Risk for HIV-1 infection is not associated with Repeat-Region polymorphism in the DC-SIGN neck domain and Novel Genetic DC-SIGN Variants among North Indians

Anurag Rathore [a], Animesh Chatterjee [a], Vikas Sood [b], Sohrab Z. Khan [b], Akhil C. Banerjea [b], Naohiko Yamamoto [c], Tapan N. Dhole [a],*

[a] Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebarelli Road, Lucknow, India
[b] Laboratory of Virology, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India
[c] Department of International Health, Nagoya University School of Medicine, Japan

Abstract

Background: Several genetic factors have been related to HIV-1 resistance, the homozygosity for a mutation in CCR5 gene (CCR5Δ 32 allele) is presently considered the most relevant one. The C-type lectin, DC-SIGN efficiently binds and transmits HIV-1 to susceptible cell *in trans* thereby augmenting the infection. A potential association of the DC-SIGN neck domain repeats polymorphism and risk of HIV-1 infection is currently under debate.

Methods: Genetic risk association study was conducted in HIV-1 exposed seronegative (HES; $n=50$) individuals, HIV-1 seronegative (HSN; $n=314$) healthy control and HIV-1 infected seropositive patients (HSP; $n=190$) for polymorphism in neck domain of DC-SIGN gene. The DC-SIGN genotypes were identified by PCR from DNA extracted from peripheral blood and confirmed by sequencing. Fisher exact or χ^2 test was used for statistical analysis.

Results: One HSN and HSP individual who were heterozygous (7/8) with respect to DC-SIGN repeat regions were found. The DC-SIGN neck repeat polymorphism among North Indian individuals was not associated with susceptibility to HIV-1 infection. Furthermore, inheritance study of heterozygous mutation (7/8) in HSN individual's family showed that one parent, two brothers, one sister and one daughter were heterozygous (7/8) for DC-SIGN mutant allele. Sequence analyses of DC-SIGN exon 4 repeat region of randomly selected 25 North Indian individuals from HSP, HSN and HES revealed four conserved intronic mutations. These mutations were at nucleotide position 1283, 1306, 1308 upstream and 1906 downstream of the DC-SIGN exon 4 repeat region when compared with the wild type sequence (NCBI Acc. No. AF209479).

Conclusion: The polymorphism in DC-SIGN neck repeats region was rare and not associated with HIV-1 susceptibility among North Indians. Sequencing analysis of DC-SIGN gene confirmed four novel genetic variants in intronic region flanking exon 4 coding region.
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1. Introduction

Genetic polymorphism in human genes can influence the risk for HIV-1 infection and disease progression [1,2]. There are some individuals who remain seronegative despite high risk and/

or multiple exposures to HIV-1 [3,4]. Although several factors have been related to HIV-1 infection resistance, the possible genetic mechanism underlying this resistance is the homozygous presence of a 32 bp deletion in CCR5 gene (CCR5 Δ32), i.e. the main co-receptor used by the macrophage (M)-tropic strain of the virus to infect peripheral blood mononuclear cells [5].

The dendritic cell receptor, DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin, encoded by CD209) is a type II membrane associated C-type lectin that binds HIV-1 envelope glycoprotein gp120 in a CD4-independent manner and function

as a Trojan horse to enhance trans-virus infection to host cells [6]. DC-SIGN is of particular interest because it recognizes a plethora of pathogens such as *Mycobacterium tuberculosis, Helicobacter pylori, Klebsiela pneumonia*, HIV-1, Ebola virus, Cytomegalovirus, Hepatitis C virus, Dengue virus, SARS-coronavirus and parasites like *Leishmania pifanoi* and *Schistosoma mansoni* [7]. Cotransfection of DC-SIGN with HIV-1 demonstrated 95–99.5% inhibition of viral production from host cells and can also effectively inhibit 90–95% of HIV-1 generation from infected cell [8]. DC-SIGN is organized into 3 domains: an N-terminal cytoplasmic region, a neck region containing 7 tandem repeats of the 23 amino acid sequence, and a C-terminal domain with homology to C-type lectins [9]. The neck region of DC-SIGN may be important in determining the orientation of the carbohydrate-binding C-type lectin domain and may therefore have an impact on ligand specificity [10]. Polymorphism analysis of the numbers of repeats present in this neck region suggested that tandem repeats in the neck region are variable and predominantly consisted of 7 repeats among the Caucasians [11]. However, novel variations in the DC-SIGN repeat region were identified which were rare. Cohort studies of HIV-1 seronegative, HIV-1 seropositive and repeatedly exposed seronegative individuals suggest that heterozygosity in the DC-SIGN repeat region may have protective effect on transmission of HIV-1 [12] whereas no associations could be established in recent studies on Thais individuals [13].

Since, the impact of DC-SIGN neck repeat polymorphism on HIV-1 disease susceptibility/transmission have yielded conflicting results, we investigated the effect of variable number of tandem repeat (VNTR) polymorphism in DC-SIGN neck region on HIV-1 susceptibility among North Indian individuals.

2. Materials and methods

2.1. Patient selection

All the subjects with similar ethnicity were randomly selected from same source population. Demographic profiles of the study groups are given in Table 1. 190 HIV-1 seropositive patients (HSP) were enrolled from the outpatients attending the clinics of Sanjay Gandhi Post Graduate Institute of Medical Sciences, India from Jan. 2004 to Nov. 2006. Most of the patients were from the state of Uttar Pradesh in North India. 320 age-matched controls were healthy staff members of institute with HIV-1 seronegative status. 50 HIV-1 seronegative individuals with history of repeated sexual intercourse (twice a week) without any protection with HIV-1 infected partners for at least 1 y were recruited in HIV-1 exposed seronegative group (HES). HIV-1 seronegative status of the HES subjects was confirmed by Western blot at the regular interval of three months till one year. After an informed consent, 5-ml blood sample was

Table 1
Demographic profile of the study groups

	HSP	HSN	HES
No. of individuals	190	320	50
Male no. (%)	169 (88.94)	275 (85.93)	44 (88)
Female no. (%)	21 (11.05)	45 (14.06)	6 (12)
Median age (range)	58 (42–74)	56 (38–60)	59 (35–55)
Heterosexual no. (%)	190 (100%)	N.D.	50 (100%)
Homosexual no. (%)	0 (0%)	N.D.	0 (0%)

HSP = HIV-1 Seropositive, HSN = HIV-1 Seronegative, HES = HIV-1 Exposed Seronegative, N.D. = Not Detected.

Table 2
Distribution of CCR5 delta 32 genotypes/allele among HIV-1 Seropositive, HIV-1 Seronegative and HIV-1 Exposed Seronegative individuals

CCR5 delta 32	HSP ($n = 190$)	HSN ($n = 320$)	HES ($n = 50$)
wt allele (%)	380 (100)	634 (99.06)	100 (100)
delta 32 allele (%)	0 (0)	6 (0.93)	0 (0)
CCR5 wt/wt (%)	190 (100)	314 (98.12)	50 (100)
CCR5 delta32/wt (%)	0 (0)	6 (1.87)	0 (0)
CCR5 delta 32/delta32 (%)	0 (0)	0 (0)	0 (0)

Percentage values given within parenthesis.
HSP: HIV-1 Seropositive; HSN: HIV-1 Seronegative; HES: HIV-1 Exposed Seronegative.

taken in EDTA for serological and polymorphism studies. Necessary institutional biosafety and ethical clearances were obtained.

2.2. Determination of HIV-1 status

Altogether 560 individuals were screened for their HIV-1 status by primarily screening with ELISA (Vironostika, HIV Uni-FormII Ag/Ab, Biomerieux, Netherlands) and subsequently confirmed with Western Blot (LAV Blot I, BioRad, France).

2.3. Genomic DNA isolation

The genomic DNA samples were obtained from 0.2 to 0.3 ml peripheral whole blood by using QIAamp Blood kit (Qiagen, San Diego CA) according to the protocols supplied by the manufacturer. Usually ~0.1 µg genomic DNA was used for the PCR.

2.4. Genotyping of CCR5 delta 32 mutation

CCR5 delta 32 genotype was determined by sizing amplicons that include the entire region of the deletion as described previously [14]. Polymerase Chain Reaction (PCR) was conducted in a 25 µl reaction containing 50 ng of genomic DNA, 5 pmol of each primer, 175 µM dNTPs, 1.5 mM MgCl$_2$, 10X PCR buffer and 0.5 U of Taq polymerase (Roche). Thermocycling procedure (PerkinElmer 9600) consisted of initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 1 min and final extension of 72 °C for 7 min. Amplicons were visualized by ultraviolet transillumination in 2% agarose gel containing ethidium bromide. The sense primer was 5′–TGTTTGCGTCTCCCAG–3′ and antisense was 5′–CAC AGC CCT GTG CCT CTT–3′, which result in a 233 bp product for the wild type amplicon and 201 bp for the deletion product.

2.5. Genotyping of DC-SIGN neck repeat

The DC-SIGN neck repeat region was amplified from genomic DNA as described previously [12]. PCR amplification was performed using forward Primer: 5′–CCA CTT TAG GGC AGG AC–3′ and reverse primer: 5′–AGC AAA CTC ACA CCA CAC AA–3′ in a volume of 25 µl containing 0.00025 µmol/µl dNTPs, 1.0 µmol/µl each primer, 1 µl Glycerol, 0.0025 µmol/µl MgCl$_2$ and 1.0 U Taq polymerase (Roche) in a 1X reaction buffer. The cycle conditions were 5 min at 94 °C, followed by 30 cycles of 15 s at 95 °C, 7 s at 61 °C, 30 s at 72 °C and then a single cycle of 7 min at 72 °C. Wild type alleles (7/7) yielded 852 bp PCR products while heterozygous individuals (8/7) was identified by the appearance of 2 closely spaced PCR products (921 bp and 852 bp) when analyzed on a of 3% agarose gel by ethidium bromide staining.

2.6. Cloning and sequencing of wild and mutant alleles DNA fragments

PCR generated DNA fragments of wild and heterozygous mutants were excised from the gel and were cloned directly in a T-tailed vector (pGEM T-Easy, Promega Biotech, Madison. WI). Positive clones were confirmed by EcoR1 digestion and sequenced on ABI Bioprism.
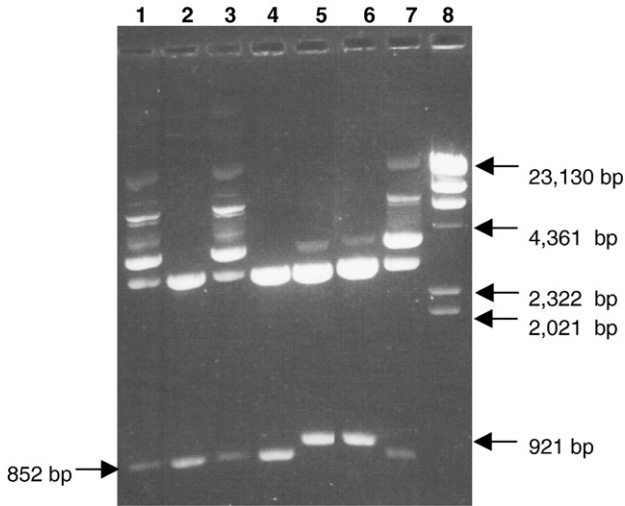
Fig. 1. Recombinant DNA clones (containing 7 or 8 repeats region) of DC-SIGN gene were analyzed by EcoR1 restriction analysis on 2% agarose gel. Lanes 1 to 4 and lane 7 show the presence of 7 repeat region whereas lanes 5 and 6 show 8 repeat regions. Lane 8 is λ Hind III marker.

2.7. Statistical analysis

Statistical analysis was done by SPSS software ver. 11.5 (SPSS, Chicago, IL). Direct gene counting method was used to determine the frequency of genotypes and alleles. The Fisher exact or χ^2 test was used to determine differences in allele/genotype frequencies of DC-SIGN neck repeat polymorphism. Odds ratios (OR) and its 95% confidence interval (C.I.) were obtained to describe the strength of association. A $p < 0.05$ was considered to be statistically significant.

3. Results

In the present study 560 individuals were screened for their HIV-1 status out of which 190 were HIV-1 seropositive and 320 were HIV-1 seronegative and 50 were HIV-1exposed seronegative as shown in Table 1.

3.1. Genotyping of CCR 5 Δ 32 mutation

In all the 3 groups the genotype frequencies observed were in equilibrium, as predicted by Hardy–Weinberg equation (Table 2). The frequency of CCR5 delta 32 allele was 0% in HSP cases, 0.93% in HSN controls and 0% in HES North

Indians. 6 of 320 (1.87%) HSN were heterozygous for the CCR5 delta 32 genotype compared to 0 of 190 (0%) HSP and 0 of 50 (0%) HES individuals. None of the cases or of controls had CCR5delta 32 homozygous genotype. All the individuals having CCR5−delta 32 allele were excluded from the study to study the impact of DC-SIGN on HIV-1 susceptibility.

3.2. Genotyping of DC-SIGN exon 4 tandem repeat polymorphism

In the studied North Indian population, the genetic polymorphism in DC-SIGN neck repeats region was rare. Frequency of DC-SIGN homozygous 7/7 genotype (wild type) was 99.72% in healthy controls. Among the entire North Indian individuals genotyped only 1 of 320 (0.31%) from HSN group and 1 of 190 (0.52%) from HSP group were found to have heterozygous 7/8 mutant genotype. The PCR amplified products (921 bp and 852 bp) of heterozygous mutant (7/8) HIV-1 seronegative individual and wild (7/7) samples (852 bp) were cloned successfully into pGEM T-Easy Vector and subjected to restriction enzyme analysis (Fig. 1). Few positive recombinants clones were selected and subjected to sequencing. An insertion of one full repeat of 69 bp was observed in heterozygous individual (Fig. 2). Statistical analysis showed that insertion of extra repeat in DC-SIGN exon 4 neck region was not associated with susceptibility to HIV-1 infection in North Indian individuals (Table 3).

3.3. Genetic variants in DC-SIGN gene

Sequence analysis of upstream and downstream region of DC-SIGN exon 4 repeat in randomly selected HSP ($n=8$), HSN ($n=12$) and HES ($n=5$) North Indian individuals revealed 4 mutations in the intronic part of DC-SIGN gene. Three upstream intronic changes were at nucleotide position 1283 (C to T), 1306 (A to T), 1308 (T to C) and 1 downstream at1906 (insertion of G) (Fig. 3A and B).

3.4. Inheritance of DC-SIGN heterozygous (7/8) mutant in a family

The mother, two brothers, sister and daughter of HIV-1 seronegative heterozygous individual were genotyped to study the inheritance pattern of the mutation. All of them were carrying this extra repeat region except the sister. All the individuals in this family were found to be HIV-1 seronegative,
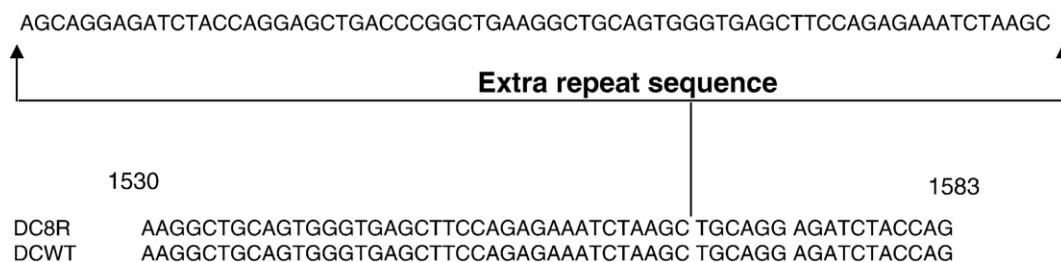

Fig. 2. Gene Alignment by Clustal W of mutant allele (DC8R) from heterozygous individual with type allele (DCWT) from the gene bank showed presence of an extra 69 bp repeat.

Table 3
Association of DC-SIGN genotypes/allele with HIV-1 susceptibility

DC-SIGN exon 4	HSP (n=190)	HSN[a] (n=314)	HES (n=50)	p value[b]	O.R.	C.I.
7Allele (%)	379 (99.73)	639 (99.84)	100 (100.00)	1.000	0.521	0.033–8.358
8Allele (%)	1 (0.26)	1 (0.15)	0 (0)	1.000	1.918	0.120–30.753
7/7Genotype (%)	189 (99.47)	313 (99.68)	50 (100.00)	1.000	0.521	0.032–8.371
7/8Genotype (%)	1 (0.52)	1 (0.31)	0 (0)	1.000	1.921	0.119–30.878

Percentage values given within parenthesis.
HSP: HIV-1 Seropositive; HSN: HIV-1 Seronegative; HES: HIV-1 Exposed Seronegative.
[a] Six samples from HSN group were excluded having CCR5 delta 32 allele.
[b] Comparison between HIV-1 Seropositive versus HIV-1 Seronegative + HIV-1 Exposed Seronegative.

physically and mentally fit. Since only one parent's sample was available for analysis, we cannot categorically say as to how this gene was inherited in the family.

4. Discussion

The DC-SIGN lectin functions as a transreceptor for HIV-1. The exon 4 neck region of DC-SIGN comprises of variable number of 69 base pair tandem repeats, encoding for parts of the extracellular neck domain. Understanding the effects of genetic polymorphism on HIV-1 susceptibility has provided essential insights in novel variants in DC-SIGN exon 4 neck gene sequences. However, no study has been done on the effect of DC-SIGN exon 4 neck repeats polymorphism on protection against HIV-1 infection among North Indian individuals.

In the present study, we analyzed the impact of the variable number of tandem-repeat polymorphism (VNTR) in exon 4 of the DC-SIGN gene with respect to the interindividual trans-mission of HIV-1 infection. Since, CCR5 delta 32 mutation is presently considered to be most relevant in conferring resistance against HIV-1 infection, the individuals having CCR5 delta 32 allele were excluded from the study. Analysis of DC-SIGN genetic variants in our studied North Indian population revealed the presence of 2 alleles and 2 genotypes of 7/7 and 7/8 type. Our data demonstrate that polymorphism in the DC-SIGN repeat region is rare and frequency of this mutant allele is <1%, which is in agreement with the previous studies [7]. Although rare among Indians, this allele was present among most of the family members of HIV-1 seronegative heterozygous individual. The restricted nature of this mutation with low level heteorozygosity suggests its recent evolutionary origin and reduced fitness of any allele other than 7-repeat allele in North

Indian population [7]. Association studies correlating the HIV-1 susceptibility with DC-SIGN neck repeat polymorphism revealed that insertion of extra repeat in the DC-SIGN neck region i.e. 7/8 have no impact on HIV-1 susceptibility, which is in agreement with the Wichukchinda et al. studies in Thais population [13]. Moreover, our results were also in agreement with Liu et al studies that confirmed the addition of a repeat does not influence the susceptibility to HIV-1 infection. In contrast, it is conceivable that the deletion of a repeat can play role in preventing the transmission of HIV-1 infection. Sequence analyses of DC-SIGN repeat region from randomly selected twenty-five North Indian individuals revealed four novel single nucleotide genetic variants, which were not described earlier in NCBI database. Genetic variants in intronic region of DC-SIGN gene (three substitutions and one insertion) reported here revealed that the North Indian population had very different allelic pattern. In addition, since these variants are present in an intronic region, it is conceivable that they influence the splicing and thus the transition from the nuclear hete-rogeneous RNA to the mRNA of DC-SIGN.

In conclusion, considering our potential limitations the present study has showed that repeat region polymorphism in DC-SIGN gene do not influence the HIV-1 susceptibility among North Indians and warrants further studies to determine the functional consequences of novel variants in intronic region on HIV/AIDS susceptibility.
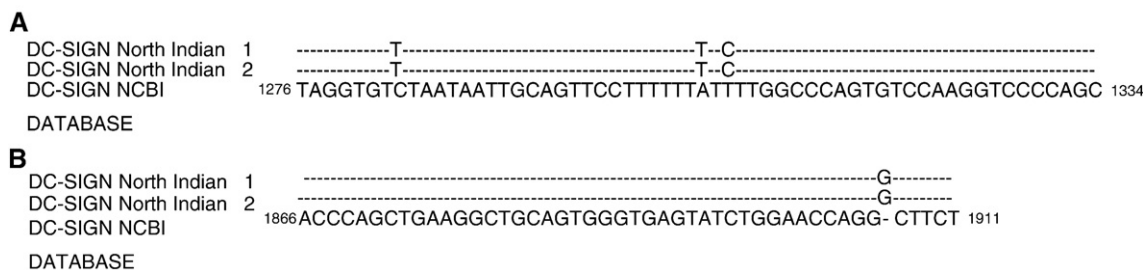
Acknowledgement

Fig. 3. Sequence comparison of two North Indian individuals and NCBI database sequence showing 3 SNPs (1283, 1306 and 1308) upstream (A) and an insertion of G nucleotide position downstream (B) of exon 4 DC-SIGN gene.

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