

Original Article

Serological and Molecular Evidence of HTLV-I Infection among Japanese Immigrants Living in the Amazon Region of Brazil

Antonio Carlos R. Vallinoto*, Nilton A. Muto, Gemilson S. Pontes, Luiz Fernando A. Machado, Vânia N. Azevedo, Sidney E. B. dos Santos¹, Ândrea K. C. Ribeiro-dos-Santos¹, Marluísa O. G. Ishak and Ricardo Ishak

Laboratório de Virologia and ¹Laboratório de Genética Humana e Médica, Departamento de Patologia, Centro de Ciências Biológicas, Universidade Federal do Pará, Para, Brasil

(Received December 25, 2003. Accepted April 19, 2004)

SUMMARY: Human T-lymphotropic virus type I (HTLV-I) infection was investigated in 168 Japanese immigrants (64 males and 104 females) living in the Tome-Acu county located in the State of Para, Brazil. The serological screening was performed using an enzyme-linked immunosorbent assay, and showed the presence of anti-HTLV in four women whose ages ranged from 50 to 88. Confirmation of infection and discrimination HTLV typing was performed using a nested PCR on the extracted DNA targeting the pX region. In three of the samples, infection was confirmed to be HTLV-I. Sequencing HTLV-I 5'LTR and the RFLP pattern using *DraI* and *SacI* endonucleases indicated that the virus is a member of the Cosmopolitan group. These three women originated from the Kyushu region, though two of the corresponding HTLV-I strains were phylogenetically related to the Japanese subgroup and the third to the Transcontinental subgroup, which probably reflects the geographical origin of the infected individuals. The Japanese community residing in the northern Brazil apparently have not contributed to increase the prevalence of HTLV-I in the country.

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I) is endemic in Japan, the Caribbean, South America, Sub-Saharan Africa, and Melanesia (1). The dissemination of the virus to different continents seems to have occurred as the result of ancient and recent human movements (2,3), and is supported by the finding of HTLV-I in a 1,500-year-old Andean mummy (4,5). There is a strong suggestion that HTLV-I was brought from Asia into the Americas by the ancestors of the present native inhabitants of the Americas (5).

The widespread distribution of the virus among indigenous communities that have been isolated from other human populations provides indirect evidence of the expansion and longtime presence of HTLV-I (6). HTLV-I was broadly disseminated during the last few centuries as a result of the great increase in human movement (1,3).

In Brazil, HTLV-I infection has been described mostly in urban areas (7-10), and its pattern has been different from that usually observed with HTLV-II; it has been detected in few individuals from Indian populations from the Brazilian Amazon region – within the Galibi, Yanomami, and Aukre (11). The origin of HTLV-I infection in Brazil has been attributed to three different sources: (i) Amerindian ancestors who arrived at the continent around 13,000 years ago, (ii) Africans brought as slaves to Brazil around the 17-18th centuries, and, more recently, (iii) Japanese immigrants who came to Brazil in the beginning of the 20th century. These hypotheses have been supported by the finding of Transcontinental and Japanese subtypes of HTLV-I in Brazil (12).

The present study describes the presence of HTLV-I

infecting Japanese immigrants residing in Tome-Acu county (State of Para), an epidemiologically closed population group in the Amazon region of Brazil.

MATERIALS AND METHODS

Population group examined and sample collection: Blood samples were collected between November and December, 1999, from 168 Japanese immigrants (64 men and 104 women, ages 40 to 90) residing in the county of Tome-Acu, State of Para. Blood samples were placed in tubes containing EDTA in order to obtain plasma and peripheral blood mononuclear cells (PBMC). Both specimens were stored at -20°C before use.

Serological assays: Samples were screened for the presence of antibodies to HTLV-I/II using an enzyme-linked immunosorbent assay (HTLV-I/II Ab-Capture ELISA Test System, Ortho Diagnostic Systems Inc., Raritan, N.J., USA). Positive samples were subjected to confirmation by nested PCR.

Nested PCR and restriction fragment length polymorphism (RFLP) patterns: DNA extraction was performed on PBMC from HTLV-seropositive subjects. Nested PCR targeted the amplification of part of the pX and 5'LTR of HTLV-I (13).

The amplification of the pX segment was performed using a nested PCR that was carried out as follows. Reactions were performed in 50 μl volumes containing 1 μg of the extracted DNA, 125 μM of each dNTP, 20 pmol/ μl of each of the two external primers, 10 \times PCR buffer-MgCl₂ and 0.5U *Taq* DNA polymerase. Reactions were carried out in a thermocycler (GeneAmp PCR System 2,400, Perkin-Elmer, Norwalk, Calif., USA) for 5 min at 94°C, followed by 35 cycles at 94°C (40 sec), 51.6°C (30 sec), 72°C (40 sec), and extended for 10 min at 72°C. Five microlitres of the amplified product were used in the nested PCR using a set of internal primers,

*Corresponding author: Mailing address: Rua Oliveira Belo n^o. 238, apt^o. 1002, Umarizal, 66.050-380, Belem, Para, Brazil. Tel: +51-91-81158578, Fax: +55-91-249-0373, E-mail: vallinoto@ufpa.br

Table 1. Serological and molecular characterization of HTLV-I infection among Japanese immigrants living in the Amazon region of Brazil

Sample code	Age	EIA	Nested PCR		RFLP (5'LTR)		Subgroup	Geographical origin
			pX	5'LTR	<i>SacI</i>	<i>DraII</i>		
JPNBR41	50	+	+	+	+	-	A	Kyushu
JPNBR81	63	+	+	+	-	+	B	Kyushu
JPNBR177	88	+	+	+	-	+	B	Kyushu
JPNBR157	50	+	-	-	NT	NT	-	Tohoku

A: Transcontinental subgroup, B: Japanese subgroup, NT: Not tested.

maintaining the same mixture (final volume of 50 μ l), temperature, and incubation periods used in the first reaction. The external primer sequences were 5'-TTCCCAGGGTTTGGACAGAG-3' (nucleotide positions 7219-7238) and 5'-GGGTAAGGACCTTGAGGGTC-3' (nucleotide positions 7483-7464). The internal primer sequences were 5'-CGGATACCCAGTCTACGTGTT-3' (nucleotide positions 7248-7268) and 5'-GAGCCGATAACGCGTCCATCG-3' (nucleotide positions 7408-7386). Amplified products were subjected to electrophoresis on 2% agarose gels.

Typing of HTLV was performed by RFLP analysis of the pX amplified product using the restriction endonuclease enzyme *TaqI*, as described elsewhere (14).

All samples characterized as HTLV-I were subjected to a nested PCR to amplify the 5'LTR region. The external primers sequences were 5'-TGACAATGACCATGAGCCCCAA-3' (LTR-I.01) and 5'-CGCGGAATAGGGCTAGCGCT-3' (LTR-I.02), corresponding to nucleotide positions 1-22 and 823-842, respectively. The internal primer sequences were 5'-GGCTTAGAGCCTCCCAGTGA-3' (LTR-I.03) and 5'-GCCTAGGGAATAAAGGGGCG-3' (LTR-I.04) corresponding to nucleotide positions 30-49 and 781-800 from HTLV-I^{ATK} strain. The PCR product was electrophoresed on a 1.5% agarose gel (200V, 120 Amp/45 min) and purified by QIA Quick Purification Kit (Qiagen Sciences Inc., Germantown, Md., USA) prior to direct sequencing of the product.

In addition, RFLP analysis of the 5'LTR from three samples amplified in the present study was performed using *DraI* and *SacI* endonucleases, which permit distinction between Japanese and Transcontinental subtypes of HTLV-I (15,16).

Sequencing: The amplified fragments were subjected to a direct sequencing assay according to the protocol of the ABI Prism Dye Terminator Cycle Sequencing Ready Kit (Perkin-Elmer); the reaction products were loaded on the ABI Prism 373 DNA Sequencer (Perkin-Elmer).

Phylogenetic analysis: The nucleotide sequences obtained in the present study originated from the 5'LTR region (Genbank Accession Number: JPNBR41, AY499185; JPNBR81, AY499186; and JPNBR177, AY499187), and were used, together with 33 HTLV-I strains described in the Genbank, to establish the phylogenetic relationship. The sequence alignments were performed using the ClustalX Sequence Editor, version 1.81 (17). The phylogenetic relationship was implemented using the Molecular Evolutionary Genetics Analysis software - MEGA version 2.1 (18). The Neighbor-Joining (NJ) method was used for the construction of the phylogenetic tree considering the standard Kimura two-parameter model (19). The statistical reliance of NJ tree was evaluated using 1,000 bootstrap samples.

RESULTS

Serological and demographic analysis: The serological assay detected the presence of antibodies to HTLV-I/II in four women (ages ranging from 50 to 88; Table 1). Demographic analysis indicated that one of them was from Tohoku region (Northern part of Honshu island of Japan); the other three from Kyushu region (Southern part of Honshu island).

Molecular characterization and phylogenetic analysis: Amplification attempts were performed targeting the pX region using PBMC of the four seropositive individuals. Three of them (immigrants from Kyushu) elicited amplification products that were typed as HTLV-I (Table 1).

Nucleotide sequencing of the 535 nt amplified product from 5'LTR, followed by phylogenetic analysis, showed a cluster phylogenetically related to the viral members of the Cosmopolitan group; two samples clustered in the Japanese sub-

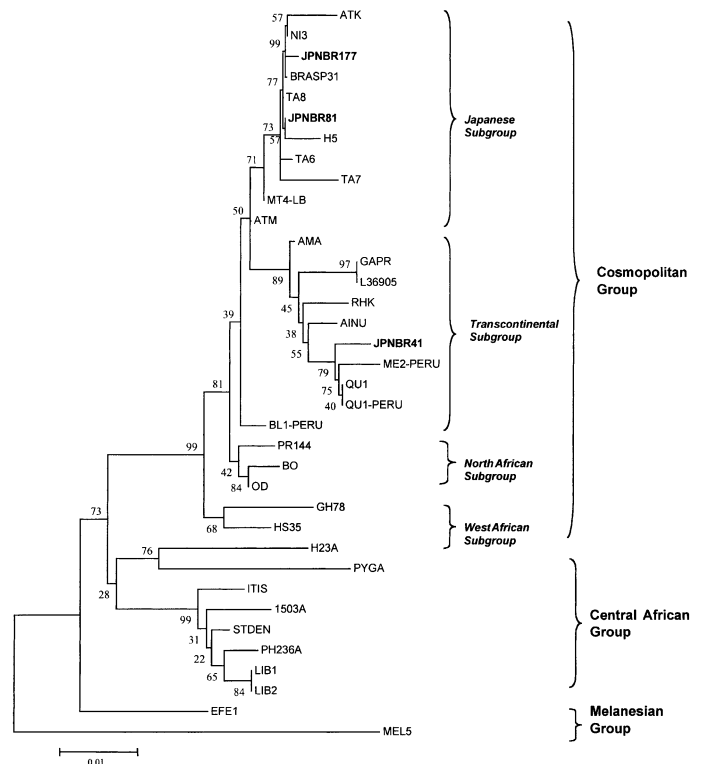


Fig. 1. Unrooted phylogenetic tree showing the evolutionary relationship of HTLV-I strains reported thus far including newly sequenced isolates from the present study (Japanese subjects: JPNBR41, JPNBR81, and JPNBR177). The tree was constructed by the NJ method after alignment of 535 nucleotides of the 5'LTR region. The statistical support was applied using 1000 bootstrap replicates. Cosmopolitan group: Transcontinental subgroup, Japanese subgroup, West African subgroup, and North African subgroup.

group, the other in the Transcontinental subgroup (Fig. 1) associated with isolates from South America. RFLP patterns using *DraI* and *SacI* endonucleases were consistent with the profile of these subgroups, supporting the results of phylogenetic analysis (Table 1).

DISCUSSION

The presence of antibodies to HTLV-I/II and the confirmation of proviral nucleic acid integrated into PBMC from Japanese immigrants residing in Tome-Acu describes the occurrence of the virus among this particular population group. Tome-Acu is a small rural village (47,273 inhabitants), located in the north of the State of Para, and a large component of its inhabitants were born from a core of Japanese immigrants who arrived in Brazil during the first wave of migration from that country in 1929.

HTLV-I was present in 3/168 (1.8%) emigree women who came from the Kyushu region, a southern part of the Honshu island, Japan, largely recognized for its high prevalence of HTLV-I and tropical spastic paraparesis (TSP/HAM) (20). A previous seroepidemiological approach did not detect any serological reaction to the virus among 44 individuals residing in the same geographic area (21). The low prevalence of HTLV-I in most parts of the world requires that a large number of individuals be examined in order to increase the chance of identifying those infected. On the other hand, the information presented herein is in agreement with the demographic situation occurring in Japan in which the prevalence of women infected is higher than that of men (22). Furthermore, the positive individuals were probably infected in Japan and brought the virus in the recent past to Brazil.

The HTLV-I isolates were analysed according to specific genomic sequences of the 5'LTR, and the phylogenetic relatedness indicated they were members of the Cosmopolitan group; two of them were included in the Japanese subgroup, one in the Transcontinental subgroup. This data is in agreement with previous information described in Brazil as well as in that described among Japanese immigrants living in Sao Paulo, where the presence of both subgroups has been described (23,24).

In Japan, HTLV-I strains of the Japanese and Transcontinental subgroups present a clear difference in their geographic distribution according to ethnic group and origin of the infected subject (24). As an ancient human virus, the distinct strains of HTLV-I accompanied different Japanese ancestors during the settlement of Japan (24,25) into the three known distinguishable ethnic groups, the Ainu (North Japan), the Ryukyuan (South Japan), and the Wajin (Central Japan). The three HTLV-I infected women described herein were immigrants from Kyushu who arrived in Brazil and took part in the development of the Tome-Acu village in the early 20th century in the State of Para, Brazil.

Mongoloid groups harboring the virus introduced it not only into Japan but also in the Americas via the ancient migratory path over the land bridge of Beringia some 10,000 to 20,000 years ago (24,25). The HTLV-I isolate JPNBR41 is phylogenetically close to other isolates from South America, which reflects a possible common ancestry.

HTLV-I is maintained in Brazil at a low level prevalence, and the Japanese immigration wave to Brazil that occurred in the beginning of the 1920s apparently did not contribute to increase the prevalence of the virus (12). Indeed, the occasional high rates of infection found in some areas of Japan were not

found among the Japanese communities examined in Brazil (12,21). Additionally, in urban and rural populations from the Amazon region, the HTLV-I Transcontinental subgroup has been found as the sole subtype circulating in this area (13,26). Although it is thought that two of the strains identified in our patients were conclusively brought from Japan (JPNBR81 and JPNBR177), the third strain (JPNBR41) derived from a younger woman could have had the same origin but could also have resulted from a later event involving infection while in Brazil.

ACKNOWLEDGMENTS

We thank all the individuals involved in the present study. The present work was partially supported by grants from the Universidade Federal do Para (UFPA/PROINT).

REFERENCES

1. Gessain, A. and Mahieux, R. (2000): Epidemiology, origin and genetic diversity of HTLV-I retrovirus and STL-1 simian affiliated retrovirus. *Bull. Soc. Exot.*, 93, 163-171.
2. Doherty, R. (1996): HTLV-I in Australia and Oceania: long term resident or recent immigration? *Med. J. Aust.*, 164, 84-86.
3. Black, F. L. (1997): Tracing prehistoric migrations by the viruses they carry: human T-cell lymphotropic viruses as markers of ethnic relationships. *Hum. Biol.*, 69, 467-482.
4. Li, H. C., Fujiyoshi, T., Lou, H., Yashika, S., Sonoda, S., Cartier, L., Nunez, L., Munoz, I., Horai, S. and Tajima, K. (1999): The presence of ancient human T-cell lymphotropic virus type I provirus DNA in an Andean mummy. *Nat. Med.*, 5, 1428-1432.
5. Sonoda, S., Li, H. C., Cartier, L., Nunez, L. and Tajima, K. (2000): Ancient HTLV type 1 provirus DNA of Andean mummy. *AIDS Res. Hum. Retroviruses*, 16, 1753-1756.
6. Miura, T., Fukunaga, T., Igarashi, T., Yamashita, M., Ido, E., Funahashi, S., Ishida, T., Washio, K., Ueda, S., Hashimoto, K., Yoshida, M., Osame, M., Singhal, B., Zaninovic, V., Cartier, L., Sonoda, S., Tajima, K., Ina, Y., Gojobori, T. and Hayami, M. (1994): Phylogenetic subtypes of human T-lymphotropic virus type I and their relations to the anthropological background. *Proc. Natl. Acad. Sci., USA*, 91, 1124-1127.
7. Castro-Costa, C. M., Vale, O. C., Goubau, P., Desmyter, J. and Carton, H. (1991): HTLV-I and tropical spastic paraparesis in Fortaleza (northeastern Brazil). *J. Trop. Geogr. Neurol.*, 1, 45-48.
8. Galvão-Castro, B., Loures, L., Rodrigues, L. G. M., Sereno, A., Ferreira, O. C., Franco, L. G. P., Muiller, M., Sampaio, D. A., Santana, A., Passos, L. M. and Proietti, F. (1997): Distribution of human T-lymphotropic virus type I among blood donors: a nationwide Brazilian study. *Transfusion*, 37, 242-243.
9. Ishak, R., Ishak, M. O. G., Azevedo, V. N., Santos, D. E. M., Vallinoto, A. C. R., Saraiva, J. C. P., Crescente, J. A. and Hall, W. W. (1998): Detection of HTLV-IIa in blood donors in an urban area of the Amazon region of Brazil (Belem, Para). *Rev. Soc. Bras. Med. Trop.*, 31, 193-197.
10. Vallinoto, A. C. R., Azevedo, V. N., Santos, D. E. M., Caniceiro, S., Mesquita, F. C. L., Hall, W. W., Ishak, M.

- O. G. and Ishak, R. (1998): Serological evidence of HTLV-I and HTLV-II co-infections in HIV-1 positive patients in Belem, State of Para, Brazil. *Mem. Inst. Oswaldo Cruz*, 93, 407-409.
11. Ishak, R., Harrington, Jr. W. J., Azevedo, V. N., Eiraku, N., Ishak, M. O. G., Guerreiro, J. F., Santos, S. E. B., Kubo, T., Monke, C., Alexander, S. and Hall, W. W. (1995): Identification of human T-cell lymphotropic virus type IIa infection in the Kayapo, an indigenous population of Brazil. *AIDS Res. Hum. Retroviruses*, 11, 813-821.
 12. Yamashita, M., Veronesi, R., Menna-Barreto, M., Harrington, Jr. W. J., Sampaio, C., Brites, C., Badaró, R., Andrade-Filho, A. S., Ohkura, S., Igarashi, T., Takehisa, J., Miura, T., Chamone, D., Bianchini, O., Jardim, C., Sonoda, S. and Hayami, M. (1999): Molecular epidemiology of human T-cell leukemia virus type I (HTLV-I) in Brazil: the predominant HTLV-Is in South America differ from HTLV-Is of Japan and Africa, as well as those of Japanese Immigrants and their relatives in Brazil. *Virology*, 261, 59-69.
 13. Pontes, G. S., Muto, N. A., Lopes, I. G. L., Azevedo, V. N., Machado, L. F. A., Ishak, M. O. G., Ishak, R. and Vallinoto, A. C. R. (2003): Seroepidemiology and molecular epidemiology of HTLV infection in rural and urban populations from the Amazon region of Brazil. *Virus Res.*, 8 (Suppl. 1), 211.
 14. Hall, W. W., Takahashi, H., Liu, C., Kaplan, M. H., Sheewind, O., Ijichi, S., Nagashima, K. and Gallo, R. C. (1992): Multiple isolates and characteristics of human T-cell leukemia virus type II. *J. Virol.*, 66, 2456-2463.
 15. Komurian-Pradel, F., Pelloquin, F., Sonoda, S., Osame, M. and De Thé, G. (1992): Geographical subtypes demonstrated by RFLP following PCR in the LTR region of HTLV-I. *AIDS Res. Hum. Retroviruses*, 8, 429-434.
 16. Segurado, A. A. C., Biasutti, C., Zeigler, R., Rodrigues, C., Damas, C. D., Jorge, M. L. S. G. and Marchiori, P. E. (2002): Identification of human T-lymphotropic virus type-I (HTLV-I) subtypes using restricted fragment length polymorphism in a cohort of asymptomatic carriers and patients with HTLV-I associated myelopathy/tropical spastic paraparesis from Sao Paulo, Brazil. *Mem. Inst. Oswaldo Cruz*, 97, 329-333.
 17. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 24, 4876-4882.
 18. Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. (2001): MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics*, 17, 1244-1245.
 19. Kimura, M. (1980): A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, 16, 111-120.
 20. Mueller, N., Okayama, A., Stuver, S. and Tachibana, N. (1996): Findings from the Miyazaki cohort study. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.*, 13 (Suppl. 1), S2-S7.
 21. Nakauchi, C. M., Maruyama, K., Kanzaki, L. I., Linahres, A. C., Azevedo, V. N., Fukushima, T., Miyauchi, M., Koshikawa, N., Tamayama, C., Mochizuki, S. and Kawamura, K. (1992): Prevalence of HTLV-I antibody among two distinct ethnic groups inhabiting the Amazon region of Brazil. *Rev. Inst. Med. Trop. Sao Paulo*, 34, 323-328.
 22. Nakashima, K., Kashiwagi, S., Kajiyama, W., Hirata, M., Hayashi, J., Nogi, A., Urabe, K., Minani, K. and Maeda, Y. (1995): Sexual transmission of human T-lymphotropic virus type I among female prostitutes and among patients with sexually transmitted diseases in Fukuoka, Kyushu, Japan. *Am. J. Epidemiol.*, 141, 305-311.
 23. Song, K., Nerurkar, V., Pereira-Cortez, A., Yamamoto, M., Taguchi, H., Miyoshi, I. and Yanagihara, R. (1995): Sequence and phylogenetic analyses of human T-cell lymphotropic virus type I from a Brazilian woman with adult T cell leukemia: comparison with virus strains from South America and the Caribbean Basin. *Am. J. Trop. Med. Hyg.*, 52, 101-108.
 24. Yamashita, M., Ishida, T., Ohkura, S., Miura, T. and Hayami, M. (2001): Phylogenetic characterization of a new HTLV type I from the Ainu in Japan. *AIDS Res. Hum. Retroviruses*, 17, 783-787.
 25. Ureta-Vidal, A., Gessain, A., Yoshida, M., Mahieux, B., Nishioka, K., Tekaia, F., Rosen, L. and De Thé, G. (1994): Molecular epidemiology of HTLV type I in Japan: Evidence for two distinct ancestral lineages with a particular geographical distribution. *AIDS Res. Hum. Retroviruses*, 10, 1557-1566.
 26. Ishak, M. O. G., Vallinoto, A. C. R., Azevedo, V. N., Machado, L. F. A., Lobato, L. and Ishak, R. (2003): Molecular characterization of HTLV coinfecting HIV-1 patients. *AIDS Res. Hum. Retroviruses*, 19 (Suppl. 1), S-35.