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Full Length Research Paper

HIV subtype and drug resistance patterns among drug naïve persons in Jos, Nigeria

P. Lar[1,5*], N. Lar[2], K. Bemis[1], J. Jelpe[2], L. Enzyguirre[1], L. Ayuba[2], D. Zella[1], P. Kanki[3], J. K. Carr[1,4], W. Blattner[1] and A. G. Abimiku[1]

[1]Institute of Human Virology, University of Maryland, Baltimore, USA.
[2]PSSH, Jos Plateau state, Nigeria,
[3]Harvard School of Public Health, Boston, MA, England.
[4]Walter Reed Army Institute of Research, Rockville, MD, USA.
[5]University of Jos, Nigeria.

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To determine HIV-1 subtypes and antiretroviral drug resistance mutations for 16 infected, pregnant women in Jos, Nigeria, part of pol (1040 bp) was amplified from patient PBMC DNA, sequenced and analyzed. Eight of the samples were subtype G, three were CRF02_AG and 2 were unique recombinant forms (URF) between G and CRF02_AG. The remaining consisted of 3 different strains: one was subtype C, and the other 2 were unrelated URF. Nearly full-length genome sequences were completed for 6 of the strains: 4 subtype G and 2 CRF02_AG. In the 14 drug-naïve subjects, no primary resistance-associated mutations were found, but secondary mutations were identified in 7 different codons of the gene coding for protease: PR K20I, M36I, L63A/P/V, V82I, L10M/I and I93L. In addition, the K238R mutation was identified in the reverse transcriptase gene of 3 viruses. The PR K20I and M36I mutations occurred in all of the strains, and the L10M and V82I mutations occurred only in subtype G. The mutation, I93L, was carried by subtype C viruses. Two of the women that had prior niverapine treatment, had primary resistance-associated mutations, RT M184V and K103N, archived in their proviral DNA several months after treatment cessation. The study reports a predominance of clade G and CRF02_AG, and provides many more examples of nearly full-length genome sequences for subtype G viruses from Nigeria. The ubiquitous presence of PI secondary resistance-associated mutations, as well as primary resistance-associated mutations in 2 previously treated women, underscores the need to ensure adherence compliance to treatment.

Key words: Characterization, CRF02_AG, baseline, niverapine, Unique Recombinant Forms, West Africa.

INTRODUCTION

Many genetic subtypes of HIV-1 have been characterized from diverse geographic regions with a growing number of inter-subtype circulating recombinant forms (CRF) (http://hiv-web.lanl.gov). There are currently 16 circulating recombinant forms (CRFs) in various geographical regions, including CRF02_AG, an AG recombinant that has been reported to account for >50% of the HIV infections in Cameroon and other West African populations Carr et al. (2001), and is present in Western Europe, South America and Central Asia (Carrion et al., 2003; Carr et al., personal communication). Subtype designations are powerful epidemiological tools that have been used to track the spread of the virus and to determine transmission patterns (Jannsens et al., 1997). Data in the literature show a heterogeneous distribution of the sub-types and a pandemic of geographically localized genetic subtypes (Kanki et al., 1997; Peeters et al., 1997). Genetic differences in the virus impact the efficiency of diagnostic assays such as viral loads assays (Coste et al., 1996; Triques et al., 1999) and the susceptibility to antiretroviral therapy. Viral genetic differences have been associated with natural resistance against antiretroviral drugs and

*Corresponding author. E-mail: larp1000ng@yahoo.com.

some subtype G samples have been reported to be less susceptible to protease inhibitors (Apetrei et al., 1998; Descamps et al., 1997) and subtype O strains are naturally resistant to non nucleoside reverse transcriptase inhibitors (Descamps et al., 1995).

In Nigeria, the first AIDS case was reported in 1986; since then seroprevalence jumped from 1.8% in 1991 to 5% in 2003 (Nigeria sentinel survey, 2003) among antennatal clinic (ANC) attendees. Several studies have shown HIV subtype A and G as well as CRF02_AG as the predominant strains causing the epidemic in Nigeria. HIV-1 from an asymptomatic and AIDS patient from Jos in the north central region of Nigeria was first isolated by Abimiku et al. (1994) and shown to have a subtype G env and gag regions. Full- length genome sequences of these isolates showed one of them to be subtype G and one an AG recombinant (Gao et al., 1998). In addition, the CRF02_AG prototype (IbNG) was described in Ibadan in the south-western region by Howard et al. (1994). McCutchan et al. (1999) identified subtype G and various forms of A/G recombinants in the complete env gene in 10 patients hospitalized in the Northeastern region of Nigeria. Further characterization of the subtype distribution conducted in several states across Nigeria indica- ted the selective prevalence of subtype A and the CRF02_AG prototype IbNG in southern Nigeria and subtype G in the north (Gao et al., 1998; Howard et al. 1994). However this regional distribution has not been confirmed by other investigators in the field neither have there been studies that describe the distribution of subtypes in specific groups targeted for intervention strategies. With the implementation of the expanded ARV program and the PMTCT in Nigeria, gaps exist with regard to information on the actual distribution of HIV-1 subtypes amongst high-risk and vulnerable groups in Nigeria such as commercial sex workers, pregnant women, young children and youths which should be targeted for intervention and control programs. Our study targets a population of pregnant women attending the antenatal clinic at the Plateau State Specialist Hospital (PSSH) and aims to genetically characterize the subtype distribution in an environment in which the HIV-1 A/G recombinant was shown to be circulating ten years ago(Abimiku et al., 1994). In addition, baseline data regarding the viral mutations associated with drug resistance obtained from this study are vital in guiding the choice of ARV therapy and in controlling the emergence of drug resistance.

METHODS

Blood was drawn from asymptomatic HIV-1 seropositive antenatal clinic attendants, after obtaining consent from them during an 18-month study (2001 – 2002) to establish baseline epidemiological data for HIV infection at the Plateau State Specialist Hospital (PSSH) in Jos, Nigeria. Plasma was collected from spun blood and peripheral blood mononuclear cells (PBMCs) were separated on ficoll gradients, after which the plasma and cells were frozen appropriately at –70^{o}C and liquid nitrogen, respectively, until required. The frozen samples were later shipped to the Institute of Human Virology in Baltimore (IHV) for molecular studies. Genomic DNA was isolated from PBMCS utilizing the QIAmp DNA Blood mini Kit (Qiagen, Valencia, CA, USA) and the DNA integrity was first determined by the amplification of the beta-globulin gene by PCR.

The region encoding the protease protein and part of the reverse transcriptase (RT) protein (Pro/RT)(1.1kb) was amplified using nested PCR with the following as outer primers: POLF1 (5`-CWTTRGARGAAATGATGACAGC-3`) and CAMPOLR2 (5`TTCTC-TGCCAATTCTAATTCTGC-3`), followed by POLF3 (5`RGARCC AAGAGCCCCAMCAGC-3`) and CAMPOLR1 (5`CCTGSATAAATC-TGACTTCC-3`) as inner primers. Cycling conditions for first round amplification were the initial denaturation of 95^{o}C for 10 min followed by 45 cycles of 94^{o}C (30 s) for denaturation, 55^{o}C (30 s) for annealing and for extension, 72^{o}C (1.5 min). This was followed by a final extension of 72^{o}C for 10 min. Two microlitre of the first round reaction served as template for the second round amplification reaction and the conditions were an initial denaturation at 95^{o}C for 10 min, and 30 cycles of 95^{o}C (30 s), 58^{o}C (30s), 72^{o}C (1.5 min), and a final extension 72^{o}C for 10 min to inactivate the reaction. In some cases several second round reactions were combined in order to obtain sufficient template for sequencing. For both reactions the concentration of the components were similar, thus all primers were diluted at final concentration of 0.1 µM, 1unit of Amplitaq gold polymerase was used per reaction, with 200 µM dNTPs, 1.5 mM $MgCl_2$ and reaction buffer. Positive amplification products were purified using Microcon YM-50 columns (AMICON Bioseparation kit) and served as template for automated sequencing. PCR Products were directly sequenced using fluorescent dye terminators and Applied Biosystems (ABI) model 3100 capillary sequencer. Both sense and antisense strands of the amplified DNA sequences were determined. Derived sequences were analyzed and edited with Sequencher v 4.1 (Gene codes Inc.) on Macintosh computers, and all sequence ambiguities were resolved.

Multiple alignments of the Pro/RT sequences with selected reference sequences of subtypes A-K plus CRF01_AE (CM240), CRF02 _ AG prototype IbNG and NG083 from Nigeria was conducted using GDE software, which uses PHYLIP software for the phylogenetic analyses (Felsenstein, 1989). A neighbor-joining phylogenetic analysis with a maximum parsimony bootstrap was performed in order to determine the genetic relationship of the Nigerian strains to other reference sequences.

The region of the Pol gene that was amplified covers coding regions for all of the protease protein (99 aa), and part of the reverse transcriptase (252 aa). The sequences of the isolates were examined for protease and RT drug resistance mutations using the HIVseq of the Stanford HIV drug resistance database at http://hivdb.stanford.edu.

RESULTS

The study population consisted mostly of married young women whose age ranged from 19 to 38 years (average 25 yrs) (Table 1). The average CD4 count was 539.85 cells/µl blood (range of 224 - 1183cells/µl blood). All but two women were drug-naive prior to sample collection but two women had had niverapine a year before to prevent mother to child transmission of HIV-1. The genotypes for the regions sequenced are presented (Table 1).

In phylogenetic analysis of the Pro/RT sequences, the majority of the strains (13/16) were grouped into two main clusters: the CRF02_AG group and the subtype G group. Situated between these two clusters, were the subtype G/CRF02_AG recombinants (Figure 1A). Four isolates clustered closely with subtype G reference isolate

Table 1. Demographic features, subtypes and susceptibility of HIV-1 infected women attending the Plateau State Specialist Hospital antenatal clinic

Sample ID	Age	CD4 Count	ARV experience	Drug resistance PI, NRTI, NNRTI	PRO/RT Subtype
01NGPL148	29	237	Naïve	Susceptible	G/CRF02_AG
01NGPL209	20	511	Naïve	Susceptible	G
01NGPL338	31	-	NVP	Resistant	G
01NGPL364	28	482	Naïve	Susceptible	C
01NGPL368	26	423	Naïve	Susceptible	G
01NGPL490	26	569	Naïve	Susceptible	A/G/CRF01_AE
01NGPL567	19	1183	Naïve	Susceptible	CRF02_AG
01NGPL601	21	ND	Naïve	Susceptible	G
01NGPL644	35	301	Naïve	Susceptible	CRF02_AG
01NGPL658	21	256	Naïve	Susceptible	C/unclassified
01NGPL674	38	1127	NVP	resistant	G
01NGPL669	30	224	Naïve	Susceptible	G
01NGPL710	26	573	Naïve	Susceptible	CRF02_AG
01NGPL754	24	453	Naïve	Susceptible	G
01NGPL760	21	506	Naïve	Susceptible	G/CRF02_AG
01NGPL800	27	407	Naïve	Susceptible	G

92NG083 which was first isolated in Jos, Nigeria, in 1992, the same location as these strains 9 years later. One (1/16) of the strains that clustered with subtype G, 01NGPL601, was actually a recombinant between sub-type G and CRF02_AG. There was another significant cluster of 4 subtype G strains (the cluster including 01NGPL674) that represented another Nigerian G cluster. Four other isolates clustered significantly with reference strains from CRF02_AG, although recombinant analysis revealed that 01NGPL148 was actually recombinant between subtype G and CRF02_AG. Two isolates clustered significantly with subtype C; one, 01NGPL364, was pure subtype C while the other, 01NGPL658, was a recombinant between subtype C and an unclassified strain. The sample for 01NGPL364 was known to be dually infected because cloned envelope sequences derived from the plasma contained both clade C and CRF02_AG (data not shown). Finally, strain 01NGPL490 was an unusual URF made up of subtypes A and G, however, the subtype A region clustered with CRF01_AE rather than CRF02_AG. Full length genome sequence analysis for 6 samples (Figure 2) confirmed the classification based on the pol region: 4 strains (01NGPL567, 669, 674 and 760) were pure subtype G strains and two other isolates (01NGPL710 and 01NGPL754) clustered with IbNG and were CRF02_AG. In conclusion, half of the strains were subtype G, and CRF02_AG and two were recombinants between two genetic forms (G/CRF02AG). The remaining isolates were other recombinants and one subtype C. The presence of different subtypes and recombinants in this small sample is an indication of the diversity of genetic forms circulating among women in Jos.

Next we examined the Protease/RT sequences for mutations that have been shown to be associated with increased resistance to PI, NRTI, and NNRTI antiretroviral (ARV) drugs in subtype B viruses (Figure 3). All but 2 of the women in this study were ARV drug-naïve. Among these women, 7 different secondary resistance amino acid substitutions were identified in the protease and RT regions at codons 10, 20, 36, 63, 82, 93 and 238. There were no significant differences in baseline mutations between subtypes G and CRF02_AG, and secondary mutations PR K20I and M36I occurred in all of the samples of both genetic forms, an observation not seen with subtype B. The V82I (62.5%) and L10M (25%) mutations occurred only in subtype G isolates and I93L occurred only in the pure subtype C and the C recombinant samples. Other mutations that were identified include L63A/P/V and K238R. Further observation of the frequency of the mutations carried by each isolate shows that most of them harbored more than 2 mutations, and some isolates had accumulated up to 4 different mutations. More than 50% of the sequences showed low level potential to develop resistance to PIs. No major mutations associated with drug resistance were detected for protease inhibitors. Two of the women had had nivirapine (NVP) treatment during an earlier birth to interrupt mother-to-child transmission. Examination for primary resistance-associated mutations in the reverse transcriptase gene of those women revealed the presence of M184V and K103N in one sample (01NGPL338) and K103N only in another (01NGPL674). Both of these strains were subtype G. These isolates each carried 3 or 4 different secondary polymorphisms respectively and were

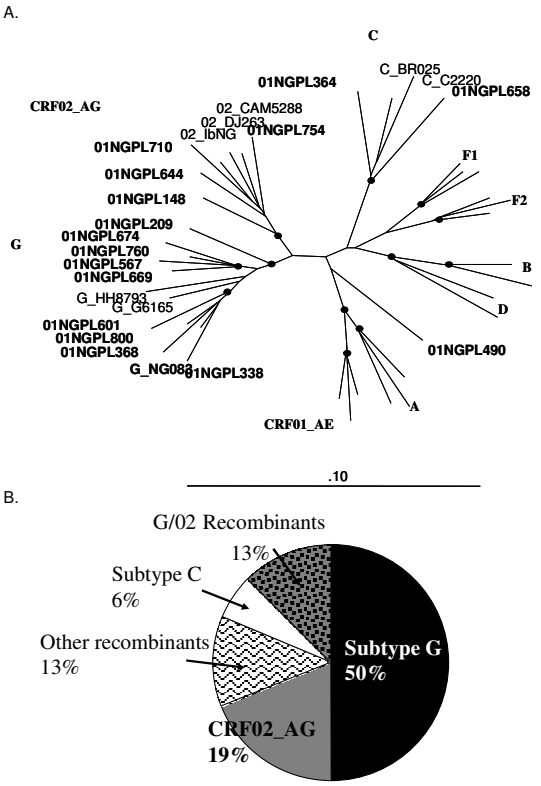

Figure 1. Phylogenetic analysis of Nigerian Pro/RT sequences from HIV-1 positive pregnant women from Jos, Nigeria. Neighbor-joining analysis was performed using the Kimura 2-parameter (gamma) method of distance estimation; bootstrap values were computed using Neighbor-joining and values > 70% is indicated with dots at the respective nodes. A) Radial phylogenetic tree of 16 Nigerian protease/RT sequences with reference sequences of different subtypes. Nigerian sequences (in Bold), reference sequences (small type). B) Distribution of HIV-1 subtypes among infected pregnant women in Jos, Nigeria. The pie chart demonstrates the proportion of the women infected with each genetic form.

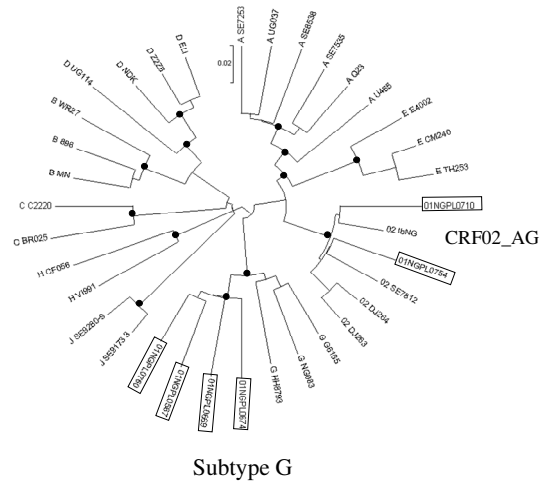

Figure 2. Circular phylogenetic tree of 6 nearly full genome sequences with reference sequences of different subtypes. Nigerian sequences (boxed), reference sequences (not boxed). Full-length genomes confirm PRO/RT classification.

isolated several months after NVP prophylaxis.

DISCUSSION

Our analysis provides evidence for the presence of subtype G and CRF02_AG isolates in Jos, nine years earlier the first isolate from Jos, Nigeria was characterized as a subtype G (Gao et al., 1998; Howard et al., 1994). This strain was distinct from the CRF02_AG proto-type IbNG isolate from Ibadan, Nigeria, which was mostly subtype A. Other studies have documented the presence of subtypes A and G, CRF02_AG and various forms of AG recombinants as well as a low level prevalence of subtype C (Peters et al., 2000; Agwale et al., 2002) in different geographic regions of Nigeria. The presence of mixed circulating recombinants in Nigeria is confirmed and extended by the contribution of 6 nearly full length genomes from Jos, Nigeria in this study (Figure 2).

This study demonstrates for the first time in this population the high prevalence of secondary resistance associated mutations in Protease present before the initiation of therapy. There were secondary mutation sites at codons 10, 20, 36, 63 and 82 for most of the strains and mutation I93L for the subtype C strains. The prevalence of these mutations in this population differs from those described for subtype B viruses in the same codons. Thus while the frequency of mutations at codons 10, 20, and 36 was 2-7% among drug naïve individuals with subtype B infection, it was higher (21.74, 100 and 105%) in this study and in other studies (Hirsch et al., 2000; Konings et al., 2004; Fonjungo et al., 2002) of non-B viruses. These data provide baseline information on the level of drug resistance in this environment, where access to antiretroviral therapy is being scaled up. Vergne et al. (2000) did not find any primary mutations associated with NRTI or PI in non-B HIV-1 isolates from 142 ARV- naïve patients, but reported the presence of many secondary mutations, especially at codons 36, 10,63,20,77 and 71. The mutation at position 63 is poly-morphic and most of the isolates harbored the L63P mutation, and three viruses (two CRF02-AG and one subtype C) showed the K238R polymorphism that might be linked to the novel mutation (K238S) associated with niverapine resistance (Hachiya et al., 2004). Gonzales et al. (2001) had shown that viral differences occur among amino acid substitutions related to drug resistance in subtype B and non-B isolates. They also revealed that secondary mutations in positions 10, 20 and 36 of the protease gene are more common in non-B viruses. Several other studies in non- subtype B viruses show that secondary mutations are common before treatment, suggesting there could be background effect on the susceptibility to ARVs. Our study is consistent with these reports on the high prevalence of secondary mutations that are associated with susceptibility to antiretroviral treatment in the Pol region in treatment naïve isolates of non B subtypes, and could impact significantly on susceptibility to PIs. The relevance of our find-

ings will need to be evaluated in these women or other persons undergoing therapy in order to understand their relationship to the evolution of primary resistance mutations in specific geographic clades. Already, it has been suggested that the presence of secondary mutation M36I at baseline has the potential for predicting the appearance of the primary mutation L90M, which is associated with resistance to all protease inhibitors, at virological failure in an Italian cohort with a high proportion of non subtype B isolates of HIV (Perno et al., 2004).

The primary mutations observed at RT codons 184 and 103 associated with 3TC and NVP resistance in the two drug-experienced women are of concern especially in the treated population where niverapine prophylaxis is already in use. It is an indication of a high probability of the evolution of widespread resistance to protease and reverse transcriptase inhibitors in this population where subtype G and its recombinants are prevalent. A preliminary study had shown that the niverapine resistance mutations (K103N) were readily selected in Ugandan women who received a single dose prophylactic regimen at the onset of labor in the Phase I/II trial HIVNET 006 (Jackson et al., 2000).

Conclusion

Our results support previous studies that subtypes G and AG recombinants are predominant in Nigeria, but the presence of new recombinant forms and other subtypes not known to commonly circulate may further complicate the epidemiology of HIV in this region. While the absence of primary resistance mutations among the treatment naïve isolates portends well for the use of PIs and other nucleotide and non-nucleoside reverse transcriptase inhibitors, care must be taken in the management of adherence and treatment administration in order to prevent the development and transmission of resistance given the high background of secondary mutations among HIV-1 isolates observed in this study. Our study clearly underscores the need for continuous monitoring of the HIV epidemic in Nigeria especially with the significant increase in HIV prevention and treatment programs such as the PMTCT and PEPFAR.

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GENE SEQUENCES

The sequences of the pro/RT are found in Genbank accession numbers DQ013268-DQ013283. Disclaimer: The views and opinions expressed herein do not necessarily reflect those of the U.S. Army or the Department of Defense.

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