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Sequence Note

Genetic Linkage of Nevirapine Resistance Mutations in HIV Type 1 Seven Days after Single-Dose Nevirapine

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ABSTRACT

The HIVNET 012 trial in Uganda demonstrated that a regimen of single-dose nevirapine (NVP) can prevent HIV-1 mother-to-child transmission. Previous studies show that HIV-1 with one or more NVP resistance (NVPR) mutations can be selected in many women as early as 7 days after single-dose NVP. We evaluated the genetic linkage of NVPR mutations in plasma from women in HIVNET 012 collected 7 days after single-dose NVP administration. The HIV-1 *pol* region was amplified and cloned from 20 plasma samples (16 with NVPR mutations detected by population sequencing and 4 with no NVPR mutations detected), and 10 clones from each sample were sequenced. Up to five different NVPR mutations were detected in clones from a single sample. K103N and Y181C were the most common mutations detected. Clones with two genetically linked mutations were detected in four samples. Different combinations of NVPR mutations were linked in individual clones, but none of the clones contained both K103N and Y181C. Further studies are needed to evaluate whether selection of minority variants with one or more NVPR mutations after single-dose NVP is clinically relevant.

In the HIVNET 012 trial in Uganda, antiretroviral drug-naive Ugandan women received a single 200-mg dose of NVP at the onset of labor, and infants received a 2-mg/kg dose within 72 hr of birth.[1,2] The women did not receive other antiretroviral therapy, consistent with the standard of care in Uganda at the time the trial was performed. The low cost, efficacy, and simplicity of this regimen make it attractive in resource-poor settings. It is now being implemented in countries around the world.

Plasma samples collected from women in HIVNET 012 seven days after NVP administration were analyzed previously using the ViroSeq HIV-1 genotyping system (ViroSeq; Celera Diagnostics, Alameda, CA).[3] This system is based on population sequencing and provides sequence information for protease amino acids 1–99 and reverse transcriptase (RT) amino acids 1–335. Among 65 women analyzed, 14 (21.5%) had one or more NVPR mutations detected, with up to four mutations detected in a single sample. The mutations were typically detected as mixtures of mutant and wild-type sequences. Y181C was the most common mutation detected, followed by K103N. V106A and G190A were also detected in some women.[3] Because genotyping was performed by population sequencing, it was not possible to determine from that analysis whether detection of multiple NVPR mutations in a single sample reflected selection of HIV-1 variants with genetically linked NVPR mutations, or independent selection of subpopulations of HIV-1 variants with different NVPR mutations.

In this study, we cloned and sequenced individual HIV-1 variants to define further the spectrum and genetic linkage of NVPR mutations in maternal plasma 7 days after single-dose NVP. Analysis included 20 samples: the 14 samples with NVPR mutations described above,[3] 2 additional samples with NVPR mutations, and 4 samples with no NVPR mutations detected by population sequencing. Among the 16 samples with NVPR mu-

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A

185-A	103	106	181	188	190	Summary
POPN						Y181C*
1						WT
2						V106A
3						Y181C
4						Y181C
5						Y181C
6						Y181C
7						Y181C
8						Y188C
9						G190A
10						G190S

790-A	103	106	181	188	190	Summary
POPN						Y181C*
1						WT
2						WT
3						WT
4						WT
5						WT
6						V106A
7						V106A
8						Y181C
9						Y181C
10						G190A

599-A	103	106	181	188	190	Summary
POPN						Y181C*
1						WT
2						WT
3						WT
4						WT
5						WT
6						WT
7						WT
8						WT
9						WT
10						Y181C

607-D	103	106	181	188	190	Summary
POPN						Y181C*
1						WT
2						WT
3						K103N
4						Y181C
5						Y181C
6						Y181C
7						Y181C
8						Y181C
9						Y181C
10					D	Y181C, (G190D)

667-A	103	106	181	188	190	Summary
POPN						Y181C*
1						WT
2						Y181C
3						Y181C
4						Y181C
5						Y181C
6						Y181C
7						Y181C
8						Y181C
9			S			(Y181S)
10						G190A

490-D	103	106	181	188	190	Summary
POPN						Y181C*
1						WT
2						WT
3						WT
4						WT
5						WT
6						Y181C
7						Y181C
8						Y181C
9						Y181C
10						Y181C

703-A	103	106	181	188	190	Summary
POPN						K103N*
1						WT
2						WT
3						WT
4						K103N
5						K103N
6						K103N
7						K103N
8						K103N
9						K103N
10						K103N

73-C	103	106	181	188	190	Summary
POPN						K103N*
1						WT
2						WT
3						WT
4						WT
5						WT
6						WT
7						WT
8						WT
9						WT
10						V106A

FIG. 1. NVPR mutations detected in plasma from women 7 days after single-dose NVP. Plasma samples collected from women 7 days after single-dose NVP were analyzed by population sequencing with the ViroSeq system, and by sequencing clones representing individual HIV-1 variants. Each table shows the results from a single woman. A reference number for each woman is shown in the upper left-hand corner of each table, followed by a letter designating the HIV-1 subtype of the sample (A, C, D, or recombinant [R], e.g., 185-A). Tables indicate which NVPR mutations were detected in each sample (103 = K103N, 106 = V106A, 181 = Y181C, 188 = Y188C/L, 190 = G190A/S). Results from population sequencing (POPN) and from analysis of 10 clones from each sample (1–10) are shown. Shaded boxes indicate the presence of NVPR mutations. Mutations detected by population sequencing were all detected as mixtures with wild-type virus (indicated with an asterisk). NVPR mutations detected in clones that were also detected by population sequencing are shaded black. NVPR mutations detected in clones that were not detected by population sequencing are shaded gray. The NVPR mutations detected by population sequencing and sequencing of clones are listed for each sample (Summary). Alternative amino acids detected at positions of NVPR mutations are indicated in the tables without shading, and are listed in the summary in parentheses. (**A**) Samples with a single NVPR mutation detected by population sequencing. (**B**) Samples with two or more NVPR mutations detected by population sequencing. (**C**) Samples with no NVPR mutations detected by population sequencing.

B

868-D	103	106	181	188	190	Summary
POPN						K103N* + Y181C*
1						WT
2						K103N
3	E					Y181C (K103E)
4						V106A + Y181C
5						Y181C
6						Y181C
7						Y181C
8						Y181C
9						Y188C
10						G190A

687-A	103	106	181	188	190	Summary
POPN						K103N* + Y181C*
1						WT
2						WT
3						WT
4						WT
5						K103N
6						K103N
7						K103N
8						K103N
9	T					Y188C (K103T)
10						Y181C

477-R	103	106	181	188	190	Summary
POPN						K103N* + Y181C*
1						WT
2						K103N
3						K103N
4						K103N
5						K103N
6						K103N
7						K103N
8						K103N
9						K103N
10						K103N

847-A	103	106	181	188	190	Summary
POPN						Y181C* + G190A*
1						WT
2						WT
3						WT
4						WT
5						K103N
6						V106A
7						Y181C
8						Y181C
9						Y181C
10						G190A

545-A	103	106	181	188	190	Summary
POPN						V106A* + Y181C*
1	R					V106A +Y181C + (K103R)
2						Y181C
3						Y181C
4						Y181C
5						Y181C
6						Y181C
7						Y181C
8						Y181C
9						Y181C
10						Y181C

638-D	103	106	181	188	190	Summary
POPN						V106A* + Y181C*
1						WT
2						K103N
3						K103N
4						K103N
5						K103N
6						V106A
7						Y181C
8						Y181C
9						Y181C
10						Y181C

164-A	103	106	181	188	190	Summary
POPN						K103N* + Y181C* + G190A*
1						WT
2						WT
3						WT
4						WT
5						K103N
6						V106A + G190A
7						Y181C
8						Y181C
9						Y181C + G190A
10						Y181C + G190A

842-A	103	106	181	188	190	Summary
POPN						K103N* + V106A* + Y181C* + G190A*
1						WT
2						K103N
3						K103N
4						K103N
5						V106A
6						V106A + Y181C
7						Y181C + G190A
8						Y188L
9						G190A
10						G190A

FIG. 1. (*continued*)

tations, 8 had one mutation detected (Fig. 1A, population sequencing [POPN]) and 8 had two or more mutations detected (Fig. 1B, POPN). HIV-1 subtyping was performed by phylogenetic analysis using *pol* region sequences, as previously described.[4] The HIV-1 subtypes of the 20 samples were as follows: 14 A, 4 D, 1 C, and 1 intersubtype recombinant.

To analyze HIV-1 variants in each sample, PCR products generated using ViroSeq were cloned into a plasmid vector using the UNG-negative *Escherichia coli* strain BD2314, as described previously.[5] Ten clones from each sample were sequenced. Phylogenetic analysis was performed to compare the population sequences (one sequence per plasma sample) with the sequences obtained from the clones (10 sequences per plasma sample). Sequences were aligned using the CLUSTAL algorithm (MegAlign; DNASTAR, Madison, WI), and trees were generated to compare the genetic relatedness of the sequences. In each case, the sequences from the clones clustered most closely with the population sequence from the same sample, confirming an absence of sample mix-ups or cross-contamination (data not shown).

Because PCR amplification produces numerous amplicons from each template, cloned PCR products may not represent sequences from individual viruses. ViroSeq uses AmpliTaq Gold DNA polymerase for PCR, which has an error rate of

C

532-A	103	106	181	188	190	Summary
POPN						WT
1						WT
2						WT
3						WT
4						WT
5						WT
6						WT
7						WT
8						WT
9						WT
10	E					(K103E)

584-A	103	106	181	188	190	Summary
POPN						WT
1						WT
2						WT
3						WT
4						WT
5						WT
6						WT
7						WT
8						WT
9						WT
10						WT

401-A	103	106	181	188	190	Summary
POPN						WT
1						WT
2						WT
3						WT
4						WT
5						WT
6						WT
7						WT
8						Y181C
9						Y188C
10						Y188C

FIG. 1. (*continued*)

1/50,000–1/100,000 nucleotides incorporated. In a 40-cycle amplification of the *pol* region (1270 nucleotides), PCR products derived from the same viral genome (e.g., PCR sibs) would be expected to differ on average at a single nucleotide position. We analyzed the sequence diversity (percentage of nucleotide differences) among the 10 clones from each sample, using the MegAlign program (pairwise analysis). For one sample, 5 of 10 clones had identical sequences, or had sequences that differed at only one nucleotide position, suggesting either that the sample had low genetic diversity or that some of the clones isolated were derived from the same virus. That sample was not analyzed further. For 18 of the other 19 samples, all 10 clones from each sample differed from one another at two or more positions (range, 2–44 nucleotide differences). The median number of nucleotide differences among the 10 clones from each sample ranged from 6 to 36. This suggested that the majority of the clones isolated were derived from independent HIV-1 variants. For the remaining sample (sample 584), all 10 clones lacked NVPR mutations (Fig. 1C). Two pairs of clones from that sample differed from one another at only one nucleotide position. This analysis suggests that most or all of the clones were derived from independent viruses. It should be noted, however, that the proportion of clones with different NVPR mutations might not reflect the true proportion of HIV-1 variants with those mutations because only 10 clones were sequenced (i.e., there may be sampling error), and because selective priming during RT-PCR could potentially bias the amplification reactions.

Analysis of the clones revealed a mixture of HIV-1 variants with and without NVPR mutations (Fig. 1A–C). More than half of the samples analyzed (12 of 19) had NVPR mutations detected by cloning that were not detected by population sequencing. Among the 16 samples that had one or more NVPR mutations detected by population sequencing, additional mutations detected by cloning included K103N (3 samples), V106A (6 samples), Y188C (3 samples), Y188L (1 sample), G190A (4 samples), and G190S (1 sample). In addition, one sample that did not have any NVPR mutations detected by population sequencing (wild type) had both Y181C and Y188C detected in clones.

Clones from four women had two genetically linked NVPR mutations (samples 868, 545, 164, and 842; Fig. 1B). A fifth woman had a clone with the NVPR mutation Y181C linked to G190D, an unusual variant at position 190 that is not known to be associated with NVPR (sample 607; Fig. 1A). All four samples that had clones with genetically linked NVPR mutations had two or more NVPR mutations detected by population sequencing: sample 868 had K103N plus Y181C; sample 545 had V106A plus Y181C; sample 164 had K103N, Y181C, and G190A; and sample 842 had K103N, V106A, Y181C, and G190A. All of those mutations were detected as mixtures with wild-type virus. The genetically linked mutations detected in those four women included Y181C plus V106A in three women (868, 545, and 842), Y181C plus G190A in two women (164 and 842), and G190A plus V106A in one woman (164). Interestingly, none of the cloned variants contained K103N plus Y181C, even though K103N and Y181C were the two most common mutations detected (detected in 31 of 190 and 56 of 190 clones, respectively), and even though 8 of 19 samples had both mutations detected among the set of 10 clones.

A database of phenotype and genotype test results from more than 34,000 samples submitted for routine resistance testing was also analyzed to identify combinations of NVPR mutations. Most of the sequences were from subtype B HIV-1. Mutations were detected by population sequencing, but samples with mixtures were excluded. Therefore, detection of multiple mutations in a sample was likely to reflect genetic linkage. The most common combinations of two NVPR mutations in samples collected since January 2003 were L100I plus K103N, and K103N plus Y181C, which were found at approximately equal frequencies (data not shown). L100I was not detected in our cohort (in population sequencing or among the clones). Furthermore, whereas K103N and Y181C were detected together as mixtures in five of the HIVNET 012 samples by population sequencing, they were not found together in any of the clones. Our failure to detect clones with K103N plus Y181C may reflect the small size of this study. Alternatively, this could reflect an influence of HIV-1 subtype on mutation selection, or a difference between variants that are rapidly selected by a single dose of NVP (in HIVNET 012, most likely preexisting minority variants) ver-

sus those selected during treatment with nonnucleoside RT inhibitors (NNRTIs; with more opportunity for evolution under drug pressure).

Although we did not find any cloned variants with K103N plus Y181C, three women had NVPR mutations linked to other mutations at position 103 (K103E, K103T, and K103R). Substitution of amino acids other than asparagine (N) at position 103 has been observed previously.[6] K103R is a polymorphism that does not appear to confer NVPR on its own, but that may have a modulatory effect in combination with other NVPR mutations. For example, the combination of K103R with V179D increases resistance to all three NNRTIs 10- to 20-fold.[7] In the database described above, K103T was detected rarely, and is associated with a 10- to 20-fold decrease in NVP susceptibility in the absence of other NNRTI mutations. In contrast, K103E was detected only as a mixture, with no effect on NNRTI susceptibility (data not shown). In clinical samples with subtype B HIV-1, Y181C has been seen in combination with K103R and K103T, and Y188L has been seen in combination with K103R and K103Q.

Most of the NVPR variants detected in women after single-dose NVP do not become the major viral species, but instead are detected as mixtures in population sequencing (i.e., wild-type virus is also present).[3,4] Other mutations, as this study shows, are present below the level of detection by population sequencing-based assays. Therefore, more sensitive assays may be particularly helpful for analysis of NVPR in this setting. Further studies are needed to assess the clinical significance of HIV-1 minority variants with drug resistance mutations. In AIDS Clinical Trials Group protocol 398 (ACTG 398), patients with and without prior NNRTI therapy were evaluated for response to a salvage regimen including the NNRTI efavirenz (EFV) plus tenofovir, abacavir, and one or two protease inhibitors.[8] NNRTI-resistant minority variants were detected at baseline, using a single genome sequencing assay, in 6 of 10 NNRTI-experienced patients and in 2 of 9 NNRTI-naive patients who lacked NNRTI resistance on standard genotyping. In some patients, those variants were genetically related to variants that emerged at the time of failure.[9] This suggests that the presence of NNRTI-resistant variants may affect response to NNRTI-based therapy even if the variants are not detected with routine genotyping assays.

It is not known whether selection of HIV-1 variants with NVPR mutations in women after single-dose NVP prophylaxis will have the same clinical consequences as selection of NVPR variants after prolonged exposure to NVP during treatment with NVP-containing regimens. A study from Thailand suggests that women who received single-dose NVP for prevention of HIV-1 mother-to-child transmission may have lower rates of maximal virologic suppression (<50 copies/ml) after 6 months of NVP-based therapy, compared with women who did not receive single-dose NVP.[10] Clinical trials are being developed to test whether use of single-dose NVP for prevention of mother-to-child transmission influences the subsequent treatment response of women or infants to antiretroviral therapy, or the efficacy of NVP prophylaxis in subsequent pregnancies. These issues are important, because the impressive efficacy, safety, and ease of administration of the HIVNET 012 regimen make it the primary choice for implementation of prevention of HIV-1 mother-to-child transmission in resource-limited countries around the world, and because NNRTI-based regimens are likely to be used for initial HIV-1 treatment in many of those countries. Analysis of NVPR by both routine genotyping assays and more sensitive assays will provide more information on the selection and fading of NVPR after single-dose NVP, and its impact on subsequent use of NNRTIs for prevention or treatment of HIV-1 infection.

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SEQUENCE DATA

GenBank accession numbers for the 20 sequences obtained using the ViroSeq system are as follows: AY428671, AY428676-8, AY428683, AY428690-1, AY428695, AY428697, AY428701, AY428705, AY428708, AY428712, AY428715, AY428721, AY428727, AY428729, AY428731, AY572850-1.

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