

Zero prevalence of primary drug resistance-associated mutations to protease inhibitors in HIV-1 drug-naive patients in and around Aligarh, India

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Abstract

Introduction: This study aimed to evaluate the prevalence of resistance mutations in the protease gene of HIV-1 strains isolated from north Indian antiretroviral (ARV) treatment-naive patients and to assess the phylogenetic relatedness of these strains with known HIV-1 strains.

Methodology: Fifty-four HIV-1 strains isolated from treatment-naive patients (n = 54) were included in this study. Resistance genotyping for the protease gene was performed using semi-nested PCR and DNA sequencing. The sequences were aligned (ClustalW) and a phylogenetic tree was built (MEGA 4 software). Drug resistance (DR) pattern was analyzed using the Stanford HIV-DR database and the IAS-USA mutation list. For subtyping purposes, all the nucleotide sequences were submitted to the REGA HIV-1 subtyping tool version 2.01.

Results: All the strains (100%) were found to belong to the C subtype and to harbor at least two secondary mutations in the protease gene. The most frequent mutations were H69K and I93L (52 of 52 strains), followed by I15V (80.7%), L19I (69.2%), M36I (67.3%), R41K (94.2%), L63P (61.5%), and L89M (82.7%).

Conclusion: This study confirms that HIV-1 subtype C predominates in northern India. Protease secondary mutations associated with drug resistance to protease inhibitors (PIs) were present with high frequency in the HIV-1 C subtype strains isolated from north Indian ARV treatment-naive patients, but no primary resistance mutations were found in this region. We suggest that resistance testing in HIV-1 infected patients should ideally be performed before the initiation of therapy to tailor the treatment for the individual to achieve the optimal therapeutic outcome

Key words: HIV; protease gene; drug resistance mutations

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Introduction

At the end of 2012, there were approximately 35.3 (32.2–38.8) million people living with HIV worldwide as per UNAIDS global report 2013[1]. It has been estimated that approximately 2.31 million people are living with HIV/AIDS in India [2] and nearly 9.7 million people are receiving anti-retroviral therapy (ART) in low- and middle-income countries by the end of 2012 as per UNAIDS global report 2013. ART is the best and only option to reduce morbidity and mortality associated with HIV infection [3]. A major obstacle to the long-term efficacy of ART is the emergence of drug resistance mutations in the polymerase gene of HIV-1, which reduces the susceptibility of the gene to anti-retroviral (ARV) drugs [4]. Factors that are associated with the development of drug resistance include use of

monotherapy, inadequate suppression of viral replication, adherence to ART drugs, and initiation of therapy late in the course of HIV infection [4].

The high mutation rate of the HIV genome is associated with the low fidelity of the reverse transcriptase (RT) and the lack of proofreading function; eventually, the high selective pressure of the specific drugs lead to increases in genetic mutation, causing resistance to current ART therapy [5]. Due to the widespread use of antiretroviral drugs, it has been hypothesized that there will be an increase in the transmission rate of drug-resistant viruses, which will raise the prevalence of resistant variants. The transmission of resistant variants to uninfected individuals will cause serious clinical and public health problems.

The variability at the HIV-1 protease gene may account for differences in drug resistance pathways and distinct susceptibility to protease inhibitors (PIs) [6]. Thus, the growing diversity of HIV-1 protease gene sequences in India highlights the need for closer epidemiological characterization of viral isolates prior to the introduction of new PIs. PIs were introduced after 2008 by the Indian government in the ART program of India as second-line treatment regimens [2]. Resistance development may differ according to primary, secondary, or polymorphic mutations [7]. Primary mutation leads to reduced drug susceptibility directly, whereas secondary mutation leads to resistance in conjunction with primary mutation [8].

The current knowledge of the prevalence of drug resistance to ARV drugs in India is limited [9]. Several studies have examined the drug resistance pattern for the subtype B population, but there is a paucity of data related to the drug resistance pattern among the HIV-1 subtype C that predominates in this region. The purpose of this study was to investigate the polymorphisms in the entire protease gene region among drug-naive HIV-1-infected patients and to assess the phylogenetic relatedness of these strains to known HIV-1 strains.

Methodology

Patients and samples

The population of this study was composed of 54 newly diagnosed individuals enrolled between 2010 and 2011 and followed at the Integrated Counseling and Testing Centre (ICTC) center in the Department of Microbiology and ART Center, Jawaharlal Nehru Medical College (JNMC), Faculty of Medicine, Aligarh Muslim University (AMU), Aligarh, India. Informed consent and detailed history was obtained from all the patients before sampling. The study was approved by the Institutional Ethics Committee, JNMC Faculty of Medicine, AMU. Five milliliters of whole blood samples were drawn into K3 EDTA vacutainers (Becton Dickinson, Franklin Lakes, USA). CD4⁺ T cell count was enumerated by flow cytometry (PartecCyFlow Counter, Munster, Germany) and cell count was established according to the instructions of the Flow Cytometer manufacturer. PCR and DNA sequencing were performed at the National Centre for Disease Control (NCDC), New Delhi. HIV viral load quantification was done at SRL diagnostic Reference Lab by real time PCR (Rotor Gene 6000) using an HI Virus-1 RG RT-PCR kit (Vela diagnostic, Singapore, Malaysia).

Pro-viral DNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples using standard HISTOPAQUE-1077 (Sigma-Aldrich, St. Louis, USA) density gradient centrifugation method per the manufacturer's instructions. Proviral DNA was extracted from the PBMCs using a QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The DNA was finally stored at -20°C.

PCR and DNA sequencing

For genotypic analysis of the protease gene, outer PCR was performed using GoTaqGreen Master Mix (Promega, Madison, USA) with forward primer Pro-1 5'-ACC AGA GCC AAC AGC CCC ACC A-3' and reverse primer Pro-2 5'-CTT TTG GGC CAT CCA TTC CTG GC -3'. Semi nested PCR was performed using Pro-3 GAA GCA GGA GCC GAT AGA CAA GG and Pro-2 primers (OPERON Biotechnologies, Cologne, Germany). The primers were slightly modified from a previously published study [10]. The cycling conditions were as follows: 95°C for 5 minutes for initial denaturation followed by 35 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 45 seconds followed by a final extension at 72°C for 7 minutes for outer PCR to amplify a 470-bp fragment. Semi-nested PCR was performed using 95°C for 5 minutes for initial denaturation followed by 27 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 60 seconds followed by a final extension at 72°C for 10 minutes for second round PCR to amplify a 394-bp fragment on Gene Amp PCR system 9700 (Applied Biosystems, Foster City, USA). After PCR, the amplified products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and evaluated under UV light.

ABI Prism Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) was used to perform sequencing PCR with Pro-3 and Pro-2 primers separately. The cycling condition was 96°C for 10 minutes, 50°C for 5 seconds, and 60°C for 4 minutes on the Gene Amp PCR system 9700 (Applied Biosystems, Foster City, USA). Sequencing of purified cycle sequencing PCR products was performed on 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA).

Phylogenetic analysis

Protease sequences were aligned with reference sequences of all known HIV-1 subtypes from the Los Alamos HIV sequence database

Table 1. Demographic and clinical data of the study population

Characteristics	Patients (n = 52)
Age (yrs)	
Mean \pm SD	33.5 \pm 7.11
Range	21-50
Sex	
Male/Female	28 (53.8%) / 24 (46.2%)
Risk factors	
Heterosexual	43 (82.7%)
Blood transfusion	8 (15.3%)
Intravenous drug user	1 (1.9%)
Educational status	
Literate/Illiterate	23 (44.2%) / 29 (55.8%)
Locality	
Urban/Rural	20 (38.5%) / 32 (61.5%)
Marital status	
Married	37
Unmarried	5
Widower	10
CD4+ cells count (cells/mm³)	
Median	194
IQR	(136.5-270)
Mean \pm SD	203.73 \pm 103.45
*HIV viral load (IU/mL)	
Median	16473
Range	72-2413

*The lower limit of detection of the assay is 4.5 IU/mL and the upper limit of quantification is 1×10^8 IU/mL. IQR: interquartile range

(<http://www.hiv.lanl.gov>). Phylogenetic analysis was performed using the software MEGA version 4 after multiple alignments of data by CLUSTAL X. Pairwise evolutionary distances were estimated using Kimura's two parameter method, and phylogenetic trees were constructed by the neighbor-joining method. The reliability of the topologies was estimated by performing bootstrap analysis (1000 replicates). All identified subtypes in this study referred only to the protease gene, not to the full length of the HIV-1 genome.

Genotypic drug resistance interpretation algorithms

The protease sequences were analyzed for drug resistance-associated mutations using the Stanford surveillance drug resistance mutation (SDRM) [11], International AIDS Society (IAS)-USA drug resistance mutation list [12], and the last rules of the French ANRS AC11 Resistance Group [13]. Primary drug resistance was also analyzed by the calibrated population resistance (CPR) tool using Stanford surveillance drug resistance mutations. Subtyping was done using the REGA HIV-1 subtyping tool version 2.01 [14].

Statistical analysis

Demographic data, frequencies, mean, median, and standard deviation (SD) of major variables and percentages were analyzed using SPSS software version 17.0.

Results

A total of 54 individuals were studied between 2010 and 2011. Protease genes were successfully amplified and sequenced in 52 patients. Two samples could not be amplified, which could have been due to low viral loads. The main characteristics of this population were as follows: the mean age was 33.5 years (range 21-50 years), 53.8% of the patients were male, the median (IQR) CD4 cell count was 194 (136.5-270) cells/mm³, and the median HIV-1 RNA load was 16473 (range 72-2413) IU/mL, as shown in Table 1. The majority of the subjects (82.7%) acquired HIV infection through sexual contact. None of the patients received ART.

After analyzing the entire protease gene, all 52 patients (100%) were found to belong to subtype C (Figure 1), and all patients had at least two secondary mutations that are associated with resistance to PIs. However, none of the patient isolates contained primary resistance mutations within the PR gene.

Figure 1: Phylogenetic tree of the protease region for HIV-1 subtype C. References sequences included in the tree are obtained from the Los Alamos HIV database. Reference sequences are marked with circles and clinical samples are marked with squares.

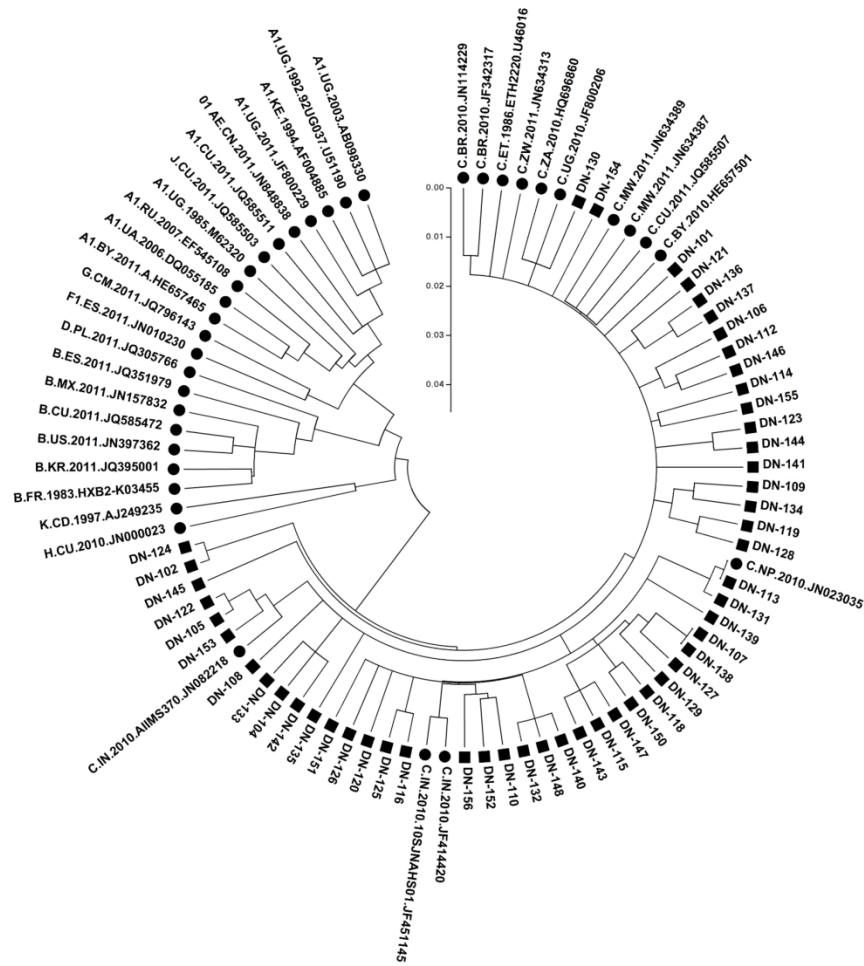
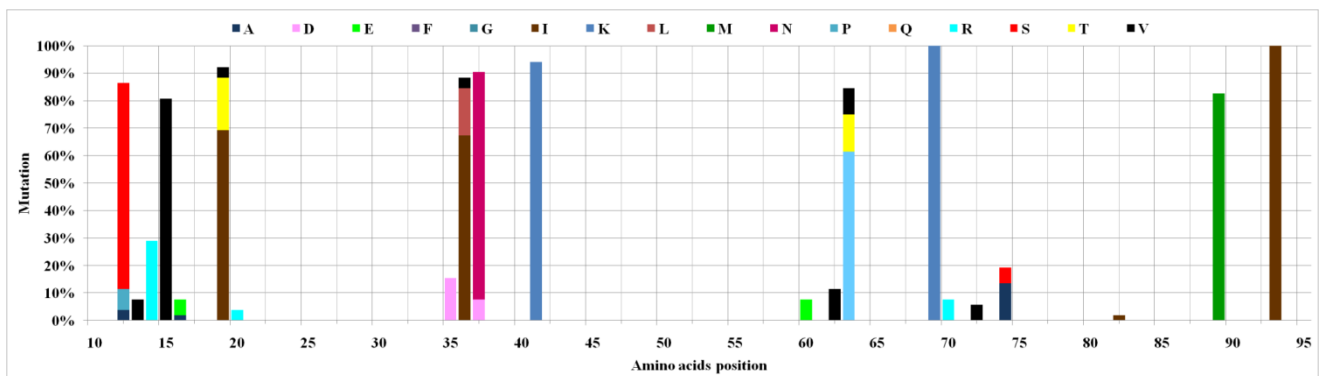


Figure 2: Frequency of mutations in the protease gene in HIV-1 subtype C drug-naive patients from position 10 to 99.



The majority of the samples had several simultaneous mutations in their PR gene. The most frequently observed positions were T12S (75%), I15V (80.7%), L19I (69.2%), M36I (67.3%), S37N (82.7%), R41K (94.2%), L63P (61.5%), H69K (100%), L89M (82.7%), and I93L (100%) (Figure 2). Among the mutations identified, three amino acids changes together, M36I/L/V, H69K, and L89M, occurred most frequently (80.7%) in the study population. Other secondary mutations were seen with lower frequencies: at positions I13V (7.7%), K14R (28.9%), G16E (5.7%), K20R/I (38%), E35D (15.4%), D60E (7.7%), I62V (11.5%), K70R (7.7%), I72V (5.7%), V82I (1.9%), as shown in Figure 2.

Discussion

Mutations conferring drug resistance in HIV-1 individuals have become a serious issue due to their transmission from treatment-experienced patients to newly infected patients in the majority of developed and developing countries, posing a major obstacle in successful antiretroviral therapy [10]. Primary drug resistance in HIV-1 has been a significant clinical and public health concern with the widespread use of antiretroviral therapy (ART) worldwide. The accessory mutations may not result in a significant decrease of sensitivity to ARV drugs, but they have been reported to be associated with increased viral fitness with primary mutations. It can therefore be hypothesized that the selection of primary mutations under ARV treatment pressure could precipitate the development of highly resistant HIV variants where one or more secondary mutations are already present [15]. In this study, we investigated the presence of ART drug-related amino acids changes in the protease gene of HIV-1 in and around the Aligarh district of north India that may harbor resistance to current PIs and may not yet be included in the current mutation list.

On analysis of the phylogenetic tree, HIV-1 protease gene sequences from our patients were found to be evolutionarily close to HIV-1 subtype C reference sequences. All the study subjects were found to be infected with HIV subtype C. Other studies also support the dominance of HIV-1 subtype C strains in India [2,10,16-18].

None of the subjects included in this study had received any antiretroviral treatment. In order to investigate the impact of HIV-1 C subtype on resistance before the onset of ARV treatment, we searched mutations known to be associated with resistance to protease inhibitors. No primary resistance

mutations were found, but every HIV-1 strain analyzed harbored at least two accessory or secondary mutations. However, the prevalence of primary HIV-DR in the protease gene has been reported to be 14.2% by Arora *et al.* in 2008 [10] and 1.4% by Sinha *et al.* in 2012 [2] in north India, while in southern and western India, it has been reported to be 20% by Balakrishnan *et al.* in 2005 [19], 2.5% by Lall *et al.* in 2008 [20], 2.7 % by Iqbal *et al.* in 2009 [21], 5.9% by Chaturbhuj *et al.* in 2010 [22], and 4% by Deshpande *et al.* in 2011 [23]. Several secondary drug resistance-associated mutations were also reported by some of these studies [2,10,19-23]. The M36I mutation in the protease gene (associated with resistance to ritonavir and nelfinavir in B subtype viruses) [15] was found in 35 (67.3%) strains. Ode *et al.* [24] found that the M36I, which is a non-active site mutation, decreases the volume of the binding cavity of the protease enzyme. Substitutions were also observed in other protease gene positions, some of them with rather high frequency: 12, 15, 19, 37, 41, 63, 69, 89, and 93.

The existence of M36I/R41K/H69K in the hinge and L89M in the α -helix of the C protease gene has been linked to increased catalytic activity [10]; this mutation pattern was seen in 31 (59.6%) strains in our study population. The other most common pattern of minor mutated positions were M36I, H69K, and L89M in 42 (80.7%) patients; these mutations are associated with reduced susceptibility and diminished virological response to TPV [6]. A recent study from Morocco reported an equally high prevalence of these mutations.

The well-recognized E35D mutation was seen in eight (15.4%) patients in our study. This mutation affects the conformational equilibrium between the closed and semi-open conformations of the free protease. It has been postulated that an E35D mutation reduces interaction with HLA B44, thus evading immune response as well conferring resistance [3].

L63P mutation strongly enhances viral replicative fitness in the presence of some ART drugs [25]; it was observed in 32 (61.5%) patients. Champenois *et al.* [26] observed that this substitution may reduce susceptibility to LPV, which is retained as a secondary resistance mutation to LPV by the ANRS algorithm [13]. Ten (19.2%) patients showed atypical T74A/S mutations that may be responsible for supplementing resistance to ATV, FSV, IDV, NFV, and saquinavir (SQV). The presence of K14R mutant may slightly increase resistance to darunavir (DRV) [27]. T74S mutation alone may reduce NFV susceptibility [28].

In conclusion, our study indicates that HIV-1 subtype C predominates in the Aligarh region of northern India and that significant secondary mutations related to resistance to PIs are also present. Based on our findings, we suggest the implementation of active screening for resistance to PIs in drug-naive populations for early detection of resistance. Drug resistance mutations in HIV-1 subtype C are only beginning to be reported, and the effects arising from the combination of these mutations with the already existing polymorphisms in subtype C are not yet clearly known. We suggest that resistance testing in HIV-1 infected patients should ideally be performed before the initiation of therapy to tailor the treatment for the individual to achieve optimal therapeutic outcome. Our study can extend the knowledge of the degree of heterogeneity present in the protease gene of HIV-1 clinical isolates and lead to a better understanding of the enzymatic properties of this viral protein, which would be invaluable for future drug development.

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