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Research article

Frequency of Drug-Resistant Variants of HIV-1 Coexistent With Wild-Type in Treatment-Naive Patients of India

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Abstract

Context: Over the past few years, reports of emergence and transmission of drug-resistant strains of HIV have increased, especially in western countries. In the context of increased widespread use of zidovudine- and lamivudine-based combinations in India, coupled with the genetic diversity of HIV, it is essential to generate preliminary data on the frequency of zidovudine- and lamivudine-resistant variants of HIV-1 in North India.

Objectives: In the present study, the authors screened for mutations in the *pol* gene of HIV-1 associated with resistance to zidovudine and lamivudine in HIV-infected treatment-naive patients from North India.

Design and Patients: The mutations were screened at codons 70 and 215 (conferring resistance to zidovudine) and at codon 184 (conferring resistance to lamivudine) by using a nested amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) approach from the proviral DNA of 60 patients.

Results: Most of the patients showed a mixture of both wild-type and mutant virus. In all but 1 patient, wild-type virus was observed with respect to each codon. Mutant variants were also observed in many patients, especially at codon 70 (48 patients [80%]) and codon 184 (19 patients [31.67%]). In contrast, the frequency of mutation at codon 215 was found to be very low (1 patient [1.67%]).

Conclusion: In this sample of treatment-naive HIV-1-infected patients in North India, a high proportion of mutant variants harbored mutations in the *pol* gene at codons- 70 and 184 coexisting with wild-type HIV-1.

Introduction

The emergence and transmission of drug-resistant variants of HIV-1 can limit the therapeutic options of newly infected patients. There are several reports on drug resistance in treatment-naive individuals infected with subtype B of HIV-1 or its recombinants. Reports from North America and Europe indicate that up to 14% of recently infected patients carry a strain of virus with either well-characterized drug resistance mutations (in 1% to 10% cases) or reduced susceptibility to a particular drug (2% to 14% of cases).[1-3] In pregnant women, primary resistance to nucleoside reverse transcriptase inhibitors has

increased up to 17%[4]. In Africa and South Asia, including India, where most of the world's HIV-infected patients and most of those infected with subtype C live, there is very little information on drug resistance in HIV-infected people.

The frequency at which drug-resistant reverse transcriptase variants occur in evolving HIV-1 populations depends on the mutation frequencies in the *pol* gene, viral load, and the number of mutations needed to confer the resistance phenotype.[5,6] For some drugs, such as zidovudine, high-level resistance requires accumulation of 3 or more mutations in a single viral genome (mainly involving codons 215 and 70 along with codons 210, 219, 41, or 67).[7,8] In other drugs, such as lamivudine, a single mutation (at codon 184) can confer high-level resistance and drug-resistance mutations can be established within weeks. The mutations associated with primary resistance persist for at least 7 years; however, M184V/I mutation may revert after 1 year because of reduced replication fitness.[9]

Our study was designed to obtain preliminary data on the frequency of drug-resistant variants of HIV-1 in a North Indian population. We performed genotyping in 60 treatment-naive patients to detect primary mutations associated with resistance to zidovudine, lamivudine, or both in the *pol* gene of HIV-1. We used a highly sensitive nested amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) approach, which allowed us to identify the minority population of the virus harboring these mutations among the predominant wild-type population. In the case of zidovudine, we targeted the 2 most common resistance-associated mutations at codons 70 and 215 of the *pol* gene, while for lamivudine we chose codon 184 to detect mutations.

Materials and methods

Patient Recruitment

Mutation studies were performed in 60 consecutive HIV-1-infected individuals from North India who came to a voluntary counseling and testing center in the Department of Immunopathology, Post Graduate Institute of Medical Education and Research, Chandigarh, India, for testing and routine measurement of CD4+/CD8+ cell counts between January 2000 and December 2002. Patients were randomly recruited without any disease status bias. None of the patients had received any antiretroviral therapy before the beginning of the study. The age of the patients ranged from 1 -to 67 years (mean, 30.8 ± 11.5 years). Twenty-nine men, 23 women (none of whom were pregnant), and 8 children were recruited. The patients' mean CD4+ cell count was 276.7 ± 238.1 cells/mcL, while their mean CD8+ cell count was 999.1 ± 598.4 cells/mcL. The mean CD4+/CD8+ ratio was 0.28 ± 0.22. Thirty patients had CD4+ cell counts above 200 cells/mcL, and 30 patients had CD4+ cell counts below 200 cells/mcL. After written consent was obtained from each patient, blood was drawn in a K_3 EDTA *Vacutainer* (Becton, Dickinson and Company, Franklin Lakes, New Jersey) and was centrifuged at 2500 rpm for 10 minutes to separate plasma and buffy coat (consisting of leukocytes).

DNA Extraction and pol *Gene Amplification*

DNA was extracted from leukocytes with some modifications.[10] In the first PCR, extracted DNA was used to amplify a 768 bp fragment of the *pol* gene (encompassing codons 70, 184, and 215) using primers POL Pr-1 and POL Pr-2.[11] We modified the primers as published for clade- B according to the sequences of consensus regions in the *pol* gene of HIV-1 clade C isolates from India[12] by extending their 3' ends.

Identification of Mutations (ARMS-PCR)

An amplified *pol* gene fragment (768 bp) was used as the template to detect wild and mutant sequences at codon 184 by ARMS-PCR using codon-specific primers. Briefly, the downstream primers, 184W, 184G, and 184I, were paired separately with 184U as the common upstream primer to detect the codons Met (wild), Val (mutant), and Ile (mutant), respectively, at codon 184 of the *pol* gene.[13] Mutations at codons 70 and 215 were also analyzed in a similar manner,[11] using primers modified from previously published sequences.[12] The PCR products were separated on 2% agarose gel (Sigma; Saint Louis, Missouri) and documented in a gel documentation system (Image Master VDS; Pharmacia Biotech; Sweden). The sequences (5' 3') of the primers (custom synthesized from SigmaGenosys; The Woodlands, Texas) were as follows:

POL Pr-1: TTC CCA TTA GTC CTA TTG AAA CTG T

POL Pr-2: TCA TTG ACA GTC CAG CTA TCC TTT T

184U: TAC AAT GTG CTT CCA CAG GG

184W: TCC TAC ATA CAA ATC ATC CAT

184G: CCT ACA TAC AAA TCA TCC AC

184I: GAT CCT ACA TAC AAA TCA TCT

Pr-2W: CT GAA ATC TAC TAA TTT TCT CCA CT

Pr-2M: CT GAA ATC TAC TAA TTT TCT CCA CC

Pr-B: GGA TGG AAA GGA TCA CCA GCA

Pr-3W: TGA TGT TTC TTG TCT GGT GTG GT

Pr-3M: TGA TGT TTC TTG TCT GGT GTG AA

The amplification of the *pol* gene and subsequent detection of wild/mutant sequences at codons 70, 184, and 215 in the *pol* gene were cross-checked by using a wild-type control (clone of HIV-1 subtype C in pNL4-3 vector, provided by Dr. Vijay Chaudhary, Department of Biochemistry, Delhi University, New Delhi, India). Also, to check whether the primers used in our study were sensitive enough to detect the subtype B strain of HIV as well, we used another clone of HIV-1 subtype B, cloned in pNL4-3 vector and provided by Dr. Shahid Jameel, International Centre for Genetic Engineering and Biology, New Delhi, India.

Sequencing

The amplified *pol* gene fragments of 10 representative samples were subjected to sequencing and were analyzed. The PCR products were purified by using a PCR product purification kit (Invitek; Berlin, Germany) according to the manufacturer's instructions. The purified PCR products were sequenced with the forward primer Pol F1 (5'-GCC TGA AAA TCC ATA TAA CAC TCC-3') on an automated DNA sequencer (ABI Prism 3100, version 3.0, Applied Biosystems Inc., Foster City, California) using the Big Dye terminator cycle sequencing kit (version 3.1) supplied by Applied Biosystems.

Statistical Analysis

We used the chi-square test (^{2} test) to analyze the significance of appearance of mutations at different codons in correlation with CD4+ cell counts and patients' age. The significance of appearance of mutations among men, women, and children was analyzed by using 1-way analysis of variance.

Results

Mutations at Codon 184

Point mutations at codon 184 (MetVal/Ile) in the *pol* gene of HIV are known to confer high-level resistance to lamivudine. While amplification of wild-type sequence (Met) was observed in all of the patients, 19 patients (31.67%) also showed presence of mutant variants (Val, Ile, or both) as well. Of these patients, 16 (84%) showed the presence of both Val and Ile variants (26.67% of the total patients) and 3 (16%) showed Val (5.0% of the total patients) coexisting with the wild type (Table 1).

Mutations at Codon 215

Mutation at codon 215 (ThrPhe) is known to be associated with resistance to zidovudine as one of the primary mutations. In case of codon 215, all patients showed the presence of wild-type virus. Mutation was seen in only 1 patient, indicating a very low frequency (1.67%) (Table 1).

Mutations at Codon 70

Mutation at codon 70 (Lys Arg) is also known to be one of the primary mutations associated with resistance to zidovudine. Of the 60 patients, 47 (78.33%) showed a mixture of both wild and mutant types of the virus, while 1 patient (1.67%) showed only the mutant type of the virus carrying the codon 70 mutation (Table 1).

The PCR products of each codon are shown in the Figure.

Sequence Analysis

The sequences of all of the samples were analyzed by using the ClustalX alignment software, version 1.81. The amplified *pol* gene sequences of all the 10 samples showed the presence of wild-type sequence at codons 70, 184, and 215, indicating that these were predominantly available variants.

Correlation of Appearance of Mutations with CD4+ Cell Counts, Age, and Sex of Patients

Nineteen patients had lamivudine-associated mutations. Of these 19 patients, 10 had CD4+ cell counts below 200 cells/mcL and 9 had CD4+ cell counts above 200 cells/mcL (Table 2). There was no significant correlation between the appearance of mutations at codon 184 and CD4+ cell counts in these patients ($^2 = 0.077$; $P > .05$). Of the 48 patients showing zidovudine-associated mutations at codon 70, 23 had CD4+ cell counts below 200 cells/

Table 1: Composite Mutation Profile of the 60 Patients, at Different Codon Positions in the *pol* Gene

Codon Position	Resistance to Drug	Number of Patients With Wild Type	Number of Patients With Mutation Type	Proportion of Patients Showing Mutations
184	Lamivudine	60 (Met)	19 (16: Val & Ile) (3: Val only)	31.67%
215	Zidovudine	60 (Thr)	1 (Phe)	1.67%
70	Zidovudine	59 (Lys)	48 (Arg)	80%

Table 2: Characteristics of Patients With Respect to Mutations Observed in the *pol* Gene

Mutation Position	Age		CD4+ Cell Counts		Gender		
	< 30.8 years (n = 32)	> 30.8 years (n = 28)	< 200 cells/ mcL (n = 30)	> 200 cells/ mcL (n = 30)	Men (n = 29)	Women (n = 23)	Children (n = 8)
Codon 184	11	8	10	9	10	7	2
Codon 70	28	20	23	25	24	21	3
Codon 215	0	1	1	0	1	0	0

mcL and 25 had CD4+ cell counts above 200 cells/mcL, which is again nonsignificant (^{2} = 0.157 P > .05). With respect to age, there were 39 patients above the mean age (ie 30.8 ± 11.5 years) and 21 patients below it; of the latter group, 8 were children. The relationship between the age of patients and the appearance of mutations at codons 70 (^{2} = 2.41; P > .05) and 184 (^{2} = 0.307; P > .05), respectively, was also found to be nonsignificant. However, for codon 70, the mutation frequency in children was low compared with adults (1-way analysis of variance = 0.03; P < .05).

When we compared the appearance of mutations at codons 70 and 184 between men and women, the difference in appearance of mutations was nonsignificant (P > .05), indicating no relation with sex.

Discussion

In a country where an estimated 5.1 million people are living with HIV, information on the prevalence as well as the transmission of drug-resistant HIV strains would aid design of optimal drug regimens and clinical management. Our study was designed to obtain preliminary data on the frequency of drug-resistant variants in a treatment-naive North Indian population. The study involved patients from North Indian states (Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir, and Chandigarh) who were not taking any antiretroviral drugs. We performed genotyping to detect mutations associated with resistance to zidovudine and lamivudine, the most common first-line drugs administered in India, as well as the rest of the world as part of antiretroviral regimens. We used a highly sensitive, nested ARMS-PCR approach to detect these mutations so that the minority population of the virus harboring the mutations could be detected among the predominant wild-type population.

The mutation T215F causes intermediate (about 10-fold) resistance to zidovudine. The point mutation K70R causes low-level (4- to 5-fold) resistance to zidovudine and is usually the first drug resistance mutation to develop in patients receiving zidovudine monotherapy.[14,15]

Table 3: *Pol* Gene Mutation Profile in Different Countries as Observed by Different Groups

Country	Codon 70 (K70 R)	Codon 215 (T215 F/Y)	Codon 184 (M184V/I)
Spain[6]	22.8%	0	
Israel[22]	--	100% (clade B)	7/14 in clade B 0/20 in clade C
United States (infants)[21]		199294 = 6.3% 199899 = 33.3%	
Poland[29]	81.8%		33.3%
Ivory Coast[34]		39.7% to any of the NRTI drugs	
Martinique (French West Indies)[24]	17.15%	2.85%	5.71%
Present study (North India)	80%	1.67%	31.67%

NRTI = nucleoside reverse transcriptase inhibitor.

Mutations at positions 70 and 215 are antagonistic in their effect on zidovudine resistance and rarely occur together unless additional nucleotide excision mutations are present.[16] Both K70R and T215F cause reproducible reductions in zidovudine susceptibility regardless of the susceptibility assay used. Until recently, zidovudine monotherapy was also offered to HIV-infected patients and pregnant women to stop vertical transmission.[17] The data obtained from our study revealed a low frequency of codon 215 mutations (1.67%) compared with codon 70 (80%) and codon 184 (31.67%). The fact that codon 215 mutations have a relatively low level of fitness may explain their low frequency.[18] Moreover, the codon 215 mutation involves substitution of 2 bases (ACCTTC). Prevalence of these mutations is also very low worldwide, varying between 1% and 8% in treatment-naive HIV-infected populations.[3,19,20] However, in a US study of mother-to-child transmission, the authors investigated codon 215 mutation by nested PCR approach in 49 HIV-1-infected infants born between 1992 and 1999.[21] They observed that 6.3% of the infants born between 1992 and 1994 showed codon 215 mutation but that this figure increased to 33.3% in infants born between 1998 and 1999 (Table 3). In a comparative study in Israel of genotypic variation of reverse transcriptase between clade C and clade B strains of HIV-1, the authors reported a 100% mutation rate (T215F/Y) at codon 215 in 14 patients who had clade B strain. However, none of the 20 patients with clade C isolates carried the codon 215 mutation.[22] This indicates that mutations at codon 215 are more prevalent in clade B than in clade C. In another study, reverse transcriptase mutations M41L, L210W, and, to a lesser extent, T215Y were less prevalent in patients infected with non-B variants[23]. In some parts of the world, the transmission of codon 215 mutant variants has decreased, possibly because of the use of improved regimens.[20] Such studies highlight the fact that significant genotypic variation may occur in certain regions of the reverse transcriptase gene of clade C and clade B strains of HIV. In Africa, South America, and the Caribbean, he prevalence of HIV strains with at least 1 primary drug-resistant mutation is low (less than 7%), as evidenced by a few reports on drug resistance based on sequencing of the *pol* gene.[24-28] In Africa and South Asia (including India), where most of the world's HIV-infected patients and most of those infected with subtype C live, there is very little information on drug resistance in HIV-infected people. With recent increases in the use of zidovudine-based combinations in India, future studies on this issue should assess the transmission pattern of clade C drug-resistant variants, which are prevalent in India.

In contrast to codon 215, the results of codon 70 PCR paint an intriguing picture. Of the 60 patients, 78.33% showed mutant variants coexisting with the wild type, while 1 patient (1.67%) carried pure mutant type (K70R). Many reports on drug resistance-associated mutations in treatment-naive patients have been published, but none of them have reported such high levels of codon 70 mutation. This may be because most of these studies have reported the genotype of the virus isolated from the plasma by sequencing methods, which normally detect the predominant viral population in the circulation and may miss the minority viral populations that may harbor the mutations. However, a study from Warsaw, Poland, has also reported high percentage of codon 70 mutations in treatment-naive patients. The authors identified K70R mutation in 54 of 66 patients (81.8%) and an M184V mutation in 22 cases (33.3%) by using a line-probe assay[29] (Table 3). In another study from Martinique in the French West Indies, genotypic resistance was studied by using a line-probe assay in samples of plasma HIV RNA collected between 1988 and 1998 from 70 antiretroviral-naive patients.[24] Most of these patients showed a mixed population, and 17 harbored mutated viruses with 1 or more mutations in the reverse transcriptase gene codons

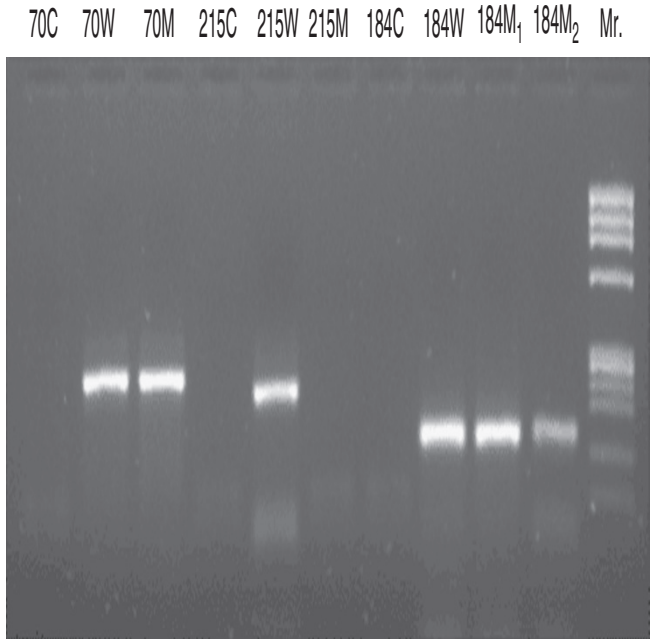

Figure 1
A 2% agarose gel stained with ethidium bromide showing products of codon-specific PCRs from a patient's pol gene fragment. The patient showed wild-type and mutated codons at position 70 (227 bp fragment in lanes 70 W and 70 M), wild type at codon 215 (210 bp fragment in lane 215 W), and both mutations along with wild type at codon 184 (134 bp fragment in lanes 184 W, 184M1, and 184M2). Mr: Molecular weight marker (phi X174, Hae III digested).

analyzed. Zidovudine resistance mutations T215Y/F, M41L, and K70R were found in 2, 5, and 12 individuals, respectively. Mutant strains of M184V were detected in 4 patients.

The results of codon 70 and 215 in our study have also confirmed that both these mutations rarely coexist. Although these mutant variants were present along with wild-type virus, such mutations would always be a threat to antiretroviral therapy because they are the reservoirs of phenotypic resistance to zidovudine. Regular resistance genotyping of patients with such mutations thus becomes important once patients begin antiretroviral therapy. Another important mutation detected in our study population was M184V/I, which is usually the first to develop in isolates from patients receiving lamivudine-containing regimens.[13] Considering that all of the patients were treatment-naive, the proportion of patients showing mutant variants (31.67%) still represents a high number even though all of them also had wild-type virus. Other studies have reported mutation rates of codon 184 ranging from of 2%,[3] 7%,[30], and 5%[1] in the United States to as high as 33.3% in Poland[29] among treatment-naive HIV-infected patients.

In contrast to the results of ARMS-PCR, the results of sequencing showed the presence of wild-type sequence at codons 70, 184, and 215 as observed in the few representative samples, indicating this to be the predominant variety. Therefore, to confirm whether the results obtained by ARMS-PCR were correct, we used another approach. We used the clones of HIV-1 subtype B and subtype C that were pure for the wild type. None of these clones showed amplification of mutant sequence in the PCR reactions of codons -70, 184, and 215. This indicates that the mutations seen in the patient samples represented the minority population of the total viral population, which is predominantly of the wild type. Such variations in the sequencing versus other genotyping approaches have been observed earlier as well; the available sequencing assays have been shown to identify only the resistance profile of the predominant viral variant in the infected patient.[6,31] Moreover, it has also been shown that the targeted genotyping approaches are much more sensitive than the common sequencing approaches.[32,33] In one such study, the sensitivity and discriminatory power of the ARMS-PCR were evaluated, and their performance for the detection of drug resistance at codons 151 and 215 in mixed genotypic populations of the reverse transcriptase gene of HIV-1 were compared with T7 cycle sequencing, the line probe assay, and the recombinant virus assay. [33] The line-probe assay and ARMS-PCR detected minor variants that in particular cases made up only 1% of the viral population.

In conclusion, our study indicates that the proportion of mutant variants harboring mutations in the *pol* gene at codons 70 and 184 coexisting with wild-type HIV-1 is high in treatment-naive patients in North India. The appearance of these mutations had no correlation with CD4+ cell counts or sex of the patients, although mutant variants seemed to be less common in children. Finally, the genetic diversity of HIV suggests that targeted genotyping resistance assays applicable for different HIV-1 subtypes can be used to determine HIV drug resistance in different parts of the world.

Authors and Disclosures

Naresh Sachdeva, MSc, has disclosed no relevant financial relationships.

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