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Prevalence and Phylogenetic Analysis of HTLV-I Isolates in Cameroon, Including Those of the Baka Pygmy

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Our previous analysis of an HTLV-I isolate (CMR229) from a Cameroonian Pygmy demonstrated that the isolate is distinct from typical HTLV-Is of the "Central African group," which has a close similarity to HTLV-I-related simian viruses (STLV-I) in Africa. In this study, we analyzed six new HTLV-Is from Cameroon consisting of three isolates from the Pygmy and three from the Bantu to examine further the genetic features of HTLV-I in Cameroon, especially in the Pygmy. A phylogenetic tree based on the long terminal repeats (LTR) region showed that all the new HTLV-Is belong to the Central African group. On the other hand, an *env*-based analysis of CMR229 confirmed the previous finding derived from LTR-based analysis that CMR229 has a similarity to African STLV-Is, but is distinct from the typical Central African group of HTLV-I. This suggests that multiple interspecies transmissions from non-human primates to humans have occurred in Central Africa, resulting in the presence of two distinct HTLV-I strains in this area. In addition, it seems likely that the Pygmy harbors the heterogeneous HTLV-I strains from which the main HTLV-I population spread into the Bantu.

Key words: HTLV-I — Phylogenetic analysis — Long terminal repeats — Pygmy — Central Africa

Human T-cell leukemia/lymphotropic virus type I (HTLV-I),[1] is the etiological agent of adult T-cell leukemia,[2] tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM),[3,4] and other diseases. The virus is recognized to be mainly endemic in southwestern Japan, the Caribbean basin, Africa, south America and the Melanesian islands.[5–11] Although HTLV-I is well conserved in its genome, extensive genetic analyses have demonstrated that molecular heterogeneity of HTLV-I is related to the geographic distribution and ethnic backgrounds of the virus-carriers rather than to the diverse manifestations of the disease.[12,13]

HTLV-I infections have been identified in Central African countries such as Zaire, Gabon and the Central African Republic.[14–18] Genetic analyses have shown that the majority of HTLV-I isolates of Central African countries are significantly different from those predominantly spread throughout the world (the Cosmopolitan group), as well as from divergent variants discovered in Australo-Melanesian areas (the Melanesian group).[12,13,19,20] Thus, they were collectively designated as "the Central African" group. Interestingly, the Central African group of HTLV-Is is closely related to simian T-cell leukemia/lymphotropic virus type I (STLV-I) from African non-human primates, especially that of the chimpanzee.[13,21–23] This fact led us to speculate on the possibility of interspecies transmission of this virus group from non-human primates to humans in Central Africa.

Recently, the presence of HTLV-I has been found in Pygmies in Central African countries such as Zaire, the Central African Republic, and Cameroon.[17,18,24,25] Since the Pygmy is considered to be one of the oldest ethnic populations in Africa, genetic analysis of HTLV-I from the Pygmy should help to clarify the origin of the virus in Central Africa. We previously reported the isolation and characterization of an HTLV-I isolate from a Pygmy in Cameroon. The isolate (CMR229) exhibited a close similarity to African STLV-I, as did the Central African HTLV-I group, yet it was clearly distinct from the latter.[26] In the present study, to better understand the phylogenetic features of HTLV-I from the Pygmy, we conducted a phylogenetic study of three HTLV-I strains from Pygmies, as well as three HTLV-I strains from Bantu, all newly isolated in Cameroon.

In 1995, we collected 346 blood samples in five provinces in Cameroon including 87 samples from the Baka Pygmy living in Yokadouma, a remote area of the equatorial forest of East Cameroon. The plasma samples were

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screened for anti-HTLV-I antibody with particle agglutination kits (Serodia HTLV-I, Fujirebio, Inc., Tokyo) and positivity was confirmed by western blotting (WB) (Problot HTLV-I, Fujirebio). Using the nested polymerase chain reaction (PCR),[27] we amplified and sequenced about 500-bp of the long terminal repeats (LTR) region which corresponds to positions 122–628 in ATK, a prototypic Japanese HTLV-I strain (GenBank accession number, J0209). The primer pairs used to amplify about 590-bp of the LTR region were as follows: ATLTR1 (5′-TGA CAC TGA CCA TGA GCC CCA AAT-3′)/ATLTR2 (3′-AAC CCC CGA GCA GGC CCT ATG CT-5′) as outer pairs and ATLTR11 (5′-ACT AAG GCT CTG ACG TCT CCC CC-3′)/ATLTR 12 (3′-GCC GAA CCGGGT GCC GGT TCA TGG C-5′) as inner pairs. For CMR229, the 522-bp *env* fragment (nucleotides 6068 to 6591 in ATK) including the carboxyl terminus of gp46 and almost the entire transmembrane protein gp21 was amplified with the outer primer pair HLpol1 (5′-CCC TAC AAT CCA ACC AGC TCA GG-3′)/SK44 (3′-GCT ACC TGC GCAATA GCC GAG-5′) and inner pair ATLenv1 (5′-ATT CAA GCT ATA GTC TCC TCCCCC TG-3′)/ATLenv2 (3′-GGA GGC AGT CGA TGC TGT GGA GGG-5′). For both LTR and *env* region, the first- and second-round PCRs were carried out in 50 μl of PCR mixture containing about 0.5 μg of chromosomal DNA, 50 m*M* KCl, 10 m*M* Tris (pH 8.3), 6 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 20 μg/ml bovine albumin, 200 ng of each primer, 0.2 m*M* of each dNTP and 2.5 U of Tth polymerase (Toyobo Co., Tokyo). The first-round PCR was performed for 35 cycles. One cycle consists of 1 min at 94°C (10 min for the initial step), 1 min at 55°C and 2 min at 72°C (5 min for the final step). The second-round PCR reaction mix-

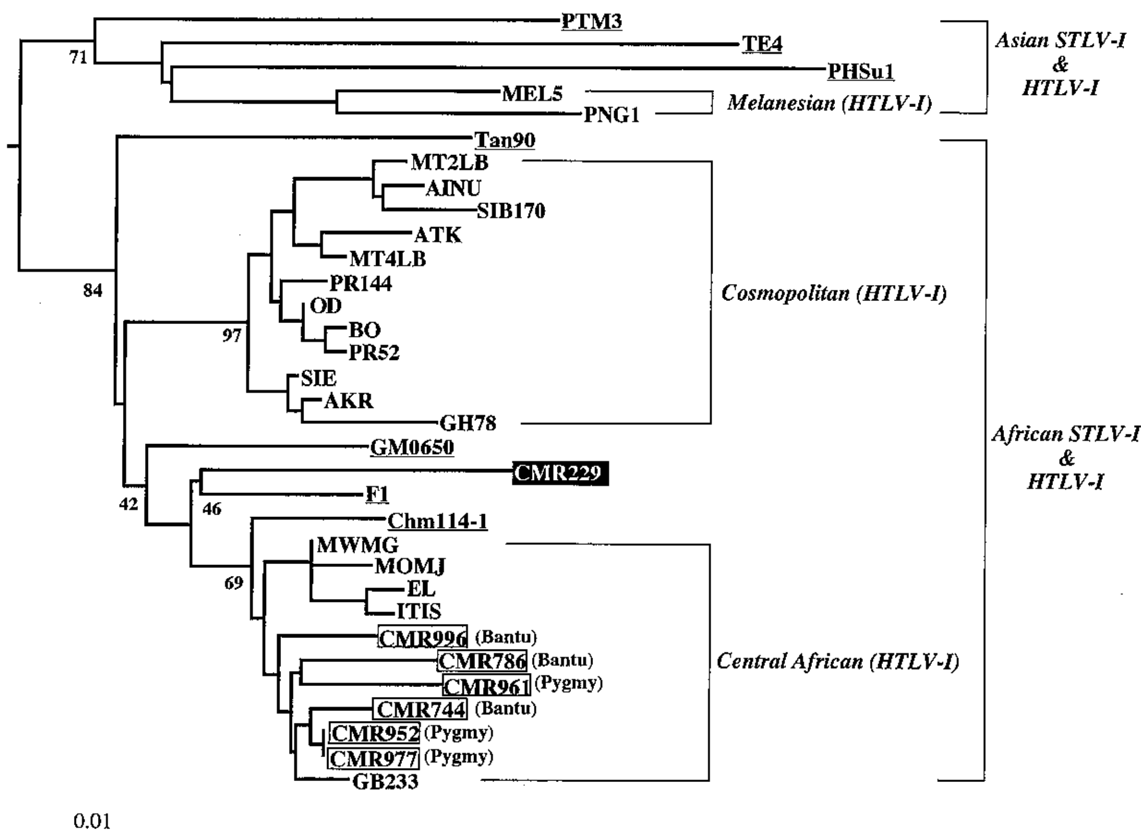

Fig. 1. A phylogenetic tree of HTLV-I/STLV-I based on partial LTR sequences. The tree was constructed by the neighbor-joining method, and statistically evaluated by bootstrap resamplings. Horizontal branch lengths are proportional to scale (the scale bar represents 0.01 nucleotide substitutions per site). Vertical branches are for clarity only. Values at nodes are the percentage of 100 bootstrap replicates. Six HTLV-I isolates newly sequenced in the present study are indicated in boxes, and an HTLV-I isolate of a Cameroonian Pygmy reported previously is shown in a black box. All the STLV-I isolates are indicated with underlines. The details for other DNA sequences have been described previously.[13, 20, 26, 35–38] A prototypic HTLV-II isolate, Mo (Acc. No. M10060), was used as the outgroup to root the tree.

tures (50 μl) contained 5 μl of the first-round PCR products and PCR was carried out under the same conditions as the first-round PCR except for the annealing temperature (at 60°C) and the number of cycles (25 cycles). The amplified DNA fragments were blunt-ended by Klenow enzyme and cloned into the *Sma* I site of pUC119. The nucleotide sequences were determined in both directions (sense and antisense) using a 373A DNA sequencer (Applied Biosystems, Foster City, CA). We sequenced one or two clones per sample. Although we found a few nucleotide substitutions among different clones of the same sample, such variations did not alter the topology of our phylogenetic trees significantly. Thus, we used one nucleotide sequence as representative of each sample for subsequent analysis. Each set of nucleotide sequences newly obtained and previously reported was aligned by using the computer program CLUSTAL W[28] with minor manual modifications. Pairwise genetic distances were estimated by Kimura's two-parameter method[29] and then phylogenetic trees were constructed by the neighbor-joining method.[30] The bootstrap estimation of phylogenetic variabilities using 100 replications was performed on the trees. These procedures were carried out with CLUSTAL W[28] and the trees were visualized by using TREEVIEW.[31]

Of 346 collected samples, seven including three from the Pygmy were judged as HTLV-I-positive by confirmative assay (WB). The seroprevalence was 2.0% (7/346) in total, 1.2% (3/259) for the Bantu and 3.4% (3/87) for the Baka Pygmy. Proviral DNAs were successfully amplified in six out of seven samples designated as CMR744, CMR786, CMR996 (from the Bantu) and CMR952, CMR961, CMR977 (from the Baka Pygmy).

The phylogenetic tree based on the LTR region indicated that all six of the sequenced HTLV-I isolates in the present study belonged to the Central African group, clustering with those from Gabon (GB233) and Zaire (MWMG, MOMJ, EL, ITIS) and differing from

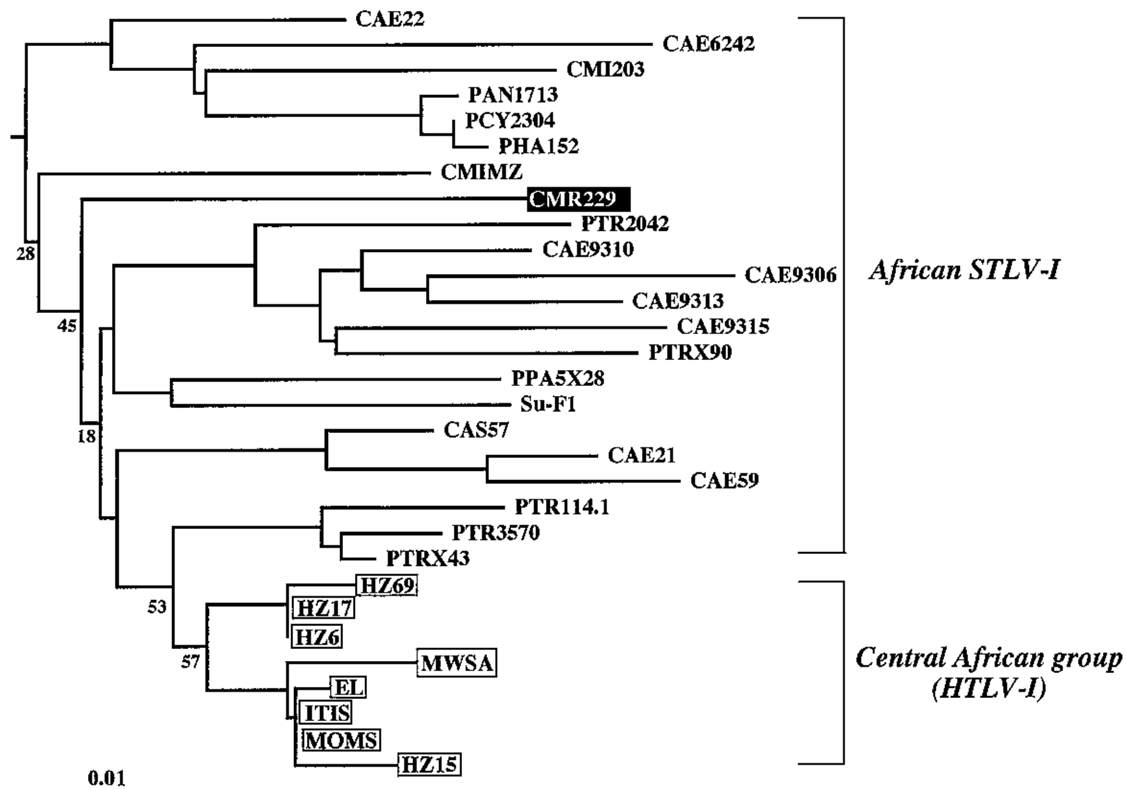

Fig. 2. A phylogenetic tree based on 522-bp of envelope sequences showing the evolutionary relationships of the Central African group of HTLV-Is and African STLV-Is. Typical isolates of the Central African group are shown in boxes, while an HTLV-I isolate from a Cameroonian Pygmy, CMR229, is shown in a black box. The others are all STLV-Is from African non-human primates. The tree was constructed as shown in Fig. 1. The details for other DNA sequences have been described previously.[23, 36, 38] A divergent isolate of HTLV-I, MEL5 (Acc No. L02534), was used as the outgroup to root the tree. All the sequences have been submitted to GenBank under the accession numbers U88718 through U88724.

CMR229 (Fig. 1). An important feature characterizing the Cameroonian HTLV-I was the nucleotide substitution of C to T at position 244 (except for CMR744), which was not present among the Zairian HTLV-I isolates (MWMG, MOMJ, EL, ITIS) or a Gabonese isolate (GB233). In contrast, the Zairian isolates possessed the same nucleotide substitution at position 571, whereas the Cameroonian isolates and Gabonese GB233 did not (data not shown). The nucleotide divergence in the partial LTR region between the HTLV-I isolates of the Baka Pygmy and the Bantu in Cameroon were 0.2 to 2.4%, whereas CMR229 diverged from them by 4.1% to 5.3% and was the most divergent strain among the African HTLV-Is, exhibiting 6.7% divergence from ATK.

As shown in Fig. 2, the *env* sequence of CMR229 did not belong to the Central African group of HTLV-Is, but was positioned within the cluster of African STLV-Is. Although CMR229 clustered with CMIMZ, which is an STLV-I isolate from the Mitis monkey in Zaire, the cluster was not strongly supported by the bootstrap estimation. In this partial *env* region, CMR229 exhibited the highest divergence from ATK (4.2%) among the African HTLV-Is. Taken together, the results based on the *env* sequence are identical to those based on the LTR sequences, confirming that CMR229 is unique among the various HTLV-I isolates.

The Pygmies have been living in Central Africa for more than 20,000 years, and are considered to be one of the oldest ethnic populations in the region. They are traditionally hunter-gatherers carrying out rudimentary agriculture and exchanging manufactured goods with neighboring farmers. It should be noted that another type of HTLV (HTLV-II) has recently been shown to be present in some Pygmy populations in the region.[18, 24, 25, 32, 33] Since the Pygmy is considered to be isolated from other ethnic populations, the HTLV group might have been present in some Pygmy populations for a long time, as has also been suggested by others.[25, 33] Also, the possibility that the virus was recently introduced into the Pygmy seems unlikely in view of the fact that another sexually transmissible retrovirus, HIV, has been found in the Bantu, but not in the Pygmy.[32] We observed higher HTLV-I seroprevalence among Baka Pygmy (3.4%) than other Pygmy groups in the region, such as the Bakola Pygmy in Cameroon (0.93%), the Bambuti Pygmy in Zaire (1.0%) and the Pygmy in the Central African Republic (0%).[18] The causes of the differences in seroprevalence in Pygmy populations of different localities remain to be determined.

From the phylogenetic analysis based on the LTR sequences, all the isolates sequenced in this study clustered with those of Central Africa. On the contrary, CMR229 was distinct from the Central African group and the most divergent isolate among the African HTLV-Is. Taken together, these results indicate that CMR229 is unique compared with other HTLV-I and STLV-I isolates from the African continent. Generally, it is believed that an interspecies transmission from African non-human primates to humans resulted in the emergence of the Central African group of HTLV-Is.[23, 34] Though we did not find any STLV-I isolate significantly similar to CMR229 in the present study, CMR229 clearly showed a closer relationship to African STLV-Is than to other HTLV-Is or Asian STLV-Is (Fig. 1), and both CMR229 and the Central African HTLV-Is were positioned within the cluster of African STLV-Is (Fig. 2). The distinctiveness of CMR229 from Central African HTLV-I isolates suggests multiple events of interspecies transmission between non-human primates and humans in Central Africa.

The origin of HTLV-I in the Bantu must be closely linked with that of the Pygmy as most HTLV-I isolates of the Pygmy were indistinguishable from those of the Bantu in Central Africa, as shown in this study. We speculate that the Pygmy have harbored heterogeneous HTLV-I strains from which the main HTLV-I population spread into the Bantu. To clarify this possibility as well as to better understand the history of HTLV, further analysis of the genomic sequences of HTLV-I isolates from other Pygmy populations is needed. These include HTLV-I isolates from the Efe Pygmy in Zaire[25] and the Pygmies in the Central African Republic,[17] for which little sequence information is available.

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