

Significance of HTLV-1 proviral load quantification by real-time PCR as a surrogate marker for HTLV-1-infected cell count

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Summary

We developed a real-time (RT) PCR quantitative assay to measure the level of the integrated viral genome of HTLV-1 in host peripheral blood-mononuclear cells (PB-MNC) from healthy carriers and patients with adult T-cell leukemia (ATL). All of the clinical specimens were serologically and molecularly characterized by enzyme-linked immunosorbent assay (ELISA) and Southern blot hybridization (SBH) analyses. The assay system for quantifying the proviral copy level was sensitive, accurate, and reproducible over a wide range of density from 100 to 0.1% with a coefficient of variation (%) of 4.5 to 9.6. The proviral load of the healthy carriers and patients with ATL was 301 ± 339 copies per 10^4 MNC ($3 \pm 3.4\%$) on average and varied depending on the ATL cell number and the SBH band-status of single or multiple bands. In ATL cases with multiple bands detected by SBH analysis, their ATL cells were shown to harbor multiple copies within one ATL cell, so that the corrected copy number interpolated by the band number in SBH was closely equivalent to the expected ATL cell number in PB, corresponding to the virus-infected cell burden. The proviral load in healthy carriers ranged from 0.1 to 15% of PB-MNC, and, in combination with the fraction (%) of ATL-like flower cells defined by PB smear morphology, enabled carriers to be sub-grouped into three categories. This result indicates that the detection of proviral load by (RT) PCR is sufficient and relevant to monitor the infected cell number in the PB and to evaluate the HTLV-1 pathologic status.

Keywords real-time PCR, HTLV-1, proviral load, ATL, integration status

Introduction

In order to understand pathologic role of the causative agent in a viral infectious disease, quantification of either viral load or cell number infected with the virus has

become indispensable, especially in the case of viruses such as human immunodeficiency virus and cytomegalovirus, that cause severe disease after a long, asymptomatic latent period, (Ho *et al.*, 1989). Human T-cell leukemia virus type-1 (HTLV-1) is also latent for 20–50 years, subsequently leading to different kinds of human disorders: adult T-cell leukemia (ATL) and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (Poiesz *et al.*, 1980; Gessain *et al.*, 1985; Osame *et al.*, 1986). This diverse pathogenesis of HTLV-1-associated diseases is thought to be due in part to differences in the virus-infected cell burden, but it is unclear whether or not proviral load reflects the virus-infected cell number.

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The HTLV-1 retrovirus mainly infects CD4 T-cells and the proviral genome is known to be randomly integrated into the human genome, with a different genomic integration site in each infected cell (Yoshida *et al.*, 1982; Seiki *et al.*, 1984). If one infected cell harbors one proviral genome, the number of proviral genomes corresponds to the number of infected CD4 T-cells. The proviral integration status, random or non-random, in the infected cells of an individual has generally been examined by Southern blot hybridization (SBH) using EcoR1. This enzyme has no cleavage site within the integrated proviral sequence, implying that it would show sharp band(s) or a smear band, respectively, according to the clonal or polyclonal proliferation status of the virus-infected cells (Seiki *et al.*, 1983). The band number following SBH using EcoR1 digestion should be identical to the copy number within one infected cell if ATL cells are monoclonal.

The ATL cells are characterized by a typical morphology, described as 'flower-cells'. These cells harbor the HTLV-1 provirus (Uchiyama *et al.*, 1977), while abnormal lymphocytes similar to flower-cells (designated ATL-like flower-cells) are observed in peripheral blood smears even in asymptomatic carriers (Shinzato *et al.*, 1993). Some carriers having ATL-like flower cells show molecularly-detectable monoclonal proliferation of the virus-infected T-cells (Ikeda *et al.*, 1993). Therefore, the existence of ATL-like flower cells in carriers may be a risk factor for the development of ATL, as the prodromal state of ATL (pre-ATL; Chen *et al.*, 1995; Hisada *et al.*, 1998).

In the present study, a real-time (RT) PCR assay for quantification of the HTLV-1 proviral load was established, using LightCycler Technology (Roche Diagnostics GmbH, Mannheim, Germany) with specific probes and the relevance of this assay was examined for determination of the virus-infected cell number, derived from the proviral load.

Materials and methods

Study samples

One hundred sixty-seven heparinized peripheral blood samples were studied. These were selected from samples sent to our department for routine HTLV-1 SBH and sero-status testing. Seropositivity for HTLV-1 was determined by an ELISA assay (EiTest-ATL, Eisai K.K., Tokyo, Japan). Peripheral blood-mononuclear cells (PB-MNC) were collected by Ficoll-Conray gradient centrifugation and genomic DNA was processed using the phenol-chloroform method. Genomic DNA and sera were stored at -80°C until use.

Diagnosis and classification of the clinical subtypes of ATL were made based on the criteria of the LSG group (Shimoyama *et al.*, 1991). The results were then confirmed in all cases by SBH analysis, with detection of monoclonal integration of HTLV-1 provirus into the genome using HTLV-1 full-length and pX probes (Kamihira *et al.*, 1997). Clinical and laboratory data of the patients providing samples were obtained from the computer filing system of our institute.

Real-time quantitative PCR

The primers and probes for the HTLV-1 provirus target were theoretically optimized sequences for this system. The primer pairs and probes were selected so that they were located in the pX region of the provirus, which is the most stable, in order to prevent false negative results. The primers used were from highly conserved sequences of the tax gene (sense, 5'-CCCACTTCCCAGGGTTTGGACAGAG; anti-sense, 5'-CGCGTTATCGGCTCAGCTCTACAG). The sequences of probes were 5'-CTTTTCCAGACCCCGGACTCCG-3' and 5'-CCCAAACCTGTACACCCTCTG-3', respectively. The former and latter were labelled with FITC as a reporter probe, and LC Red 640 as a quencher probe, respectively. The PCR reaction was carried out using the LightCycler Technology assay instrument (Wittier *et al.*, 1997; Kreuzer *et al.*, 1999). This consists of three main components: a PCR amplification system using capillary tubes, a detection system for the amplicon, and a quantifying system using a standard curve generated for each assay target. In the detection system, the reaction is characterized by the point during cycling at which amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. For each test sample, 30 ng genomic DNA was used as a template for amplification with Taq DNA polymerase and TaqStartTM antibody (CLONTECH Laboratories, Palo Alto, CA, USA) in the LightCycler PCR Instrument, and the amplified products were automatically measured according to the manufacturer's instructions.

The reaction conditions were 95°C for 2 min for activation of the Taq polymerase and then 40 cycles of 10 s at 95°C (denaturation) followed by 5 s at 68°C (annealing) and by 10 s at 72°C (extension). An HTLV-1 standard curve was generated using serial dilutions as the template for the real-time PCR from 10^6 to 10^2 copies of the proviral DNA, derived from a clone in pBR322 vector as follows. A 9 kb proviral DNA fragment was cloned into pBR322 and propagated. After digestion with EcoR1, the insert was purified and quantified with a PicoGreen dsDNA Quantification kit (Molecular Probes, Eugene, OR, USA).

Molecular concentrations were calculated and the purified insert was used for generation of the standard curve.

The copy numbers in the samples were estimated by interpolation from the plasmid control regression curve and an internal control of β -globin to correct for sample DNA quality differences. Copy numbers were reported as copy equivalents per 10^4 MNC, mainly lymphocytes.

Southern blot hybridization analysis

To detect clonal integration and to determine the status of the provirus integrated into the genomic DNA derived from leukemic cells, SBH analysis was carried out using pX and whole viral genomic probes for HTLV-1, as described previously (Kamihira *et al.*, 1997). Briefly, an aliquot of 10 μ g genomic DNA was digested with EcoR1 or Pst-1 and electrophoresis on a 0.7% agarose gel was followed by blotting onto a positively charged Nylon membrane (Boehringer, Mannheim, Germany). After annealing with the digoxigenin-labelled whole HTLV-1 probe, band signals were visualized using CDP-Star (Boehringer Mannheim) as the chemiluminescence substrate for alkaline phosphatase. This SBH has the potential to detect 5% monoclonal cells as a visual band. Provirus status in the genomic DNA from leukemic cells, i.e. the presence or absence of clonal integration and complete or defective provirus, was determined based on our previously described criteria (Tamiya *et al.*, 1996; Tsukasaki *et al.*, 1997).

Statistical analysis

Values of clinical, biological, and laboratory parameters were compared using Wilcoxon analysis and the chi-square test. Differences between the two populations were assessed as significant at confidence levels greater than 95% ($P < 0.05$). Correlation analysis was estimated by the one-sample *t*-test.

Results

Characteristics of the study samples

Fifty-nine samples were found to be positive for monoclonal integration of the provirus by SBH. The SBH bands were single in 52 samples, double in six samples, and triple in one sample, as shown in Figure 1. All patients showing clonal band(s) were clinical ATL cases, with 11 smoldering, 23 chronic, and 25 acute subtypes. In the remaining 108 samples, no clonal band was detectable. The HTLV-1 sero-status for these samples was negative in 20, and



Figure 1. Band patterns by HTLV-1 Southern blot hybridization analysis in three representative adult T-cell leukemia (ATL) cases. Two bands in case 1 and one band in cases 2 and 3 were detected as shown in lanes 2, 4, and 6, of which genomic DNAs were digested by EcoR1 enzyme. M: marker. Lane 1; positive control using Pst-1 digested genomic DNAs from ATL cell-lines harboring the two proviral copies. Lanes 1, 3, 5, and 7; Pst-1 digestion. Lanes 2, 4, and 6, EcoR1 digestion.

positive in 88. Of the 88 seropositive cases, morphological examination of the PB smear revealed ATL-like flower cells comprising 1 to 5% of MNC fraction in 28 samples, but not clinically ATL.

Quantification of HTLV-1 proviral copy load

The HTLV-1 proviral copy load is thought to range widely from 100 to 0.1% or less of the MNC fraction in clinical practice, so our (RT) PCR system was first evaluated for accuracy and reproducibility at different cell concentrations of 10^0 – 10^{-3} . This was equivalent to about 100 to 0.1% infected cells/ 10^4 MNC in which 10^4 ATL cells harboring a single proviral copy were diluted with normal lymphocytes. As shown in Figure 2, the mean copy number/ 10^4 MNC and coefficients of variation (CV) at each concentration were 8913 ± 570 , 655 ± 32 , 70 ± 7 and, 5 ± 2 , and 6.4, 4.8, 9.6 and 51.8%, respectively. The between-day precision using MNC from a seropositive healthy individual was reproducible, with a CV of 15.9% and a mean of 189 copies, as shown in Figure 3.

Next, the proviral load was quantified in 108 samples without ATL, of which 20 were seronegative (Sn) and 88 were seropositive (Sp), as well as in 59 samples from ATL

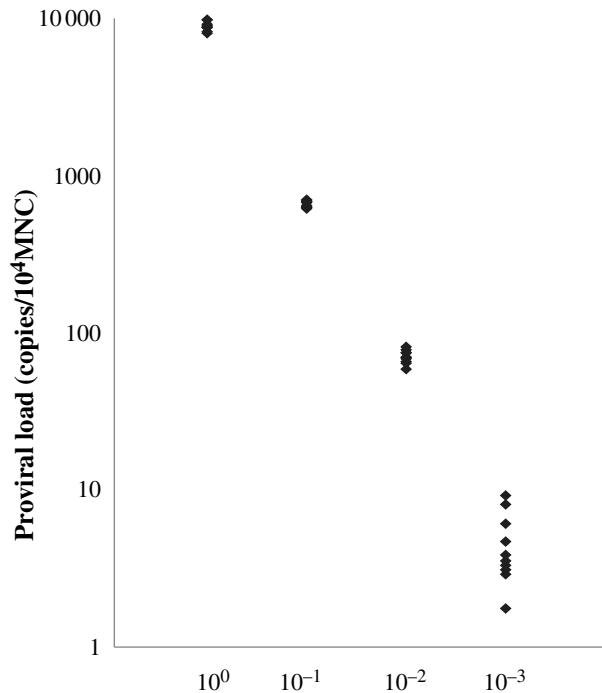


Figure 2. Reproducibility and accuracy within-run depend on different levels of proviral load. The proviral load was measured 10 times in each sample diluted from 10^0 to 10^{-3} with normal lymphocytes. The original 10^0 sample contained about 10^4 freshly isolated ATL cells showing one band pattern by SBH analysis.

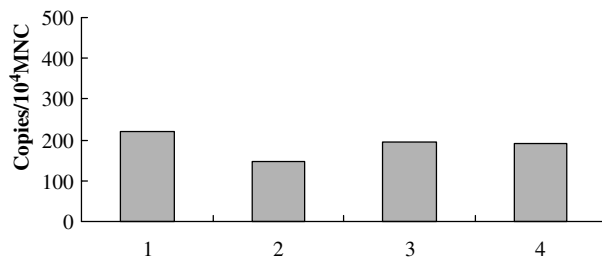


Figure 3. Reproducibility between-day. The proviral load was measured using blood samples drawn from the same person on four different days.

patients. The individual results are shown in Figure 4. Provirus was undetectable in all of the Sn group and detectable in all but one of the Sp group, with a proviral load of 301 ± 339 copies. Provirus was detectable in all patients with ATL, ranging from 1.72×10^2 to 1.53×10^4 . A seropositive case that was negative by PCR was ATL, status-post-BMT. As shown in Figure 4, the proviral load of the seven cases indicated by open diamonds was generally higher than the expected ATL cell counts by morphology. All cases showing discrepancies between the proviral load and the expected cell

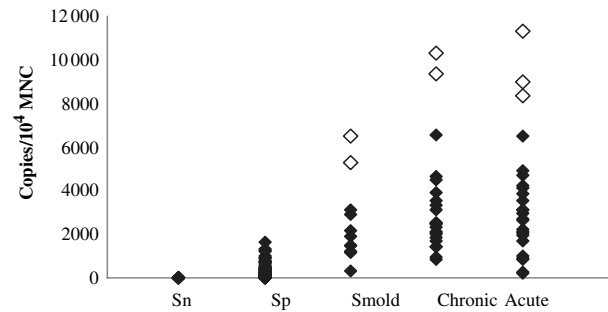


Figure 4. Distribution of copy number of the proviral load among 10^4 mononuclear cells in relation to sero-negative (Sn) and sero-positive (Sp) status, and subtypes of adult T-cell leukemia. Closed and open diamonds represent single band, and multiple band cases, respectively.

numbers had two or three bands by SBH analysis (Table 1). Furthermore, the ratio of the proviral load to cell number was closely equivalent to the band number by SBH, as summarized in Table 1, indicating that ATL cells from these cases harbored multiple copies within a single ATL cell.

Circulating proviral load surrogating tumor burden or infectious cells

To clarify the relevance of our quantification method, we first studied the relationship between ATL cell counts (tumor burden) and infected cell counts interpolated by the proviral load and the SBH band pattern in ATL. The majority of ATL cases had single bands by SBH, and the remaining cases with multiple bands harbored multiple copies within one ATL cell. Thus the corrected copy number interpolated by the SBH band status (the raw copy number divided by the band numbers) should correspond to the infected cell number in ATL. There was in fact a significant correlation between ATL cell counts and the copy number including the corrected copy number in the 7 'aberrant' cases, as shown in Figure 5 ($r = 0.6835$, $P < 0.0001$).

Next, we compared the correlation between the proviral load and the presence (%) of ATL-like flower cells in the PB of healthy carriers, on the assumption that each infected cell in such carriers generally contains a single proviral copy. The proviral load of seropositives with no detectable ATL-like flower cells was lower than that of the remaining seropositives with 1 to 5% of such cells (271 ± 314 vs. 384 ± 308 copies). In a twin plot graph (Fig. 6) comparing the relationship between the numbers of ATL-like flower cells and the proviral load, it is notable that the points corresponding to individual cases were characteristically distributed into three groups. In group-A, the copy

Table 1. Comparison of the copy number vs. infected cell number ratio and band status by Southern blot hybridization in cases where there were discrepancies between the raw copy number proviral load and the predicted adult T-cell leukemia (ATL) cell number within the DNA template

ID	Proviral copies	ATL cell counts	Ratio (copy/cell)	Number of band
375	5721	2167	2.4	2
121	6510	3380	1.9	2
69	12289	8450	1.5	2
89	9379	4120	2.3	3
529	11290	5140	1.4	2
382	8984	6647	1.4	2
399	8340	5667	1.5	2

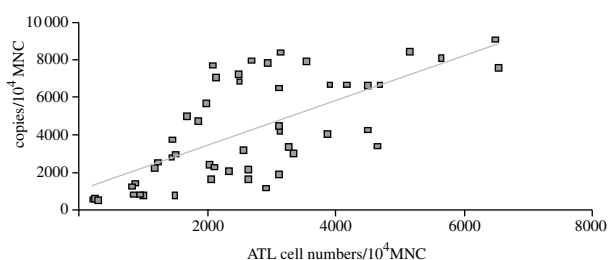


Figure 5. Correlation between number of adult T-cell leukemia cells (*y*-axis) and copy number of proviral load (*x*-axis). The proviral load correlated significantly with the cell number ($r = 0.6825$, $y = 990 + 1.21x$, $P < 0.0001$).

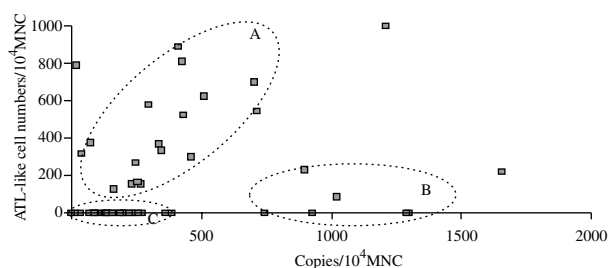


Figure 6. Twin plot graph showing the correlation between the number of adult T-cell leukemia-like flower cells and the proviral load in healthy carriers without clonal band by Southern blot hybridization. The points corresponding to individual cases tend to fall into three groups (A, B, and C).

number was correlated to the number of ATL-like ATL cells; in group-B, the copy number alone was highly detectable; and in group-C, both were faintly detectable.

Discussion

Only 3 to 0.1% of peripheral lymphocytes have been reported to be persistently infected with HTLV-1 in asymptomatic carriers of the virus (Shinzato *et al.*, 1993;

Ono *et al.*, 1998; Etoh *et al.*, 1999). Therefore, an increase in the number of virus-infected cells in the PB after a long latency has been considered to be an important marker for the development of ATL and HAM (Jeffery *et al.*, 1999, Abet *et al.*, 2000). In contrast to virus-infected cells, the quantitation of proviral load has been greatly simplified by RT-PCR technology, although it remains unclear whether the proviral load is a surrogate for the viral-infected cell burden. Accordingly, we developed a real-time quantitative PCR for HTLV-1 performed in the LightCycler analyzer using two adjacent fluorescently labelled probes and applied the method to the evaluation of samples defined by ELISA and SBH analyses. We then used this approach to examine the relevance of interpolation from the proviral load to the virus-infected cell burden in HTLV-1 infection.

Our assay system was sensitive, accurate and reproducible for quantification of the proviral load over a wide range (100–0.1% infected cells per 10^4 MNC. It is noteworthy that our CV (%) for within-run and between-day reproducibility was 10% or less, even at the 0.1% level of proviral copy number. This indicates that it is suitable for sequential monitoring of the proviral load in asymptomatic carriers, who usually have a low levels (0.1–5%, corresponding to 10–500 copies/ 10^4 MNC by our data). The diagnostic validity of our assay system was excellent qualitatively, our results being completely consistent with sero-status, as defined by the serological method.

The question of whether the proviral load can be used to predict the infected cell number was examined using freshly isolated ATL cells whose provirus-integration status was defined by SBH analysis. Interestingly, seven of 59 ATL cases were found to show discrepant results between the proviral copy load and the expected ATL cell number. In all seven cases, however, two or three bands were demonstrated in the SBH analysis, implying that their ATL cells harbored multiple copies in one genome. The proviral load corrected for the SBH band status was correlated closely to the expected ATL cell number, i.e. the virus-infected cell burden. These results indicate that the proviral load can be used to predict the virus-infected cell number, but it is important to consider the SBH band status.

There are few reports documenting the provirus-integration status of non-malignant infected cells from asymptomatic carriers. One of these reports (Wattle *et al.*, 1995) stated that an increased proviral load from a stable dose results from clonal expansion of the virus-infected cells, rather than virus replication and re-infection. Supporting this view, the morphological and molecular results of our previous studies (Ikeda *et al.*, 1993; Chen *et al.*, 1995)

demonstrated ATL-like flower cells, some of which are clonal at the asymptomatic stage, indicating a preleukemic state for ATL. We therefore examined the relationship between proviral load and the presence of ATL-like flower cells as distinct from the virus-infected cell burden. Our previous conclusions were supported, as there was a close correlation between the proviral load and the number of ATL-like flower cells expanding oligoclonally at the healthy carrier stage, as shown in Figure 6, group-A. These ATL-like flower cells are also thought to precede the development of ATL, so it is useful to monitor the proviral load to detect early transformation to ATL. Healthy carriers were found to be subgrouped into three categories based on two parameters: the proviral load and the presence of ATL-like flower cells in the PB. We are now conducting a prospective follow-up study to determine which subgroup(s) is at risk for the development of ATL or other HTLV-1 associated-diseases.

In conclusion, we developed a real-time quantitative PCR assay using suitable primers and probes, and then confirmed the relevance of this assay for determination of the virus-infected cell number in healthy individuals and individuals infected with HTLV-1. The virus-infected cell number was calculated from the proviral load, using samples defined by SBH analysis.

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