GENETIC STUDY OF *VPU* VARIANTS AMONG THE HIV-1 INFECTED INTRAVENOUS DRUG USERS OF MANIPUR PREDICTS HIGHLY PATHOGENIC VIRUS

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Abstract

HIV-1 mutation at amino acid position 61 influences the stability, release of virus and toxicity. We have conducted a population based study on variability of vpu gene of HIV-1 among HIV-1 infected the intravenous drug users (IDUs) of Manipur, northeastern region of India. Forty three numbers of HIV infected IDUs blood samples have been studied by amplification and sequencing of vpu gene. 95.34% of samples have been found to possess S61 wild type and 4.65% of samples have mutation at S61 amino acid. S61 of vpu is highly conserved among the HIV infected IDUs of Manipur which has been known to enhance CD4 down regulation and degradation leading to high cell toxicity and moderate virus release. Phylogenetic tree analysis revealed that virus is circulated among the IDUs of Manipur. These findings enhance our understanding of HIV-1 pathogenicity and are valuable for the development and implementation of a comprehensive public health approach to HIV-1 prevention in the country.

Keywords: HIV, vpu, IDU, Manipur, AIDS

1. INTRODUCTION

HIV/AIDS was detected for first time in Manipur, a northeastern part of India, in the year 1990 from the blood sample of an intravenous drug user (IDU) individual. Eighteen year later, the estimated number of IDUs infected with HIV-1 was 28.65% (Manipur AIDS Control Society). Geographically, Manipur is an important gateway of India to "golden triangle" of Southeast Asia, the second largest opium producer in the world, enabling migration of people, cultures and religions. In the early stages, the epidemic was concentrated among injecting drug users, but now it is spreading fast among the general population through risk behaviors ("Manipur AIDS Control Society,"). People in the 21-30 age-groups were at the highest risk and vulnerable of contracting this deadly disease. According to epidemiological data released by the Manipur State AIDS Control Society, as of May 2008, 10,213 men and women between the ages of 21-30 years were HIV-positive. Risk behaviors among HIV-positive IDUs pose dangers to themselves, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, re-infection with new HIV and HCV subtypes, and super-infection with antiretroviral drug-resistant HIV strains, as well as dangers of transmission of HIV, HBV and HCV to their sexual and drug-using partners. In fact, extremely high rates of HBV (100%) and HCV (92%) infection have been documented among HIV-1 positive IDUs in Manipur. In September 2013, a total of 43,882 HIV/AIDS positive cases have been detected which include 24,711 males, 12,411 females and 2919 children. Since then the morality rate with HIV/AIDS related disease has been tremendously increased among the risk group in the state ("Manipur AIDS Control Society,").

HIV-1 has six accessory gene in addition to main *gag, pol* and *env* genes, a characteristic features of lentivirus (Cullen & Greene, 1990). Among these genes, *vpu* gene remains exclusive features and makes them differ from HIV-2, SIV-SMM, or other SIVs in terms of pathogenicity and virulence. Vpu is a 16-kd (81-amino acid) type I integral membrane protein with at least two different biological functions (Maldarelli, Chen, Willey, & Strebel, 1993): (a) enhancement of virion release from the plasma membrane of HIV-1-infected cells and (b) degradation of CD4 in the endoplasmic reticulum. Vpu possesses an N-terminal hydrophobic membrane anchor and a hydrophilic moiety. It is phosphorylated by casein kinase II at positions Ser52 and Ser56. Moreover, *vpu* locus is considered as one of the most variable regions in HIV-1genome. It is not known whether any of these variations have any role in viral replication or disease progression. However, substitution of serine by alanine at amino acid position 61 influence cell stability and release of virus (Verma, Ronsard, Kapoor, & Banerjea, 2013). Mutation at this particular site plays a major role in controlling viral release as well as apoptosis, both of which are important for HIV-1 pathogenesis (Estrabaud et al., 2007; Verma et al., 2013)

In spite of these facts, Manipur among highest HIV/AIDS cases in the country, there are no studies available on variability of *vpu* which influence the level toxicity to infected cell among HIV-1 infected IDUs of Manipur till date. In this study we investigate for the first time the variability of HIV-1 *vpu* among the IDUs HIV-1 infected individuals of this region.

2. MATERIAL AND METHODS

2.1. Ethics Statement

With the approval from ethical committee, the intravenous drug users' (IDUs) groups of HIV-1 infected individuals were recruited for the present study during the period of two year, 2011 and 2013. Forty three numbers of HIV blood samples have been collected after getting being signed the informed consent form by the participants from Anti-Retroviral Therapy (ART) Centre at Regional Institute of Medical Sciences (RIMS), Manipur. Clinical information of the participants was also collected at the time of collection and personal interview.

2.2. **DNA Extraction, Gene Amplification and Sequencing**

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood samples using the Ficoll hypaque (GE healthcare, USA) density gradient centrifugation method as per manufacturer's instructions. Genomic DNA was extracted from the PBMCs through QIAamp DNA blood mini kit (Qiagen GmbH, Germany), according to the manufacturers protocol. The complete open reading frames (ORFs) corresponding to the *vpu* locus of HIV-1 genome, (HXB2 position 5861-6352, Genbank Acc No: K03455) was amplified from genomic DNA with forward primer (MUHIVVF1- AGARGAYAGATGGAACAAGCCCCAG) and reverse primer (MUHIVVR1, GTGTGTAGTTCTGCCAATCAGGGAA) in pre-nested PCR and forward primer (MUHIVVF2, TGGAAGCATCCRGGAAGTCAGCCT) and reverse primer (MUHIVVR2, CTCTCATTGCCACTGTCTTCTGCTC) in nested polymerase chain reaction (PCR) technique using high fidelity Taq polymerase (Invitrogen, USA) (Nadai et al., 2008).The PCR reaction conditions are as follows: 250µM dNTPs, 200 nM of each primer, 2 mM of $MgCl_2$, and 6U of Taq polymerase in a total volume of 50 µl. The following cycling conditions of touchdown PCR: One cycle of denaturation at 94^{o}C for 5 minutes and then add 6U of PCR enzyme as a manual hot-start; followed by 10 cycles of: 94^{o}C for 30s, 60^{0}C for 30s, and 68^{o}C for 4 mins; then 20 cycles of: 94^{0}C for 30s, 55^{0}C for 30s and 68^{0}C for 4 mins; one final extension at 68^{o}C for 10 minutes for outer PCR. Nested PCR was performed using the same conditions described above for second-round PCR except the extension time for 45 seconds to amplify a 490-bp fragment. After PCR, the amplified products were electrophoresed on a 0.8 % agarose gel, stained with ethidium bromide, and evaluated under UV light. The desired band was excise from the agarose gel and purified using QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacture's manual. Each DNA fragments was sequenced on an ABI PRISM 3730XL DNA Analyzer using BigDye terminators (Applied Biosystems, Foster City, California, USA).

2.3. Sequence Comparison and phylogenetic tree construction

The sequences chromatograms were viewed in Chromas 2.4.1 (http://www.technelysium.com.au) , manually edited, trimmed of low quality end, and analysed using HIV Blast to search the sequenced sample with previously reported sequences. Reference sequences of subtype B and subtype C were downloaded from HIV website (http://www.hiv.lanl.gov.com). All sequences were trimmed so as to contain only the complete *vpu* of HIV-1. The sequence were translated and aligned with translated reference sequence using clustalW in BioEdit software (BioEdit software version 7. 1.11). Phylogenetic tree was also interfered among the studied sample using the UPGMA based on the bootstrap method of 1000 replicate and Poisson model of amino-acid substitution type in MEGA software (Version 6.0).

3. RESULT

3.1. Clinical and demographic characteristics of Study Subject:

The entire studied samples were intravenous drug users (IDUs) male from Manipur, a state of northeastern India. The median age of the participants was 35 years. 58.13% (25/43) were Co-infected with hepatitis B or C. The patients who were on ART at the time of blood collection were 86.61%.

3.2. Origin, dissemination paths and Phylogenetic tree of HIV infected IDUs:

BLAST search similarity score revealed that among *vpu* sequences of the HIV-1 infected intra venous drug users (IDUs) of Manipur, 77% showed highest similarity score with the HIV-1 variants originated from China, 7% of each were originated from Myanmar, India and Zambia and 2% from Thailand (Figure 1).

Phylogenetic tree of the *vpu* sequences of HIV-1 infected IDUs of Manipur displayed a cluster except two sequences. This finding is indicative of virus transmission among IDUs of Manipur (Figure 2).

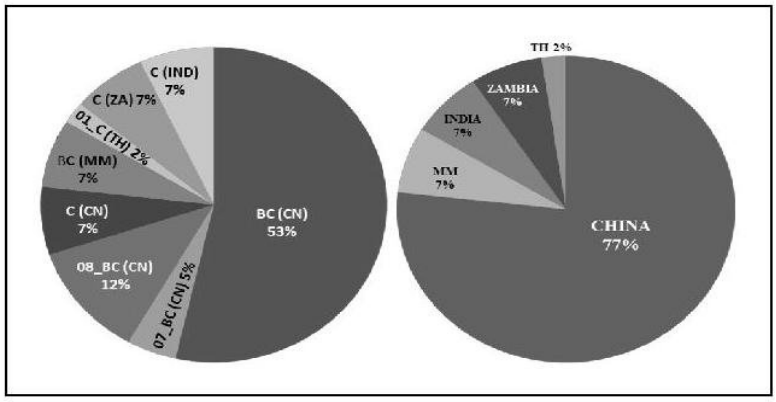

Figure 1. BLAST search for origin based on complete vpu region of HIV-1 isolate from IDUs group of Manipur. Variants (left side) and countries (right side) wise distribution among *vpu* sequence, India (IND), China (CN), Myanmar (MM), Thailand (TH) and Zambia (ZA).

3.3. Genetic variants of *Vpu*

To study the nature of natural variations exhibited by *vpu* from IDUs HIV infected individuals in this region, all sequence were translated in to amino acid and aligned with reference amino acid sequences of subtype B and subtype C. It is worth to be mentioned that the size of the open reading frame (ORF) of *vpu* found in this region was different due to the extensive polymorphisms. 95.34% (41/43) of HIV isolated from IDUs of Manipur has been found to possess serine amino acid (wild type) at position number 61 indicating high level of cell toxicity with moderate level of virus release. On other hand, 4.65% (2/43) HIV infected IDU individuals were found to possess amino acid mutant variant at position number 61. Sample number MU031, and MU057 display amino acid substitutions at position number 61 with alanine and leucine respectively.

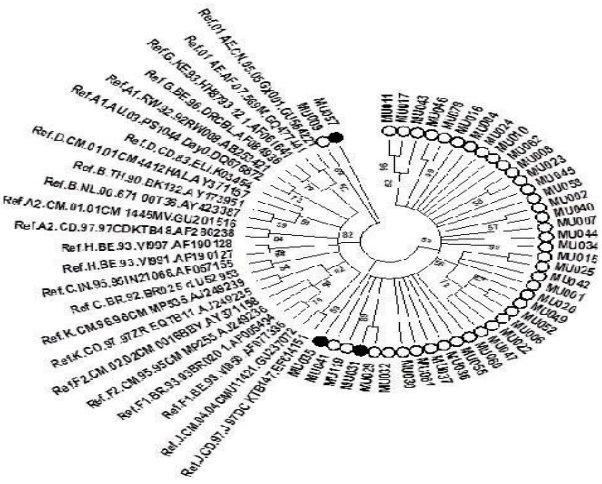

Figure 2. Phylogenetic tree of HIV-1 vpu sequence isolate from IDUs of Manipur were constructed along with reference sequences using UPGMA tree in MEGA 6.0. The sequences from Manipur are labeled by circles (solid circle; S61 mutation, open circle; wild type).

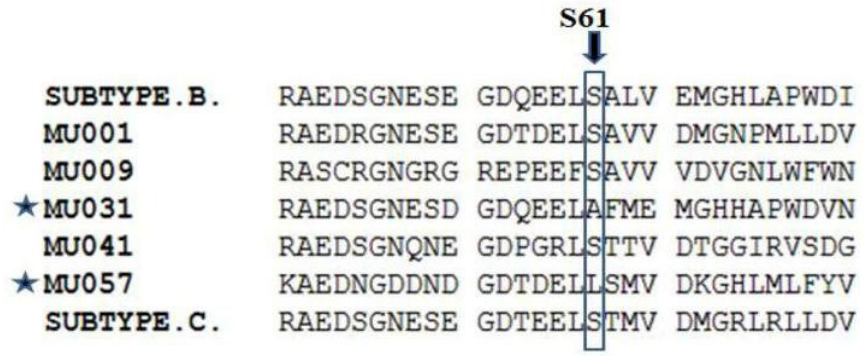

Figure 3. Multiple sequence alignment of HIV-1 *vpu* isolates of HIV-1 infected individuals from Manipur. Amino acid position number 61 is denoted by arrow and a labeled asterisk denotes sequences which are mutated at amino acid position 61.

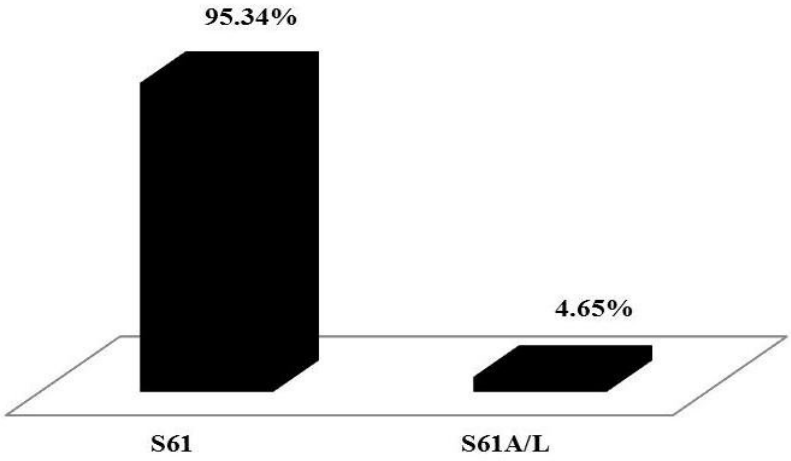

Figure 4. Percentage wise distribution of wild type serine amino acid at position number 61 and mutation of serine substituted by alanine or leucine among vpu sequence of HIV-1 isolate from Intra venous drug users of Manipur.

4. DISCUSSION

HIV-1 encodes a small integral membrane vpu protein and this protein is a subset of related simian immunodeficiency viruses (SIV) and has no homolog HIV-2 virus (less pathogenic) (Cohen, Terwilliger, Sodroski, & Haseltine, 1988; Strebel, Klimkait, & Martin, 1988). The high pathogenic of HIV-1 is due to inclusion of vpu protein (Hout et al., 2004). Vpu performs two major functions in HIV-1 replication (Bour & Strebel, 2003). First, vpu enhances the release of retroviral particles from most human cells, including lymphocytes and primary macrophages (Strebel, Klimkait, Maldarelli, & Martin, 1989; Terwilliger, Cohen, Lu, Sodroski, & Haseltine, 1989). Second, vpu induces the degradation of the CD4 viral receptor and therefore participates in the general down-regulation of CD4 expression during the course of HIV infection (Levesque, Zhao, & Cohen, 2003). Vpu locus is considered one of the most variable regions in HIV-1genome. It is not known whether these variations have any role in viral replication or disease progression. However, it was reported that variation at serine 61 determined the virus pathogenicity by controlling viral release as well as apoptosis.

Our study reveals that 95.34% (41/43) of the HIV-1 infected IDUs found in this region have highly conserved vpu at amino acid serine (wild type) position number 61 which are known to enhance CD4 down-regulation and degradation leading to target cell toxicity and virus virulence (Gomez et al., 2005).

Moreover, 4.65% (2/43) of our samples show substitution of serine at amino acid position 61 by alanine, or leucine. According to data published in north India, such substitution of a phosphorylable serine residue to alanine at amino acid position 61 in *vpu* variants showed enhanced intracellular expression and intracellular stability. These variants retain moderate cell death potential compared to wild type at position number 61 of *vpu* (Verma et al., 2013).

Thus overall finding of our study reports that the circulating form of virus prevails in this region have highly conserved serine residue at amino acid position 61 of *vpu* site which predicts highly virulence form of virus.

Competing Interests:
The authors declare no competing financial interests.

Accession Numbers:
The GeneBank accession numbers reported in this study are available under KM359873-KM359880, KM359883-KM359885, KM359889-KM3598900, KM3598902-KM359918, KM359926, KM406315 and KM406319

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