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

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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)*§	—

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

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 (■).

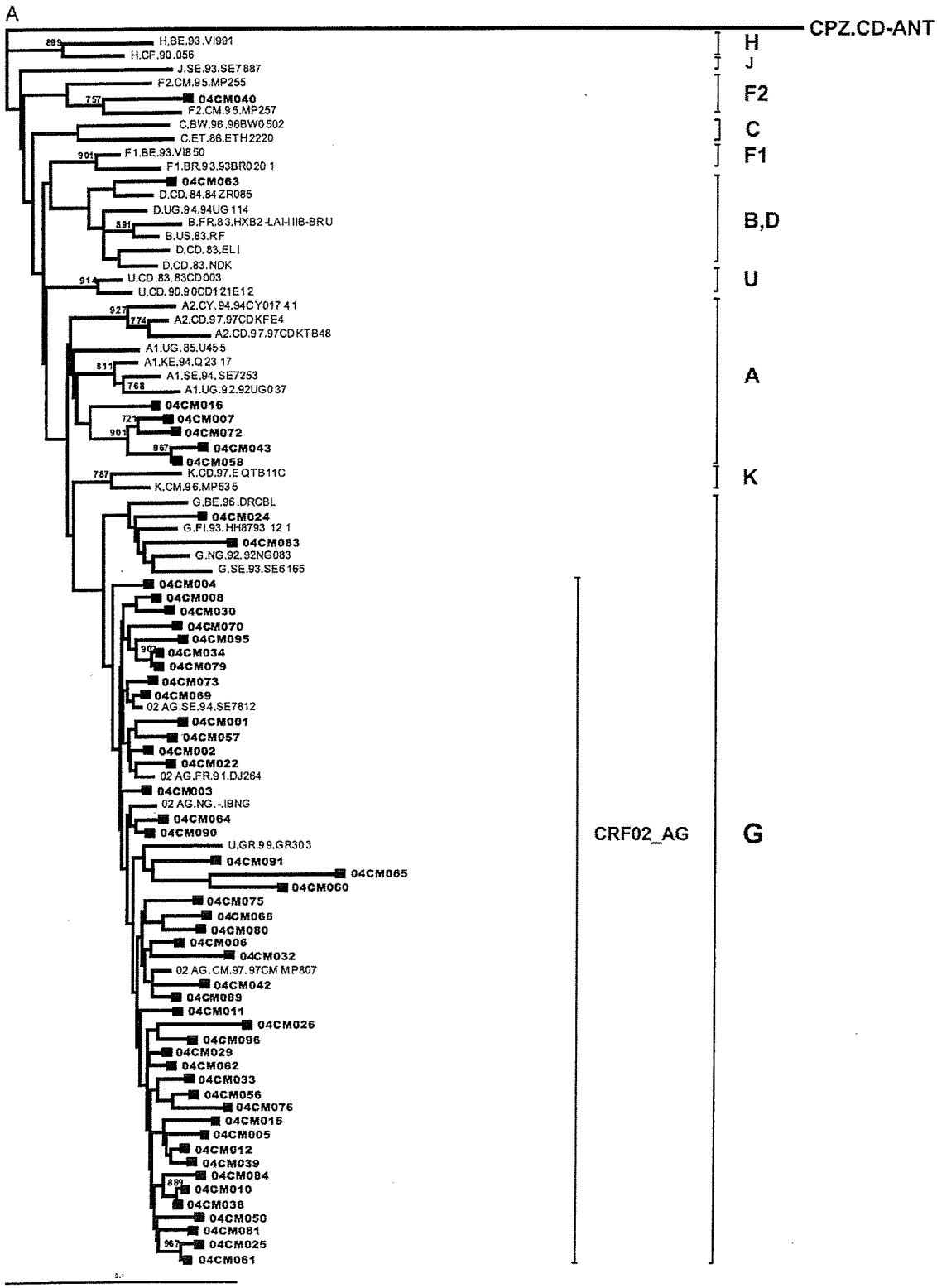

FIGURE 1. *(continued)*

As for secondary mutations, *E44D*, which is associated with multi-NRTI resistance,[29] was detected in 1 case with full clones; yet there were no coexistent primary mutations.

Secondary mutations as minor populations were omitted from the figure because they were numerous, and their importance is likely much less than that of the other mutations.

DISCUSSION

In the current study, we found PI and RTI resistance–associated primary mutations in 7.4% and 9.8%, respectively, of drug-naive HIV-1–infected individuals in Cameroon when drug-resistant minor populations were included. This is one of the first reports of the emergence of primary ART resistance–associated mutations among drug-naive, non-B subtype HIV-1–infected individuals in Cameroon.

The current study was designed to evaluate the prevalence of potential resistant strains as minor populations, so we used proviral DNA extracted from PBMCs and analyzed several clones per sample. There is no doubt that direct sequencing of plasma RNA is the gold standard for drug resistance surveillance.[31] Recently, however, it has been reported that conventional genotype testing may overlook minor virus populations if their frequencies are less than 25%.[19,20] Minor resistant strains that eventually overgrow and affect the clinical course of disease can emerge from levels of virus that are undetectable by conventional assays.[32,33] Therefore, we focused on the baseline frequency of the dormant hazard in a newly ART-promoting country.

Unlike developed countries where PI-including regimens are frequently administered, the current first line of ART in Cameroon is the combination of 2 NRTIs and 1 NNRTI, which means that PI use is limited compared with that of industrialized countries. Konings et al[23] reported that only secondary mutations associated with PI resistance were detected among drug-naive HIV-1 patients in Cameroon during 2000 to 2002. Nonetheless, in our study, primary mutations of PI resistance were detected in drug-naive patients at the rate of 7.4% as of February 2004. This is consistent with findings by Ndembi et al suggesting the emergence of the drug resistance primary mutations during the last few years (personal communication). The HIV-1 strains with primary mutations were found to be CRF02_AG, subtypes A1 and F2, which circulate in west central Africa. It would, therefore, be natural to explain this phenomenon by simple transmission of the resistant strains from patients treated with PIs in Cameroon, although PI use has been limited in this country. However, the possibility that genetic diversity gave birth to resistant strains in the drug-naive individuals cannot be excluded. In addition, the possibility of laboratory artifact that was induced by PCR error cannot be fully excluded either.

As for RTI resistance, previous reports have not detected primary mutations in sub-Saharan Africa during 1999 to 2003,[34,35] including the report by Konings et al[36] that was based on samples collected in Cameroon between 2000 and 2002. In our study, samples were collected in western Cameroon in February 2004, and 5 cases (9.8%) yielded HIV-1 strains with primary mutations, although all were found as minor populations. Recently, there have been several reports noting the importance of drug-resistant strains detected as minor populations. Minor drug-resistant HIV-1 populations have been detected both in the early phase of treatment failure[32,33] and during successful structured treatment interruption.[21] Minor drug-resistant populations undetectable by conventional assays can eventually overgrow and affect the clinical course. They also have been found to persist longer than previously expected in untreated patients, a favorable condition for wild-type virus to overgrow,[37–39] which also indicates the risk of resistance transmission even from minor strains. Thus, careful follow-up studies should be conducted to assess whether the drug-resistant mutants found in our study as minor populations might impact future ART.

Most of the published data focusing on minor populations of resistant strains are based on the analysis of subtype B strains using quantitative real-time PCR with specific primers for *V82A*, *L90M*, or *M184V* of RT.[21,40] In our study, the entire regions of PR and RT genes were amplified, cloned, and genetically analyzed to identify mutation sites other than these 3. This is particularly important in the context of analyzing non-B subtype HIV-1 strains, which often contain polymorphisms. As a result, we detected some other important mutations as minor populations, such as *V82A* in the PR gene and *V75I/Y188C* in the RT gene.

In our study, several non-B subtype-specific polymorphisms were confirmed in the protease gene. First, we detected *K20I/N* and *M36I/N* at relatively higher frequencies than previous reports. Because Holguin et al reported that *K20I* and *M36I* were detected in all cases of subtype G strains,[30] the higher rate can be explained by the fact that most of our samples were CRF02_AG, whose *Pol-PR* is subtype G. Other secondary mutation sites that we detected, such as L10, L63, and V77, are consistent with previous reports that consider these mutations non-B subtype natural polymorphisms. Secondly, *V82I* was detected in 2 cases, one of which belonged to subtype G; and the amino acid change could be regarded as a subtype-specific natural polymorphism.[28] In this study, there were no other subtype-specific polymorphisms that could cause considerable natural drug resistance.

The subtype distribution of HIV-1 circulating in western Cameroon was found to be slightly different from those of other districts.[16–18,23,36] The predominance of CRF02_AG was much more conspicuous in the western district (85%) than in other districts (about 60%), although the prevalences of other constituents such as A1, G, F2, and D were almost identical to those in other reports. As expected, independent recombinants were detected between CRF02_AG and subtype A1, D, or G, although the rate of recombination in this area was slightly lower (16.7%) than in other areas of Cameroon (about 21%).[18] Still, the independent recombinants carry the potential hazard of developing natural resistance.

In conclusion, as of February 2004, primary mutations of the HIV-1 protease exist in drug-naive patients in rural western Cameroon, despite little use of protease inhibitors. As the scheduled rapid provision of ART can give rise to resistant strains in developing countries, we should be careful not to overlook the emergence of resistant strains. More importantly, we also observed the emergence of minor populations of both RTI- and PI-resistant strains, which could have been

underestimated by conventional surveillance. Thus, careful drug regimen design is needed to prevent the rise of overt resistant strains from minor populations.

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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

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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,

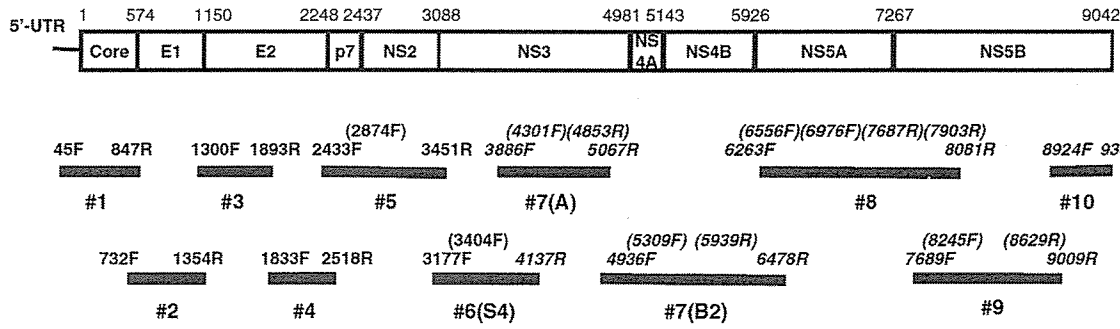

Fig. 1. Strategy for the sequence determination of an hepatitis C virus (HCV) strain, SE-03-07-1689. A genetic organization of HCV is indicated with blocks (coding regions) and a flanking bar (5′-untranslated region, 5′-UTR). The numbers on the block denote the starting positions of the coding regions 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-

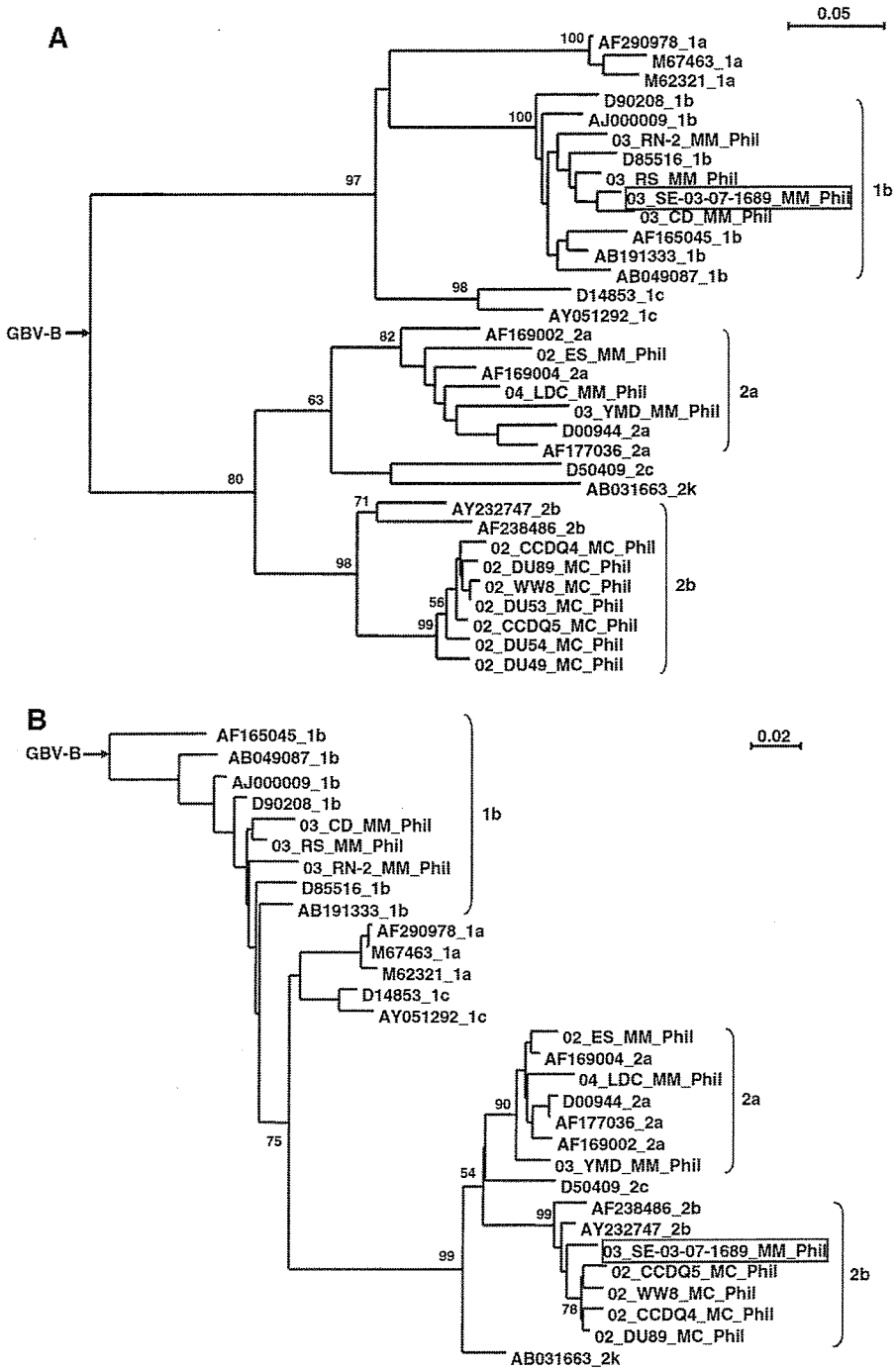

Fig. 2. Phylogenetic analysis of the HCV strains on 222 and 538 nucleotides within the NS5B (**A**) and 5′-UTR-Core (**B**) regions. The tree was constructed by the neighbor-joining method using ClustalW program with GBV-B (accession number; NC001655) as the outgroup. Strains including the SE-03-07-1689 (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].

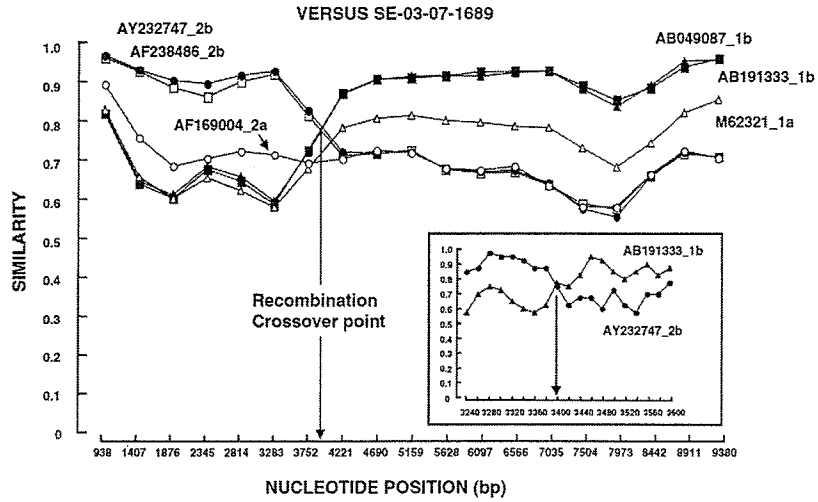

Fig. 3. Similarity plots for a putative recombinant strain, SE-03-07-1689, and reference strains based on the 9.4 kb sequences and a #6(S4) fragment (inset). The plot was performed with the Recombination Analysis Tool (RAT). Four reference sequences of HCV genotype-1b (AB049087 and AB191333) and -2b (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

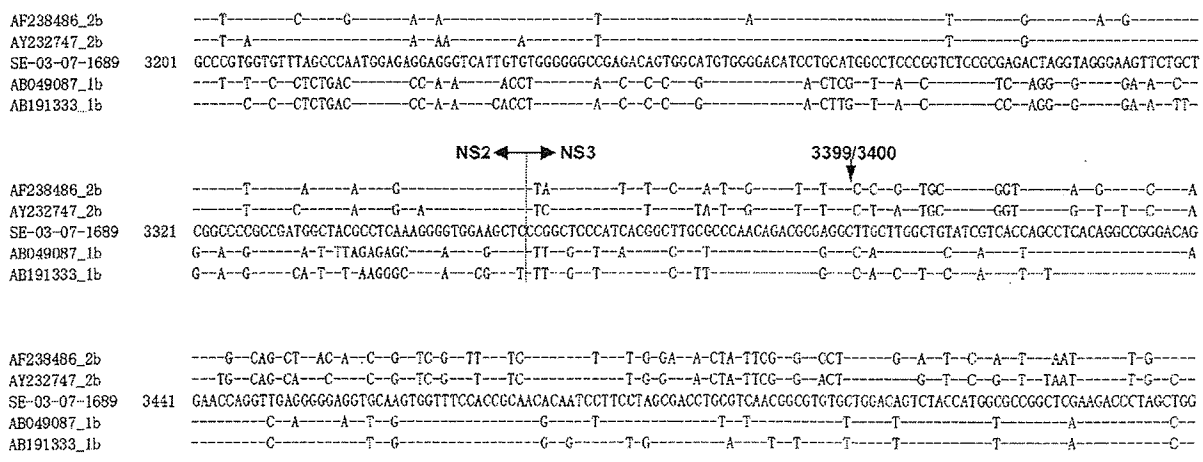

Fig. 4. Alignment of the nucleotide sequences of SE-03-07-1689 and four reference sequences: AF238486, AY232747, AB049087, and AB191333 within #6(S4) fragment. Arrow indicates the possible crossover point (3399/3400). The borderline between NS2 and NS3 was determined on Los Alamos HCV sequences database.

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.

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