Identification of Attenuated Variants of HIV-1 Circulating Recombinant Form 01_AE That Are Associated with Slow Disease Progression Due to Gross Genetic Alterations in the nef/Long Terminal Repeat Sequences

Makiko Kondo, Takako Shima, Masako Nishizawa, Koji Sudo, Shinya Iwamuro, Takeshi Okabe, Yutaka Takebe, and Mitsunobu Imai

We identified an unusual case of human immunodeficiency virus type 1 (HIV-1) infection in a patient (GM43) who exhibited a persistently low antibody response and undetectable viral load during a 5-year follow-up period. GM43 harbored HIV-1 circulating recombinant form 01_AE with gross deletions in the nef/long terminal repeat (LTR) region. The sizes of the deletions increased progressively from 84 to >400 bp during the 5-year period. GM43 appeared to have acquired defective variants from her husband. The genetic alterations in the nef/LTR region were remarkably similar to those that have been reported in slow progressors (such as the slow progressors in the Sydney Blood Bank Cohort). The present study is the first report of slow disease progression due to gross genetic alterations in the nef/LTR region in a person infected with an HIV-1 non–subtype B strain.

Rates of disease progression vary among individuals infected with HIV-1, because of the complex interplay between host genetic and immunologic factors and the pathogenic potential of the infecting virus. The viral nef gene is one of the crucial determinants of disease progression, as has been demonstrated in animal models [1–3]. That the nef gene is a key factor for disease progression in humans is strongly supported by the finding that some long-term nonprogressors (LTNPs) with low viral loads (despite 10–14 years of HIV-1 infection) carry viruses with gross deletions [4–6] or small structural defects and mutations [7] in the nef gene.

The nef gene is known to have pleiotropic functions, including down-regulation of the cell-surface expression of CD4 and class I major histocompatibility complex (MHC) molecules, enhancement of viral replication and infectivity, induction of cytokine and chemokine expression by T cells and macrophages, and blockage of proapoptotic signaling by HIV-1–infected cells (reviewed in Geyer et al. [8]). A large number of cellular interaction partners critical to nef gene functions have been identified, and the binding sites have been mapped to distinct locations within the Nef protein (reviewed in Geyer et al. [8]).

Although the genetic alterations in the nef gene that are associated with slow disease progression have been identified in HIV-1 subtype B in US and European populations [4–7], it remains unclear whether these alterations are found only in the subtype B lineage. In the present study, we identified attenuated variants of HIV-1 circulating recombinant form 01_AE (CRF01_AE) that harbored gross deletions in the nef/LTR region in an asymptomatic patient (GM43) who had an unusually weak antibody response and an undetectable viral load during a 5-year follow-up period, demonstrating the association between nef/LTR deletions and slow disease progression with respect to infection with a non–subtype B strain.

Patients, materials, and methods. Informed consent was obtained from the patients, and the study was conducted in accordance with the clinical research guidelines of Japan. Antibodies to HIV-1 were detected by use of the Serodia HIV-1 gelatin particle agglutination (PA) test (Fujirebio), and Western-blot (WB) analysis (LAV Blot I; Bio-Rad) was used for confirmation. Plasma HIV-1 RNA loads were measured by the ultrasensitive method with the Amplicor HIV-1 Monitor Kit (version 1.5; Roche Diagnostics), which allows the sensitive
Figure 1. Changes in serological and virological parameters in patient GM43. A, Profiles of serological and virological parameters. Top, Detection of proviral HIV-1 DNA by nested polymerase chain reaction (PCR) for gag (p24), env (C2/V3), and the nef/long terminal repeat (LTR) region. Antibody (Ab) titers (determined by the Serodia HIV-1 gelatin particle agglutination [PA] test) are also shown. —, negative; +, positive; Δ, the size (in base pairs) of the deletion in the nef/LTR region in major PCR products; WB, Western blot. Middle, CD4+ cell count, in cells per microliter of blood (white circles). Bottom, Plasma HIV-1 RNA load, in copies per milliliter of blood (log scale) (black diamonds). HIV-1 proviral genomes were analyzed by use of the serum samples collected at the indicated time points (I–V). HIV-1 was isolated from GM43 for the first time in February 2002 (time point V). B, WB analysis (LAV Blot I; Bio-Rad) for GM43 and her husband, GM46. Strips 1–10 are for serum samples serially collected between January and October 1996, and strips I and II are for serum samples collected in July 1997 (time point I) and December 1998 (time point II). d, day; m, month.
Figure 2. Genomic organization of the nef/long terminal repeat (LTR) region. A, Schematic drawing of the genomic structure of HIV-1 circulating recombinant form (CRF) 01_AE CM240 [12] for the corresponding region, at top. The genomic organizations of HIV-1 isolates from patient GM43 and her husband, GM46, and of HIV-1 variant C18 [an attenuated variant of HIV-1 subtype B detected in the Sydney Blood Bank Cohort [5]] are shown for comparison. Black bars represent amplified sequences, and white bars represent deletions. The nucleotide positions are shown relative to CM240. The numbers in the white bars represent the sizes of the deletions. m, month; PPT, polypurine tract. B, Comparison of the genetic organization of the nef/LTR region in GM43-20, a major quasispecies (Δ391) found in GM43, with that of C18 [5]. C, Sequence landmarks and the sites of deletions in the nef/LTR region in GM43-20. C18 carries a 23-bp duplication comprised of a single set of NF-κB and Sp1 sequences.
detection of HIV-1 RNA from both HIV-1 subtype B and non-subtype B strains, including CRF01_AE [9]. Replication-competent HIV-1 strains were isolated by cocultivation with CD8-depleted peripheral-blood mononuclear cells (PBMCs) from healthy donors. PBMCs were activated by use of anti-CD3 antibody (CLB-CD3; PeliCluster), instead of the standard activation stimuli of phytohemagglutinin and interleukin-2, to improve the efficiency of isolation [10, 11].

The nef/LTR regions of HIV-1 provirus genomes were amplified by nested polymerase chain reaction (PCR), with the outer primers Env43F14 (sense; 5′-GAGTATTGGCAGGGATACTCAC-3′; positions 7892–7912 of the genome of CM240, the HIV-1 CRF01_AE reference strain [12]) and 3′-LTR43R16 (antisense; 5′-TAAGCACTCAAGGCAAGC-3′; positions 9202–9185 of the genome of CM240). The nucleotide sequences of both strands were determined by the BigDye Terminator cycle sequencing method, using a Prism 310 DNA Sequencer (Applied Biosystems). Nucleotide sequences (GenBank accession numbers AB193797–AB193800) were aligned by use of CLUSTAL W (version 1.4). Phylogenetic trees were constructed by the neighbor-joining method, based on Kimura’s 2-parameter distance matrix with 100 bootstrap replicates. Analyses were implemented by use of PHYLIP (version 3.573) [13].

Results. GM43 is a 28-year-old Thai woman who has lived in Japan for the past 6 years. GM43 was infected with HIV-1 via her husband, GM46. GM46 had contracted HIV-1 via heterosexual contact before 1995, presumably in Thailand. Both GM43 and GM46 remain healthy and do not have any clinical symptoms. There is no indication of HIV-2 infection. In January 1996, serologic tests conducted for GM43 at her 18th week of pregnancy (her first visit) showed low marginal HIV-1 seropositivity, with a PA antibody titer of 1:16 (figure 1A). Indeterminate WB results (1+ reactivity for gp160 only) persisted for GM43 throughout an 18-month observation period, whereas GM46 was unequivocally positive by WB (figure 1B). Empirically, in most HIV-1–infected patients, PA antibody titers
exceed 1·10⁶ within 2 weeks of seroconversion and reach 1·10⁷ one month after seroconversion. However, in GM43, low PA antibody titers (<1·10³) persisted for 1·5 years, and it took >4·5 years until her PA antibody titer reached 1·10⁴ (figure 1A).

In parallel with this slow process of seroconversion, GM43’s plasma HIV-1 RNA load was persistently below the limit of detection by PCR (<50 copies/mL) during a 4·5-year period (figure 1A). HIV-1 proviral DNAs were amplified by nested PCR for the env (C2/V3) region only in July 1997 and for both the gag (p17) and env (C2/V3) regions in December 1998 (figure 1A), providing conclusive evidence that GM43 was infected with HIV-1. During the first 3 years after seroconversion, GM43’s CD4⁺ cell count gradually decreased, from 1074 to 600 cells/µL, as her plasma HIV-1 RNA load gradually increased, but her CD4⁺ cell count remained stable thereafter, at 400–600 cells/µL (figure 1A).

To investigate the mechanism of this unusual clinical course, we examined the structural features of the nef region that are known to be associated with slow disease progression [4–6]. The nef/LTR regions of HIV-1 proviral genomes were amplified by nested PCR from the PBMC DNAs sampled at 5 different time points between July 1997 and February 2002 (time points I–V, as shown in figure 1). Although the expected size of amplicons of the nef/LTR region of the intact HIV-1 genome is 1140 bp, the resulting amplified fragments ranged in size from 500 to 1000 bp, indicating the existence of deletions of ∼100–500 bp in the nef/LTR region.

To further characterize the genetic alterations in the nef/LTR region for GM43, we molecularly cloned (by the TA cloning method) the PCR products and determined the nucleotide sequences of 16–39 independent PCR clones at each time point. Nucleotide-sequence alignment revealed progressive deletions in the nef/LTR region over time (figure 2). Plasma HIV-1 RNA load was detectable after the increased deletions in the nef/LTR region (time points III–V). A replication-competent HIV-1 strain (GM43-23) was isolated from the first time point (V). The HIV-1 quasispecies with the 391-bp nef/LTR deletion (∆391) appeared to constitute a major functional (replication-competent) segment of the proviral population in GM43.

We next analyzed the structural characteristics of the HIV-1 genomes for GM46. We attempted to amplify the nef/LTR region by PCR at 7 time points (time points 46-1 through 46-7, as shown in figure 2A) between 1996 and 1997 (GM46 then dropped out of the follow-up). At all sampling points except the first (46-1), both full-sized and smaller-sized PCR products (containing deletions ranging in size from 12 to 472 bp) were amplified (figure 2A) in independent PCR amplification experiments. Although we were not able to establish the exact frequency of defective genomes in GM46, considerable proportions of the HIV-1 quasispecies in GM46 appeared to contain gross genetic alterations in the nef/LTR region, especially at later time points. In contrast, no appreciable defects in the nef/LTR region were detected among 73 other CRF01_AE–infected individuals (of both Japanese and Thai nationality) in Japan. Phylogenetic-tree analysis based on nucleotide sequences of env (C2/V3) and the nef/LTR region revealed that HIV-1 sequences from GM43 and GM46 formed a monophyletic cluster within CRF01_AE, with high bootstrap support (100% and 98%, respectively) (data not shown). Furthermore, this GM43/GM46 cluster was distinct from other CRF01_AE local control sequences sampled in the same geographical region. These findings strongly suggest that GM43 was indeed infected via her husband. However, none of the deletions detected in GM46 were identical to those detected in GM43 (figure 2).

The genetic organization of nef/LTR deletions detected in GM43 and GM46 are summarized in figure 2. Two large deletions were detected. The first large deletion was located in the amino-terminal half of the nef gene that does not overlap with the LTR sequences. The second large deletion was mapped to the nef/U3-overlapping region. One or 2 small, additional deletions followed the 2 large deletions. Most of the first large deletion removed the highly conserved acidic (EEEE) domain and (Pxx), motif, which are essential for Nef function. However, the downstream deletions located in the nef/U3 region left intact the polypurine tract (3′-PPT), NF-κB, and SpI binding sites and the TATA box (figure 2), which are indispensable for HIV-1 replication, as was reported in the previous studies of defective subtype B variants in US and European populations [4–6].

The overall structural configuration of the nef/LTR deletions found in the CRF01_AE variant infecting GM43 was remarkably similar to that of the attenuated HIV-1 variant C18 (which belongs to HIV-1 subtype B) detected in the Sydney Blood Bank Cohort [5] (figure 2A and 2B). Of note, the sequence features unique to CRF01_AE—including the GABP motif (5′-ACTT-CGG-3′), a single NF-κB [14], an unusual TATA box (5′-TAAA-3′), and a 2-nt bulge in TAR stems (figure 2)—were detected in GM43. No appreciable direct repeats that may have caused the deletion in the nef/LTR region [6] were detected in GM43.

**Discussion.** We have identified a unique case of CRF01_AE infection, in which a patient, GM43, experienced an unusually slow increase in HIV-1 antibody titers and had an undetectable viral load over a prolonged period of time. GM43 carried attenuated viral variants with a range of nef/LTR deletions that were similar to those found in LTNPs infected with HIV-1 subtype B [4–6]. The present study is the first report demonstrating the association between gross nef/LTR deletions and slow disease progression in a patient infected with a non–subtype B strain.

As can be seen in figure 2, striking similarities in the alteration of the nef/LTR region between subtype B and CRF01_AE were observed. The genetic alterations observed in the nef/LTR region of an attenuated subtype B variant detected in the Sydney
Blood Bank Cohort (isolate C18 [5]) and the CRF01_AE variants found in GM43 in the present study removed most of the sequence elements essential for Nef functions—including the highly conserved acidic (EEEE) domain that is required for the down-regulation of class I MHC molecules and the (Pxx)₄ motif mediating the interaction between Nef and signaling molecules—and placed downstream sequences out of frame (figure 2). Although a number of deletions were present in the nef region that overlapped U3 in the LTR region, none of these alterations affected cis-acting elements known to be critical for viral replication, including the 3′-PPT, the U3 terminal sequences, the TATA box, and the NF-κB and Sp1 binding sites. This convergent manner of evolution of such genetic alterations in the nef/LTR sequences implies the presence of the strong selection pressures that maintain the replication capacities in defective HIV-1 genomes.

Phylogenetic-tree analysis demonstrated that GM43 acquired CRF01_AE from her husband, GM46. Interestingly, GM46 was also found to harbor unique sets of nef/LTR deletions, although the profiles of the deletions detected in GM46 were not identical to those detected in GM43 (figure 2). It is tempting to speculate that the defective genomes detected in GM43 may have evolved from a minor viral quasispecies carried by GM46 that was not detected in the present study or that was present only transiently at the time of transmission to GM43. If this is the case, the lack of selection for functional nef alleles in GM43 during transmission and/or establishment of infection from GM46 is rather surprising, because functional forms of nef alleles are quickly and efficiently selected for in rhesus monkeys infected experimentally with nef-defective simian immunodeficiency virus [1]. This suggests that, in certain patients, attenuated viral variants might have a selective advantage over HIV-1 strains with an intact nef allele. For instance, an efficient immune response may contribute to the selection of nef-defective viruses that could escape the cytotoxic T lymphocyte recognition that is critical to the effective control of viral replication [15]. In light of the identification of this unique case of CRF01_AE infection, a systematic search for the viral and host factors that influence disease progression may be warranted—especially in less-studied regions of the HIV-1 epidemic, such as in developing countries in Asia—with a slow increase in antibody titer used as a convenient marker.

Acknowledgments

We thank the staffs of the participating clinics for their help and Dr. Shingo Kato for his valuable advice on virus isolation.

References