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Polymorphisms in HIV-1 Subtype C Reverse Transcriptase and Protease Genes in a Patient Cohort from Mumbai

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Abstract

Objectives: Developed countries have been alarmed at the rates of primary/transmitted drug resistance of HIV-1. There is a debate on the validity of certain polymorphisms in HIV-1 Subtype C mutations related to consensus Subtype B sequences being associated with and sometimes mistaken as, primary resistance. In this preliminary study, we have determined polymorphisms in reverse transcriptase (RT) and protease (PR) genes of HIV-1 Subtype C from a patient cohort in Mumbai, India.

Methods: The study was performed with plasma samples from twenty-four patients (antiretroviral therapy experienced as well as drug-naive) employing a 'home-brew' semi-nested reverse-transcriptase-PCR followed by sequencing and sequence analysis. The Stanford HIV drug-resistance database was used for analysis and interpretation of polymorphisms and other drug-resistance mutations. We also analysed Surveillance Drug Resistance Mutations (SDRMs) for *PR* gene.

Results: Polymorphisms were determined at a mutational frequency of 0.1337 ± 0.042 in *PR* gene and 0.067 ± 0.014 in RT gene, while 16.6% and 12.5% samples harboured drug-resistance mutations in PR and RT genes respectively. Substitutions greater than 50% were at positions at L19, V82, M36, R41, L63, H69, L89 and I93 for *PR* gene and D121, K122, T165, K166, K173, D177, T200, Q207 and R211 for *RT* gene. Additionally, *PR* gene SDRMs were observed in 15.0% samples.

Conclusions: Our findings concur with previous findings that polymorphisms in HIV-1 Subtype C from India exist and re-iterate that these polymorphisms may also include a number of major and minor/accessory mutations associated with resistance. Most of the drug-resistance databases available online are based on Subtype B; hence, we recommend that HIV Subtype-specific drug-resistance databases be created to empower routine and unambiguous surveillance of drug-resistance prior to initiating antiretroviral therapy, especially while including Protease Inhibitors.

Keywords: HIV-1 Subtype C; Polymorphisms; Drug resistance database; Reverse transcriptase; Polymerase

Introduction

The emergence of drug resistance is a threat to effective anti-retroviral therapy (ART). Mono-therapy and combination therapy using currently available drugs are implicative of emergence of drug-resistance or cross-resistant variants. A majority of the studies carried out on HIV are primarily on HIV subtype B and in developed countries. This is probably due to the lack of access to ART drugs in several developing countries where non-B subtypes are more prevalent. It has also been suggested that certain naturally occurring polymorphisms among different non-B subtypes can affect HIV-1 susceptibility to antiretroviral drugs (ARVs), although known mutations exist [1]. These findings have supported by both enzymatic and virological data and suggest that such polymorphisms can affect both the magnitude of resistance conferred by major mutations as well as the propensity to acquire certain resistance mutations, even though such differences are sometimes difficult to demonstrate in phenotypic assays [2].

Protease Inhibitors (PIs) are a class of drugs that have not been extensively used in India as yet and are not part of the national ART roll-out for first line therapy [3]. It is imperative that more effective drugs such as PIs are included in the future to curb the HIV-1 epidemic; there is a growing fear of primary or transmitted drug resistance, both in developed and developing countries. Although rates of transmitted drug-resistance in developing countries are currently still low [4], the presence of resistant isolates is likely to raise a discussion on the possible mechanisms of resistance to anti-retroviral drugs in inherently drug naive patients.

The detection of drug-resistant variants in the patient cohort in Mumbai serves as an indicator of the status of drug-resistant HIV-1 prevalent in the Indian sub-population; however, only a limited number of studies focusing on HIV-1 Subtype C protease drug resistance mutations and polymorphisms in India that have revealed that some of the mutations may not be drug-resistance associated [1,5-7].

Hence, we have made a preliminary attempt to determine the extent of polymorphisms in HIV-1 subtype C protease (PR) and reverse transcriptase (RT) genes in the patient cohort from Mumbai.

Materials and Methods

Study population

The present preliminary study was conducted with plasma specimens from 25 HIV sero-positive individuals, of whom 17 were receiving anti-retroviral therapy (ART group) and 8 were anti-retroviral drug naive (DN group). Inclusion criteria: all individuals were greater than 18 years of age, belonging to both sexes and attending the outpatient department of AIDS Research and Control Centre (ARCON), Sir JJ Hospital Complex, Mumbai. Exclusion criteria: individuals who were under the age of 18 and pregnant women were excluded from the study. Institutional Ethics Committee approval and written informed consent was acquired prior to blood collection.

Sample collection and processing

Whole blood was collected into vacuum collection tubes (Purple capped; K_3 EDTA Vacutainers; Becton, Dickinson and Company, Gurgaon, India). All samples were subject to confirmation of HIV sero-status as per national guidelines available for resource limited settings (NACO Guidelines on HIV Testing, 2007 [8]) prior to admission to ARCON and/or initiation of therapy. The samples were screened by at least two HIV-1/2 Rapid tests and/or further confirmed by HIV-1/2 ELISA using manufacturers' instructions. The whole blood was used immediately for the determination of CD4/CD8 counts using a BD FACSCount™ (BD Biosciences) and separated into plasma aliquots.

HIV Polymorphism Assay

HIV polymorphism assay was carried out using an in-house 'home-brew' method involving a semi-nested PCR and DNA Sequencing for determination of polymorphisms and drug-resistance associated mutations. The HIV RNA was extracted manually using a QIAamp® Viral RNA mini kit (Qiagen India Pvt. Ltd., New Delhi, India). The first round PCR was carried out using a One-step RT-PCR kit (Qiagen) with outer primers *PR1*and *PR2* for the *PR* gene that yielded a 476-bp amplicon and Pol PR-1 and Pol PR-2 for the *RT* gene (Table 1) that yielded a 768-bp amplicon as described previously [9,10]. The PCR cycling conditions used for the outer round PCR were: 50°C/30 min; 95°C/10 min; [94°C/30 s; 59°C/30 s; 72°C/40 s]×40 cycles; 72°C/10 min. The second round PCR was carried out using a PCR Master Mix (Fermentas Inc., Maryland, USA) and semi-nested inner primers PR3 and PR2 for the *PR* gene that yielded a 395-bp amplicon and nested inner primers POL F1 and POL F2 for the *RT* gene (Table 2) that yielded a 540-bp amplicon as described previously [9,10]. The PCR cycling conditions for the inner round PCR were: 95°C/10 min; [94°C/30 s; 59°C/30 s; 72°C/30 s]× 25-35 cycles; 72°C/10 min. A non-template control (NTC) was included by the addition of nuclease-free water in place of template RNA or first round amplicon to serve as a negative control and a 'pIndie' plasmid (generously provided by Dr. Udaykumar Ranga; Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru, India) appropriately diluted to yield minimum 103 copies/mL was used as a positive control for each reaction.

Primer name	Region	Sequence 5'–3' (OUTER)
PR1 (forward)	PR	5'-ACC AGA GCC AAC AGC CCC ACC A-3'
PR2 (reverse)		5'-CTT TTG GGC CAT CCA TTC CTG GC-3'
POL Pr-1 (forward)	RT	5'-TTC CAA TTA GTC CCA TTG AAA CTG T-3'
POL Pr-2 (reverse)		5'-TCA TTG ACA GTC CAG CTA TCC TTT T-3'

Table 1: Outer primers used for PR and RT genes of HIV-1 subtype C

Primer name	Region	Sequence 5'–3'
PR3 (forward)	PR	5'-GAA GCA GGA GCC GAA AGA CAG GG-3'
PR2 (reverse)		5'-CTT TTG GGC CAT CCA TTC CTG GC-3'
POL F1 (forward)	RT	5'-GCC TGA AAA TCC ATA TAA CAC TCC-3'
POL F2 (reverse)		5'-CCA TCC AAA GAA ATG GGG GTT C -3'

Table 2: Inner primers used for PR and RT genes of HIV-1 subtype C

Purification of PCR products from the inner nested PCR was carried out using a QIAquick® Spin PCR Purification Kit (Qiagen) and sequencing of the purified PCR products was carried out on a third-party commercial basis (M/s Ocimum Biosolutions India Pvt. Ltd., Hyderabad) using an ABI 3730 X1 DNA analyzer (Applied Biosystems). The raw data of the sequences was analyzed using Chromas v2.33 (Technelysium Pty Ltd.) and Sequence Scanner v1.0 (Applied Biosystems) and saved in the FASTA format. The sequences were also analyzed for quality assessment using the Quality Control Tool [11], and manually edited using MEGA 5 software [12] before submitting to GenBank.

Mutation analyses

The sequences obtained were subjected to a National Center for Biological Information (NCBI; NIH) BLAST (Basic Local Alignment Search Tool [13]), HIV BLAST search (Los Alamos National Laboratory; LANL [14]) and REGA HIV-1 Automated Subtyping Tool [15] to confirm their subtypes.

The HIV polymorphism and drug-resistance mutations were analyzed and interpreted using the Stanford HIV drug resistance database [16]. The sequences were also analyzed using the Calibrated Population Resistance (CPR) tool available on the Stanford HIV drug resistance database and the HIVdb algorithm tool [17] to determine Surveillance Drug Resistance Mutations (SDRMs) and other polymorphisms.

Results

Of the total 25 sequences, 1 sequence could not be analyzed by the database and was eliminated from the study. All the remaining sequences were found to be HIV-1 Subtype C according to the REGA tool. The HIV polymorphism and drug-resistance mutations for 24 PR regions and 8 RT regions were analysed using the Stanford HIV drug resistance database and we determined polymorphisms at a mutational frequency of 0.1337 ± 0.042 in the PR gene and 0.0670 ± 0.014 in the RT gene. The mutation frequency is defined as the proportion of mutant positions relative to the consensus nucleotide sequence from each sample group; the frequencies have been calculated by dividing the number of mutations (relative to the consensus) by the total number of nucleotides sequenced.

The patterns of polymorphisms and mutations associated with drug resistance in the *PR* gene are depicted in Figure 1 and *RT* gene obtained in the sequences is depicted in Figure 2. Substitutions >50% were at positions T12, L19, M36, R41, L63, H69, V82, L89 & I93 for *PR* gene and at positions D121, K122, T165, K166, K173, D177, T200, Q207 and R211 for *RT* gene.

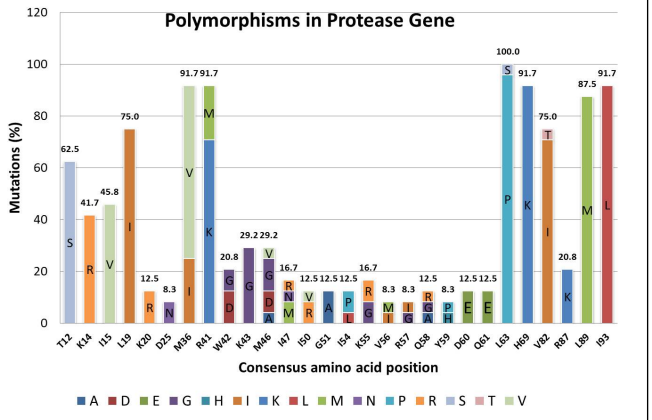

Figure 1: Patterns of polymorphisms and mutations associated with drug resistance in the *PR* gene of HIV-1 Subtype C. The alphabets represent single letter amino acid codes.

Additionally, we also determined that 16.6% harboured drug-resistance associated mutations in *PR* with minor amino acid substitutions M46V/D/G, I47M/N, G48R, and major amino acid substitutions I50V, I54L/P and V82T conferring resistance to PIs while 12.5% samples harboured drug-resistance associated mutations in *RT* genes with amino acid substitutions T69I, F77L, K219N conferring Nucleoside Reverse Transcriptase Inhibitor (NRTI) resistance and V179F conferring Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) resistance. Further, *PR* gene SDRMs were observed in 15.0% samples.

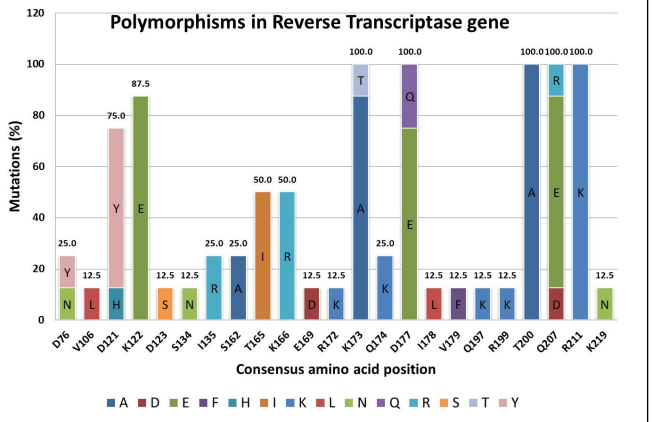

Figure 2: Patterns of polymorphisms and mutations associated with drug resistance in the *RT* gene of HIV-1 Subtype C. The alphabets represent single letter amino acid codes.

Discussion

The polymorphisms obtained were found to be concurrent with studies carried out previously in different regions of India-North India (Chandigarh [9], Delhi [18], Aligarh [19]), West India (Mumbai [20], Pune [21-23]) and South India (Bengaluru [1,24], Chennai [10,25-27] and Vellore [28]).

We also observed that some of the polymorphisms observed in other parts of the country were different than our findings. A study from Delhi showed major amino acid substitutions conferring resistance in the *RT* gene at positions M41, V90, A98, K103, V106, V108, E138, Y181, M184, G190, T215 and K219 [29].

It needs to be clarified that high prevalence rates may not reflect true cases of resistance because HIV-1 subtype C protease has several polymorphisms that are considered to be drug resistance mutation positions in subtype B (e.g., M36I, L63P and L89M which have been selected in Indian subtype C sequences [24]).

While this is a preliminary study, limited to 25 HIV sero-positive individuals, it may be deduced that in general that HIV-1 subtype C viruses in India have been exhibiting varying levels of primary or transmitted PI drug resistance. Although it has been observed that considerable genetic variability exists at subtype B drug resistance positions, treatment trials and phenotypic studies need to be carried out to ensure that baseline mutations have no bearing on antiretroviral drug resistance. Our study has shown that the Protease gene of HIV-1 subtype C is more prone to mutation than the Reverse Transcriptase gene. These results may be rationalized as a possibility of a greater fitness of the subtype C protease as depicted by a relatively high average mutational frequency. Our study has also shown a high level of primary drug resistance, and it may be deduced in general that HIV-1 subtype C viruses in India have been exhibiting varying levels of primary or transmitted PI drug resistance.

We concur with previous findings that it is now time that tools are optimized to assure accurate measurements of drug susceptibility in non-B subtypes and to recognize that each subtype may have a polymorphisms that may or may not affect their distinct resistance profiles.

Conclusion

In conclusion, our study presents preliminary data from 25 ART experienced and drug-naive patients, underlining the issue of the relatively high prevalence of polymorphism and transmitted drug resistance being reported in HIV-1 subtype C in India especially South India and Mumbai. We recommend that routine monitoring and surveillance of HIV drug resistance is increased such that the most appropriate treatment strategies may be developed prior to the inclusion of PIs in the treatment regimen. Our study re-iterates that polymorphisms in HIV-1 Subtype C from India may also include a number of major and minor/accessory mutations associated with resistance.

We further recommend that HIV Subtype-specific drug-resistance databases be created to empower routine and unambiguous surveillance of drug-resistance prior to initiating antiretroviral therapy.

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Author Disclosure Statement

No competing financial interests exist.

Sequence Data

GenBank accession numbers for the sequences reported in our study are JX982834 to JX982858 (PR gene) and KC710205 to KC710212 (RT gene).

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