridized to the sample at 70°C for 5 min, followed by incubation at 37°C overnight. The probe was stained with anti-DIG-Cy3 antibody. Signals were detected by a Leica DMRA2 system and analyzed with Leica CW4000 fluorescent *in situ* hybridization (FISH) software (Wetzlar, Germany).

Splinkerette PCR analysis. Splinkerette PCR was performed as previously reported (20). The first-round PCR was performed as indicated in reference 20. The second-round, nested PCR was performed using the HTLV-1 long-terminal-repeat (LTR)-specific primer. The nested PCR product was loaded onto 3% Tris-acetate-EDTA buffer (TAE) agarose gels. Two distinct DNA bands were cut from the agarose gel and purified using a QIAquick gel extraction kit (Qiagen). After thymine and adenine (TA) cloning, each band was sequenced by the Sanger method (21).

Inverse PCR analysis. TL-Om1 gDNA was digested with BamHI or XbaI. Digested DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Briefly, 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol were added to the sample. After centrifugation at $2 \times 10^4 \times g$ for 15 min, the DNA pellet was washed with 70% ethanol and then air dried. Purified DNA was self-ligated using a Ligation-Convenience kit (Nippon Gene, Tokyo, Japan). Ligated DNA was purified again by phenol-chloroform extraction followed by ethanol precipitation. PCR was performed with KOD FX (Toyobo, Osaka, Japan). The PCR mixture contained 20 ng gDNA, 0.4 mM forward and reverse primers, 1 mM deoxynucleoside triphosphate (dNTP), 1× KOD FX buffer, and 0.5 U KOD FX in a total volume of 25 μl, in duplicate. The forward primer sequence was 5′-ACAAATACACCTTGCAATCCTATG G-3′, and the reverse primer sequence was 5′-CGCTTGGGAGACTTCT TGCT-3′. PCR mixtures were denatured at 94°C for 2 min, followed by 34 cycles of 98°C for 10 s and 68°C for 10 min. PCR products were loaded onto 0.8% agarose gels and detected by LAS-3000 (Fujifilm, Tokyo, Japan).

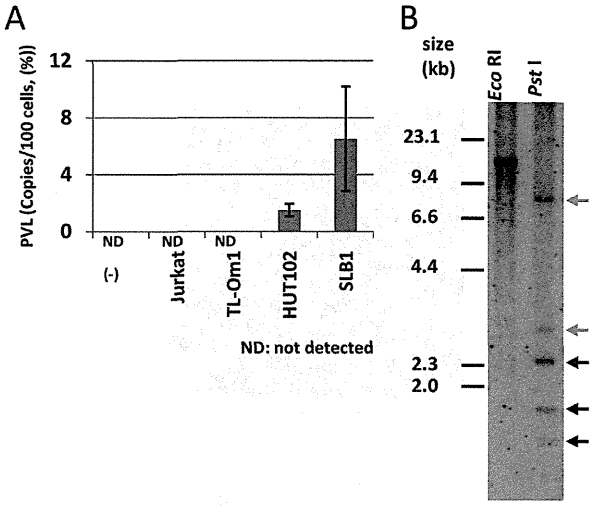
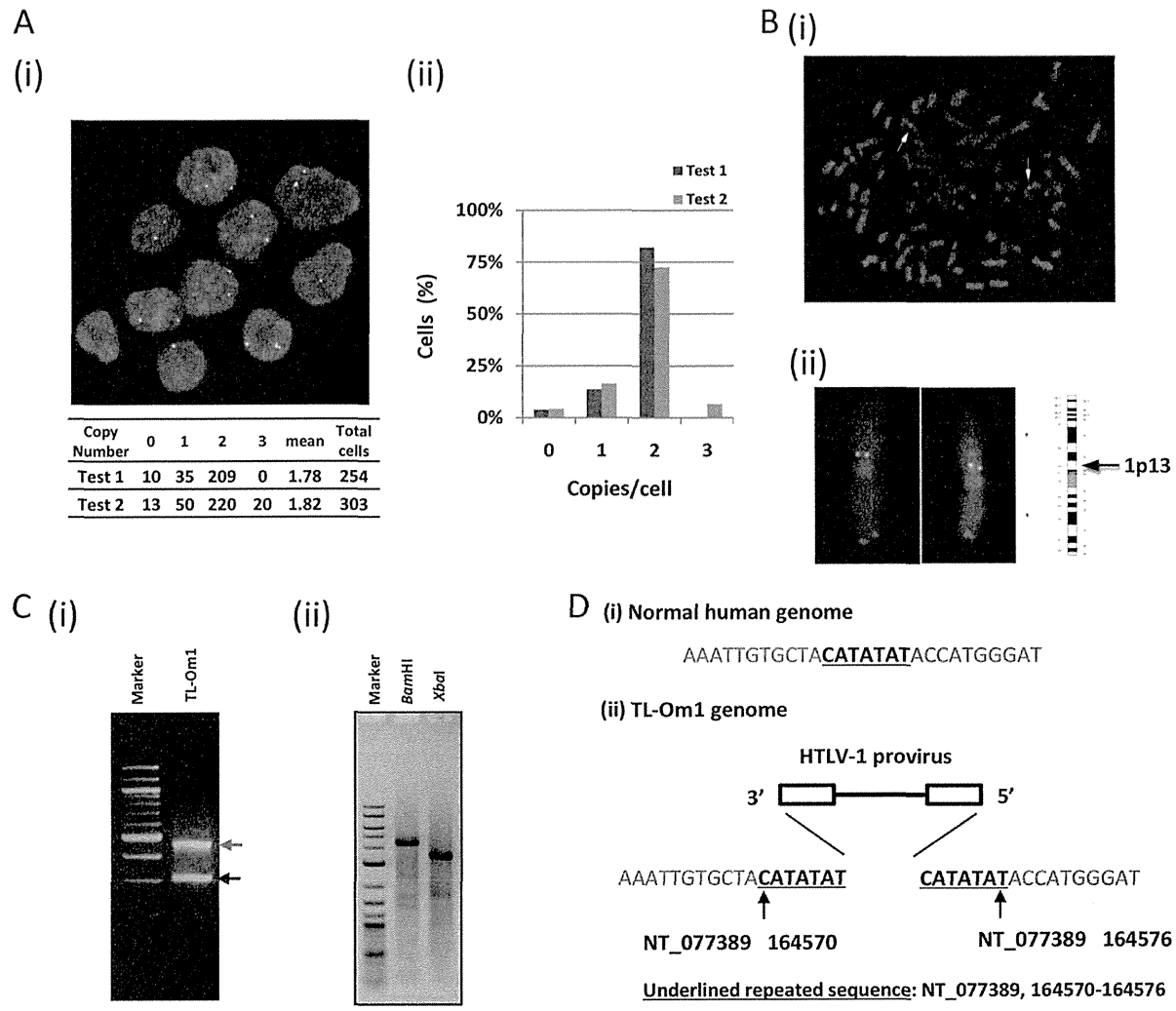


FIG 1 Infectivity and clonality of HTLV-1 provirus in TL-Om1 cells. (A) Mitomycin C-treated Jurkat, TL-Om1, HUT102, and SLB1 cells were cocultured with Jurkat cells. PVL (%) was measured 2 weeks later by qPCR. (B) gDNA from TL-Om1 cells digested with EcoRI or PstI was subjected to Southern blotting probed by the full HTLV-1 genome. Three black arrows show bands for typical HTLV-1 genomic sequences; two gray arrows show bands for host genomic sequences ligated to the HTLV-1 genome. Because the EcoRI site is not included in the HTLV-1 sequence, the number of bands indicates the number of clones in the cells. Detection of two gray bands indicates that there is a pair of 5′ and 3′ HTLV-1 genomes conjugated with the host genome, signifying that the HTLV-1 provirus is monoclonal. On the other hand, detection of more than two gray bands indicates that it is multiclonal.

Genomic long PCR. Genomic long PCRs were performed using KOD FX (Toyobo). Primers are listed in Table S1 in the supplemental material. The conditions for the PCR mixture and thermal cycling program were the same as those for the inverse PCR analysis.

DNA sequencing analysis. The genomic long PCR and inverse PCR products were purified by a GenElute PCR Clean Up kit (Sigma). Direct sequencing was performed using a BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence primers are listed in Table S2 in the supplemental material. Sequences were read and analyzed using a 3120× genetic analyzer (Applied Biosystems).

Synchronized qPCR analysis. The primers used for the synchronized qPCR amplification are listed in Table 1. The PCR mixture was prepared with SYBR premix *Ex Taq* II (TaKaRa, Tokyo, Japan) containing 100 ng gDNA and 0.4 mM forward and reverse primers in a total volume of 15 μl, in triplicate. PCR was performed according to the manufacturer's protocol. The ΔC_T(RPPH1) value (where C_T is threshold cycle) was calculated by the following equation: ΔC_T(RPPH1) = average C_T of target gene primer results − average C_T of RPPH1. The gene copy number was calculated by the following equation: target gene copy number (N) = copy number determined by FISH × $2^{-\Delta CT(\mathrm{RPPH1})}$. Using normal PBMCs or plasmids, the primer correction factor, which can compensate for small differences in amplification efficiency among different primers, was calculated. The correction factor was determined by the difference of each C_T



Copy Number	0	1	2	3	mean	Total cells
Test 1	10	35	209	0	1.78	254
Test 2	13	50	220	20	1.82	303

FIG 2 Clonality, copy number, and integration site of HTLV-1 in TL-Om1 cells. (A) HTLV-1 proviral copy number per cell was determined by FISH using an HTLV-1 full-genome probe. (i) Yellow signals indicate the HTLV-1 probe. Lower table shows the counts of HTLV-1 signals per cell. (ii) Vertical axis indicates percentage counts of each fraction in relation to total cells. Data were the results from two independent analyses. (B) Number of HTLV-1 integrated chromosomes was determined in metaphase cells with the HTLV-1 and 1q44 probes. (i, ii) Yellow signals indicate the HTLV-1 probe, and red signals indicate the 1q44 probe. All HTLV-1 signals were located on chromosome 1. HTLV-1 signals on chromosome 1 were positioned at 1p13. (C) Determination of the HTLV-1 integration site in TL-Om1 cells. (i) The 3′ integration site was determined by Splinkerette PCR with an HTLV-1-specific primer. PCR products were subjected to agarose gel electrophoresis. (ii) BamHI- or XbaI-digested TL-Om1 genomes were self-ligated and subjected to inverse PCR with an HTLV-1-specific primer set. PCR products were subjected to agarose gel electrophoresis. (D) 5′ and 3′ HTLV-1 integration sites were determined by a sequencing analysis of DNA fragments from both Splinkerette and inverse PCR. (i) Normal human sequence; (ii) determined HTLV-1 integration site. HTLV-1 was inversely integrated at chromosome 1: NT_077389, 164570 to 164576.

value of target gene primers from the average C_T value of RPPH1 primers (Table 1). The correction value was calculated as follows: correction C_T value = correction factor × actual C_T value. By applying the correction factors, we reduced the limits of error of the C_T values to 0.1 cycles with normal PBMCs (data not shown).

Digital PCR analysis. Primers and probes for digital PCR analysis of HTLV-1 were previously reported (11, 15). In brief, the primers and probe for HTLV-1 were as follows: forward, 5′-CGGATACCCAGTCTACGTG TT-3′; reverse, 5′-CAGTAGGGCGTGACGATGTA-3′; probe, FAM-5′-CTGTGTACAAGGCGACTGGTGCC-3′-TAMRA (where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). The primers and probe for albumin were as follows: forward, 5′-TGTCAT CTCTTGTGGGCTGT-3′; reverse, 5′-GGTTCTCTTTCACTGACATC TGC-3′; probe, FAM-5′-CCTGTCATGCCCACACAAATCTCTCC-3′-TAMRA. The mixture of primers and probe for RPPH1 was purchased from Applied Biosystems. The PCR mixture was prepared using 2× digital droplet PCR (ddPCR) supermix for probes (Bio-Rad, Hercules, CA, USA). Droplets were prepared on a QX100 droplet generator (Bio-Rad). PCR was performed with a LifePro thermal cycler (Bio-Rad) and detected with a QX100 droplet reader (Bio-Rad). Data were means of triplicate analysis.

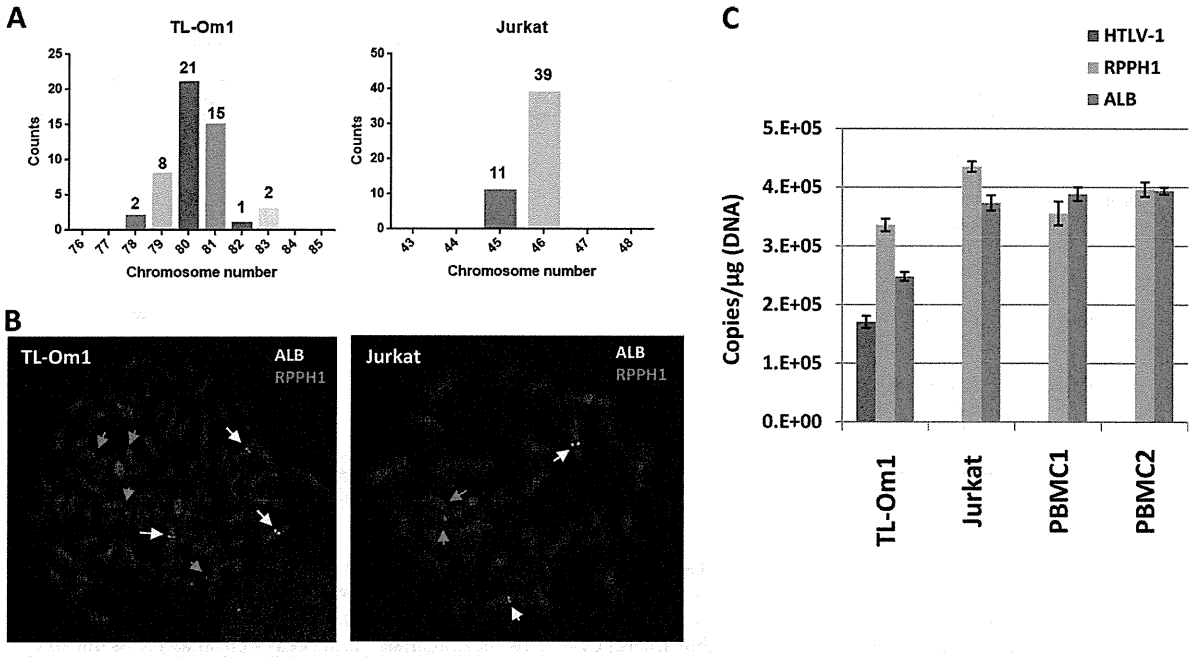


FIG 3 Gene copy number of IC cellular genes for HTLV-1 qPCR. (A) The number of chromosomes in TL-Om1 and Jurkat cells at metaphase was counted. Horizontal line indicates the number of chromosomes per cell. (B) Representative FISH images of TL-Om1 and Jurkat cells at metaphase. Yellow and red arrows indicate signals for ALB and RPPH1 probes, respectively. Left panel shows three signals for ALB and four for RPPH1; right panel shows two signals for ALB and two for RPPH1. (C) Determination of the gene copy number of HTLV-1, RPPH1, and ALB genes by digital PCR. gDNA of TL-Om1 and Jurkat cells and of PBMCs from two healthy donors were subjected to digital PCR. Data show the absolute copy number of HTLV-1, RPPH1, and ALB genes per microgram of gDNA. Bars are means from triplicate analyses.

***In vitro* HTLV-1 infectivity test.** Frozen cells were thawed and immediately cultured for a week. Exponentially growing cells were used for the assay. Jurkat, TL-Om1, SLB1, and HUT102 cells were treated with 50 μg/ml mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) and incubated for 1 h at 37°C. After being washed twice with 2% FBS-PBS, 1×10^5 cells were added to culture medium containing 1×10^6 Jurkat cells. Mitomycin C was used to block the growth of ATL cell lines added to Jurkat cells. Cells were cocultured for 2 weeks and then subjected to qPCR to determine PVL, as described previously (11).

RESULTS

HTLV-1 infectivity in TL-Om1 cells. We investigated the production potential of infective virus to ascertain the clonal stability of HTLV-1 integration *in vitro*. Mitomycin C-treated TL-Om1 cells were cocultured with Jurkat cells for 2 weeks. At the end of the 2 weeks, no HTLV-1 integration was observed in the Jurkat cells that were cocultured with TL-Om1 cells, while HTLV-1 integration was observed when Jurkat cells were similarly cocultured with SLB-1 and HUT102 cells (Fig. 1A). These findings suggested that the production of infective HTLV-1 particles from TL-Om1 cells was low or diminished; thus, the increase in copy number over the course of cell culture was thought to be negligible. If TL-Om1 cells had infectious potential, the clonality of HTLV-1 provirus in them would vary because of the mutual HTLV-1 infections between cells. To evaluate the clonality of HTLV-1 provirus in TL-Om1 cells, TL-Om1 gDNA was analyzed by Southern blotting. EcoRI-digested gDNA showed a single band, while PstI digestion produced five DNA bands that contained an HTLV-1 sequence (Fig. 1B). Three of the five DNA bands were HTVL-1 internal sequences. The other two DNA bands contained either 5′ or 3′ HTLV-1 sequences ligated with the host genome (Fig. 1B). These fragment patterns indicated that HTLV-1 provirus integration in TL-Om1 cells was monoclonal.

Determination of copy number and integration site of HTLV-1 provirus by FISH. To confirm the clonality and copy number of HTLV-1 provirus and of IC genes in detail, we performed a FISH analysis. There were one or two signals of HTLV-1 provirus in the cells. The mean proviral copy number was calculated at 1.8 copies/cell from the count of signals with >250 cells in two independent analyses (Fig. 2Ai and ii). Double-staining of the TL-Om1 genome with both HTLV-1 and 1q44 probes in meta-

TABLE 2 Gene copy number of IC genes determined by FISH

Karyotype	Gene copy no.			
	TL-Om1 (20 analyzed cells)		Jurkat (20 analyzed cells)	
	RPPH1 gene	ALB gene	RPPH1 gene	ALB gene
2N	0	0	20	20
3N	1	20	0	0
4N	19	0	0	0
Average	3.95	3	2	2
Ratio to the RPPH1 gene	1	0.76	1	1

TABLE 3 Summary of ratio of gene copy numbers to the RPPH1 gene

Method	Cell line	Gene copy no. ratio to the RPPH1 gene							
		RPPH1 gene	ALB gene	ACTB gene	CD81 gene	HBB gene	RAG-1 gene	HTLV-1 gene	LTR gene
FISH	TL-Om1	1.00	0.76					0.46	
	Jurkat	1.00	1.00						
Digital PCR	TL-Om1	1.00	0.74					0.51	
	Jurkat	1.00	0.86						
	PBMC1	1.00	1.09						
	PBMC2	1.00	0.99						
qPCR (plasmid)	TL-Om1	1.00	0.74					0.48	1.02
	Jurkat	1.00	0.92						
qPCR (gDNA)	TL-Om1	1.00	0.74	1.18	0.99	0.92	0.94		
	Jurkat	1.00	0.95	1.07	0.99	0.90	1.08		
	PBMC 1	1.00	0.99	1.00	0.98	0.99	1.00		
	PBMC 2	1.00	1.01	1.01	0.99	1.00	1.01		

phase showed that all HTLV-1 DNA signals were located on chromosome 1 (Fig. 2Bi). When the number of copies of chromosome 1 was 1, 2, 3, or 4 per cell, the number of HTLV-1 proviruses per cell was 1, 1, 2, and 2, respectively (data not shown). HTLV-1 signals on chromosome 1 were positioned on the band of 1p13 (Fig. 2Bii). These results correlated well with the Southern blotting results that showed monoclonal integration.

Confirmation of integration site of HTLV-1 in TL-Om1 cells. To identify the integration site of monoclonal HTLV-1 provirus, Splinkerette PCR was performed with TL-Om1 gDNA. Two specific PCR products were obtained by gel electrophoresis (Fig. 2Ci). The DNA fragments were analyzed by direct sequencing. Sequencing analysis of the lower-molecular-weight DNA fragments (Fig. 2Ci, lower band) showed that they were provirus genomic sequences. Sequencing analysis of the higher-molecular-weight band showed that it contained host gDNA ligated to the 3′ LTR of HTLV-1. We also performed inverse PCR with TL-Om1 gDNA that was digested with BamHI or XbaI followed by self-ligation. Single DNA bands were obtained from both BamHI and XbaI self-ligated templates (Fig. 2Cii). Sequencing analysis demonstrated that both bands contained the same sequences. A BLAST search revealed that the sequence was located on chromosome 1. The integration site was identified, and the HTLV-1 provirus was integrated inversely in between the CATATAT repetitive sequences at the region of NT_077389 from nucleotides (nt) 164570 to 164576 on chromosome 1 (Fig. 2Di and ii).

We determined the full-length sequence of HTLV-1 provirus in TL-Om1 cells by genomic long PCR followed by direct sequencing. The length of HTLV-1 provirus was determined to be 8,941 bp (GenBank accession no. AB979451; see also Text S1 in the supplemental material). The percent identity to the HTLV-1 genomic sequence of the ATK-1 strain (accession no. J02029) was 98.7%. Compared with the full-length HTLV-1 genomic sequence of ATK-1, there was a 93-nt deletion in the *env* gene. The region that was deleted was equivalent to nt 5547 to 5669 of ATK-1. The deduced amino acid sequence of the deletion was 31 in-frame amino acids (Δ125–155 of Env). The deleted region was located on the receptor binding domain of Env (see Fig. S1 in the supplemental material).

Calculation of chromosome and gene copy numbers of HTLV-1, RPPH1, and ALB genes in TL-Om1 and Jurkat cells. We counted the chromosome number in TL-Om1 and Jurkat cells by FISH analysis. Jurkat cells were analyzed as one of the control cell lines. The chromosome number differed from 78 to 83 in TL-Om1 cells (Fig. 3A). The mean chromosome number was estimated at 80.2, which indicated that the karyotype of TL-Om1 cells was about 4*N*. There were 45 or 46 chromosomes in Jurkat cells, indicating that their karyotype is near that of normal human diploid cells (Fig. 3A and B and Table 2).

The absolute gene copy number of HTLV-1 provirus and IC genes was measured using digital PCR. gDNA from TL-Om1 cells, Jurkat cells, and PBMCs from two healthy donors was subjected to digital PCR and used to calculate the copy numbers of these genes (Fig. 3C). Although the ALB-to-RPPH1 gene copy number ratios in the two PBMC samples were 1.09 and 0.99, the ALB-to-RPPH1 gene copy number ratio in TL-Om1 cells was low (ratio of 0.74) (Table 3). The provirus-to-RPPH1 gene copy number ratio in TL-Om1 cells was 0.51 (Table 3). These results were consistent with the provirus- and ALB-to-RPPH1 gene copy number ratios estimated by FISH, which were 0.46 and 0.76, respectively (Table 3). The usefulness of TL-Om1 as a reference standard is strongly supported by the consistent results from the FISH and digital PCR analyses (Table 4).

Estimation of the gene copy number of HTLV-1 and IC genes by synchronized qPCR. We previously developed a method to determine inherited allelic deletions by using qPCR with primer sets that can amplify fragments synchronously, even though the

TABLE 4 Absolute gene copy number per microgram gDNA determined by digital PCR

Cell line	Gene copy no./μg gDNA[a]		
	HTLV-1 gene	RPPH1 gene	ALB gene
TL-Om1	170,171.1	335,452.3	248,410.8
Jurkat	NT	434,529.6	373,423.9
PBMC1	NT	355,116.1	388,650.0
PBMC2	NT	397,260.3	394,520.5

[a] Data are means of triplicate analysis. NT, not tested.

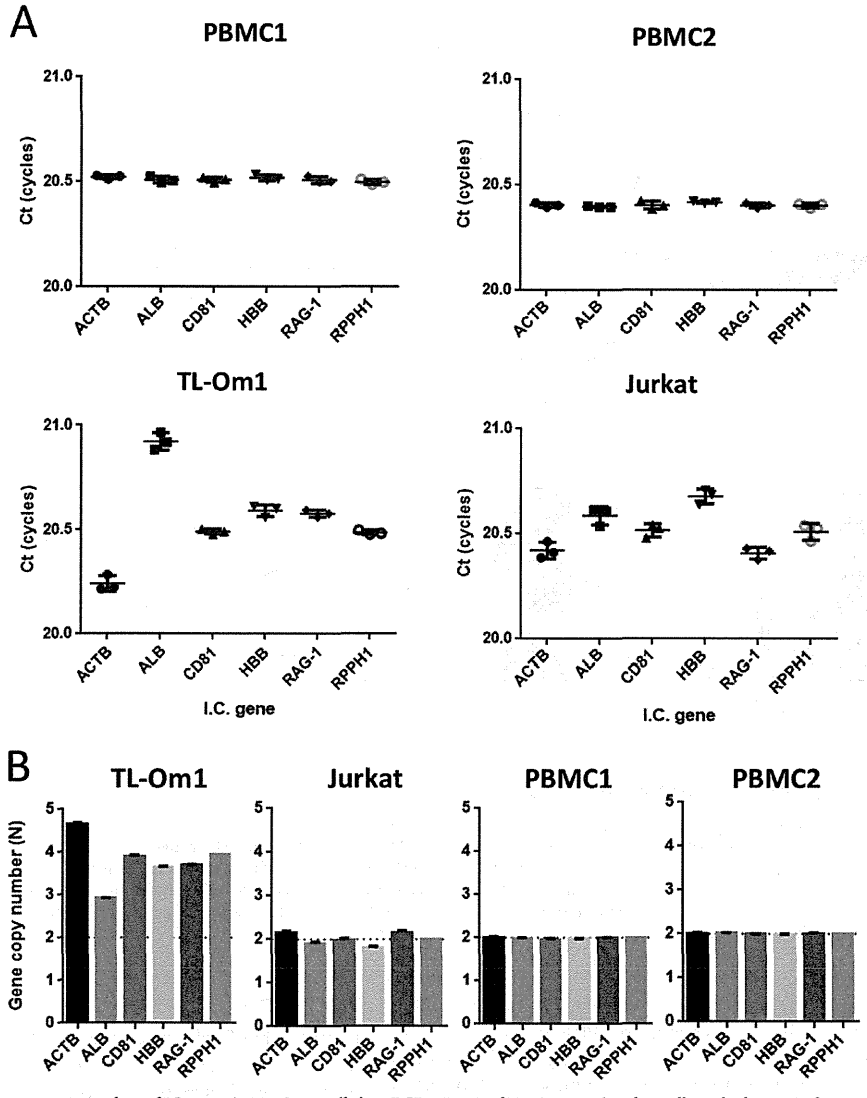


FIG 4 Estimation of gene copy number of IC genes in TL-Om1 cells by qPCR. gDNA of TL-Om1 and Jurkat cells and of PBMCs from two healthy donors was tested by qPCR with synchronous amplification primer sets for IC genes. (A) C_T scores (cycles) of each primer set for IC genes. Each dot indicates the mean from triplicate analyses. The C_T scores in the graph were the results of correction by the factors described in Table 1. (B) Estimated gene copy number of IC genes calculated using the difference in C_T scores from RPPH1. The copy numbers of IC genes of TL-Om1 and Jurkat cells were calculated based on FISH analysis for the RPPH1 gene. RPPH1 gene copy number from PBMCs was set as 2*N*. Equation for the estimation of gene copy number was as follows: gene copy number (N) = RPPH1 gene copy number determined by FISH analysis $\times 2^{-\Delta CT}$, $\Delta C_T = C_T$(target gene) $- C_T$(RPPH1).

target genes are different. The method shows that the difference in C_T value determines the difference in gene copy number. We used primer sets for HTLV-1 genes (LTR and coding regions) and ACTB, ALB, CD81, HBB, and RAG-1 IC genes (Table 1). To increase the specificity, we used primer correction factors, which compensate for the slight difference in PCR amplification efficiency between different primers for target genes. As shown in Fig. 4A, TL-Om1 and Jurkat cells did not show the complete synchronized amplifications that were observed in normal PBMCs. By setting the PCR amplification efficiency of all primer sets per cycle to approximately 2-fold, the ratio of the gene copy number against the RPPH1 gene was estimated using the difference in the mean C_T scores of the IC gene primer sets from the mean of those for the RPPH1 gene. The ratios of the gene copy number of the ALB gene to that of the RPPH1 gene in TL-Om1 and Jurkat cells were 0.74 and 0.92, respectively (Table 3). When the copy number of the RPPH1 gene in TL-Om1 cells was set at 3.95, which was determined by FISH analysis, the copy number of the IC genes was at least 2.9 (ALB gene) and at most 4.7 (ACTB gene) (Fig. 4B and Table 3).

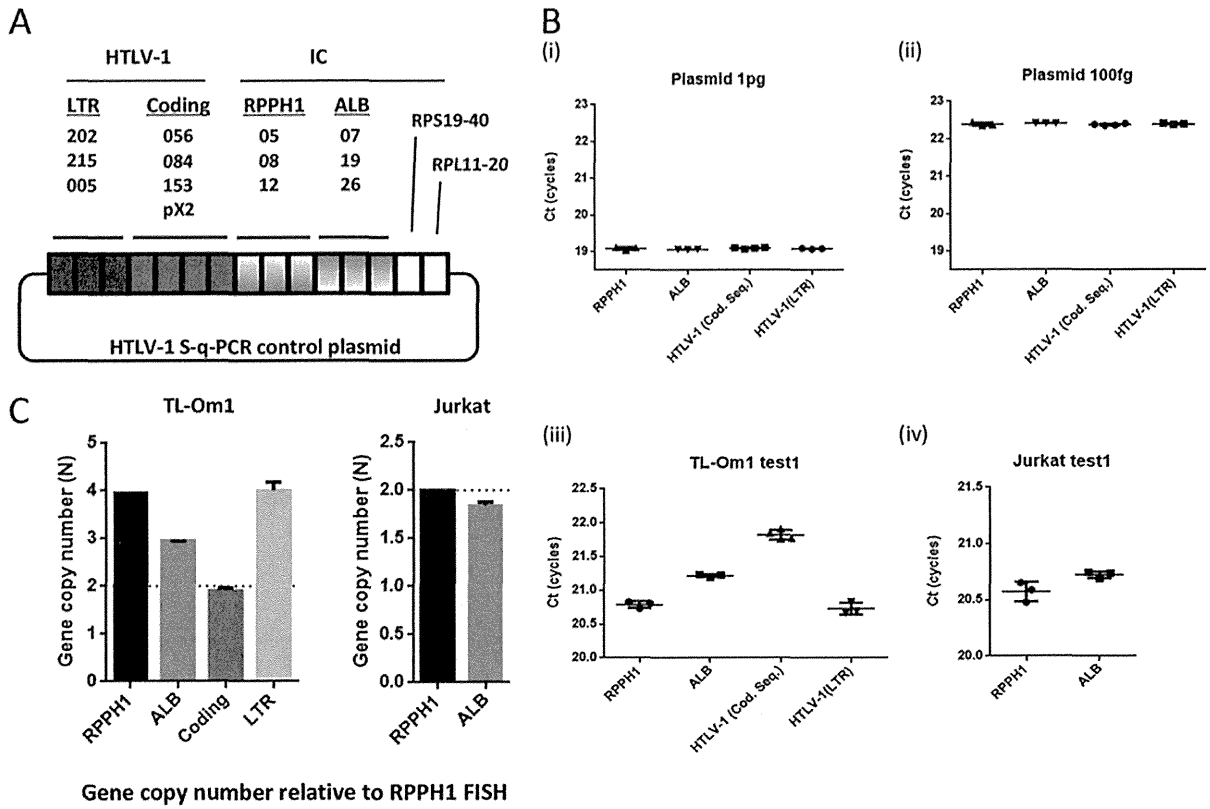


FIG 5 Estimation of the HTLV-1 gene copy number in TL-Om1 cells by synchronized qPCR. gDNA of TL-Om1 and Jurkat cells and of PBMCs from two healthy donors was tested for qPCR with synchronous amplification primer sets for HTLV-1, RPPH1, and ALB genes. (A) Construction of control plasmid with a single copy of each target sequence; (B) data indicate C_T scores of HTLV-1, RPPH1, and ALB genes for control plasmid at 1 pg, 100 fg/reaction, and for TL-Om1 and Jurkat cells. qPCR with the plasmid showed synchronous amplification of all primer sets. Each dot indicates the mean from triplicate analyses. The C_T scores in the graph are the results of correction by the factors described in Table 1. (C) Estimated HTLV-1 and ALB gene copy number in TL-Om1 and Jurkat cells. Data were estimated using the difference in C_T scores between target genes and RPPH1 genes.

Additionally, we tried to determine the HTLV-1 copy number in TL-Om1 cells using a synchronized qPCR method. We prepared a plasmid that had one copy of every target PCR amplicon (Fig. 5A). The plasmid had the same copy number as all the target regions. Using the plasmid as a template, we performed qPCR and confirmed the synchronized amplification of primer sets for HTLV-1, RPPH1, and ALB genes (Fig. 5B). The difference in mean C_T scores for the HTLV-1 gene to the RPPH1 gene was 1.05 cycles on average in TL-Om1 cells (Fig. 5C and Table 3). As with the sequencing analysis, use of the synchronized qPCR method also estimated the copy number of the LTR to be 4.01, indicating that TL-Om1 cells have two LTRs (Fig. 5C and Table 3).

Comparison of HTLV-1 copy number from different calculation methods. We compared the results of HTLV-1 and ALB gene copy number obtained from FISH, digital PCR, and synchronized qPCR. The copy number ratios of the HTLV-1 gene to the RPPH1 gene in TL-Om1 cells were 0.46, 0.51, and 0.48, from FISH, digital PCR, and synchronized qPCR, respectively, and those for the ALB gene were 0.76, 0.74, and 0.74 (Fig. 6 and Table 3). The results from these varied assays strongly support one another, indicating that TL-Om1 cells are suitable for use as a reference material for HTLV-1 qPCR.

DISCUSSION

Recently, NAT reference materials have been established for the safety of blood and blood products, such as international standards for HIV, hepatitis B virus, and hepatitis C virus (22–24). These materials have been frequently used for the purpose of calibration and validation of test systems, preparation of secondary reference materials, and comparison of multicenter results, which have helped improve the consistency of the results. Most international standards for blood-transmitted viruses use plasma from infected human blood, because the test target is extracted from human plasma. With regard to HTLV-1 NAT, it may be better to use a cell line as a reference material to standardize the qPCR results, because this test uses cells obtained from peripheral blood. An example of NAT reference material using cell lines is reported in a test for quantitation of BCR-ABL mRNA. Panels of K562 cells combined with HL60 cells were set as standards, which have been approved by the WHO Expert Committee of Biological Standardization (25). Although a variety of cell lines harboring HTLV-1 provirus in their genomes has been established, detailed characterization of the candidate cell lines with regard to their suitability as reference materials for HTLV-1 NATs has not yet been per-

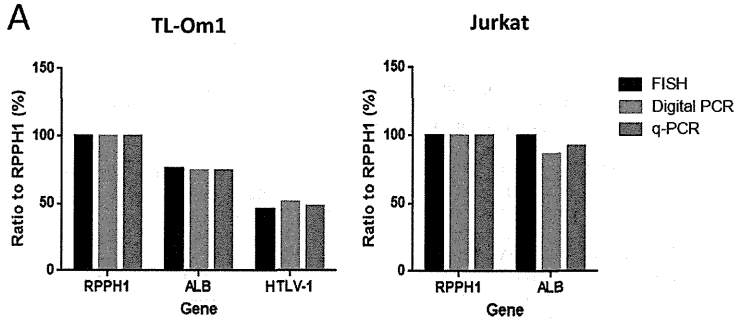


FIG 6 HTLV-1- and ALB-to-RPPH1 gene copy number ratios. (A) Comparison of the HTLV-1- and ALB-to-RPPH1 gene copy number ratios determined by FISH, digital PCR, and qPCR. Data indicate percentages of gene copy number ratio to the RPPH1 gene.

formed. Among the HTLV-1 cell lines, TL-Om1 is well known to be latently infected with HTLV-1 and is thought to be stable for HTLV-1 clonality (17, 26). Transcription from HTLV-1 provirus in TL-Om1 cells is blocked by the highly methylated LTR (27).

In this study, we evaluated the distinct genomic properties of HTLV-1 and IC genes in TL-Om1 cells with regard to their suitability as reference materials for HTLV-1 NATs. Precise information about HTLV-1 infectivity, karyotype, and absolute copy number of HTLV-1 and cellular control genes of TL-Om1 is useful for applying TL-Om1 as a reference material for HTLV-1 qPCR. As such, for this use, TL-Om1 has advantages over other cell lines, such as the human ATL cell line MT2 and the rat T-cell line TARL-2. A recent study of HTLV-1 testing in Japanese blood donor screening revealed that virus prevalence is not limited to areas where HTLV-1 is endemic but has shifted to the entire country, especially the Tokyo metropolitan area (6). Nationwide HTLV-1 tests have been performed on pregnant women in Japan since the end of 2010. The frequent occurrence of seroindeterminate results after Western blotting is one weakness of the HTLV-1 antibody tests. HTLV-1 qPCR is thought to be a solution for decreasing the number of seroindeterminate results; therefore, an accurate measurement of HTLV-1 proviral DNA by qPCR is needed. Additionally, a PVL value of >4% in PBMCs is reported to be a risk factor for ATL development from HTLV-1 asymptomatic carriers, which emphasizes the importance of measuring PVL by qPCR (7). PVL monitoring also provides a risk indicator for HAM/TSP (8).

An attempt to minimize the differences between laboratories by using a common plasmid that included the pX region has been reported. When standard curves were constructed by utilizing the common plasmid in all participating laboratories, the differences in median intralaboratory coefficient of variation (CV) could be reduced by about half (16). Although the attempt worked well among participating laboratories with in-house qPCR methods, the transferability of utilizing common plasmids for standard curves to other methods for PVL determination, for example, digital PCR, is uncertain.

To standardize HTLV-1 qPCR, we advocate the use of TL-Om1 cells with finely elucidated HTLV-1 genomic information as reference material. A previous report showed that PVL values of males and females, on average, are 1.39% and 2.10%, respectively (7). Thus, a dilution or a serial dilution of TL-Om1 with PBMCs or Jurkat cells at a PVL value of around 2% would be an appropriate material for the standardization of HTLV-1 qPCR. These kinds of references can be easily prepared, because the absolute gene copy number is determined from the dilution rate of TL-Om1. TL-Om1 cells were also used as a control in a deep-sequencing-based method for the quantification of the clone size of HTLV-1-infected cells in HTLV-1 carrier or ATL patients (28).

We conclude that TL-Om1 cells can be used as a useful reference material for HTLV-1 NATs. By using TL-Om1 cells, researchers will be able to define the exact values of HTLV-1 by quantifying the copy numbers of provirus and IC genes. In the future, we hope that other laboratories will utilize the features of TL-Om1 cells to standardize the HTLV-1 qPCR.

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We declare that we do not have any competing interests.

Ethical approval was not required for this study.

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ORIGINAL ARTICLE: CLINICAL

The incidence of adult T-cell leukemia/lymphoma among human T-lymphotropic virus type 1 carriers in Japan

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Abstract

Human T-lymphotropic virus type 1 (HTLV-1) is highly endemic in the Kyushu/Okinawa region of Japan. A nationwide investigation verified the frequency of HTLV-1 carriers among first-time blood donors and the occurrence of newly diagnosed adult T-cell leukemia/lymphoma (ATL) cases from 2007 through 2008. After adjusting for differences in capture rate between areas, the age-, sex- and area-specific incidence of ATL among carriers was determined. Annual ATL incidence among 10 000 carriers was 7.7 and 8.7 for the Kyushu and non-Kyushu/Okinawa regions, respectively. The incidence increased sharply for men from their 40s to their 70s, but the rate in females remained unchanged through their 40s to 70s. ATL incidence in middle-aged females was still low, even if female carrier frequency was assumed to be identical to that of males. Patients with ATL in their 60s and 70s will comprise two-thirds of all patients with ATL for the next 15 years in Japan.

Keywords: Adult T-cell leukemia/lymphoma, disease incidence, epidemiology, human T-lymphotropic virus type 1, virus carrier

Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a causative agent for adult T-cell leukemia/lymphoma (ATL) and infects approximately 15–20 million people worldwide. After a long incubation period, ATL arises in HTLV-1 carriers and has one of the most aggressive clinical courses of the various leukemias. HTLV-1 is highly endemic in southwestern Japan (namely, in the Kyushu region and Okinawa Prefecture), the Caribbean Islands, regions of South America and tropical Africa [1,2]. Detailed data on ATL incidence among HTLV-1 carriers in the whole of Japan were published in 1990 [3]. There has been no systematic study of ATL incidence among carriers since then, although records of patients with ATL were collected biennially until 1997 [4]. In 2008, a new nationwide study was initiated to investigate HTLV-1 prevalence among blood donors and the incidence of ATL. The study verified that the number of HTLV-1 carriers was 1.08 million in the whole of Japan and has increased outside the Kyushu region [5], indicating that the issue of HTLV-1 carriers and ATL development has to be considered nationwide in Japan. That study also collected records of patients with ATL who were newly diagnosed during 2 years (2007 and 2008), yielding estimates for the prevalence and incidence of patients with ATL in relation to geographic area and patient age [6].

Progress continues to be made in characterizing the molecular mechanisms of gene alterations, signaling deregulation and cell cycle disorder in HTLV-1-infected cells [7,8]. However, the etiologic factors that promote transition to the transformed leukemic state, whether biologic or socioeconomic, remain unknown. In contrast, the environmental conditions that contribute to the occurrence of carriers and hence patients with ATL are relatively clear from the public health point of view: the routes of HTLV-1 infection are well defined (breastfeeding, blood transfusion and sexual behavior); measures for preventing HTLV-1 transmission are well established (bottle feeding and blood screening); carriers of the virus can be easily identified by testing for anti-HTLV-1 antibodies; and the cost for establishing these strategies can be calculated without much difficulty. Therefore, the introduction of measures effective for the eradication of HTLV-1 transmission might be considered a political matter, requiring a solution that would involve medical facilities, health officials and health authorities nationwide [9,10]. To conduct such organized measures, epidemiologic studies elucidating the exact number and distribution of HTLV-1 carriers and patients with ATL are essential.

In this article, we provide an analysis of HTLV-1 prevalence and ATL incidence in Japan, verifying the incidence of ATL among carriers in relation to geographic area and carrier sex and age.

*These authors contributed equally to the work.

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Materials and methods

Nationwide study of HTLV-1 carriers and patients with ATL

An investigation of the frequency of HTLV-1 carriers and patients with ATL in Japan was organized in 2008 with the aid of the Japanese Ministry of Health, Labor and Welfare. Methods used and results for the number of HTLV-1 carriers have been described in detail elsewhere [5]. Briefly, we examined the number of individuals positive for anti-HTLV-1 antibody with a confirmatory immunofluorescence test among first-time blood donors during the 2 years from 2007 to 2008. Based on the frequency obtained, we calculated the number of HTLV-1 carriers in categories defined by gender, age and geographic area. The study team also collected the number of patients with ATL newly registered during this 2-year period [6]. Almost one-third of medical institutions with hematologic faculties in Japan ($n = 156$) participated in the investigation. The results indicated a mean annual ATL occurrence of 455 in those institutions. The investigation included a survey for newly registered patients with B-cell non-Hodgkin lymphoma (B-NHL), which served as an internal control (see below).

Capture rate for patients with B-NHL and ATL in the survey

Our survey likely captured only a subset of the population of patients with ATL, given that only one-third of medical institutions with hematological faculties participated in this study. The frequency of participation by medical facilities differed greatly among areas. The distribution of HTLV-1 carriers, moreover, is geographically biased; nearly half of the carriers dwell in the southwestern region of Japan [5]. These factors would have compromised the precise enumeration of patients with ATL if the observed numbers for ATL were directly extrapolated using the ratios of population size or the number of facilities participating. To avoid this problem, we collected data for patients with B-cell non-Hodgkin lymphoma (B-NHL) as well. It is generally accepted that the frequency of B-NHL occurrence is independent of geography in Japan [11,12]. The number of patients with B-NHL in an area, therefore, is expected to correlate with the size of the population of the area studied.

The statistics for cause of death published by the Japanese Ministry indicate that the annual number of deaths from B-NHL has remained constant over the past 3 years, with a mean value of 9020 [6]. Considering the malignancy of the diseases included in this disease category, the number of annual occurrences of B-NHL is expected to be similar to that of deaths from the disease. We thus estimated the B-NHL incidence in a given area by allocating the total number of deaths in Japan (9020) based on the proportion of the population of a given area to that of the whole of Japan. Comparing the expected number and the observed number in a given area, we calculated the capture rate of B-NHL occurrence in each region covered by this survey. Applying this capture rate to the observed number of patients with ATL, we deduced the number of patients with ATL that would occur in each area.

Estimation of sex-, age- and area-specific ATL occurrence and future occurrence of patients with ATL

The number of patients with ATL is known to be exceptionally high in Okinawa and all prefectures in the Kyushu region, but very low in other areas. Therefore, figures for ATL were deduced for the geographic areas of Okinawa Prefecture, each of the seven individual prefectures in the Kyushu region, the Kyushu region (all seven prefectures combined) and all non-endemic regions combined (non-Okinawa/Kyushu region) (Figure 1). The total of the number from each area that was deduced using the capture rate described above was defined as that expected to occur in a year in the whole of Japan. We assigned the total number obtained to each age category based on the proportion of observed ATL incidence among age categories. Age category was defined by dividing the 0–99 age-span into 10-year intervals. ATL incidence in an age category was defined as the proportion of the deduced number of patients with ATL among HTLV-1 carriers in the age category. Cumulative lifetime frequency of ATL incidence as expressed by percent was obtained by summing the decennial incidences for ages 0–79 years and multiplying the total by 10.

Using the projected estimates for the number of HTLV-1 carriers that have been published [5] and the ATL incidences in age categories obtained in this study, we calculated the number of cases of ATL expected to occur in the next 15 years.

Statistical analysis

Data were analyzed using SSRI software (Excel Statistics ver. 8; Social Survey Research Information, Tokyo, Japan) for Windows (Microsoft Excel 2007; Tokyo, Japan). Statistical analysis was performed using the χ^2 test. p-Values of less than 0.05 were considered significant.

Results

We first evaluated the B-NHL capture rate in each area studied. We allocated the annual number of deaths from

Figure 1. Geographic areas of Japan covered in this article.

Table I. Survey for B-cell non-Hodgkin lymphoma.

	B-NHL observed*	Population (thousand)	Population ratio	B-NHL expected†	B-NHL capture rate‡ (95% CI)
Whole country	3582	127 771	1	9020	0.397 (0.387-0.407)
All regions except Kyushu/Okinawa	2870.5	113 107	0.8852	7985	0.359 (0.349-0.370)
Kyushu region§	704	13 295	0.1040	939	0.750 (0.722-0.777)
Fukuoka	265	5055	0.0396	357	0.742 (0.697-0.788)
Saga	31.5	861	0.0067	61	0.518 (0.391-0.642)
Nagasaki	125.5	1460	0.0114	103	1.218
Oh-ita	30.5	1205	0.0094	85	0.359 (0.257-0.461)
Kumamoto	63.5	1832	0.0143	129	0.491 (0.406-0.579)
Miyazaki	68.5	1146	0.0090	81	0.847 (0.767-0.924)
Kagoshima	119.5	1737	0.0136	123	0.975 (0.942-1.001)
Okinawa	7.5	1371	0.0107	97	0.078 (0.024-0.130)

B-NHL, B-cell non-Hodgkin lymphoma; CI, confidence interval.
*Average annual number of patients with B-NHL observed in 2008 and 2009.
†Expected annual number of patients with B-NHL assuming 9020 deaths from B-NHL in whole country per annum.
‡Ratio of number of observed patients with B-NHL to that of expected patients with B-NHL.
§Kyushu region includes prefectures of Fukuoka, Saga, Nagasaki, Oh-ita, Kumamoto, Miyazaki and Kagoshima.

B-NHL (9020) among the defined areas according to the proportion of the population in each of the areas studied. For example, as the population of Fukuoka Prefecture accounts for 3.96% of the Japanese population, the expected annual incidence of B-NHL in the area was calculated to be 357 (9020×0.0396) (Table I). Because the present study identified 265 patients with B-NHL in Fukuoka, the B-NHL capture rate in Fukuoka was determined to be 0.742 (265/357). Assuming that we identified patients with ATL with the same capture rate as for B-NHL in this area, the expected number of new patients with ATL in Fukuoka was calculated to be 76 (56.5/0.742), with the observed number of patients being 56.5 (Table II). Compared with the estimated carrier number in Fukuoka (102 700) [5], the annual incidence for ATL among carriers in Fukuoka Prefecture was calculated to be 0.074% (76/102 700). Using the same method, we deduced the annual occurrence of ATL cases and ATL incidence among carriers for each of the defined areas (Table II).

The numbers for expected ATL occurrence in Okinawa Prefecture, the Kyushu region as the total of those in the seven individual prefectures, and the non-Okinawa/Kyushu region were 148, 325 and 510 per annum, respectively. In Okinawa Prefecture, however, only two institutions participated in this study, and reported only 15 and 23 cases for B-NHL and ATL, respectively, during the study period. This yielded wide confidence intervals (CIs) for both the B-NHL capture rate and annual ATL incidence. We therefore described the total number of patients with ATL as being 835 occurring annually in the whole of Japan excluding Okinawa Prefecture (Table II).

The annual incidence of ATL among 10 000 HTLV-1 carriers in Okinawa was 21.8, and in the individual prefectures in the Kyushu region the annual incidence ranged between 6.0 and 9.0. Thus, although the rate in Okinawa was significantly higher than any of those in the prefectures in Kyushu ($p < 0.01$), it had a broad CI, as explained above. The rate in the Kyushu region, 7.7, was not statistically different from that in the combined non-Okinawa/Kyushu region (8.7).

The age distribution of HTLV-1 carriers and ATL incidence among carriers are shown in Figures 2(a) and 2(b) by adjusting the total number of ATL occurrences to 983 per annum including the number for Okinawa. The incidence increases sharply for men in their 40s, peaks in their 70s, and decreases sharply thereafter. The rate also increases for women in

Table II. Survey for adult T-cell leukemia/lymphoma.

	ATL observed†	ATL expected‡	Number of HTLV-1 carriers (thousand)	Annual ATL incidence per 10 000§ (95% CI)
Whole country	455	(983)*	1078.7	—
Whole country except Okinawa	443.5	835*	1010.7	8.3 (7.9-8.8)*
All regions except Kyushu/Okinawa	183	510	586.1	8.7 (8.4-8.9)
Kyushu region¶	260.5	325*	424.5	7.7 (7.1-8.6)*
Fukuoka	56.5	76	103.1	7.4 (7.0-7.9)
Saga	6.5	13	18.4	7.1 (5.5-9.0)
Nagasaki	58	48	55.4	8.7
Oh-ita	14.5	40	44.6	9.0 (7.1-12.7)
Kumamoto	15.5	32	46.4	6.9 (5.8-8.2)
Miyazaki	27.5	32	53.5	6.0 (5.6-6.7)
Kagoshima	82	84	103.1	8.1 (7.9-8.4)
Okinawa	11.5	148	68.0	21.8 (13.0-70.0)

ATL, adult T-cell leukemia/lymphoma; B-NHL, B-cell non-Hodgkin lymphoma; CI, confidence interval; HTLV-1, human T-lymphotropic virus type 1.
*Cumulative sum.
†Average annual number of patients with ATL observed in 2008 and 2009.
‡Expected annual number of patients with ATL deduced using B-NHL capture rate.
§Annual ATL incidence among 10 000 HTLV-1 carriers.
¶Kyushu region includes the prefectures of Fukuoka, Saga, Nagasaki, Oh-ita, Kumamoto, Miyazaki and Kagoshima.

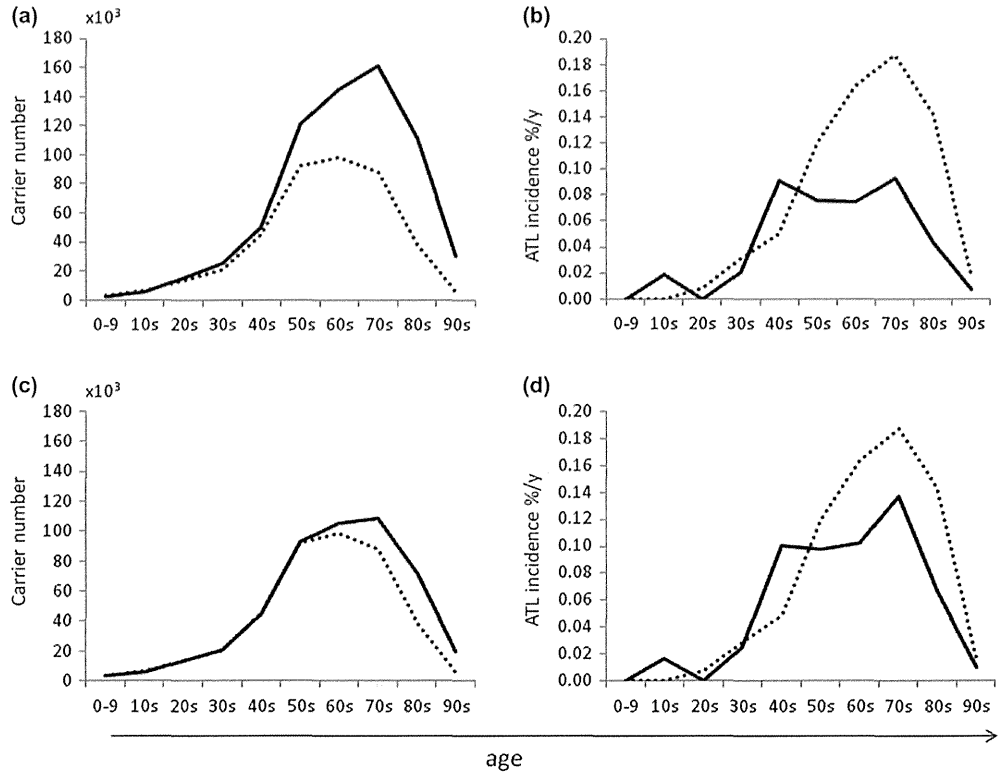

Figure 2. Sex-specific HTLV-1 carriers and ATL incidence in relation to age. Solid and broken lines indicate data for females and males, respectively (a-d). (a, b) Data obtained in this study: (a) carrier number, (b) ATL incidence among carriers (percent per annum). (c, d) Data deduced from calculations assuming that the carrier rate in females is identical to that in males: (c) carrier number, (d) ATL incidence among carriers (percent per annum). ATL incidence for females is higher in their 40s and lower in their 60s to 80s compared to males.

their 40s but remains at approximately the same level until it decreases in their 70s. The rate is significantly higher in females than in males in their 40s ($p<0.01$), whereas it is significantly lower in females than in males in their 50s to 80s ($p<0.01$). Cumulative lifetime incidence of ATL at the age of 79 was 7.2% and 4.2% for male and female carriers, respectively.

It has been argued that ATL occurs almost exclusively among carriers who acquired HTLV-1 infection during their neonatal period or infancy [13,14], and that horizontal transmission after adolescence occurs preferentially from males to females [15–17]. Adult female carriers, therefore, include a considerable proportion of individuals who acquired infection horizontally and thus have little possibility of developing ATL, which might correspond to the lower ATL incidence in females in their 50s to 80s. To examine this possibility, we recalculated the female carrier number by applying the male carrier rate to the female population in each age category and then recalculated the ATL incidence in females. Based on this adjustment, the total number of female carriers is predicted to decrease by 27.1% (from 667 000 to 486 000) for the whole of Japan [Figure 2(c)]. Using this recalculated carrier number, the adjusted ATL incidences for female carriers are predicted to increase but still show differences from those for males [Figure 2(d)]: rates for females are significantly higher in their 40s and lower in their 60s to 80s ($p<0.01$ for all comparisons).

To estimate the number of patients with ATL expected in the future, we applied the ATL incidence in each age category to the estimated number of HTLV-1 carriers in each age category [5] for the next 15 years in Japan. We thus expect that the number of patients with ATL will decrease continuously from 940 in 2012 to 530 in 2027 (Figure 3). Patients in their 60s and 70s are expected to account for the increasing plurality (27% and 39%, respectively) of patients during the next 15 years.

Discussion

We evaluated the incidence of ATL among HTLV-1 carriers in the categories defined by sex and age of carriers and geographic area in Japan. To compensate for the geographic variation in participation frequency by medical facilities, we normalized values using the B-NHL capture rate for each area, given that the B-NHL incidence in Japan appears to be independent of geography [11,12]. This strategy of data collection, which was previously adopted by Tajima [3], is essential, particularly for the epidemiologic study of

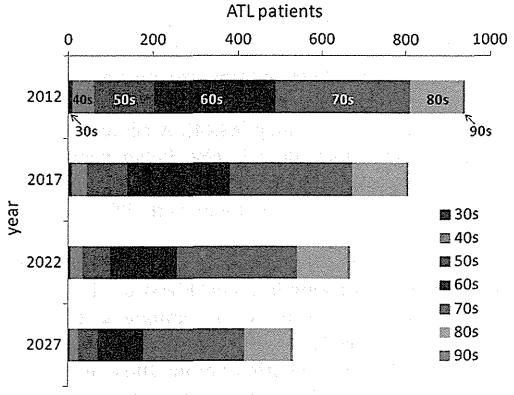

Figure 3. Projected ATL occurrence over the next 15 years. Bars show the expected number of patients with ATL for each 10-year age category.

a phenomenon with geographic clustering of subjects. We also assumed that the number of cases of disease is similar to that of deaths for B-NHL. Comparing the total number of observed B-NHL cases with the average annual number of B-NHL deaths registered nationwide, the B-NHL capture rate in this study was calculated to be 39.7%, a figure reflecting the data collection efficiency of this study. The capture rate was higher in the Kyushu region (0.750) than in non-Kyushu regions (0.359), which might have resulted from greater interest in an HTLV-1-related study among physicians in the endemic area, Kyushu, than among physicians in the non-endemic area.

Annual occurrence of ATL as the total in seven individual prefectures in the Kyushu region and non-Kyushu/Okinawa region was deduced to be 835 (95% CI, 795–887). The number for Okinawa Prefecture was not included in this calculation because of the wide CI (95% CI, 88–476). If, however, the number for Okinawa is added, the total number of cases of ATL in the whole of Japan amounts to around 1000, which is close to the number of deaths from ATL reported by the Japanese Ministry of Health, Labor and Welfare (1075 and 1048 in 2007 and 2008, respectively) [18]. This could support the adequacy of the estimation of ATL occurrence based on the B-NHL capture rate in the present study. Thirty-nine percent of patients with ATL were expected to be found in the Kyushu region, and more than 350 were expected in the metropolitan areas of Tokyo, Osaka and Nagoya combined (data not shown).

We did not detect a significant difference in ATL incidence among carriers when comparing Kyushu to non-Okinawa/Kyushu regions, despite the large differences in HTLV-1 prevalence. This result suggests that local environmental factors that may influence ATL development are shared between these areas. The ATL incidence in Okinawa was shown to be more than twice as high as those in other regions, including the Kyushu region, which is geographically proximal to Okinawa. The transcontinental HTLV-1 subgroup (subgroup A) accounts for 20–35% of virus infecting residents in Okinawa, but for very few cases in other regions of Japan, including Kyushu, where the Japanese subgroup (subgroup B) predominates [19,20]. The ATL-causative potency of subgroup A might be higher than that of subgroup B, although there is, to our knowledge, no published data showing distinguishable differences in ATL incidence between HTLV-1 variants. Background genetic diversity including HLA in carriers also may contribute to differences in ATL incidence [21–23]. In fact, several genome-wide association studies have verified a considerable difference in the genetic background between residents of Okinawa and mainland Japan [24,25]. Comorbidity or differences in lifestyle, socio-economic status and natural environment also may play a role [26–28].

Although these questions are worthy of inquiry, we are, for the present study, limited in the data collection we can do in the Okinawa region. We estimated annual ATL incidence on the basis of B-NHL capture rate. Accordingly, the estimate likely becomes less confident when the number of observed cases of B-NHL decreases. This is the case for Okinawa, where small numbers of cases were reported for B-NHL and ATL during the study period. Future study participation by a larger number of medical institutions and physicians in Okinawa will be needed to draw conclusions on geographic differences in ATL incidence among carriers.

Another limitation of this study is that all demographic data on ALT incidence presented in this article were deduced using, as the denominator, the HTLV-1 carrier number determined among blood donors. However, the HTLV-1 prevalence among blood donors often is reduced compared to values obtained in other populations, such as pregnant women, outpatients, inpatients or even local residents [29,30]. Therefore, the actual numbers of HTLV-1 carriers for each demographic category are expected to be greater than those used in this study. This effect is expected to lessen the actual ATL incidence and lifetime incidence compared to the results derived in this article.

Figure 2 shows that the ATL incidence rate starts to increase for patients in their 30s and peaks in their 70s before subsequently decreasing [3,31]. This trend was seen for both sexes. The decreased incidence in very elderly patients may reflect comorbidity with other age-related diseases such as cardiovascular disease or malignancy, such that those other diseases are registered as the cause of death. Alternatively, carriers with more intrinsic risk factors for ATL might have already developed the disease before their 80s, effectively selecting for living carriers in their 80s and 90s with low risk of developing ATL.

Another finding in this study was that ATL incidence in males increased with age, with peak values observed among men in their 70s; in contrast, incidences were largely unchanged in women from their 40s to 80s. The rate in males in their 70s was almost twice as high as that for females in their 70s. This sex-specific change of incidence with age was previously documented in the study conducted in Ehime Prefecture [31]. As explained above, carriers who acquired infection via horizontal transmission after adolescence are believed to be devoid of risk for ATL development [13,14]. Assuming that vertical transmission occurs equally for male and female children and that horizontal transmission occurs preferentially from males to females, we recalculated

the number of female carriers by applying the male HTLV-1 prevalence to the female population, and then used this adjusted value to reevaluate ATL incidence. This is a strategy that had previously been employed by Tokudome *et al.* [32]. This modification permitted the comparison of sex-specific ATL incidence mainly among carriers who nominally acquired infection vertically, although some proportion of male carriers might also have been infected horizontally. The recalculated ATL incidence for females was increased by this assumption, but the significant differences between sexes remained valid except for patients in their 50s. Thus, male to female transmission of HTLV-1 with proposed reduced risk for future ATL development does not fully explain the lower ATL incidence observed in middle-aged female carriers [32].

The low ATL incidence peculiarly found among females in their 50s to 70s may suggest that women in their 50s to 70s might have had some condition around birth that decreased the risk of ATL development. However, it is difficult to envision such a condition that would have affected only the female population. Sex-specific differences in immune surveillance potency or tumorigenicity may affect the incidence. It was reported that men were significantly less likely than women to have reduced reactivity to purified protein derivative among HTLV-1-negative individuals, whereas this gender difference was not apparent among HTLV-1 carriers [33]. Habitual smoking or alcohol intake, both of which are seen more frequently among males, may also increase their risk of developing ATL. A significant increase in HTLV-1 viral load in subjects with moderate alcohol consumption has been described [34]. Gender differences in lung and gastric cancer incidence and mortality have been attributed to the higher rate of smoking and drinking in males compared to females [35,36]. Alternatively, the low incidence of ATL specifically found among females through their 50s to 70s might reflect the influence of sex hormones or menopausal status on disease development. To our knowledge, studies have rarely mentioned the effect of sex hormones on the molecular mechanism described for ATL development [37–39]. It will be important to examine any possibility of sex differences in the molecular or cellular mechanism(s) of ATL development.

Previous work has shown that the lifetime ATL incidence is between 2 and 5% [13,31,32,40] among carriers. The result in our study, 5.2% for both sexes combined, is in line with reported values. The rate for men (7.2%) was higher than that for women (4.2%), which could partly be explained by the hypotheses described above, namely, the absence of ATL development among horizontally infected carriers and preferential horizontal transmission of HTLV-1 from men to women. These views, however, are based on relatively small cohort studies and deserve verification by a nationwide cohort study wherein seroconverted individuals are followed for life.

Our analysis predicts that the number of patients with ATL will decrease continuously over the next 15 years. In contrast, the proportion of patients in their 60s and 70s will increase year by year, with senior citizens expected to account for two-thirds of new cases detected over the next 15 years. Patients with ATL in their 60s and 70s are generally considered intolerant of aggressive chemotherapy or hematopoietic stem cell transplant with a conventional myeloablative regimen [41,42]. Recently, hematopoietic stem cell transplant following a reduced intensity conditioning regimen has been proposed for use in elderly patients with hematologic malignancy [43,44]. A clinical trial using interferon-α and zidovudine is also being conducted in Japan [45]. Demonstration of the efficacy and safety of these new approaches for elderly patients with ATL will require further study.

This article defines a nationwide ATL incidence among HTLV-1 carriers by taking into consideration the expected differences in disease capture rate among areas. Among Caribbean and Brazilian carriers, a demographic peak of patients with ATL was reported among those in their 40s, with little sex-specific difference in incidence [46–48], which is in contrast with the data presented in this article. Studies in endemic areas outside of Japan for the occurrence of ATL among carriers by age, sex and geographic area using disease capture rate will be required to understand local factors that contribute to disease pathogenesis.

In conclusion, ATL develops in nearly 1000 HTLV-1 carriers annually in Japan. ATL incidence among carriers is similar among all regions of Japan, including Kyushu. The incidence is significantly lower among middle-aged females compared to males. The number of patients with ATL is predicted to decrease over the course of the next 15 years, but patients in their 60s and 70s will constitute two-thirds of new cases. The development of treatment strategies and supportive interventions applicable to elderly patients is needed.

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System Vaccinology for the Evaluation of Influenza Vaccine Safety by Multiplex Gene Detection of Novel Biomarkers in a Preclinical Study and Batch Release Test

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Abstract

Vaccines are beneficial and universal tools to prevent infectious disease. Thus, safety of vaccines is strictly evaluated in the preclinical phase of trials and every vaccine batch must be tested by the National Control Laboratories according to the guidelines published by each country. Despite many vaccine production platforms and methods, animal testing for safety evaluation is unchanged thus far. We recently developed a systems biological approach to vaccine safety evaluation where identification of specific biomarkers in a rat pre-clinical study evaluated the safety of vaccines for pandemic H5N1 influenza including *Irf7, Lgals9, Lgalsbp3, Cxcl11, Timp1, Tap2, Psmb9, Psme1, Tapbp, C2, Csf1, Mx2, Zbp1, Ifrd1, Trafd1, Cxcl9, β2m, Npc1, Ngfr* and *Ifi47*. The current study evaluated whether these 20 biomarkers could evaluate the safety, batch-to-batch and manufacturer-to-manufacturer consistency of seasonal trivalent influenza vaccine using a multiplex gene detection system. When we evaluated the influenza HA vaccine (HAv) from four different manufactures, the biomarker analysis correlated to findings from conventional animal use tests, such as abnormal toxicity test. In addition, sensitivity of toxicity detection and differences in HAvs were higher and more accurate than with conventional methods. Despite a slight decrease in body weight caused by HAv from manufacturer B that was not statistically significant, our results suggest that HAv from manufacturer B is significantly different than the other HAvs tested with regard to *Lgals3bp, Tapbp, Lgals9, Irf7* and *C2* gene expression in rat lungs. Using the biomarkers confirmed in this study, we predicted batch-to-batch consistency and safety of influenza vaccines within 2 days compared with the conventional safety test, which takes longer. These biomarkers will facilitate the future development of new influenza vaccines and provide an opportunity to develop *in vitro* methods of evaluating batch-to-batch consistency and vaccine safety as an alternative to animal testing.

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Introduction

Vaccination is a beneficial and universal tool to prevent infectious disease [1]. Because most vaccines are derived from inactivated virus, bacteria or toxoids, contamination by incomplete inactivation can cause serious adverse events. Thus, historically, the safety of vaccines is strictly regulated by law and each batch of vaccine must be tested by the National Control Laboratories according to the guidelines published in each country, *e.g.* the European Pharmacopeia, United States Pharmacopeia and World Health Organization guidelines [2]. After the diphtheria toxoid (DT) immunization incident in Japan in 1950 that caused the death of 68 children and illness in over 600 infants owing to contamination by incomplete inactivation of DT [3], the abnormal toxicity test (ATT) (also known as general safety test) was introduced to the Japanese guidelines. This stated that the minimum requirement of biological products (MRBP) and all inactivated vaccines and toxoids was mandatory safety evaluation by ATT and other specific toxicity tests.

Influenza vaccine is one of the most widely used commercially available vaccines worldwide for preventing seasonal influenza and its complications. Influenza virus vaccine is mainly produced using embryonated fertilized chicken eggs and inactivated with formaldehyde. Whole particle influenza virus vaccine [WPv] was first licensed as an influenza vaccine in the US in 1945 [4] and is still used in some countries. Although WPv contains all the components of the influenza virus and induces strong immunity in the vaccinated individual, a high incidence of adverse events, including local reactions at the site of injection and febrile illness, particularly among children have been reported [5,6]. Thus, most recent vaccines manufactured since the 1970s have been subvirion vaccines. The subvirion influenza HA vaccine [HAv] showed a marked reduction of pyrogenicity compared with WPv [7]. The trivalent influenza vaccine [TIV] is a recently developed subvirion

influenza vaccine with components selected and updated each year to protect against one of the three main groups of circulating influenza virus strains in humans. TIV may be administered every year. Vaccine adjuvant, e.g. alum, MF59 and AS03, was also used to enhance immunity in preparation for the H5N1 pandemic [8]. To improve immunogenicity and reduce toxicity in addition to batch-to-batch quality assurance of influenza vaccine, seed lot systems, recombinant DNA technology, as well as animal and insect cell culture inactivated vaccine production systems were introduced. Despite the increase in many vaccine production platforms, adjuvants, additives and vaccine types, safety evaluation tests in the preclinical phase and batch release have been unchanged in most countries, including in Japan.

We previously reported that improved ATT could evaluate and assure the batch-to-batch consistency of vaccines more strictly compared with conventional methods [9]. In addition, we recently introduced a system biological approach to vaccine safety evaluation and demonstrated that specific biomarkers could be used to evaluate batch-to-batch consistency and safety of vaccines to diphtheria-pertussis-tetanus (DPT) [10,11] and Japanese encephalitis virus (JEV) [12]. Most recently, we showed that a system biological approach could evaluate the safety of pandemic H5N1 influenza vaccine [13]. We found 20 biomarkers for the evaluation of batch-to-batch consistency and the safety of H5N1 vaccine compared with HAv.

In this study, we tested whether these biomarkers could evaluate batch-to-batch consistency and the safety of seasonal HAv, as well as adjuvanted whole virion-derived influenza vaccine, using a multiplex gene detection system. This method might facilitate the evaluation of batch-to-batch consistency of HAv and reduce the time required for batch release compared with conventional ATT. These biomarkers will help the future development of new *in vitro* methods to evaluate vaccine safety as an alternative to animal testing.

Materials and Methods

1. Animals and Ethics statement

Eight-week-old male Fischer (F334/N) rats weighing 160–200 g were obtained from SLC (Tokyo, Japan). All animals were housed in rooms maintained at 23±1°C, with 50±10% relative humidity, and 12-h light/dark cycles for at least 1 week prior to the test use. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. The study was approved by the Institutional Animal Care and Use Committee of NIID.

2. Vaccines

The following vaccines were used in this study: (1) PDv: inactivated monovalent A/H5N1 whole-virion influenza vaccine (derived from NIBRG-14: A/Vietnam/1194/2004) adjuvanted with aluminum hydroxide, containing 30 μg HA/ml; (2) WPv: inactivated whole trivalent influenza vaccine (A/Newcaledonia/20/99 (H1N1), A/Hiroshima/52/2005 (H3N2), and B/Malaysia/2506/2004); HAV: trivalent HA influenza vaccine (A/Solomon Island/3/2006 (H1N1), A/Hiroshima/52/2005 (H3N2), and B/Malaysia/2506/2004), containing 30 μg HA/ml each strain. For evaluation of commercially distributed HAV in Japan, we used trivalent HA influenza vaccine (A/Solomon Island/3/2006 (H1N1), A/Hiroshima/52/2005 (H3N2) and B/Malaysia/2506/2004), containing 30 μg HA/ml per strain. PDv and WPv were produced, and manufactured by the Chemo-Sero-Therapeutic Research Institute, Kaketsuken (Kumamoto, Japan). Licensed and authorized HAvs were purchased from four different manufactur-

Table 1. Biomarkers to evaluate influenza vaccine safety.

Official Symbol	Official Full Name	Gene ID
Irf7	Interferon regulatory factor 7	293624
Lgals9	Lectin, galactoside-binding, soluble, 9	25476
Lgalsbp3	Lectin, galactoside-binding, soluble, 3 binding protein	245955
Cxcl11	Chemokine (C-X-C motif) ligand 11	305236
Timp1	TIMP metallopeptidase inhibitor 1	116510
Tap2	Transporter 2, ATP-binding cassette, sub-family B	24812
Psmb9	Proteasome (prosome, macropain) subunit, beta type, 9	24967
Psme1	Proteasome (prosome, macropain) activator subunit 1	29630
Tapbp	TAP binding protein (tapasin)	25217
C2	Complement component 2	24231
Csf1	Colony stimulating factor 1 (macrophage)	78965
Mx2	Myxovirus (influenza virus) resistance 2	286918
Zbp1	Z-DNA binding protein 1	171091
Ifrd1	Interferon-related developmental regulator 1	29596
Trafd1	TRAF type zinc finger domain containing 1	114635
Cxcl9	Chemokine (C-X-C motif) ligand 9	246759
β2m	Beta-2 microglobulin	24223
Npc1	Niemann-Pick disease, type C1	266732
Ngfr	Nerve growth factor receptor	24596
Ifi47	Interferon gamma inducible protein 47	246208

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