Journal of Infection (2006) 53, e167–e170

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CASE REPORT

AIDS-related cerebral toxoplasmosis with hyperintense foci on T1-weighted MR images: A case report

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Accepted 8 December 2005
Available online 26 January 2006

KEYWORDS
Toxoplasmosis;
MRI;
AIDS

Summary The neuroradiological findings are helpful for the diagnosis of toxoplasmic encephalitis. The T1 hypersignal intensity foci on brain magnetic resonance (MR) images without contrast enhancement are presented and can be a pathognomonic sign of this disease.
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Introduction

Most toxoplasmic encephalitis is opportunistic infection complicated with the acquired immunodeficiency syndrome (AIDS) and immunosuppressive conditions. The diagnosis of this disease is difficult because of the incompetence of the serological examination for the immunocompromised patients.[1] Although the direct detection method for the pathogen by polymerase chain reaction (PCR) using the cerebrospinal fluid (CSF) has high specificity, the sensitivity of this method is insufficient for definitive diagnosis.[2] We, therefore, have to synthetically diagnose with clinical symptoms, signs, laboratory data, neuroradiological images and the response to anti-toxoplasmosis therapy.

We report here our experience of a unique MR imaging finding of toxoplasmic encephalitis in an

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doi:10.1016/j.jinf.2005.12.006

AIDS patient and emphasize the hyperintense foci on T1-weighted MR images that can be one of the pathognomonic MR images of this disease.

Case report

A 44-year-old man with disturbance of consciousness and respiratory insufficiency was admitted to our hospital in April 2005. His consciousness had been rapidly deteriorated and he developed coma 2—3 days before hospitalization. Serological tests of HIV antibodies and *Toxoplasma gondii* IgG antibody were positive, but the *T. gondii* IgM antibody was not detected. The concentration of HIV RNA in plasma was 120,000 copies/ml and the CD 4 cell count was 8 μl. The chest X-ray showed bilateral ground glass shadow and *Pneumocystis jiroveci* (carinii) was detected from bronchoalveolar lavage (BAL) fluid. CSF showed mild elevated protein level of 65 mg/dl and pleocytosis, and the opening pressure was over 300 mmH_2O. No malignant cells or microorganisms were detected. *T. gondii* B1-gene fragment was detected by PCR using CSF, therefore, the diagnosis of an AIDS case with toxoplasmic encephalitis was made.[3]

MRI of the brain showed multiple high intensity lesions on T2-weighted image (Fig. 1a) and the corresponding T1-wighted image showed low intensity lesions. Contrast enhanced T1-weighted images showed multiple nodular and ring enhancement lesions.

The chemotherapy with trimethoprim/sulfamethoxazole (TMP/SMX) was very effective and the patient's consciousness level was improved gradually. *P. jiroveci* pneumonia was also cured. MR imaging after 4 weeks of treatment demonstrated that the multiple nodular lesions on T1 and T2-weighted images had significantly been reduced. After 8 weeks of treatment, the contrast enhanced T1-weighted images showed only residual small lesions without contrast enhancement. Interestingly, the hypersignal intensity foci appeared at bilateral basal ganglia obviously after 2 weeks of treatment on the non-enhanced T1-weighted images (Fig. 1b). Corresponding computed tomography (CT) image did not show hemorrhagic or calcified densities (Fig. 1c). These T1 hypersignal intensity foci regressed gradually along with anti-toxoplasmic chemotherapy in proportion to other mass lesions. The T2* (star)-weighted image, which can detect the hemosiderin deposition as hypointensity lesion, operated after 12 weeks of treatment showed no hypointensity at corresponding T1 hypersignal intensity foci on basal ganglia (Fig. 1d).[4] We concluded that the toxoplasmic encephalitis showed the hypersignal intensity foci on T1-weighted MR imaging without hemorrhage or calcification.

Discussion

Toxoplasmic encephalitis progresses rapidly and is life threatening to immunocompromised patients. Therefore, we often have to start the anti-toxoplasmosis therapy when this encephalitis is suspected on the neuroradiologic images and laboratory data. Typically, the toxoplasmic encephalitis lesions on MRI studies appear as T2 hypersignal intensity foci and T1 hypo-isosignal intensity foci, and reveal a rim of enhancement surrounding the edema on contrast enhanced T1-weighted images. Nevertheless, even characteristic foci on these MR images are not pathognomonic. Since the differential diagnosis of toxoplasmic encephalitis from other infections or CNS lymphoma is difficult, improvement in the diagnostic methods is an urgent necessity.

In our case, the toxoplasmic encephalitis was diagnosed with the highly specific PCR and confirmed by the response to anti-toxoplasmosis therapy. Brain MRI revealed unusual findings, T1 hypersignal intensity foci, accompanied by typical multiple high intense lesions on T2-weighted image during the treatment. These unique MR findings have been reported on only a few cases of non-HIV/AIDS-related toxoplasmic encephalitis. Terada et al.[5] reported a case of toxoplasmic encephalitis after stem cell transplantation with T1 hypersignal intensity foci. Autopsy revealed the disseminated toxoplasmosis, and coagulative necrosis without hemorrhage or calcification was revealed at corresponding T1 hypersignal intensity foci by neuropathological study. In another post-bone marrow transplantation case, inflammatory and vascular changes without hemorrhage appeared to be the cause of iso or hypersignal intensity rings by the stereotactic biopsy of T1 hypersignal intensity foci.[6] On the other hand, Navia et al.[7] demonstrated that the T1 hypersignal intensity foci were caused by coagulative necrosis with lipid-laden macrophages. The pathophysiological and neuroradiological mechanisms to create these MRI findings are far from clear yet. The reason why the T1 hypersignal intensity foci tend to localize in the basal ganglia is not clear either.[5,6]

CNS lymphoma, which is important for the distinction from toxoplasmic encephalitis, shows T1 hypo-isosignal intensity foci and never shows T1 hypersignal intensity foci except subacute

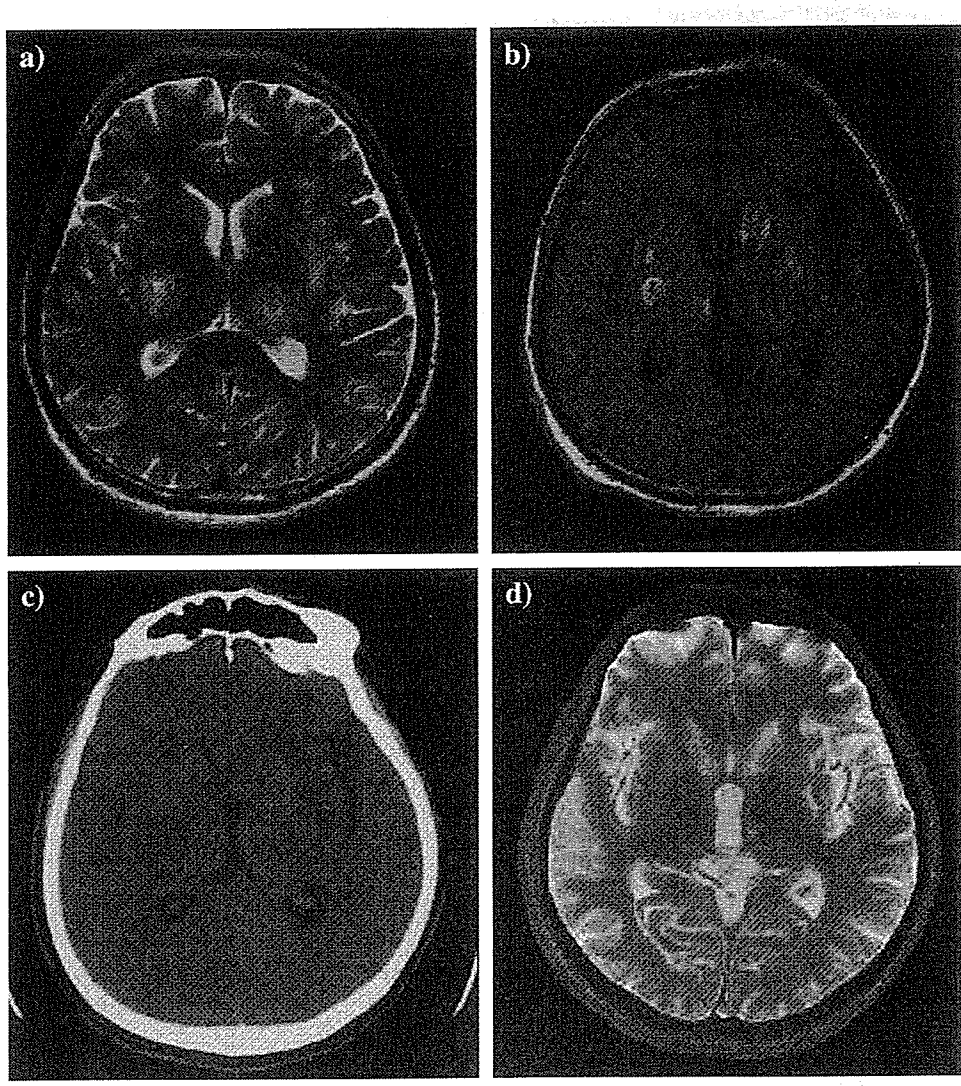

Figure 1 (a) The T2-weighted magnetic resonance image presented multiple high intense lesions. (b) Non-enhanced T1-weighted image showed hypersignal intensity foci at bilateral basal ganglia. (c) The corresponding CT image showed non-hemorrhagic or non-calcified density. (d) The T2* (star)-weighted image showed non-hemorrhagic observations at basal ganglia.

hemorrhage with hypervascular CNS lymphoma.[8,9] However, the CT imaging and T2* (star)-weighted MR imaging can simply distinguish it from the toxoplasmic T1 hypersignal intensity foci without hemorrhage or calcification.

We reported here the unique MRI findings, T1 hypersignal intensity foci, without hemorrhage or calcification on HIV/AIDS-related toxoplasmic encephalitis. It will be helpful for the diagnosis of toxoplasmic encephalitis and may be a pathognomonic finding. Unfortunately, since this report we are yet to experience another case of toxoplasmic encephalitis, but we would like to continue to explore this unique MRI findings of this disease.

Acknowledgement

This work was partly supported by the Special Coordination Funds for Promoting Science and Technology of MEXT: strategic cooperation to control emerging and reemerging infections and grants for AIDS research from the Ministry of Health, Labor and Welfare of Japan.

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The pro-apoptotic human BH3-only peptide harakiri is expressed in cryptococcus-infected perivascular macrophages in HIV-1 encephalitis patients

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Received 5 July 2005; received in revised form 1 September 2005; accepted 21 September 2005

Abstract

In the central nervous system (CNS), HIV-1 targets mainly microglia/macrophages. Like the CD4+ T cell depletion and neuronal loss in AIDS, apoptosis is thought to be involved in eliminating infected macrophages. In this study, we examined the expression of the pro-apoptotic BH3-peptide harakiri (Hrk) in brain tissues of AIDS patients. Immunoreactivity against Hrk was positive in perivascular macrophages infiltrated into some restricted lesions. Most of these immunopositive cells contained small inclusions positive for Grocott's methenamine silver staining. Confocal laser microscopy demonstrated that Hrk expression coincided with immunoreactivities against HIV-1 and *Cryptococcus neoformans*. Expression of Hrk mRNA was demonstrated in these cells by in situ hybridization, which indicated that Hrk is not phagocytosed material. Some pro-apoptotic bcl-family members, including Hrk, may contribute to the delayed hypersensitive reaction in AIDS, in macrophages eliminating opportunistic infection.
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Keywords: Apoptosis; Delayed hypersensitivity; *C. neoformans*; Hrk; HIVE

Central nervous system (CNS) involvement in HIV-1 infection is represented by the "AIDS dementia complex", which primarily affects subcortical white matter [13,12]. However, HIV-1 infection is restricted to capillary endothelium, macrophages, and multinucleated giant cells (MGC) in most cases, while astrocytes and neurons are infected only in severe cases [20]. The parenchymal cells (neurons and astrocytes) are probably spared in exchange for the infected perivascular macrophages. Like CD4+ T cell depletion and cortical neuronal loss in AIDS, apoptosis may play an important role in the elimination of infected macrophages. In fact, cell death was demonstrated in HIV-1 infected cultured microglia [19], and some TUNEL-positive macrophages were observed in HIV encephalitis (HIVE) samples [15]. However, this mechanism is not fully understood.

Bcl-2 is an anti-apoptotic peptide cloned from human lymphoma with a chromosomal translocation, t(14;18) [18]. Bcl-2 family members share some of the four homologous domains with bcl-2 (bcl-2 homology regions 1-4; BH1-4). Of these, the BH3 domain is closely linked to pro-apoptotic activity, and the BH3-only proteins Bik/Nbk, Bim, Bod, BLK, and Bnip3 have pro-apoptotic functions.

The bcl-2 family was also demonstrated in the macrophages in HIVE samples. Krajewski et al. reported the expression of Bax, a pro-apoptotic bcl-family peptide, in the microglia/macrophages in HIVE samples [10]. This expression was

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doi:10.1016/j.neulet.2005.09.052

Table 1
Clinical data of autopsied AIDS patients

Patient no.	Age, sex	*C. neoformans*	Additional infection
1	36, M	–	CMV retinitis
2	20, M	–	Toxoplasma meningitis
3	36, M	–	CMV pneumonia
4	37, F	–	Lung toxoplasmosis
5	32, M	–	Lung tuberculosis
6	66, M	–	Glioblastoma
7	69, F	–	PML
8	50, M	–	Lung toxoplasmosis
9	42, M	+	MAI pneumonia
10	30, M	+	PC pneumonia
11	49, M	+	PC pneumonia, CMV retinitis
12	30, M	+	Anal herpes
13	38, M	+	–
14	38, M	+	–
15	34, F	+	–
16	37, M	+	CMV ventriculitis
17	30, M	+	CMV retinitis
18	32, F	+	PC pneumonia, CMV ventriculitis, PML
19	54, M	+	–

CMV: cytomegalovirus MAI: *Mycobacterium avium-intracellulare*; PC: *Pneumocystis carinii*; PML: progressive multifocal leukoencephalopathy.

prominent in the perivascular macrophages, especially in the basal ganglia.

The human protein harakiri (Hrk) and its murine ortholog DP5 belong to this pro-apoptotic BH3-only protein group [8,7]. Both have the cell-death promoting function and heterodimerize with Bcl-2 or Bcl-XL in vitro [8,7]. The expression of Hrk has been demonstrated in lymphoid tissue, pancreas, kidney, liver, lung, and brain. DP5 was originally isolated from an in vitro apoptosis model of the sympathetic neuron [7], and it is expressed in the developing nervous system [9]. Considering these expression patterns, Hrk and DP5 are possibly involved in CNS pathologies. In fact, the upregulation of Hrk and its heterodimer formation with bcl-2 were demonstrated in the anterior horn cells of amyotrophic lateral sclerosis patients [17].

In this study, we examined the expression of Hrk, and discussed its possible contribution to the pathophysiology of HIVE.

The brains of six successive AIDS patients collected at the Institute of Medical Science of the University of Tokyo between 1992 and 1996 were studied. After the preliminary study, we added 13 patients with AIDS (seven patients with opportunistic *Cryptococcus neoformans* (*C. neoformans*) infection were included) from *the Brain Bank of Pitié-Salpêtriére Hospital*, who died between 1991 and 2001. The clinical diagnoses were confirmed by the clinical courses and hematological and virological or bacteriological investigations. The clinical information of the patients is summarized in Table 1. As control cases, we examined 15 patients (Table 2). Controls 1–3 died from systemic disorders and showed no remarkable CNS lesion. Controls 4–11 suffered from cerebral infarction and Nos. 12–15 from inflammatory CNS disorders. Unfortunately, there was no case of *C. neoformans* infection without AIDS in the databases of either institute. All samples were examined for the pathological diagnosis and neuroscience research with the consent of the patient's family.

Table 2
Control cases

Control no.	Age	Sex	Clinical diagnosis
1	28	F	Anorexia nervosa
2	19	M	Duchenne muscular dystrophy
3	76	M	Esophageal carcinoma
4	55	M	Cerebral infarction
5	61	M	Cerebral infarction
6	64	F	Cerebral infarction
7	66	F	Cerebral infarction
8	69	F	Cerebral infarction
9	73	M	Cerebral infarction
10	80	M	Cerebral infarction
11	83	F	Cerebral infarction
12	21	M	Chronic encephalitis
13	43	M	Neuro-Behçet disease
14	35	M	Multiple sclerosis
15	35	F	Multiple sclerosis

Formalin-fixed paraffin-embedded blocks were cut into 3 μm-thick sections for the standard staining methods (hematoxylin and eosin (H&E), Klüver-Barrera, and Grocott), immunohistochemistry, and in situ hybridization. Antisera against human Hrk was raised in immunized rabbits as described elsewhere [7,17]. Other commercial antibodies are summarized in Table 3.

For the immunohistochemistry of HIV core protein p24, deparaffinized samples underwent 15 min of microwave irradiation in citrate buffer (BioGenex, HK086-9K) for antigen retrieval and then were incubated with 0.01% protease type XXIV (Sigma, P8038) for 15 min.

All samples were incubated in 3% hydrogen peroxide in methanol to block endogenous peroxide activity. After incubation in 10% normal bovine serum for 30 min, primary antibody diluted in PBS were was applied for 14 h at 4 °C. After incubation with biotinylated secondary antibodies for 1 h at room temperature, immunoreactivity was made visible using the ABC system (Vector, PK6100) with diaminobenzidine tetrahydrochloride as the substrate.

To investigate the co-localization of Hrk or *C. neoformans* and other antigens (HLA-DR, HIV p24, CMV), we visualized

Table 3
Commercial antibodies

		Clone	Dilution
Mouse monoclonal antisera			
Anti-HIV p24	DAKO	Kal-1	1:50
Anti-cytomagalovirus	MONOSAN	BM204	1:50
Anti-*P. carinii*	DAKO	3F6	1:50
Anti-*Toxoplasma gondii*	NeoMarkers	RB-282-A	1:100
Anti-human HLA-DR	DAKO	CR3/43	1:100
		Lot no.	Dilution
Rabbit polyclonal antisera			
Anti-*C. neoformans*	DAKO	E0123	1:100

the immunoreactivity using laser confocal microscopy. Because the monoclonal antibody against *C. neoformans* (NeoMarkers, CSFi) did not react with our paraffin-embedded samples, we could not confirm the co-localization of *C. neoformans* with Hrk or other rabbit-derived antibodies. For double labeling, sections were incubated with anti-Hrk and another antibody diluted in TBS for 14 h at 4 °C. FITC-conjugated anti-rabbit immunoglobulin was used to visualize the immunoreactivity of Hrk, and Cy3-conjugated anti-mouse immunoglobulin to visualize the second immunoreactivity. Photographs were taken using a Leica TCS 4D system.

In situ hybridization using digoxigenin-labeled probes (sense and antisense) of human Hrk cDNA was described elsewhere [17]. cRNA probes were labeled by the alkaline phosphatase-conjugated anti-digoxigenin antibody, and color was developed with NBT and X-phosphate solutions.

To quantitatively check the local expression patterns of Hrk and its relationship with *C. neoformans* infection, we counted the Hrk-positive macrophages in six cases with *C. neoformans* infection. We randomly selected five vessels with Hrk-positive cells in each case for this purpose. Counting was done under a light microscope instead of a confocal laser microscope, because macrophages are easily identified as foamy cells with continuous cellular membrane. And in the case of counting the *C. neoformans*-positive cells, it was technically difficult to demonstrate the co-localization of two rabbit antibodies against *C. neoformans* and Hrk in single sections. *C. neoformans* positive cells almost always showed granular materials in their cytoplasm and negative macrophages did not contain such substances. We therefore, counted foamy cells with granular materials as *C. neoformans*-positive macrophages.

We first examined the expression of Hrk protein in six Japanese AIDS patients by immunohistochemistry. No Hrk-immunoreactivity was observed in the control cases (cerebral infarction, multiple sclerosis, and encephalitis), even in infiltrated foamy macrophages (data not shown). In contrast, three samples (Nos. 9, 10, 11) from HIVE patients showed strong reactivity against Hrk. Most of the cells exhibiting Hrk immunoreactivity were accumulated around the blood vessels (Fig. 1a). In the brain parenchyma, little immunoreactivity was

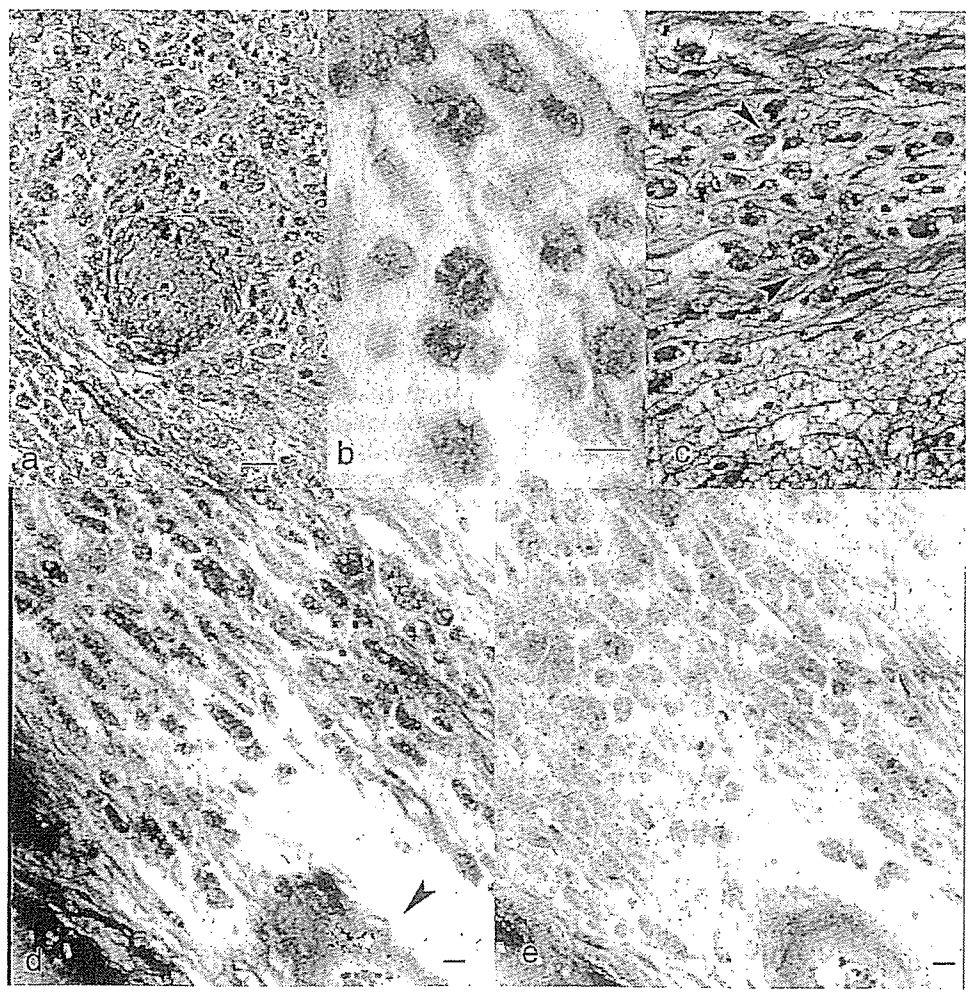

Fig. 1. Immunohistochemistry of Hrk in the basal ganglia of the AIDS patients. (patient 11): (a) immunoreactivity against Hrk was strong in the infiltrated cells around the vessels (bar = 40 μm); (b) these Hrk positive cells had foamy cytoplasm (bar = 10 μm); (c) H&E staining of the same sample. Hrk positive cells were large sized and have foamy cytoplasm. Many cells have small cytoplasmic inclusions (arrow heads) (bar = 20 μm) Hrk mRNA expression in the basal ganglia of the AIDS patients; (d) in situ hybridization of Hrk. Signal was strong in the foamy macrophages around the vessels (arrowhead) (bar = 10 μm); and (e) sense probe (bar = 10 μm).

detected in various other cell types. Morphologically, many of the Hrk-positive cells were relatively large with foamy cytoplasm (Fig. 1b), and some of them were multi-nucleated. Most of the cells contained many small cytoplasmic inclusions that were stained clearly with H&E (Fig. 1c).

We examined the Hrk mRNA expression by in situ hybridization. Consistent with the immunohistochemical analysis, the Hrk mRNA signal was positive in the foamy large perivascular cells of the cases positive for the protein expression (Fig. 1d and e).

These Hrk expressing cells were accumulated around blood vessels and contained some inclusions within the cytoplasm. It should be noted that the Hrk-positive cells were not always detected in perivascular spaces but rather were restricted to the area around only several vessels. They often contained inclusions, and this finding prompted us to test whether an immune response of macrophages against opportunistic infection is involved in Hrk induction. The multiple cytoplasmic inclusions were stained darkly by Grocott technique (Fig. 2a). When viewed using confocal laser scan microscopy, these cells contained immunoreactivity against *C. neoformans* and were positive for HLA-DR antigen (Fig. 2b). These findings indicated that Hrk immunoreactivity was positive in the perivascular macrophages that phagocytose *C. neoformans* in the HIV-1-infected CNS (Fig. 2c), but in other lesions, the expression was faint and scant even in cryptococcus-laden macrophages. This result was also consistent with the clinical information about complicating opportunistic infections. Granular immunoreactivity against HIV p24 was also confirmed, especially in the large foamy cells (Fig. 2d).

However, not all the HIV-positive macrophages were doubly positive for Hrk. We then counted the numbers of total macrophages, those with Hrk immunoreactivity, and those with C. neoformans in autopsied AIDS patients with cryptococcus infection (Fig. 2e). Hrk-positive macrophages almost always contained cryptococcus inclusions, but some cryptococcus-positive macrophages were devoid of Hrk immunoreactivity (Fig. 2e). Close relationship between Hrk expression and opportunistic crypotococcal infection was also implied by the fact that Hrk was virtually negative in the HIV cases without *C. neoformans* infection (data not shown). The proportion of Hrk-positive macrophages in cryptococcus-positive ones varied between vessels and patients (Fig. 2e), suggesting that cryptococcus infection may not directly regulate Hrk expression. In immunocompromised hosts, opportunistic infections of *Toxoplasma gondii*, Cytomegalovirus, *P. carinii*, and *C. neoformans* are frequent, but we did not confirm the common coincident infections of toxoplasma, *P. carinii*, or CMV by immunohistochemistry (data not shown). Some unknown factor(s), in addition to opportunistic infection, may influence Hrk expression in macrophages.

The presence of the Hrk immunoreactivity indicated that: (1) Hrk protein was synthesized in the infected macrophages or (2) the macrophages phagocytosed Hrk-positive cells (e.g., neurons). To test these possibilities, we examined Hrk mRNA expression by in situ hybridization. Consistent with the former hypothesis, the Hrk mRNA signal was positive in the perivascular macrophages of the cases positive for the protein expression. Again, although many macrophages were scattered throughout the brain parenchyma, Hrk mRNA expression was restricted to some perivascular lesions.

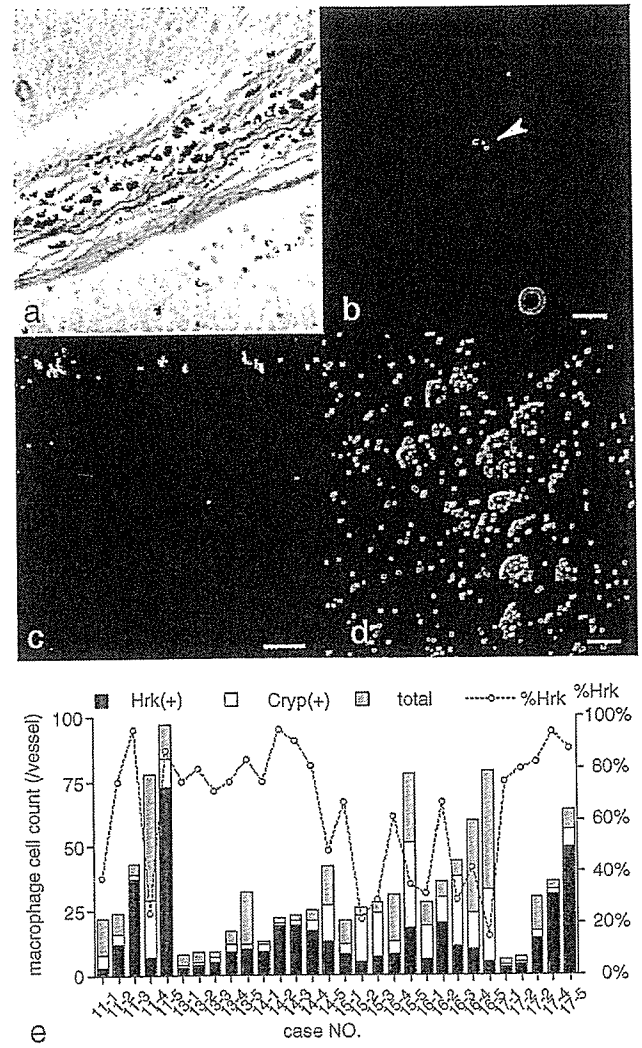

Fig. 2. Immunohistochemistry for infection of the Hrk expressing cells: (a) Grocott staining in the Hrk expressing perivascular cells. Most of the cytoplasmic inclusions were strongly stained with Groco (patient 11: bar = 20 μm); (b) immunoreactivity against *C. neoformans* and HLA-DR (CR3/43). Small circular immunoreactivity against *C. neoformans* (arrow head, Green fluorescence; FITC) was co-localized in the CR3/43 (Red fluorescence; Cy3) expressing macrophages (patient 19: overlay image, bar = 10 μm); (c) The immunoreactivity against Hrk (Green fluorescence; FITC) in a HLA-DR (Red fluorescence: Cy3) presenting cell (patient 12: overlay image, bar = 10 μm) The immunoreacticity agaisnt Hrk was diffusely distributed in the cytoplasm of HLA-DR expressing macrophages (overlay image: bar = 10 μm); (d) Co-localization of HIV p24 and Hrk in the perivascular cells. Small granular immunoreactivity against HIV p24 (red fluorescence; Cy3) was scattered in the Hrk positive (green fluorescence; FITC) perivascular cells (patient 9: overlay image: bar = 20 μm); and (e) the quantitative analysis of Hrk positive cells around cerebral vessels. (A total of six patients were subjected to the analysis. Five vessels were randomly picked up from a patient. Patient's ID numbers (11, 12, 13, 14, 16, 17) and vessel specimen numbers (1–5) are indicated). There is no apparent relationship between the numbers of total infiltrated macrophages (total; blue column) and the number of Hrk(+) macrophages (red column), even in the same patient. The proportion of Hrk expressing cells (Hrk(+)) in *C. neoformans*-positive cells (%Hrk = Hrk(+)/Cryp(+)) is variable in each vessels or patients.

Our results demonstrate that a pro-apoptotic BH3 protein, Hrk, is upregulated in the HIV-1 infected macrophages in HIVE.

These macrophages were distributed around the vessels, and Hrk mRNA expression was also demonstrated in macrophages with the same pattern of distribution. These results indicate that Hrk was not a phagocytosed substance but a product of the macrophages. In addition, these perivascular macrophages constituted a specific group that was co-infected with *C. neoformans* and HIV-1.

In the CNS, the perivascular space is continuous with the subarachnoid space and provides a site for antigen presentation from macrophages to lymphocytes. Perivascular macrophages are derived from bone marrow and have the same antigens as peripheral macrophages, and they easily induce class II MHC antigens [5]. In HIVE, MGCs, which are the morphological hallmark of HIV-1 infection, and HIV-1-infected perivascular macrophages were plentiful in perivascular spaces, especially in the basal ganglia and white matter [12]. Therefore, these results suggest that HIV-1-infected peripheral monocytes penetrate the small vessels and result in the neuroinvasion of HIV-1 that causes encephalitis.

Immune response in *C. neoformans* infection is classified as a delayed hypersensitivity. In the pathogenesis of delayed hypersensitivity, apoptosis also plays important roles. Macrophages infected with some microorganisms are known to be eliminated by apoptosis to resolve the inflammation. For example, apoptosis was demonstrated in cultured alveolar macrophages from AIDS patients with disseminated pulmonary tuberculosis [16] and cultured macrophages with *Mycobacterium avium* infection [3]. In the immune response in *C. neoformans* infection, CD4+ lymphocytes play a major role in activating infected macrophages to kill intracellular bacilli [2,3,5,7–10,15–18]. Experimental studies have demonstrated that macrophage activation is required to eliminate *C. neoformans* infection in murine macrophages [2,3,5,7,9,16,17], and experimental CD4+ depletion resulted in failure to clear *C. neoformans* in pulmonary infection of rats [6]. Within un-activated macrophages, *C. neoformans* were protected from other phagocytes and continued to replicate. After the activation, TUNEL-positive cells were also observed in the *C. neoformans*-infected macrophages in liver [11] and meningitis lesions [4] in rats.

Krajewski et al. reported that the expression of the pro-apoptotic member of the bcl-family, Bax, was prominent in the perivascular microglia/macrophages in HIVE samples [10]. According to their description, productive HIV-1 infection renders macrophages more vulnerable to apoptosis, which is consistent with the limitation of microglial proliferation and activation, and with the spread of productive viral infection in the CNS with HIVE [10]. In fact, Olsen reported that bcl-2 blocked influenza virus-induced apoptosis and reduced the level of infection, virus production, and spread of the virus [14].

In the immunosuppressive state of AIDS, one cannot expect the contribution of CD4+ T cells in macrophage activation. Macrophages, charging foreign bodies for elimination, express Fas, and CD4+ T cells expressing FasL induce apoptosis [1]. Our present study suggests that when macrophages phagocytose cryptococci but fail to eliminate them, Hrk might be involved in elimination of the HIV- and *C. neoformans*-infected macrophages. Further, Hrk expression may depend on the processing stage of macrophages. Considering that some viral proteins have anti-apoptotic effects, for example CMV produces immediate-early genes, IE-1 and -2, that inhibit apoptosis, multiple pro-apoptotic factors may be required to eliminate infected macrophages. Taken together, expression of the pro-apoptotic BH3 peptide Hrk may contribute to the immune reaction against *C. neoformans* infection under the immunosuppressive state of HIV-1 infection.

Acknowledgement

We are grateful to Mrs. Nobuko Naoi, Mrs. Azusa Uchinokura and Mr. Yoshio Sato for providing outstanding technical assistance.

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Journal of Medical Virology 78:1423–1428 (2006)

A Natural Inter-Genotypic (2b/1b) Recombinant of Hepatitis C Virus in the Philippines

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The prevalence study and the characterization of hepatitis C virus (HCV) was carried out in the Philippines and the sequence determination of the 5′-untranslated region (5′-UTR)-Core and the NS5B regions of HCV was carried out in this study. An HCV strain (SE-03-07-1689) collected in Metro Manila, Philippines, belonged to discordant subtypes, 2b and 1b in 5′-UTR-Core and NS5B regions, respectively. The 9.3 kb sequence of this strain including the entire open reading frame was compared with those of the reference strains retrieved from the HCV sequences database (GenBank/EMBL/DDBJ) and indicated a recombination event. The computation of the sequence similarity mapped a crossover point within the NS3 region. This is the second report on the inter-genotype recombinant of HCV and the third when an intra-genotype recombinant is included. This recombinant strain, SE-03-07-1689, is designated tentatively as RF3_2b/1b according to the suggestions used for the other two HCV recombinants. ***J. Med. Virol. 78: 1423–1428, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: HCV; recombination; subtype-2b; subtype-1b; NS3 protein; Philippines

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease worldwide and a potential cause of substantial morbidity and mortality in the future. The prevalence of HCV infection is estimated to be 2%, representing 123 million people in the world [Shepard et al., 2005].

Hepatitis C virus is characterized by a high degree of genetic heterogeneity like human immunodeficiency virus (HIV) [Zein, 2000]. Although it is well known that recombinant forms of HIV-1 have been prevalent in the world [Peeters, 2001], there have been few reports on recombination of HCV between different genotypes/subtypes, suggesting that these events are rare in vivo and that the HCV recombinant form is not viable usually [Simmonds et al., 1994; Viazov et al., 2000]. However, there was a report on the genotype discrepancy between the Core and the NS5B regions although the recombination crossover point was not clear [Yun et al., 1996]. Besides, the evidences of super-infection have been demonstrated among human population [Kao et al., 1996; Herring et al., 2004] and experimental animals [Okamoto et al., 1994], suggesting the possible occurrence of recombination. In fact, the natural inter- and intra-genotypic recombinants of HCV (RF1_2k/1b and RF2_1a/1b) were found recently and the crossover points were clearly indicated in the NS2 and NS5B regions, respectively [Kalinina et al., 2002; Colina et al., 2004]. HCV recombination has been reported only in these cases to date [Simmonds et al., 2005].

Several studies showed that the positive rate for anti-HCV was 2.2% among blood donors (Metro Davao, 1990) [Arguillas et al., 1991], and 4.6% among inmates (Metro Manila) [Katayama et al., 1996] in the Philippines. Recently, it was shown in a study (Metro Cebu, 2002) that the HCV prevalence was extremely high among injecting drug users (70%), especially among those except the trainees in the rehabilitation centers

Grant sponsor: Japan Society for the Promotion of Science; Grant numbers: 16406014, DOST-10417.

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Accepted 13 June 2006
DOI 10.1002/jmv.20714
Published online in Wiley InterScience (www.interscience.wiley.com)

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(100%), as compared to other populations, such as sex workers, antenatal clinic patients, students, and health care workers (0–2%) [Agdamag et al., 2005]. Most common HCV genotypes were 1a (55%) followed by 1b (27%), 2a (6%), 2b (3%) in Metro Manila (n = 33) [Katayama et al., 1996] and were 1a (65%) followed by 2b (35%) in Metro Cebu (n = 23) [Agdamag et al., 2005].

In this study, a natural inter-genotypic recombinant was identified by the determination of the crossover point in the 9.3 kb sequence of a Philippine strain of HCV.

MATERIALS AND METHODS

Patients

Hepatitis C virus genotyping has been conducted at the National Reference Laboratory-STD AIDS Cooperative Central laboratory, San Lazaro Hospital (Metro Manila, Philippines). Samples and/or patients were referred to this laboratory for serologic, viral load testing and for genotyping in some limited cases. The researchers explained the objectives and the procedures of the study in the local language, and confirmed the patient's intent to join the study by his/her signing an informed consent form for the referred patients. The test was done anonymously under an unlinked procedure for the referred samples.

Only seven samples from Metro Manila were available for the genotype test on NS5B and 5′-untranslated region (5′-UTR)-Core regions, and the possible recombination was analyzed on these samples. Risk factors and the travel history were not documented in the sample profile sheet. Nucleotide sequences of the strains found at Metro Cebu were from the database of STD AIDS Cooperative Central laboratory [Agdamag et al., 2005].

RNA Extraction, cDNA Synthesis and Amplification

HCV-RNA was extracted from 100 μl of plasma using SMITEST EX-R&D (Genome Science Laboratories, Fukushima, Japan), and reverse-transcribed with random primers according to First-Strand cDNA Synthesis protocol (Invitrogen, Carlsbad, CA).

cDNAs were amplified by nested PCR with the primers hep31b/hep32 and hep33b/hep34b in the first and second rounds, respectively, for the phylogenetic analysis of NS5B as reported before [White et al., 2000]. The nested PCR for the region of 5′-UTR-Core was done with the primers of KY80/C0751R (5′-ATGTACCCCAT-GAGGTCGGC-3′) and hep21b/C0727R (5′-CCACACG-TAATGGTATCGATGAC-3′) [White et al., 2000]. These amplifications were performed with 20 μl reaction mixture containing 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM primers, and 1 unit of Amplitaq Gold® (Applied Biosystems, Foster City, CA). The thermal profile of the first-round PCR included 1 cycle of 94°C for 10 min; 40 cycles of 94°C for 30 sec, 55°C for NS5B, and 50°C for 5′-UTR-Core for 30 sec, and 72°C for 1 min per kb; and a final extension of 72°C for 10 min, respectively. The profile of the second-round PCR was done in the same condition except for the annealing temperature at 60°C for NS5B and 50°C for 5′-UTR-Core, respectively. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel.

Similarly, a putative recombinant HCV-RNA was reverse-transcribed with random primer (Invitrogen) and the cDNAs were amplified with several gene specific primers for the 11 regions (Fig. 1).

Determination of Nucleotide Sequences

A PCR product was subjected to the determination of nucleotide sequence directly with the primers of hep33b/hep34b for NS5B, and hep21b/C0727R for 5′-UTR-Core, respectively, for the phylogenetic analysis of NS5B and 5′-UTR-Core regions. The sequences were aligned with those retrieved from the HCV sequences database (GenBank/EMBL/DDBJ) through ClustalW after the subsequent inspection and manual modification [Thompson et al., 1994]. The frequency of nucleotide substitution in each base of the sequences was estimated by the Kimura two-parameter method. A phylogenetic tree was constructed by the neighbor-joining method,

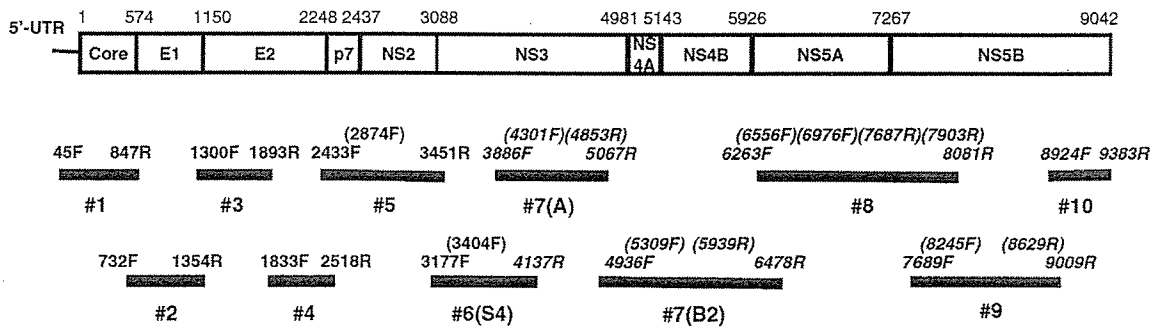

Fig. 1. Strategy for the sequence determination of an hepatitis C virus (HCV) strain, SE-03-07-1689. A genetic organization of HCV is indicated with blocks (coding regions) and a flanking bar (5′-untranslated region, 5′-UTR). The numbers on the block denote the starting positions of the coding regions except the last one (the last nucleotide position), where the position (+1) corresponds to the 5′-end of Core region. The bar under the block defines the complementary DNA that was subjected to polymerase chain reaction (PCR), cloning into a vector, and a sequence determination. A primer ID was named after the 5′-end position of the nucleotide sequences of the reference strains (accession numbers; D10988 (genotype-2b) and D90208 (genotype-1b) (italic)) used for the primer design. The forward and reverse directions are indicated with F and R, respectively. Primers used for the sequence determination are shown in the parentheses.

and its reliability was estimated by 1,000 bootstrap replications. The profile of the tree was visualized with the program of Njplot [Perriere and Gouy, 1996].

Several PCR products were cloned into pCR2.1®-TOPO® vector in TOPO TA Cloning® kit (Invitrogen) and the nucleotide sequences were determined with the M13 primers in the kit (Invitrogen), the primers used for PCR, and newly synthesized ones to determine the sequence of a possible recombinant HCV strain (Fig. 1). Three plasmids were subjected to the nucleotide sequence determination per each fragment of total 11 parts (Fig. 1), and the representative sequence was considered to be 1 of the 11 tandem parts of the recombinant HCV sequence (9,315 bp; 3,014 amino acids including the entire open reading frame). The reliability of the determined nucleotide sequences was assessed by the 'Quality Value' in ABI PRIZM® Sequence Analysis Software (Applied Biosystems).

Recombination Analysis

The sequence of the recombinant candidate, SE-03-07-1689, was aligned with reference sequences obtained from HCV sequence database, M62321_1a, AB049087_1b, AB191333_1b, AF169004_2a, AY232747_2b, and AF238486_2b (named after the accession numbers and subtypes), through ClustalW after the subsequent inspection and manual modification [Thompson et al., 1994]. The Single-Sequence Viewer of Recombination Analysis Tool (RAT) was used to examine recombination crossover point according to the user's guide available in the RAT home page (http://cbr.jic.ac.uk/dicks/software/RAT/index.html) [Etherington et al., 2005].

Nucleotide Sequence Accession Number

The sequences described in this article have been deposited in GenBank/EMBL/DDBJ under accession numbers of DQ364460 for SE-03-07-1689, DQ648505 to DQ648517 for 13 NS5B sequences (Fig. 2A), and DQ648495 to DQ648504 for 10 5′-UTR-Core sequences (Fig. 2B).

RESULTS

Phylogenetic Analysis of the NS5B and 5′-UTR-Core Regions

The NS5B and 5′-UTR-Core regions of seven strains collected in Metro Manila were analyzed (Fig. 2). Those seven strains were classified into subtype-1b (n = 4) and -2a (n = 3) based on NS5B (Fig. 2A), and subtype-1b (n = 3), -2a (n = 3), and -2b (n = 1) on 5′-UTR-Core (Fig. 2B). One strain (SE-03-07-1689) belonged to the discordant subtypes, 2b (5′-UTR-Core, Fig. 2B) and 1b (NS5B, Fig. 2A).

Identification of the Recombination Crossover Point

The sequence of SE-03-07-1689 from 5′-UTR-Core to the 5′ part of NS3 was similar to that of the subtype-2b reference strains (Fig. 3). However, the 3′-part sequence of this region was no more belonging to subtype-2b and was rather similar to the sequence of subtype-1b. The computation of similarity was performed to identify the recombination crossover point with RAT application based on the 9.4 kb sequence (1–9394, start–end; 939, window size; 469, increment size), and gave a crossover point within NS3 region. The shorter sequences within the single insert, #6(S4) (Fig. 1), were compared with the corresponding ones of reference strains through RAT to analyze more accurately the crossover point. The window size and the target sequence length in this analysis were decreased from 939 to 40 and from 9394 to 400, respectively. The crossover point was then located in the position of 3399/3400, corresponding to 3466/3467 of the pj6CF strain [Yanagi et al., 1999] (Fig. 3, inset; Fig. 4).

DISCUSSION

An HCV strain (SE-03-07-1689) belonging to the discordant subtypes, 2b and 1b, based on the sequences of 5′-UTR-Core and NS5B regions, respectively was examined. Subsequent analysis of the genome sequence coding for the entire open reading frame identified the recombination crossover point within NS3 region and demonstrated the existence of natural inter-genotypic HCV recombinant strain (2b/1b, SE-03-07-1689) in the Philippine population. The parental strains of SE-03-07-1689 might exist in the Philippines because the phylogenetic analyses (Fig. 2) suggested that some HCV strains in the Philippines belonged to the same cluster with SE-03-07-1689. However, it is important to perform more intensive investigation on the prevalence of recombinant HCV in Metro Manila and other areas of the Philippines.

The identification of the recombinant crossover point provided the final proof for the existence of a natural recombinant strain [Kalinina et al., 2002]. The recombinant crossover point was located in the NS3 region of the SE-03-07-1689 strain through RAT analysis, and was apparently mapped at 3399/3400 corresponding to 3466/3467 of pj6CF strain [Yanagi et al., 1999] between Glycine and Leucine residues in this study. However, only one strain (SE-03-07-1689) was used for the determination of recombination crossover point. The flanking sequences of the point derived from other recombinants and possible parental strains circulating in Metro Manila are needed to identify a more accurate crossover point of the SE-03-07-1689 strain.

The recombination point had been found often in the genome coding the first non-structural protein, for example, NS2 of HCV recombinants [Kalinina et al., 2002] and NS1 of Dengue viruses, also members of *Flaviviriae* [Tolou et al., 2001]. However, analysis of the crossover junction of the SE-03-07-1689 was in the NS3 next to NS2 and this is discordant with previous findings. The recombination crossover point was NS5B in the intra-genotype recombination case found in Peru, not in the genome coding the first non-

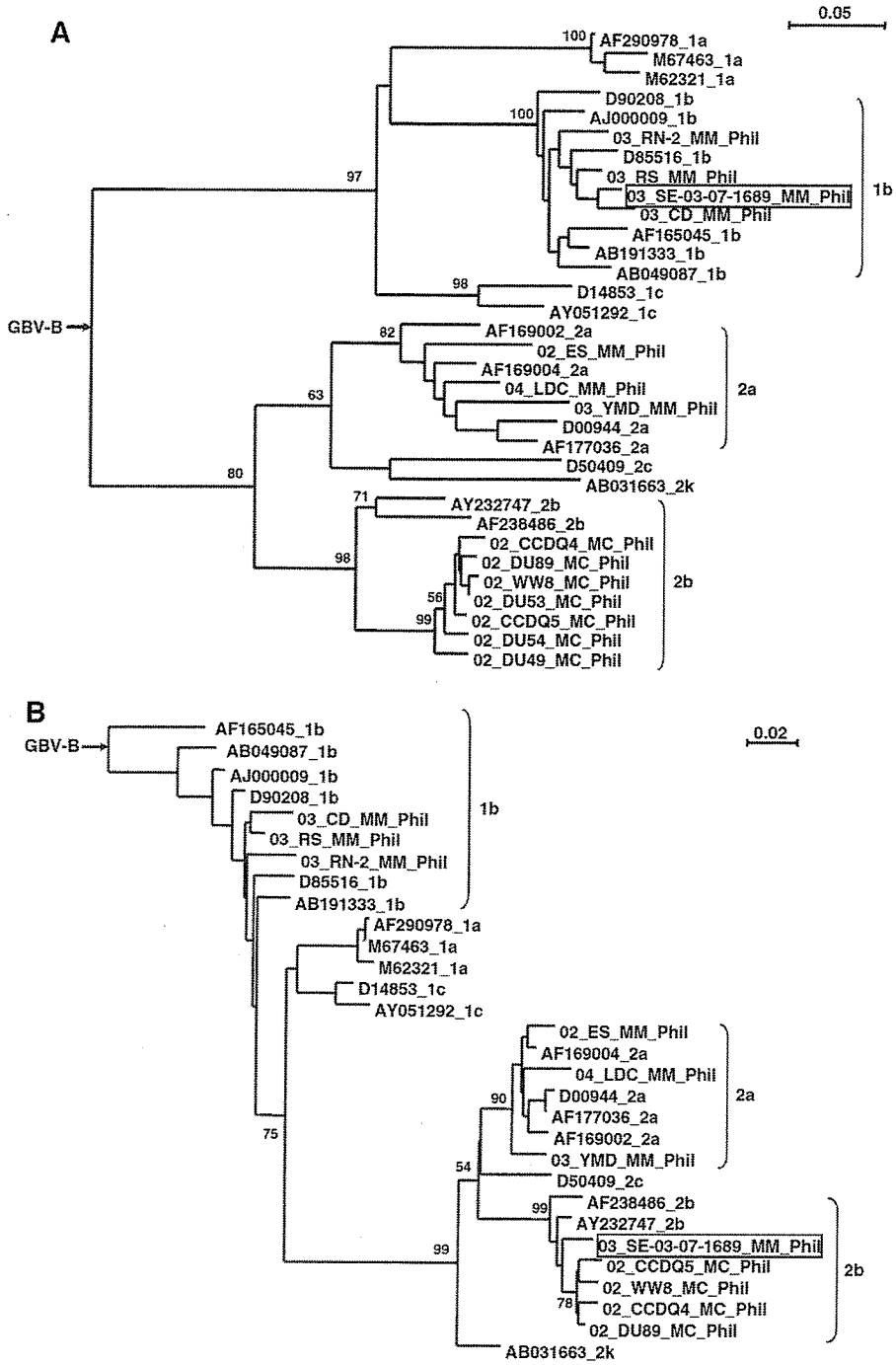

Fig. 2. Phylogenetic analysis of the HCV strains on 222 and 538 nucleotides within the NS5B (**A**) and 5′-UTR-Core (**B**) regions. The tree was constructed by the neighbor-joining method using ClustalW program with GBV-B (accession number; NC001655) as the outgroup. Strains including the SE-03-07-1689 (boxed) are indicated with two digits of the collecting year and the location, such as MM Phil and MC Phil, denoting the Metro Manila and Metro Cebu, Philippines (e.g., 03_CD_MM_Phil and 02_CCDQ4_MC_Phil). Accession numbers are used for the reference strains with two digits indicating genotypes at the end of the number. Bootstrap values (>70%) are given on blanches as percentages from 1,000 replicates.

structural protein [Colina et al., 2004]. Further demonstrations and comparisons must be required to elucidate the regularity principle of an HCV recombination. It is expected that analysis of the recombination cross-over points would be accelerated using the cell culture system developed recently [Heller et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005].

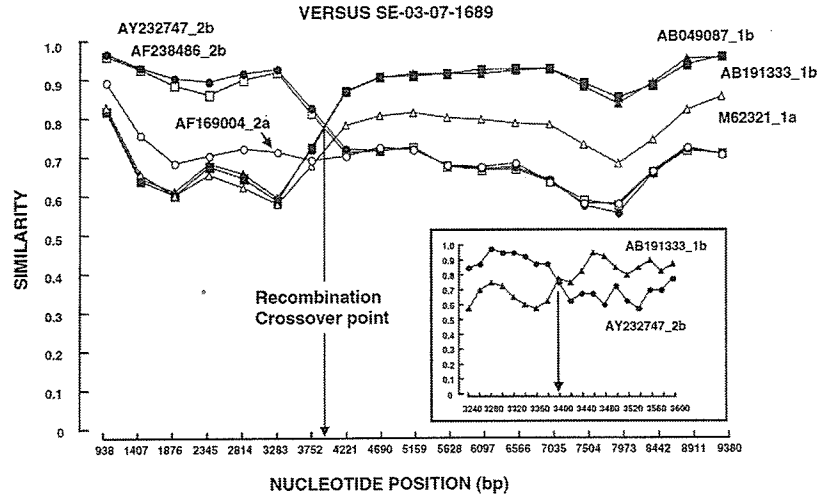

Fig. 3. Similarity plots for a putative recombinant strain, SE-03-07-1689, and reference strains based on the 9.4 kb sequences and a #6(S4) fragment (inset). The plot was performed with the Recombination Analysis Tool (RAT). Four reference sequences of HCV genotype-1b (AB049087 and AB191333) and -2b (AY232747 and AF238486) with the highest sequence scores (through the BLAST program) on the similarity to the sequence of SE-03-07-1689 were selected from GenBank/EMBL/DDBJ sequence database. Two sequences of M62321 (genotype-1a) and AF169004 (genotype-2a) were also selected from 'Ready made alignments' of the Los Alamos HCV sequences database. The recombination analysis was performed throughout 9,384 bases with a window size of 938 and a step increment of 469, and also 400 bases in the #6(S4) fragment with a window size of 40 and an increment of 20 (inset), respectively. An arrow indicates the crossover point between the nucleotide positions of 3399 and 3400 (inset).

The genotype of HCV influences the outcome of interferon treatment [Zein, 2000] and interferon sensitivity has been attributed to the sequence variability of NS5A and E2 [Gale et al., 1997, 1998; Taylor et al., 1999, 2005; Polyak et al., 2001; Pavio et al., 2002]. The recombination events at the NS2 region in St. Petersburg [Kalinina et al., 2002] and NS3 in Metro Manila implies that the genotype determination through the analysis of one subgenomic region, such as NS5B, may not be sufficient to assess the anti-HCV activity of interferon. It might be necessary to analyze the responsible nucleotide sequences in E2 and NS5A regions themselves for the assessment of antiviral activity. This assessment procedure beyond the simple genotyping would become essential when the frequency of HCV recombination proves to be a common event.

Hepatitis C virus strains are divided into genotypes, subtypes, and quasispecies in the current HCV classification system and the recent consensus proposals [Zein, 2000; Simmonds et al., 2005]. However, recombination has not been considered in this classification. Therefore, this recombinant strain found in the Metro Manila of the Philippines, SE-03-07-1689, is designated tentatively as RF3_2b/1b according to the suggestions

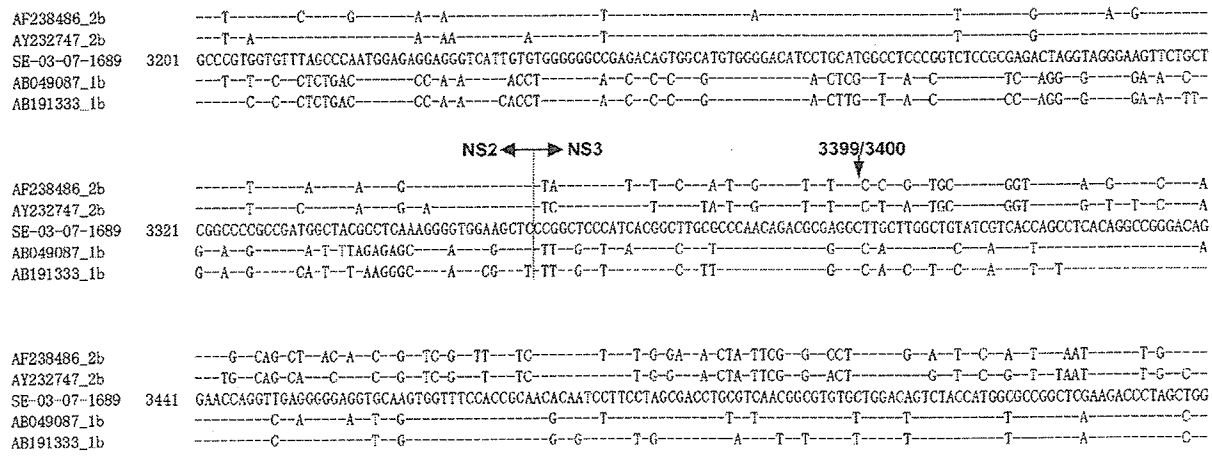

Fig. 4. Alignment of the nucleotide sequences of SE-03-07-1689 and four reference sequences: AF238486, AY232747, AB049087, and AB191333 within #6(S4) fragment. Arrow indicates the possible crossover point (3399/3400). The borderline between NS2 and NS3 was determined on Los Alamos HCV sequences database.

used for the other two strains (RF1_2k/1b and RF2_1a/1b) already reported as the HCV recombinants [Kalinina et al., 2002; Colina et al., 2004]. The existence of an HCV recombinant in the Philippines in addition to recent findings in St. Petersburg and Lima implies that HCV recombination may have an important implication for the pathogenesis, diagnosis, and treatment of HCV infection. Further prevalence studies of HCV recombinants may have an important role for considering the clinical impact of HCV recombination.

ACKNOWLEDGMENTS

Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research, 16406014 and Ronpaku Program, DOST-10417) supported this work.

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Emergence of Antiretroviral Therapy Resistance–Associated Primary Mutations Among Drug-Naive HIV-1–Infected Individuals in Rural Western Cameroon

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Summary: The prevalence of antiretroviral therapy (ART) resistance–associated mutations among HIV-1 strains in western Cameroon was evaluated by genotypically analyzing strains isolated from drug-naive individuals. Proviral DNA was extracted from 54 blood samples and amplified by polymerase chain reaction of protease, reverse transcriptase, integrase, and envelope genes. At least 4 clones per sample were analyzed. Of 54 HIV-1 strains, 45 (83.3%) had a concordant subtype or circulating recombinant form (CRF) designation: 40 CRF02_AG, 2 subtype A1, 2 G, and 1 F2. The remaining 9 (16.7%) had a discordant subtype: 6 subtype A1/CRF02_AG, 2 D/CRF02, and 1 G/CRF02. Protease inhibitor–associated primary resistance mutations were found in 4 (7.4%) cases: *M46L* with full clones in 1 case, and *M46I*, *M46L*, and *V82A* as minor populations in 1 case each. Reverse transcriptase inhibitor–associated primary resistance mutations were found in 5 (9.8%) samples: *Y188C* in 2 cases, and *L100I*, *M184V*, and *V75I* in 1 case each, although all of these mutations were found as minor populations. This is one of the first reports of the emergence of primary ART resistance mutations among drug-naive, non-B subtype HIV-1–infected individuals in Cameroon. Follow-up studies should be conducted to assess whether these drug-resistant mutants found as minor populations might impact future ART.

Key Words: non-B subtype HIV-1, antiretroviral therapy, drug resistance–associated mutations, drug-naive patients, Cameroon

(*J Acquir Immune Defic Syndr* 2006;43:15–22)

Received for publication January 9, 2006; accepted April 24, 2006.

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Supported in part by International Scientific Research Program grant 14256005 from Monbu-kagakusho (Ministry of Education and Science).

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The current mainstream antiretroviral therapy (ART) in developed countries is a combination of reverse transcriptase inhibitors (RTIs) and protease inhibitors (PIs). Since its establishment in the late 1990s, ART has benefited many HIV-1–infected patients[1,2] and has recently become prevalent even in developing countries. On the other hand, the emergence of mutants resistant to these antiviral agents has become a serious concern. In countries with sufficient treatment modalities, the prevalence of drug-resistant variants has ranged from 10% to 20% among drug-naive patients,[3–10] whereas in developing countries, resistance has rarely been reported. Recently, the World Health Organization's (WHO) "3 by 5" policy has promoted ART coverage in low- and middle-income countries. As of June 2005, about 500,000 people were receiving ART in sub-Saharan Africa, although the regional coverage rate was still only 11% of the estimated need. In countries with rapidly scaled-up ART provision such as Kenya, Uganda, and Cameroon, the threat of resistance now exists (3 by 5 progress report, June 2005, WHO/UNAIDS). In Kenya, for example, where ART has been provided for 12% to 17% of the estimated need, the prevalence of resistant strains among drug-naive patients has recently risen from 1% (2002) to 11% (2003) (personal communication). In Botswana, the prevalence of primary mutations against PIs was found to be 4% among drug-naive patients.[11]

In Cameroon, where all representative major groups and subtypes of HIV-1 cocirculate,[12–18] there have as yet been no reports suggesting the emergence of ART resistance–associated primary mutations among drug-naive, HIV-1–infected patients. According to WHO/UNAIDS, as of October 2004, the prevalence of HIV-1 infection in Cameroon was estimated to be 4.8% to 9.8% in adults; the estimated number of people needing treatment was about 95,000; and 12,896 people were reported to have received ART. With a firm political leadership, Cameroon set a national target of providing ART based on WHO guidelines [i.e., a combination of 2 nucleoside reverse transcriptase inhibitors (NRTIs) and 1 nonnucleoside reverse transcriptase inhibitor (NNRTI)] to 36,000 people by the end of 2005, which means that the ART-treated population would nearly triple in 1 year (Summary country profile for HIV/AIDS treatment scale-up, WHO/UNAIDS). With such a rapid introduction of ART, this and other developing countries might suffer a phase of drug resistance in the near future.

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TABLE 1. Overview of the Genetic Data for HIV-1–Infected Subjects Studied in Western Cameroon

			Genetic Subtype*				Drug Resistance–Associated Mutations			
Sample ID	Age (y)	Sex	*Pol-PR*	*Pol-RT*	*Pol-IN*	*Env(C2V3)*	1° *PR*	2° *PR*	1° *RT*	2° *RT*
04CM001	24	F	02(G)	02(G)	02(G)		—	*L10V*	—	—
04CM002	23	F	02(G)	02(G)	02(G)	02(A1)	—	—	*(M184V)*§	—
04CM003	24	M	02(G)	02(G)		02(A1)	—	—	—	—
04CM004	32	M	02(G)	02(G)	02(G)		—	—	—	—
04CM005	26	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM006	35	M	02(G)	02(G)	02(G)	02(A1)	—	*L10V*	—	—
04CM007*	44	F	A1	A1	02(G)	A1	—	—	—	—
04CM008	30	F	02(G)	A1	A1	02(A1)	—	—	—	—
04CM010	46	M	02(G)	02(G)	02(G)		—	—	—	—
04CM011	32	M	02(G)	02(G)	02(G)		—	—	—	—
04CM012	28	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM015	27	F	02(G)	02(G)	A1	02(A1)	—	—	—	—
04CM016*	29	F	A1	A1	A1	A1	*M46L*	—	—	—
04CM022	30	F	02(G)	02(G)	02(G)		—	—	—	—
04CM024	28	M	G	G		G	*V82I*	*L10I*	—	—
04CM025	30	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM026	32	F	02(G)	02(G)			*V82I*	*V77I*	—	—
04CM029	20	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM030	30	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM032	28	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM033	25	F	02(G)	02(G)	02(G)		—	—	—	—
04CM034	53	M	02(G)	02(G)	02(G)		—	—	—	—
04CM038	42	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM039	28	F	02(G)		02(G)	02(A1)	—	—	ND	ND
04CM040	26	F	F2	F2	F2	F2	*(V82A)*‖	*L10V*	—	—
04CM042	40	F	02(G)	02(G)	02(G)	02(A1)	—	*L10V*	—	—
04CM043**	21	M	A1	02(G)	02(G)	A1	—	*L63P*	—	—
04CM050	49	M	02(G)	02(G)		02(A1)	—	—	—	—
04CM056	35	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM057	58	M	02(G)	02(G)	02(G)	02(A1)	—	*L10V*	—	—
04CM058**	21	F	A1	02(G)	02(G)	A1	—	—	*(Y188C)*§	—
04CM060	30	F	02(G)	02(G)	02(G)	02(A1)	*(M46L)*§	*V77I*	—	—
04CM061	32	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM062	25	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM063	21	F	D	D	02(G)	D	—	—	—	—
04CM064	18	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	*E44D*
04CM065**	28	F	02(G)	D/02†	02(G)	D	—	*G73S*	—	—
04CM066	32	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM069	45	M	02(G)	02(G)	02(G)		*(M46I)*§	—	*(L100I)*§	—
04CM070	46	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM072††	36	F	A1	A1	A1	A1	—	—	—	—
04CM073	29	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM075	25	F	02(G)	02(G)	02(G)	02(A1)	—	*L63P*	—	—
04CM076	44	F	02(G)	02(G)	02(G)	02(A1)	—	*L10V*	—	—
04CM079	56	M	02(G)	02(G)	02(G)	02(A1)	—	*L63P*	—	—
04CM080	54	F	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM081‡‡	33	F	02(G)			02(A1)	—	—	ND	ND
04CM083	20	F	G	G	G	G	—	—	—	—
04CM084	54	M	02(G)	02(G)			—	—	ND	ND
04CM089	33	F	02(G)	02(G)	02(G)	02(A1)	—	—	*(V75I)*§	—

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TABLE 1. *(continued)*

Sample ID	Age (y)	Sex	Genetic Subtype*				Drug Resistance–Associated Mutations			
			Pol-PR	*Pol-RT*	*Pol-IN*	*Env(C2V3)*	1° *PR*	2° *PR*	1° *RT*	2° *RT*
04CM090	27	M	02(G)	02(G)	A1/02‡	02(A1)	—	—	—	—
04CM091	27	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—
04CM095	33	M	02(G)	02(G)	G	G	*(V82I)*‖	*V77I*	*(Y188C)*¶	—
04CM096	32	M	02(G)	02(G)	02(G)	02(A1)	—	—	—	—

Amino acid change without parenthesis denotes full-clone mutations, whereas amino acid change with parenthesis stands for presence of resistant strains as minor populations.
*Genotyping of *Pol-PR* [297 base pairs (bp)], *Pol-RT* (697 bp), a part of *Pol-Int* (288 bp), and *Env-C2V3* domain; 02, CRF02_AG.
†Possible recombination between subtype D and CRF02_AG within the region.
‡Possible recombination between subtype A1 and CRF02_AG within the region.
§Detected in 1 of 4 clones.
‖Detected in 1 of 5 clones.
¶Detected in 1 of 7 clones.
All samples contained both *K20I* and *M36I* of *Pol-PR*, secondary mutations as polymorphism, except: **mutation only at *M36I/V*, with no polymorphism at K20; ††without mutations at K20 and M36; ‡‡*K20N* and *M36N*, both of which previously unreported.
1° indicates primary mutation of drug resistance; 2°, secondary mutation of drug resistance; ND, not detected.

In the current study, we evaluated the prevalence of drug-resistant strains in previously untreated HIV-1–infected patients at the dawn of the ART era in rural western Cameroon. It has been reported that conventional genotyping tests can underestimate the overall prevalence of resistant strains,[19,20] and the importance of minor resistant mutants has been discussed recently.[21,22] Therefore, we also investigated the rate of drug-resistant strains found as minor populations.

METHODS

Study Population

Fifty-four HIV-1–infected individuals (24 men and 30 women; mean age ± SD, 32.9 ± 10.1 years) attending antenatal/STD clinics [Infectious Disease Wards of the Maternal and Child Health Clinic (Nkwen) and the Azire Integrated Health Center (Kumbo), both in the northwestern province of Cameroon] were enrolled in this study (Table 1). With thorough ethical clearance and informed consent, blood samples were collected from the individuals in February 2004, when none had been treated with ART. The presence of plasma anti–HIV-1 antibody was screened with an immunochromatography assay kit (Determine HIV 1/2; Abbott, Tokyo, Japan) and confirmed with a microparticle enzyme immunoassay kit (AxSYM HIV1/2; Abbott). Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Genomic DNA was extracted from the PBMCs using a DNA extraction kit (Qiagen, Hilden, Germany).

Polymerase Chain Reaction, Cloning, and Sequencing

A region of the HIV-1 group M *pol* gene including the protease sequence (*Pol-PR*; corresponding to nt 2265–2555 in HIV-1_{HXB2}) was amplified by nested polymerase chain reaction (PCR) with primers NYUPOL7 (5′-GGGAATTT TCTTCAGAGCAG-3′) and NYUPOL8 (5′-TCTTCTGTCA ATGGCCATTGT-3′) in the first round, and NYUPOL9 (5′-TC CTTAACTTCCCTCAAATCACT-3′) and NYUPOL10 (5′-CTGGCACGGTTTCAATAGGACT-3′) in the second round.[23] A region of the HIV-1 *pol* gene including the reverse transcriptase sequence (*Pol-RT*; corresponding to nt 2513–3209 in HIV-1_{HXB2}) was amplified by nested PCR with primers RT18 (5′-GGAAACCAAAAATGATAGGGGG AATTGGAGG-3′) and K104 (5′-TGACTTGCCCAATT TAGTTTTCCCACTAA-3′) in the first round, and K101 (5′-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3′) and K102 (5′-CCCATCCAAAGAAATGGAGGAGGTTCT TTCTGATG-3′) in the second round. A region of the HIV-1 *pol* gene including the integrase sequence (*Pol-IN*; corresponding to nt 4493–4780 in HIV-1_{HXB2}) was amplified with primers unipol5 (5′-TGGGTACCAGCACACAAAGGAA TAGGAGGAAA-3′) and unipol6 (5′-CCACAGCTGATCT CTGCCTTCTCTGTAATAGACC-3′) in the first round, and unipol1 (5′-AGTGGATTCATAGAAGCAGAAGT-3′) and unipol2 (5′-CCCCTATTCCTTCCCCTTCTTTTAAAA-3′) in the second round.[24] A region of the HIV-1 *env* gene including the *C2V3* sequence (corresponding to nt 6975–7520 in HIV-1_{HXB2}) was amplified with primers M5 (5′-CCAATTCC CATACATTATTGTGCCCC AGCTGG-3′) and M10 (5′-CC AATTGTCCCTCATATCTCCTCCTCCAGG-3′) in the first round, and M3 (5′-GTCAGCACAGTACAATGCACACAT GG-3′) and M8 (5′-TCCTTGGATGGGAGGGGCATACATT GC-3′) in the second round.[24]

Nested PCR was performed using the AmpliTaq Gold PCR kit (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions. Amplification was done with 1 cycle of 95°C for 10 minutes and 35 cycles of 95°C for 30 seconds, 45°C to 55°C for 30 seconds, and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. PCR amplification was confirmed by ethidium bromide staining of samples electrophoresed on an agarose gel. The PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced as described previously (Applied Biosystems, Foster City, CA).

Phylogenetic Analysis and Subtyping

The samples were aligned with subtype reference sequences from the Los Alamos database by CLUSTAL W (version 1.81) with minor manual adjustments. Phylogenetic trees were constructed and visualized as described previously.[25,26] To improve the accuracy, we used the genotyping

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TABLE 2. Protease Secondary Mutations (Including Polymorphisms) With Subtype Information

Site of Mutation	Frequency		Other Subtypes
	Total (n = 54)	CRF02 (n = 41)	(n = 13)
M36I	51 (94.4%)	39 (95.1%)	A1(3),G(3),F2(1),U (5)‡
V	1 (1.9%)	1 (2.4%)	—
*N**	1 (1.9%)	1 (2.4%)	—
K20I	46 (85.2%)	40 (97.6%)	G(3),U (3)‡
R	1 (1.9%)	—	F2(1)
N†	1 (1.9%)	1 (2.4%)	—
L10V	6 (11.1%)	5 (12.2%)	F2(1)
I	1 (1.9%)	—	G(1)
L63P	3 (5.6%)	2 (4.9%)	A1(1)
V77I	3 (5.6%)	2 (4.9%)	G(1)
G73S	1 (1.9%)	2 (4.9%)	02/D(1)

*†Previously unreported.
‡*U* includes different independent recombinants with different components.

tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi), the RIP 2.0 system (http://www.hiv.lanl.gov/content/hiv-db/RIPPER/RIP.html), and the REGA subtyping tool (http://dbpartners.stanford.edu/RegaSubtyping/) as needed.

PI and RTI Resistance–Associated Mutations

The PR and RT sequences (297 bp and 697 bp, respectively) were translated into the corresponding amino acids (99 and 232 amino acids, respectively) and analyzed for previously reported drug resistance–associated mutations in subtype B strains.[27] For each sample, at least 4 clones were obtained and genotyped to evaluate minor populations.

Nucleotide Sequence Accession Numbers

GenBank accession numbers of the sequences reported in this study are as follows: DQ461820 to DQ461873 for *Pol-PR*, DQ461874 to DQ461925 for *Pol-RT*, DQ461926 to DQ461973 for *Pol-IN*, and DQ464287 to DQ 464330 for *Env-C2V3*.

RESULTS

Subtype Distribution

All 54 samples could be analyzed in the *PR* region. In the *RT*, *IN*, and *Env* regions, 51, 48, and 44 samples were analyzed, respectively. The subtype of each sample was identified. Of the 54 HIV-1 strains, 45 (83.3%) had a concordant subtype or circulating recombinant form (CRF) designation: 40 (74.1%) CRF02_AG, 2 (3.7%) subtype A1, 2 (3.7%) subtype G, and 1 (1.8%) subtype F2. The remaining 9 (16.7%) strains had a discordant subtype or CRF: 6 (11.1%) subtype A1/CRF02_AG, 2 (3.7%) subtype D/CRF02, and 1 subtype (1.8%) G/CRF02. Thus, these 9 HIV-1 strains were considered to be recombinant; and in all cases, CRF02_AG was involved in the recombination.

PI Resistance–Associated Mutations

The overall prevalence of primary PI resistance–associated mutations was 7.4% (4 of 54 cases) (Table 1), although the prevalence of full-clone primary mutations was only 1.9% (1 of 54 cases). There were 3 cases that had strains with primary mutations as minor populations (1 of 4, 1 of 4, and 1 of 5 clones). One patient carried a strain with primary mutation *M46L* in full clones, and its determined subtype was A1. A mutation at codon 82 (*V82I*) was detected in full clones from 2 patients, but *V82I* confers minimal resistance to available PIs. These 2 cases were subtype G and CRF02_AG, in which *V82I* is regarded as a natural polymorphism.[28,29]

As minor populations, *V82A*, one of the most important "cleft" mutations, was detected in 1 case (1 of 5 clones); and the "flap" mutations *M46L* and *M46I*[29] were observed in 1 case each (both 1 of 4 clones).

As for secondary mutations, most were detected at polymorphic sites such as codons 10, 20, 36, 63, and 77 (Table 2).[29] Of the 54 cases, 51 (94.4%) had the *M36I* mutation, 1 had *M36V*, and 1 had *M36N*. Thus, all except 1 case had a mutation at this site. *K20I* appeared with a frequency of 85.2% (46 of 54 cases); and together with *K20R* and *K20N*, 87.0% of cases had a mutation at this site. These findings are consistent with previous reports showing mutation of codon 36 in more than 80% of non-B subtypes and mutation of codon 20 in more than 50% of subtype A and in more than 80% of subtype G and CRF02_AG.[28,30] Other secondary mutations at frequent polymorphic sites were *L10V/I* (7 cases, 13.0%), *L63P* (3 cases, 5.6%), and *V77I* (3 cases, 5.6%). *G73S* was detected in 1 case (1.9%).

RTI Resistance–Associated Mutations

Of the 51 cases analyzed, 5 (9.8%) had strains with primary RTI resistance mutations as minor populations (Table 1). No case was found to have primary mutations in full clones.

The prevalence of primary mutations associated with NRTI resistance was 3.9% (2 of 51 cases). *M184V*, a potent inducer of resistance to lamivudine and emtricitabine with less potency against multiple drugs,[29] was detected in 1 patient (1 of 4 clones), and *V75I* was detected in 1 case as a minor population (1 of 4 clones).

The prevalence of primary mutations associated with NNRTI resistance was 5.9% (3 of 51 cases). *Y188C*, which causes intermediate-to-high resistance to nevirapine,[29] was detected in 2 cases (04CM058 and 04CM095) (1 of 4 and 1 of 7 clones, respectively). *L100I* was detected as a minor population (1 of 4 clones) in 1 case. HIV-1 subtype/CRF of the 2 strains with *Y188C* were different as shown in Table 1 and Figures 1A, B, suggesting that these 2 persons were epidemiologically unrelated.

FIGURE 1. Phylogenetic trees based on the *Pol-PR* gene (297 bp) of 54 HIV-1 strains (A) and the *Pol-RT* gene (697 bp) of 51 strains (B) from western Cameroon with reference sequences of representative subtypes/CRF. The bootstrap value at each node represents the number among 1000 bootstrap replicates that support the branching order. Bootstrap values higher than 70% are shown. The bracket on the right represents the major group M subtypes. Newly analyzed sequences are marked with a filled square (■).

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