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Sequence Note

Analysis of Nevirapine Resistance Mutations in Cloned HIV Type 1 Variants from HIV-Infected Ugandan Infants Using a Single-Step Amplification-Sequencing Method (AmpliSeq)

William Ian Towler, Jessica D. Church, James R. Eshleman, Mary Glenn Fowler, Laura A. Guay, J. Brooks Jackson, and Susan H. Eshleman

Abstract

We analyzed the genetic linkage of nevirapine (NVP) resistance mutations and the genetic complexity of HIV-1 variants in Ugandan infants who were HIV infected despite single dose (SD) prophylaxis. Plasma samples were obtained from six HIV-infected infants who had two or more NVP resistance mutations detected by population sequencing (ViroSeq). ViroSeq PCR products were cloned and transformed, and a single-step amplification-sequencing reaction (AmpliSeq) was used to analyze NVP resistance mutations in cloned HIV-1 variants directly from bacterial colonies. Fifty clones were analyzed for each infant sample. This analysis revealed numerous NVP resistance mutations not detected by population sequencing, genetically linked NVP resistance mutations, and a high degree of genetic complexity at codons that influence NVP susceptibility.

Single dose (SD) nevirapine (NVP) and other NVP-containing regimens are used in resource-limited settings for prevention of HIV-1 mother-to-child transmission. These regimens are effective, but are associated with the emergence and persistence of NVP-resistant HIV-1 variants in some women and in some infants who are HIV infected despite prophylaxis.[1] Low-level NVP-resistant HIV-1 variants can persist [2] and may affect treatment outcome.[3]

Several methods can be used to analyze NVP resistance mutations. However, each method has some limitations. HIV-1 genotyping methods based on population (bulk) sequencing are relatively insensitive for the detection of low-level HIV-1 variants with resistance mutations. Point mutation assays, such as allele-specific polymerase chain reaction (PCR), the LigAmp, and the oligonucleotide ligation (OLA), are more sensitive than routine genotyping assays, but may be labor intensive if multiple mutations are analyzed.[4,5] Variations in the HIV-1 nucleotide sequence at oligonucleotide or primer binding sites can interfere with hybridization, ligation, and/or priming in these assays.

In addition to the limitations mentioned, the assays described above do not provide information about the genetic linkage of NVP resistance mutations in individual HIV-1 variants. This information can be obtained by sequencing viral genomes after cloning[6] by single genome sequencing (SGS),[7] by ultradeep pyrosequencing,[8] or by parallel allele-specific sequencing (PASS).[9] Of these methods, the first three (traditional cloning/sequencing, SGS, and ultradeep pyrosequencing) provide sequence information. In contrast, PASS provides information for individual codons (point mutations). For each method, the sensitivity of mutation detection depends on the number of HIV-1 templates analyzed. Pyrosequencing and PASS require specialized equipment, but can be used to screen large numbers of templates, enhancing the sensitivity of mutation detection.

In this report, we used a single-step amplification-sequencing reaction (AmpliSeq)[10] to analyze the genetic linkage of NVP resistance mutations and genetic complexity at HIV-1 reverse transcriptase codons associated with NVP resistance in six 6-week-old Ugandan infants who were HIV-1 infected despite SD NVP prophylaxis and who had two or more NVP resistance mutations detected by population sequencing (Table 1). The samples were collected in three clinical trials conducted in Kampala, Uganda: (1) the HIVNET 012 trial,[11,12] (2) the Repeat Pregnancy Study,[13] and (3) the Breast Feeding Study ("Pathobiology of Breast Milk among

Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

Table 1. Samples Used for Analysis[a]

Clinical study	Sample	Mutations detected by ViroSeq	HIV-1 subtype
HIVNET 012	147770	K103N + Y181C	D
	147858	Y181C + Y188C	D
Breast Feeding Study	274581	Y181C + Y188C	D
	288407	K103R + V179D + Y181C[b]	D
	289005	K101E + Y181C + G190A	A
Repeat Pregnancy Study	324698	K103N + Y181C	D

[a]The table shows the source of samples analyzed (Clinical study), the mutations detected by the ViroSeq system, and the HIV-1 subtype (*pol* region).
[b]Note that mutations K103R and V179D are associated with NVP resistance only when they occur together.

HIV-1 Infected Ugandan Women Receiving Intrapartum Nevirapine"). HIV-1 genotyping was performed using the ViroSeq HIV Genotyping System (ViroSeq, Celera Diagnostics, Alameda, CA) using 0.1 ml of infant plasma. The HIV-1 subtype of each sample was determined by phylogenetic analysis of *pol* region sequences.

DNA cloning was accomplished as follows. Using the ViroSeq system, RNA was extracted from plasma, and the HIV-1 RNA was reverse transcribed to generate complementary DNA. *Pol* region DNA (encoding the 3′ portion of HIV-1 *gag*, HIV-1 protease, and the 5′ portion of HIV-1 reverse transcriptase) was amplified using Research Use Only PCR mix provided by Celera Diagnosics, which was identical to the PCR mix included in the ViroSeq system, except that dTTP was substituted for dUTP. Amplification was performed without dUTP, because dUTP-containing PCR products cannot be cloned using commercially available competent cells. The PCR products were purified using spin columns, and were incubated with Taq polymerase and dNTPs for the addition of terminal adenosine-phosphate groups, and transformed into TOP 10 electrocompetent *Escherichia coli* cells (Invitrogen Corp., Carlsbad, CA). Colonies were isolated on plates containing ampicillin. S-Gal (Sigma Corp., St. Louis, MO) was used to indicate the presence of a DNA insert in the plasmid vector. White colonies were selected for analysis with AmpliSeq.

AmpliSeq uses commercially available DNA sequencing reagents supplemented with additional dNTPs for the combined amplification/sequencing reaction. Two oligonucleotides are added to each reaction, with one in molar excess. During the initial reaction cycles, the low-concentration oligonucleotide and the dNTPs are incorporated into amplification products. In later reaction cycles, DNA sequencing termination products (generated by priming from the high-concentration oligonucleotide and incorporating ddNTPs) predominate. To perform the analysis, a small inoculum (barely visible on a pipette tip) of each bacterial colony was added directly to a 20 μl reaction containing 4 μl Reaction Ready Mix (Applied Biosystems, Foster City, CA), 1× Sequencing Buffer (Applied Biosystems), 0.15 mM dNTPs, 0.05 μM forward primer (5′-AGATTTCAGGGAACTCAATAAAAGAACTCA-3′), and 0.25 μM reverse primer (5′-GGTTCTTTCTGATGCTTTTTGTCTGGTGT-3′). These primers were designed to bind to regions of HIV-1 that are conserved in HIV-1 subtypes A, C, and D. The AmpliSeq reaction was performed using an Applied Biosystems 9700 thermal cycler as follows: 95°C for 5 min, followed by 25 three-step cycles of

Table 2. NVP Resistance Mutations Detected in Clones[a]

Sample	Number of mutations	Number of clones	Mutations identified
147770	0	12	
	1	25	**Y181C**
	1	9	Y188C
	2	3	**K103N** + Y188C
	2	1	Y188C + G190A
147858	0	4	
	1	1	V106A
	1	27	**Y181C**
	1	17	**Y188C**
	2	1	V106A + **Y188C**
274581	0	9	
	1	2	K103N
	1	1	V108I
	1	20	**Y181C**
	1	12	**Y188C**
	1	5	G190A
	2	1	K103N + **Y181C**
288407	0	4	
	1	21	**Y181C**
	2	1	K101E + **Y181C**
	2	24	**K103R + V179D**[b]
289005	0	11	
	1	12	**K101E**
	1	4	K103N
	1	5	**Y181C**
	1	1	Y188C
	1	15	**G190A**
	2	2	**K103R + G190A**[b]
324698	0	6	
	1	4	**K103N**
		40	**Y181C**

[a]Fifty HIV-1 clones were analyzed by AmpliSeq from each infant sample. The number of clones with 0, 1, or 2 mutations is shown. Mutations shown in bold were detected using the ViroSeq HIV-1 Genotyping System (population sequencing). The following amino acid polymorphisms (not shown in this table) were also detected in the samples at codons in HIV-1 reverse transcriptase that are the sites of NVP resistance mutations: 147770: V179A (1 clone), V106S + V108S (1 clone). 147858: V181H (1 clone). 274581: L100V + A98C (1 clone). 288407: K103R (50 clones), V179E (26 clones). 289005: V179I (49 clones), V179M (1 clone), K103E + A98S (2 clones). 324698: K101R (1 clone).
[b]Note that the mutations K103R and V179D are associated with NVP resistance only when they occur together.

94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 40 three-step cycles of 95°C for 15 s, 50°C for 15 s, and 60°C 4 min. The reactions were ethanol precipitated, resuspended in 20 μl of HiDi formamide (Applied Biosystems), and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Fifty sequences were analyzed for each infant sample (total 300 sequences).

HIV-1 sequence data generated using the AmpliSeq method were analyzed using BioEdit Sequence Alignment Editor.[14] Sequences included nucleotides encoding HIV-1 reverse transcriptase amino acids 95–195. Sequences were examined for the following mutations: **A98G**, ***L100I, K101E/P, K103N/S***/Q/T, **K103R+V179D**, **V106A/M**, V108I, E138K, V179D/E/F, ***Y181C/I/V, Y188C/H/L,*** and ***G190A/E/S***/Q. Mutations shown in bold are associated with reduced NVP susceptibility; mutations in bold and italic are associated with high-level phenotypic NVP resistance and/or reduced virologic response to a clinical regimen.[15] DNAStar MegAlign (DNAStar, Inc., Madison, WI) was used to generate sequence alignments. Phylogenetic trees based on 500 bootstraps were generated using DNAStar MegAlign (DNAStar, Inc., Madison, WI). The 50 clonal HIV-1 sequences from each infant grouped together with the corresponding maternal sequence, as expected (not shown).

We first analyzed the amino acid sequences from the 50 clones isolated from each infant sample for NVP resistance mutations (Table 2). For each infant, NVP resistance mutations that were identified in the population sequence were also identified in one or more of the 50 clonal sequences. Clones from five of the six infants also had NVP resistance mutations that were not identified by population sequencing. Clones with two genetically linked NVP resistance mutations were identified in five of the six infants (Table 2).

We next analyzed the nucleotide sequences of the 50 clones from each infant to determine which codons were present at positions of NVP resistance mutations (Table 3). Up to six different amino acids were detected at a single position (e.g., at codon 179). Furthermore, at 5 of the 10 positions analyzed, we detected two different codons encoding the same amino acid (e.g., GCA and GCG for Ala at codon 98). In one infant, all 50 clones had K103R, and 24 of those clones had V179D; these two mutations are associated with NVP resistance only when they occur together.[15,16] Excluding K103R and V179D in that infant, the portion of clones that had NVP resistance mutations varied from one position to another (from 0 to 140 clones out of 300 clones examined).

The methods described in this report can be performed using DNA remaining from HIV-1 genotyping, which is ideal for analysis of samples that are limited in volume, such as those from pediatric studies. We were able to obtain read

TABLE 3. CODONS DETECTED IN CLONES AT POSITIONS IN HIV-1 REVERSE TRANSCRIPTASE ASSOCIATED WITH NVP RESISTANCE[a]

	Codon 98				*Codon 100*			*Codon 101*					*Codon 103*					*Codon 106*		
	Ala A *GCA*	*Ala* A *GCG*	Cys C TGC	Ser S AGC	*Leu* L *CTA*	*Leu* L *TTA*	Val V GTA	*Lys* K *AAA*	Arg R AGA	**Glu E GAA**	**Glu E GAG**	Gln Q AAG	*Lys* K *AAA*	**Asn N AAC**	**Asn N AAT**	**Arg[b] R AAG**	Glu E GAA	*Val* V *GTA*	**Ala A GCA**	Ser S AGT
147770	*50*	*0*	0	0	*50*	*0*	0	*50*	0	**0**	**0**	0	*47*	**0**	**3**	**0**	0	*49*	**0**	1
147858	*49*	*1*	0	0	*50*	*0*	0	*50*	0	**0**	**0**	0	*50*	**0**	**0**	**0**	0	*48*	**2**	0
274581	*49*	*0*	1	0	*49*	*0*	1	*50*	0	**0**	**0**	0	*47*	**3**	**0**	**0**	0	50	**0**	0
288407	*50*	*0*	0	0	*50*	*0*	0	*49*	0	**0**	**0**	1	*0*	**0**	**0**	**50**	0	*50*	**0**	0
289005	*0*	*48*	0	2	*0*	*50*	0	*36*	0	**13**	**1**	0	*44*	**3**	**1**	**0**	2	*50*	**0**	0
324698	*50*	*0*	0	0	*50*	*0*	0	*49*	1	**0**	**0**	0	*46*	**1**	**3**	**0**	0	*50*	**0**	0
Total	*248*	*49*	1	2	*249*	*50*	1	*284*	1	**13**	**1**	1	*234*	**7**	**7**	**50**	2	*297*	**2**	1

	Codon 108				*Codon 179*						*Codon 181*			*Codon 188*		*Codon 190*	
	Val V *GTA*	*Val* V *GTG*	**Ile I ATA**	Ser S AGT	*Val* V *GTT*	Ala A GCT	**Asp[b] D GAT**	Ile I ATT	Glu E GAA	Met M ATG	*Tyr* Y *TAT*	**Cys C TGT**	His H CAT	*Tyr* Y *TAT*	**Cys C TGT**	*Gly* G *GGA*	**Ala A GCA**
147770	*49*	*0*	**0**	1	*49*	1	**0**	0	0	0	*25*	**25**	0	*40*	**10**	*49*	**1**
147858	*50*	*0*	**0**	0	*50*	0	**0**	0	0	0	*22*	**27**	1	*32*	**18**	*50*	**0**
274581	*49*	*0*	**1**	0	*50*	0	**0**	0	0	0	*29*	**21**	0	*38*	**12**	*45*	**5**
288407	*50*	*0*	**0**	0	*0*	0	**24**	0	26	0	*28*	**22**	0	*50*	**0**	*50*	**0**
289005	*45*	*5*	**0**	0	*0*	0	**0**	49	0	1	*45*	**5**	0	*49*	**1**	*33*	**17**
324698	*0*	*50*	**0**	0	*50*	0	**0**	0	0	0	*10*	**40**	0	*50*	**0**	*50*	**0**
Total	*243*	*55*	**1**	1	*199*	1	**24**	49	26	1	*159*	**140**	1	*259*	**41**	*277*	**23**

[a]Fifty HIV-1 clones were analyzed from each of the six infant samples (300 clones total). Sample numbers are indicated on the left. NVP resistance mutations occur at codons 98, 100, 101, 103, 106, 108, 179, 181, 188, and 190 in HIV-1 reverse transcriptase. The table shows the nucleotide sequences and corresponding amino acids detected in clones from each infant sample at each position. The number of clones with each nucleotide sequence is indicated. Reference sequences/amino acids (those found in reference strain HXB2) are in italics. Sequences/mutations associated with NVP resistance are shown in bold.

[b]Note that mutations K103R and V179D are associated with NVP resistance only when they occur together.

lengths of approximately 300 bases with a single AmpliSeq reaction, enabling us to analyze the two major regions where NVP resistance mutations occur (i.e., at codons 98–108 and codons 179–190). AmpliSeq has been optimized for read lengths of approximately 500 bases using purified human and bacterial genomic templates.[10] The methods described in this report also produce cloned plasmids, which can be used for further characterization of specific HIV-1 variants.

The samples used in this study were selected from six infants who had two or more NVP resistance mutations detected by population sequencing. Clonal analysis revealed that five of the six infants had some HIV-1 variants with two or more genetically linked mutations. HIV-1 variants with multiple NVP resistance mutations may have altered fitness, different patterns of cross-resistance to other nonnucleoside reverse transcriptase inhibitors compared to HIV-1 variants with single NVP resistance mutations.[15] With the exception of one infant who had the K103R polymorphism in all clones and V179D in a high portion of clones, HIV-1 variants with two genetically linked mutations represented a small portion of the viral population (one to four clones out of 50 analyzed in each infant). In three of the five infants with genetically linked mutations, each of the individual mutations was also detected separately in some clones. Because AmpliSeq amplifies a mixed population of HIV variants, there is some possibility that recombination occurred during the amplification reaction. This potential problem can be avoided using methods such as SGS, PASS, or pyrosequencing.

Analysis of HIV-1 clones from these infants also revealed a high degree of genetic complexity at codons that influence NVP susceptibility. Among the clones from six infants, we detected at least four different codons at 5 of the 10 positions analyzed, and 5 of the 10 positions analyzed had two different codons that encoded the same amino acid. This high degree of diversity, which was also seen at codons in HIV-1 reverse transcriptase that are not associated with NVP resistance, underscores the challenges faced in designing oligonucleotide reagents for point mutation assays and other assays that rely on hybridization of olignonucleotides to HIV-1 DNA.

Sequence Data

The GenBank accession numbers for the population sequences from the six infants in the study are EU715589–EU71594.

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References

1. McConnell MS, Stringer JS, Kourtis AP, Weidle PJ, and Eshleman SH: Use of single-dose nevirapine for the prevention of mother-to-child transmission of HIV-1: Does development of resistance matter? Am J Obstet Gynecol 2007;197: S56–63.
2. Flys TS, Donnell D, Mwatha A, *et al.*: Persistence of K103N-containing HIV-1 variants after single-dose nevirapine for prevention of HIV-1 mother-to-child transmission. J Infect Dis 2007;195:711–715.
3. Johnson J, Li JF, Wei X, *et al.*: Low-frequency mutations substantially increase the prevalence of transmitted drug resistance and greatly strengthen the relationship between resistance mutations and virologic failure. 14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, February 25–28, 2007, Abstract #639.
4. Ellis GM, Mahalanabis M, Beck IA, *et al.*: Comparison of oligonucleotide ligation assay and consensus sequencing for detection of drug-resistant mutants of human immunodeficiency virus type 1 in peripheral blood mononuclear cells and plasma. J Clin Microbiol 2004;42:3670–3674.
5. Palmer S, Boltz V, Martinson N, *et al.*: Persistence of nevirapine-resistant HIV-1 in women after single-dose nevirapine therapy for prevention of maternal-to-fetal HIV-1 transmission. Proc Natl Acad Sci USA 2006;103:7094–7099.
6. Eshleman SH, Jones D, Flys T, Petrauskene O, and Jackson JB: Analysis of HIV-1 variants by cloning DNA generated with the ViroSeq HIV-1 Genotyping System. Biotechniques 2003;35:614–618, 620, 622.
7. Palmer S, Kearney M, Maldarelli F, *et al.*: Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. J Clin Microbiol 2005;43: 406–413.
8. Hoffmann C, Minkah N, Leipzig J, *et al.*: DNA bar coding and pyrosequencing to identify rare HIV drug resistance mutations. Nucleic Acids Res 2007;35:e91.
9. Cai F, Chen H, Hicks CB, Bartlett JA, Zhu J, and Gao F: Detection of minor drug-resistant populations by parallel allele-specific sequencing. Nat Methods 2007;4:123–125.
10. Murphy KM, Berg KD, and Eshleman JR: Sequencing of genomic DNA by combined amplification and cycle sequencing reaction. Clin Chem 2005;51:35–39.
11. Guay LA, Musoke P, Fleming T, *et al.*: Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in

Kampala, Uganda: HIVNET 012 randomised trial. Lancet 1999;354:795–802.
12. Jackson JB, Musoke P, Fleming T, *et al.*: Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: 18 months follow-up of the HIVNET 012 randomised trial. Lancet 2003;362:859–868.
13. McConnell MS, Bakaki P, Eure C, *et al.*: Effectiveness of repeat single-dose nevirapine for prevention of mother-to-child transmission of HIV-1 in repeat pregnancies, Uganda. J Acquir Immune Defic Syndr 2007;46:291–296.
14. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999;41:95–98.
15. Stanford University: Stanford University HIV Drug Resistance Database. http://hivdb.stanford.edu/cgi-bin/NNRTIResiNote.cgi: Accessed 04/08.
16. Parkin NT, Gupta S, Chappey C, and Petropoulos CJ: The K101P and K103R/V179D mutations in human immunodeficiency virus type 1 reverse transcriptase confer resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrob Agents Chemother 2006;50:351–354.

Address reprint requests to:
Susan H. Eshleman
Department of Pathology
HIV Genotyping Laboratory
The Johns Hopkins Medical Institutions
Ross Building 646
720 Rutland Ave.
Baltimore, Maryland 21205

E-mail: seshlem@jhmi.edu