

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*, *Klebsiella pneumoniae*, 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 MgCl₂, 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'-TGTTTGGCTCTCCAG-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 MgCl₂ 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 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 EcoRI digestion and sequenced on ABI Bioprism.

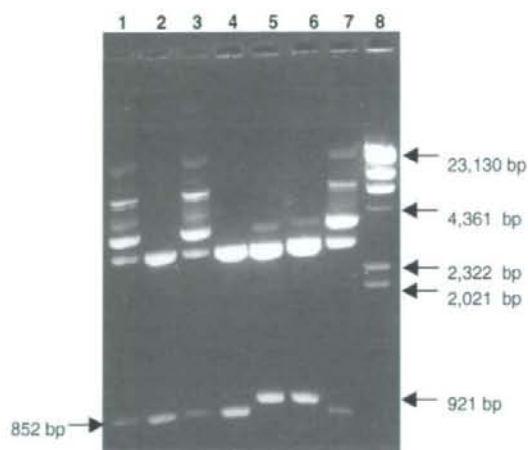


Fig. 1. Recombinant DNA clones (containing 7 or 8 repeats region) of DC-SIGN gene were analyzed by EcoRI restriction analysis on 2% agarose gel. Lanes 1 to 4 and lane 7 show the presence of 7 repeat region whereas lanes 5 and 6 show 8 repeat regions. Lane 8 is λ Hind III marker.

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-1 exposed seronegative as shown in Table 1.

3.1. Genotyping of CCR5 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 at 1906 (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,

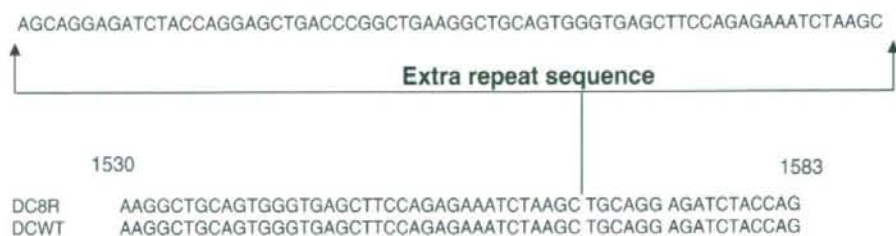


Fig. 2. Gene Alignment by Clustal W of mutant allele (DC8R) from heterozygous individual with type allele (DCWT) from the gene bank showed presence of an extra 69 bp repeat.

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 transmission 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 heterozygosity 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 Thai 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 heterogeneous 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

We thank Mr. Pradeep K. Singhal Professional Biotech Pvt. Ltd., New Delhi for his unwavering support and insightful advice. This work is part of the Ph.D. thesis of the first author

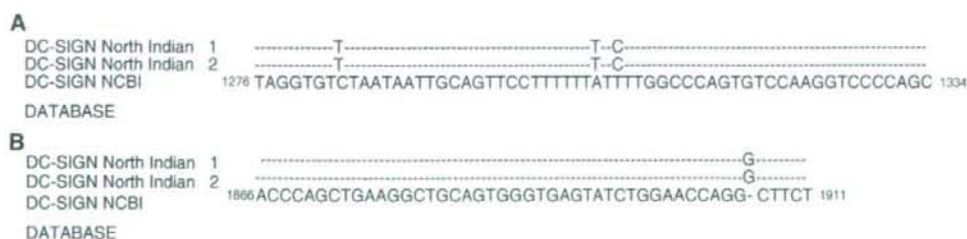


Fig. 3. Sequence comparison of two North Indian individuals and NCBI database sequence showing 3 SNPs (1283, 1306 and 1308) upstream (A) and an insertion of G nucleotide position downstream (B) of exon 4 DC-SIGN gene.

(Anurag Rathore) and is partially funded by the Indian Council of Medical Research, New Delhi, India. Work carried out by Akhil C. Banerjee, Sohrab Z. Khan and Vikas Sood at the National Institute of Immunology, New Delhi, was supported by the Dept. of Biotechnology, Govt. of India.

References

- [1] O'Brien SJ, Nelson GW. Human genes that limit AIDS. *Nat Genet* 2004;36(6): 565–74.
- [2] Shioda T, Nakayama EE. Human genetic polymorphisms affecting HIV-1 diseases. *Int J Hematol* Jul 2006;84:12–7. Review.
- [3] Fowke KR, Nagelkerke NJ, Kimani J, et al. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *Lancet* 1996;348:1347–51.
- [4] Lopaleo L, Pastori C, Barassi C, et al. Heterogeneity in exposed uninfected individuals. *J Biol Regul Homeost Agents* 1997;11:27–31. Review.
- [5] Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply exposed individuals to HIV-1 infection. *Cell* 1996;86:367–77.
- [6] Geijtenbeek TB, Kwon DS, Torensma R, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 2000;100:587–9.
- [7] Barreiro Luis B, Patin Etienne, Nyrolles Oliver, Cann Howard M, Gicquel Brigitte, Quintana-Murci. The heritage of pathogen pressure and ancient demography in the human innate immunity CD 209/CD 209 L region. *Am J Hum Genet* 2005;77:869–86.
- [8] Wang Q, Pang S. An intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) efficiently blocks HIV viral budding. *FASEB J* Nov 2007;5.
- [9] Soilleux EJ, Barten R, Trowsdale J. DC-SIGN; a related gene, DC-SIGNR; and CD23 from a cluster on 19p13. *J Immunol* 2000;165:2937–42.
- [10] Feinberg H, Guo Y, Mitchell DA, Drickamer K, Weis WI. Extended neck regions stabilize tetramers of the receptors DC-SIGN and DC-SIGNR. *J Biol Chem* 2005;280:1327–35.
- [11] Bashirova AA, Geijtenbeek TB, Van Duijnhoven GC, et al. A dendritic cell specific intracellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. *J Exp Med* 2001;193:671–8.
- [12] Liu H, Hwangbo Y, Holte S, et al. Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecular-3-grabbing nonintegrin in seronegative individual repeatedly exposed to HIV-1. *J Infect Dis* 2004;190: 1055–8.
- [13] Wichukhinda N, Kitamura Y, Rojanawiwat A, et al. The polymorphism in DC-SIGNR affect susceptibility to HIV type 1 infection. *AIDS Res Hum Retrovir* 2007;23:686–92.
- [14] Zimmerman PA, Buckler-White A, Alkhatib G, et al. Inherited resistant to HIV-1 conferred by an inactivating mutations in CC Chemokine receptor 5: studies in populations with contrasting clinical phenotypes, defined racial background and quantified risk. *Mol Med* 1997;3:23–36.

Sequence Analysis of the Capsid Gene of Aichi Viruses Detected From Japan, Bangladesh, Thailand, and Vietnam

Ngan Thi Kim Pham,¹ Quang Duy Trinh,¹ Pattara Khamrin,¹ Tuan Anh Nguyen,¹ Shuvra Kanti Dey,¹ Tung Gia Phan,¹ Le Phuc Hoang,² Niwat Maneeekarn,³ Shoko Okitsu,⁴ Masashi Mizuguchi,¹ and Hiroshi Ushijima^{1,4*}

¹Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

²Department of Gastroenterology, Children's Hospital 1, Ho Chi Minh City, Vietnam

³Faculty of Medicine, Department of Microbiology, Chiang Mai University, Chiang Mai, Thailand

⁴Aino Health Research Center, Aino University, Tokyo, Japan

Sequence analysis of the capsid gene of Aichi viruses was performed on 12 strains detected in Japan, Bangladesh, Thailand, and Vietnam during 2002–2005. The phylogenetic tree constructed from 17 nucleotide sequences of the capsid gene of the strains studied and reference strains demonstrated that Aichi virus strains clustered into two branches. A classification of Aichi viruses based on the capsid gene was proposed, in which lineage I consists of the Aichi virus strains detected from Japan, Thailand, Vietnam, and Germany, and lineage II includes Bangladeshi strains and a Brazilian strain. *J. Med. Virol.* 80:1222–1227, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: sequence analysis; Aichi viruses; capsid gene

INTRODUCTION

Acute gastroenteritis is a common cause of morbidity and mortality worldwide. Rotaviruses, adenoviruses, astroviruses, and caliciviruses have been established as the most important etiologic agents [Glass et al., 2001]. However, there remains a “diagnostic gap,” which has been attributed to less explored viral pathogens. It was suggested that unknown agents account for approximately 81% of foodborne illnesses and hospitalizations and 64% of deaths in United States [Mead et al., 1999]. Identifying new causes of enteric illness and defining the public health importance of known agents would improve foodborne disease prevention. Among the less explored viral pathogens, Aichi virus was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in Japanese patients. It is a small round virus with about 30 nm in diameter [Yamashita et al., 1991]. The virus was classified into a new genus named Kobuvirus of the family

Picornaviridae which contains nine genera: Aphthovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus (which includes Aichi virus and bovine kobuvirus), Parechovirus, Rhinovirus, and Teschovirus according to the International Committee on Taxonomy of Viruses [Yamashita et al., 1998; Pringle, 1999].

By using a neutralization test, the prevalence rate for antibody against Aichi strain in healthy adults and children in Aichi Prefecture, Japan was estimated and found to be 7.2% for infants and children aged 7 months to 4 years and increased with age to about 80% in persons 35 years old [Yamashita et al., 1993]. Aichi virus was then isolated in Vero cells from 6 (12.3%) of 47 patients in 5 gastroenteritis outbreaks in Japan. In addition, 5 (0.7%) of 722 Japanese travelers returning from tours to Southeast Asian countries and complaining of gastrointestinal symptoms at the quarantine station of Nagoya International Airport in Japan, and 5 (2.3%) of 222 Pakistani children with gastroenteritis, were found to be positive for Aichi virus [Yamashita et al., 1995].

The complete Aichi virus genome was determined in 1998 and proved to be a single-stranded positive-sense RNA molecule with 8,251 bases excluding a poly (A) tail; it contains a large open reading frame (ORF) with 7,302 nucleotides that encodes a potential polyprotein

Grant sponsor: Ministries of Education and Sciences, and Health, Labor and Welfare, Japan; Grant sponsor: Asian Development Bank.

*Correspondence to: Prof. Hiroshi Ushijima, MD, PhD, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-0033, Japan.
E-mail: ushijima-hiroshi@jeom.home.ne.jp

Accepted 6 March 2008

DOI 10.1002/jmv.21193

Published online in Wiley InterScience
(www.interscience.wiley.com)

precursor of 2,433 amino acids [Yamashita et al., 1998]. In the year 2000, a reverse transcription-polymerase chain reaction (RT-PCR) method for the detection of Aichi virus was developed and the genetic analysis was performed using the 519 base RNA sequences at the putative junction between the C terminus of 3C and the N terminus of 3D. As a result, Aichi virus isolates have been divided into two groups: group 1 (genotype A) and group 2 (genotype B) [Yamashita et al., 2000]. Since then, basic researches into Aichi virus were consecutively performed [Sasaki et al., 2001, 2003; Yamashita et al., 2001; Nagashima et al., 2003, 2005]. Recently, Aichi virus was also detected in Brazil, Germany, Thailand, Bangladesh, and Vietnam [Oh et al., 2006; Pham et al., 2007].

It is well established that highly specific serological tests used for the diagnosis of viral infections could be developed on the basis of heterologously expressed viral structural proteins. In previous studies, however, the detection and classification of Aichi virus were based on the 3CD region coding for the non-structural protein [Yamashita et al., 2000; Oh et al., 2006; Pham et al., 2007]. Therefore, in order to research further into Aichi virus genome, this study was performed to characterize the capsid region coding for structural proteins which express the antigenicity of the virus.

MATERIALS AND METHODS

Clinical Specimens

Twelve fecal samples which have already known to be infected with Aichi virus determined by PCR using the Aichi virus-specific primers [Yamashita et al., 2000; Pham et al., 2007] were examined in this study. Those samples were collected from epidemiological studies/surveillances during the period of 2002–2005 in the following countries: Japan (six samples collected from July 2002 to June 2003), Bangladesh (three samples, October 2004 to September 2005), Thailand (one sample, March 2002 to December 2004), and Vietnam (two samples, October 2002 to September 2003) [Phan et al., 2005; Dey et al., 2007; Khamrin et al., 2007; Nguyen et al., 2007].

RNA Extraction, RT-PCR, and Primer Designation

RNA extraction was first done using the QIAamp viral RNA Mini kit (QIAGEN, Inc., GmbH Hilden, Germany). The RT-PCR was then performed. To obtain the full length of 2,616 bases of the capsid gene, 5 segments were constituted and 10 primers were newly developed based on the 5 complete genomes of Aichi viruses available from GenBank and the sequence data obtained during the capsid gene characterization of Aichi virus strains detected from the four countries. Five forward primers Cap F, Cap2, Cap3, Cap4, and CapE were used in combination with reverse primers Cap1R, Cap2R1, Cap3R, Cap4R, and CapER, respectively (Table I).

Sequence and Phylogenetic Analysis

The PCR amplicons were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The primers designed for PCR were used as sequencing primers. The sequence data were collected by an ABI Prism 310 Genetic Analyzer (Perkin Elmer-Applied Biosystems, Inc.).

The comparison analysis was conducted between the obtained Aichi virus strains and other Aichi virus reference strains from Japan, Germany, and Brazil available in GenBank database. The sequence data and the phylogenesis were analyzed using BioEdit v7.0.5. A parsimony analysis was also conducted using MEGA version 3.1 to determine the evolutionary relationship among studied sequences [Kumar et al., 2004]. The method was performed using close-neighbor interchange with a random option and with 1,000 bootstrap repetitions.

The nucleotide sequences of the Aichi virus strains described in this study have been deposited in GenBank under accession numbers: EU143271 to EU143279, EU143282, EU143286, and EU143287. Reference Aichi virus strains and their accession numbers are as follows: AB010145, AB040749, AY747174, NC001918, and DQ028632.

TABLE I. Oligonucleotide Sequences of the Five Primer Pairs Used in This Study

Primer	Sequence 5'–3'	Sense	Position ^a
Cap F	CAG GTG CCT ACC AAG CAA AGA C	+	1104–1125
Cap1R	GGT GAA CTC CTG GGA CCA G	–	1786–1768
Cap2	CCT CGC CTA CCC CAC CGC C	+	1666–1684
Cap2R1	GAG ACC GTG GAA RGA GGA GTC	–	2317–2297
Cap3	CAT AGA GGT CCC YTA YAT CTC	+	2149–2169
Cap3R	CAT ACK GTG TAT GTT CCG CGC	–	2760–2740
Cap4	CAG TGG CGY GGT GRA CTC G	+	2618–2636
Cap4R	GCG ATG TAY GTG AAG CAC G	–	3303 3285
Cap E	CTA GTC GGA CCC CAC ACC GC	+	2897–2915
CapER	GGA TGG CCC AGT GGA CGT AG	–	3854–3835

R: A or G.

Y: C or T.

K: T or G.

^aBased on the Japanese reference strain with the accession number of AB010145.

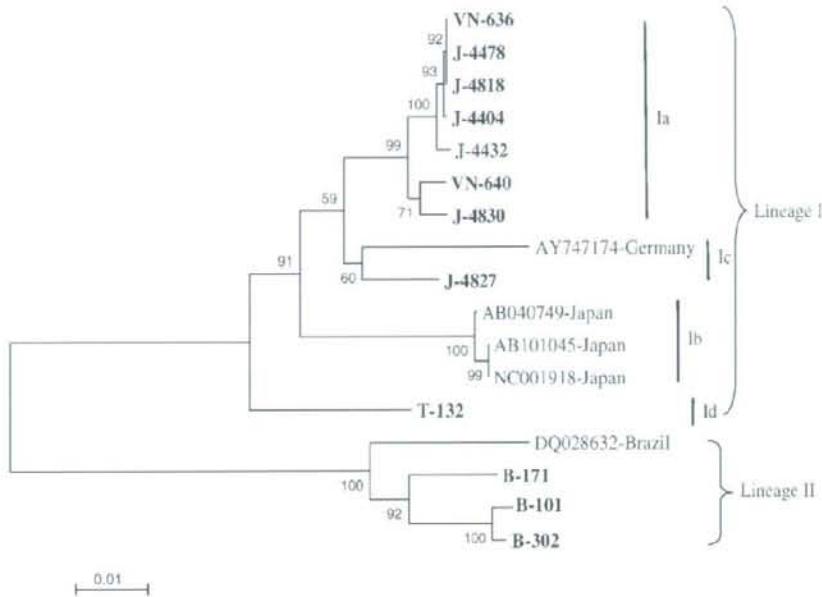


Fig. 1. Phylogenetic tree constructed from the full length sequences of the capsid gene with 1,000 bootstrap repetitions. The strains in this study are in boldface type. Abbreviations for locations: J, Japan; B, Bangladesh; VN, Vietnam; T, Thailand.

RESULTS

Twelve full lengths of Aichi virus capsid gene sequences from the four countries including Japan (6 strains), Bangladesh (3 strains), Thailand (1 strain), and Vietnam (2 strains) were obtained for comparison with global Aichi virus strains determined previously. The full length of the capsid gene of 9 strains from Japan, Thailand, and Vietnam was 2,616 bases, while that of 3 Bangladeshi sequences contained 2,619 nucleotides.

The phylogenetic tree in Figure 1 clearly showed that all of Aichi virus strains were clustered into two major branches. One branch consisted of four sub-branches containing the studied strains from Japan, Thailand,

Vietnam and reference strains from Japan, and Germany. The remaining branch comprised of the Bangladeshi strains and one reference strain from Brazil.

Distance matrix analysis of nucleotide sequences of the capsid gene incorporated with their phylogenetic tree demonstrated two major lineages which could be designated as I and II (Fig. 1 and Table II). The relationship among lineages is clearly shown in Table II. The same lineages were defined by 3.1% and 3.3% nucleotide distances, while distances between lineages were equal to or higher than 12.7%. In addition, the mean nucleotide distances among all Aichi virus strains of three structural domains of VP0, VP3, and VP1 of the capsid gene were calculated separately and found to be 12.1%, 13.3%, and 15.2%, respectively (data not shown).

A comparison of the deduced amino acid sequences of the whole capsid gene of all Aichi virus strains showed that there were at least 14 amino acid substitutions which were lineage-specific. There was a different triple of amino acids at positions 220–222 (TSS (Threonine-Serine-Serine) located within the VP0 region of lineage I and STN (Serine-Threonine-Asparagine) in case of lineage II, and an insertion of Proline or Serine at position 224 of lineage II sequences which could not be found within the lineage I sequences (Fig. 2).

DISCUSSION

Aichi virus virions contain three (VP0, VP3, and VP1), not four as other picornaviruses (VP1, VP2, VP3, and VP4), capsid proteins of 42, 30, and 22 kDa. No protein band corresponding to VP4 (usually 7–8 kDa) was

TABLE II. Percentages of Nucleotide Distances Among Aichi Lineages and Sub-Lineages

Aichi virus lineage	Mean% nucleotide distance				
	Lineage I				Lineage II
	Ia	Ib	Ic	Id	
Lineage I		(3.3)			13.3
Ia	0.6				
Ib	4.5	0.2			
Ic	4.3	5.8	3.4		
Id	4.8	6.6	5.1	NA	
Lineage II	13.4	13.5	13.4	12.7	(3.1)

Nucleotide distances within lineage I and lineage II are presented in parentheses, while distances between lineage II and every sub-branch of lineage I are in boldface type. NA, not applicable.

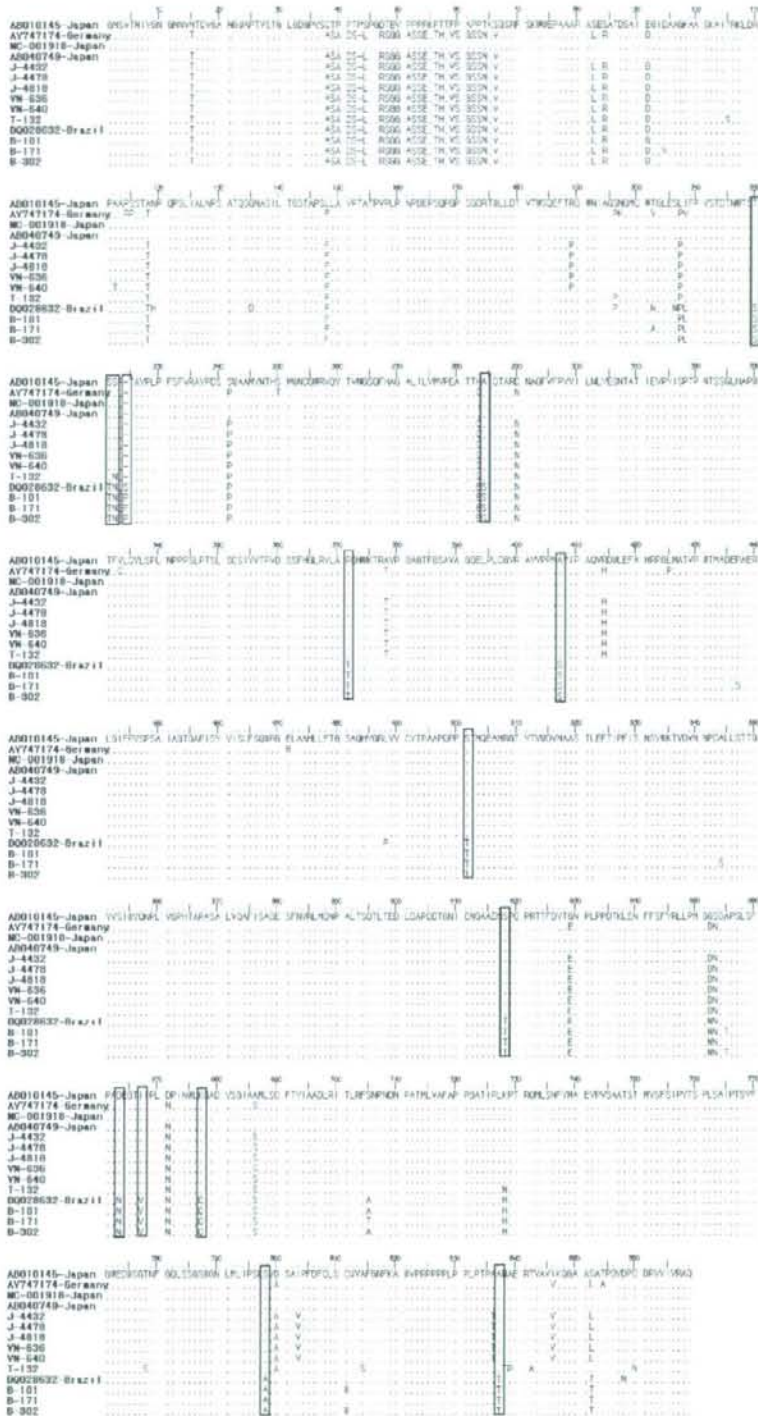


Fig. 2. Alignment of the deduced amino acid sequences of the capsid gene between the studied strains and the reference strains. Different amino acids between the two major lineages are in boxes. Abbreviations for locations: J, Japan; B, Bangladesh; VN, Vietnam; T, Thailand.

observed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [Yamashita et al., 1998]. In one previous study, the comparison of nucleotide sequences between the German and Brazilian Aichi strains with the Japanese strains revealed the VP1 coding region to be the most variable of the structural protein domains [Oh et al., 2006]. Nucleotide identities in this region were lower (86–87%) than in the 3CD section (minimum 88%). That finding was in agreement with the presumption of Yamashita et al. [2000], who suggested that sequence diversity in the structural protein coding regions might be higher than in the 3CD region. In this region, as well as over the whole length of the ORF, the genotype A sequences were more closely related to each other than to that of the Brazilian type B isolate [Oh et al., 2006]. In this study, regarding the mean nucleotide distances among all Aichi virus strains of the three structural domains of the capsid gene, the VP1 domain showed the highest percentage of 15.2% when compared to those of the VP0 and VP3 domains (12.1% and 13.3%, respectively). This finding confirms that the VP1 domain is the most variable region of the capsid gene of Aichi virus.

In this study, full lengths of the capsid gene of 12 Aichi virus strains isolated in the four countries were successfully obtained. The phylogenetic tree constructed based on the capsid sequences of global Aichi strains including the strains studied and the reference strains showed that Aichi viruses could be divided into two major lineages: lineage I includes the Aichi virus strains isolated in Japan, Germany, Thailand, and Vietnam, lineage II comprises of Aichi strains from Bangladesh and Brazil. This proposal was also supported by the nucleotide distance variations up to more than 12.7% among Aichi strains between the two lineages (Table II). Lineage I could be further divided into four sub-lineages from a to d, in which, sub-lineage Ia consists of Japanese and Vietnamese strains in this study, sub-lineage Ib contains Japanese reference strains, sub-lineages Ic comprises of the German reference strain and one studied Japanese strain, and sub-lineage Id includes the studied Thai strain (Fig. 1 and Table II). In addition, three phylogenetic trees constructed from each of three nucleotide segments of the VP0, VP3, and VP1 domains were similar to one another and to the phylogenetic tree constructed from the whole capsid gene (data not shown). Taken together, the findings indicated that the VP1 domain is the most variable region of the capsid gene and appropriate for classification of Aichi viruses.

In comparison to the phylogenetic tree constructed from nucleotide sequences of the 3CD junction region in previous studies, it is noteworthy that all the Aichi virus strains of lineages I and II were clustered into the same genotypes A and B, respectively. Based on the 12 strains studied and the 5 reference strains, the phylogenetic tree constructed from the capsid gene was quite similar to that of the 3CD junction region, in which, the Japanese, Thai, and Vietnamese strains used in this study belonged to genotype A and the three Bangladeshi strains were included in genotype B [Yamashita et al.,

2000; Oh et al., 2006; Pham et al., 2007]. Therefore, the genetic differences between the two Aichi virus lineages seem to be not only due to the variation of the capsid gene, but also of the other regions of Aichi virus genome. However, in agreement with previous findings and suggestion, the sequence diversity of the capsid gene was higher than in the 3CD region. The nucleotide distances between Aichi virus strains of the two lineages I and II based on the capsid gene were equal to or higher than 12.7% (Table II), while those of the two genotypes A and B based on 3CD region were less than 10% (data not shown).

By Figure 2, the different amino acids between Aichi virus strains of lineages I and II could be found throughout the whole deduced amino acid sequences. Those amino acid substitutions which were considered to be lineage-specific, especially the different triple of amino acids at positions 220–222 located within the VP0 region, can be used as a useful reference to develop primers for genotyping. Also, it could be presumed that the different antigenicity between strains of the two lineages might attribute to the differences of all the three capsid proteins. This hypothesis should be elucidated in future studies on epitopes of capsid proteins of Aichi viruses.

Despite of the limited number of the strains used, this study may be useful in providing the classification of Aichi virus based on the capsid gene and contributing background data for future researches into Aichi viruses. Since Aichi virus is a new virus discovered recently, it should be recognized more widely in other countries, and further genetic research is necessary.

REFERENCES

- Dey SK, Nguyen TA, Phan TG, Nishio O, Salim AF, Rahman M, Yagyu F, Okitsu S, Ushijima H. 2007. Molecular and epidemiological trend of norovirus associated gastroenteritis in Dhaka City, Bangladesh. *J Clin Virol* 40:218–223.
- Glass RI, Bresee J, Jiang B, Gentsch J, Ando T, Fankhauser R, Noel J, Parashar U, Rosen B, Monroe SS. 2001. Gastroenteritis viruses: An overview. *Novartis Found Symp* 238:5–19; discussion 19–25.
- Khamrin P, Manekarn N, Peerakome S, Tonusin S, Malaso R, Mizuguchi M, Okitsu S, Ushijima H. 2007. Genetic diversity of noroviruses and sapoviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand. *J Med Virol* 79:1921–1926.
- Kumar S, Tamura K, Nei M. 2004. MEGA A3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5:150–163.
- Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. *Emerg Infect Dis* 5:607–625.
- Nagashima S, Sasaki J, Taniguchi K. 2003. Functional analysis of the stem-loop structures at the 5' end of the Aichi virus genome. *Virology* 313:56–65.
- Nagashima S, Sasaki J, Taniguchi K. 2005. The 5'-terminal region of the Aichi virus genome encodes cis-acting replication elements required for positive- and negative-strand RNA synthesis. *J Virol* 79:6918–6931.
- Nguyen TA, Yagyu F, Okame M, Phan TG, Trinh QD, Yan H, Hoang KT, Cao AT, Le Hoang P, Okitsu S, Ushijima H. 2007. Diversity of viruses associated with acute gastroenteritis in children hospitalized with diarrhea in Ho Chi Minh City, Vietnam. *J Med Virol* 79:582–590.
- Oh DY, Silva PA, Hauroeder B, Diedrich S, Cardoso DD, Schreier E. 2006. Molecular characterization of the first Aichi viruses

- isolated in Europe and in South America. *Arch Virol* 151:1199-1206.
- Pham NT, Khamrin P, Nguyen TA, Kanti DS, Phan TG, Okitsu S, Ushijima H. 2007. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. *J Clin Microbiol* 45:2287-2288.
- Phan TG, Nguyen TA, Kuroiwa T, Kaneshi K, Ueda Y, Nakaya S, Nishimura S, Nishimura T, Yamamoto A, Okitsu S, Ushijima H. 2005. Viral diarrhea in Japanese children: Results from a one-year epidemiologic study. *Clin Lab* 51:183-191.
- Pringle CR. 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Arch Virol* 144:2065-2070.
- Sasaki J, Kusuhara Y, Maeno Y, Kobayashi N, Yamashita T, Sakae K, Takeda N, Taniguchi K. 2001. Construction of an infectious cDNA clone of Aichi virus (a new member of the family Picornaviridae) and mutational analysis of a stem-loop structure at the 5' end of the genome. *J Virol* 75:8021-8030.
- Sasaki J, Nagashima S, Taniguchi K. 2003. Aichi virus leader protein is involved in viral RNA replication and encapsidation. *J Virol* 77:10799-10807.
- Yamashita T, Kobayashi S, Sakae K, Nakata S, Chiba S, Ishihara Y, Isomura S. 1991. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *J Infect Dis* 164:954-957.
- Yamashita T, Sakae K, Ishihara Y, Isomura S, Utagawa E. 1993. Prevalence of newly isolated, cytopathic small round virus (Aichi strain) in Japan. *J Clin Microbiol* 31:2938-2943.
- Yamashita T, Sakae K, Kobayashi S, Ishihara Y, Miyake T, Mubina A, Isomura S. 1995. Isolation of cytopathic small round virus (Aichi virus) from Pakistani children and Japanese travelers from South-east Asia. *Microbiol Immunol* 39:433-435.
- Yamashita T, Sakae K, Tsuzuki H, Suzuki Y, Ishikawa N, Takeda N, Miyamura T, Yamazaki S. 1998. Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the Picornaviridae associated with acute gastroenteritis in humans. *J Virol* 72:8408-8412.
- Yamashita T, Sugiyama M, Tsuzuki H, Sakae K, Suzuki Y, Miyazaki Y. 2000. Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the Picornavirus family associated with gastroenteritis in humans. *J Clin Microbiol* 38:2955-2961.
- Yamashita T, Ito M, Tsuzuki H, Sakae K. 2001. Identification of Aichi virus infection by measurement of immunoglobulin responses in an enzyme-linked immunosorbent assay. *J Clin Microbiol* 39:4178-4180.

Association of RANTES –403 G/A, –28 C/G and In1.1 T/C Polymorphism With HIV-1 Transmission and Progression Among North Indians

Anurag Rathore,¹ Animesh Chatterjee,¹ P. Sivarama,² Naohiko Yamamoto,³ Pradeep K. Singhal,⁴ and Tapan N. Dhole^{1*}

¹Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India

²AIDS Counseling and Treatment Center, Belgaum, India

³Department of International Health, Nagoya University School of Medicine, Nagoya, Japan

⁴Professional Biotech Pvt. Ltd., New Delhi, India

The relationships between host immune factors and HIV-1 disease progression are still in dispute. The RANTES SNPs exhibit distinct ethnic distribution and are associated with different effects on the course of HIV infection. Therefore, impact of RANTES gene polymorphism on HIV-1 transmission and progression needs to be evaluated. The RANTES genotypes were identified by PCR-RFLP method and confirmed by sequencing in HIV-1 seronegative (HSN; $n = 315$), HIV-1 exposed seronegative (HES; $n = 47$) and HIV-1 seropositive (HSP; $n = 196$) patients classified into different clinical stages (i.e. Stages I, II, III). Fisher exact test was used for statistical analysis and Arlequin software for haplotype analysis. RANTES allele –403G, –28C and In1.1 T were the predominant allele in the subject studied. HSP group have higher frequency of RANTES In1.1 T allele compared with HSN (91.32% vs. 86.19%; $P = 0.013$) and HES (91.32% vs. 78.72%; $P = 0.001$). Higher frequency of RANTES In1.1 C allele in Stage III was observed, compared with Stage I (14.28% vs. 6.39%) and was significantly associated with high risk ($P = 0.047$, OR = 2.439, C.I. = 1.061–5.609). Haplotype II (ACT) was significantly higher in HSP compared with HSN (9.69% vs. 1.58%) and associated with high risk ($P < 0.001$, OR = 6.655, C.I. = 2.443–18.132). There were no significant differences in RANTES –403 A/G and –28 C/G genotype and allele distribution in all the groups compared. Our results implicate that RANTES In1.1 T allele and haplotype II (ACT) may be a risk factor for HIV-1 transmission while RANTES In1.1 C allele may be risk factor for disease progression among North Indians. *J. Med. Virol.* **80**: 1133–1141, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: RANTES polymorphism; HIV-1; transmission; progression; risk factors

INTRODUCTION

Among genes that influence human susceptibility to HIV infection or AIDS progression, chemokine-receptor and chemokine genes were extensively studied because of their role as HIV co-receptor or co-receptor competitor, respectively. RANTES (regulated upon activation, normal T cell expressed and secreted) has emerged as one of the most important co-receptor competitor molecule of the human immune system involved in the pathogenesis of HIV infection. RANTES is an endogenous ligand for CCR5, the key co-receptor for M-tropic HIV-1 strains commonly seen in early infection [Koning et al., 2003]. RANTES acts by blocking of the HIV envelope glycoprotein gp120 to CCR5 and by reducing surface levels of CCR5 co-receptor [Mack et al., 1998]. However, there is evidence that RANTES might promote the replication of some HIV strains, particularly at high concentrations [Kelly et al., 1998; Kinter et al., 1998]. With respect to RANTES, CD4 T cells from highly exposed-uninfected individuals produce increased amounts compared with random blood donor controls [Paxton et al., 1996; Furci et al., 1997; Suresh et al., 2007]. Whereas, immortalized

*Correspondence to: Tapan N. Dhole, Professor, Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raebareilly Road, Lucknow 226014, India.
E-mail: tapandhole@yahoo.co.in

Accepted 13 March 2008

DOI 10.1002/jmv.21201

Published online in Wiley InterScience
(www.interscience.wiley.com)

CD4 T cells from AIDS patients produce much less RANTES than those from long-term non-progressors [Saha et al., 1998; Paxton et al., 1999]. Moreover, defective CCR5 expression in CCR5 Δ 32 homozygotes results in the downregulation of RANTES expression thus affecting the RANTES level [Samson et al., 1996; Clegg et al., 2000; Paxton et al., 2001].

The study of host genetic polymorphism studies implicating RANTES -403 G/A, RANTES -28 C/G in the promoter region and RANTES In1.1 T/C in the first intron region has provided important evidence that control of RANTES expression might influence the risk and outcome of HIV-1 infection. The RANTES -28G variant was shown to elevate promoter activity *in vitro* [Liu et al., 1999a; An et al., 2002] and is associated with a slower rate of CD4 cell depletion in HIV-1 infected Japanese patients [Liu et al., 1999a]. Although these results were not confirmed in subsequent studies in other ethnic groups in which the RANTES -28G frequency was lower than in the Japanese [Gonzalez et al., 2001; An et al., 2002; Zhao et al., 2004], McDermott et al. reported that patients with RANTES -28G showed a clear trend towards slower rate of CD4 cell depletion in HIV-1 infected European Americans [McDermott et al., 2000]. Recent reports have shown the protective effect of RANTES -28G on HIV disease progression in Thais [Wichukchinda et al., 2006] while, delayed AIDS progression in HIV-1 infected Japanese hemophiliacs [Koizumi et al., 2007]. With respects to RANTES -403A variants, HIV-1 infected European American with RANTES -403A were initially reported to progress more slowly to AIDS [McDermott et al., 2000]. However, subsequent studies with a larger sample size showed that RANTES -403A or RANTES In1.1 C, a RANTES intron polymorphism which is in a strong linkage disequilibrium with RANTES -403A and which negatively affects RANTES transcription *in vitro*, was reported to be associated with an increased rate of disease progression [Gonzalez et al., 2001; An et al., 2002]. Recent studies of other populations reported that -403G allele may be associated with increased susceptibility to HIV infection [Zhao et al., 2004] whereas, no such association of RANTES -403 A/G and -28C/G polymorphism could be established in HIV-1 infected Ugandans [Cooke et al., 2006], North Indians [Suresh et al., 2006], and Spanish populations [Vidal et al., 2006]. *In vitro* functional data, acquired using luciferase reporter assays in transfected jurkat cells, demonstrated that the RANTES In1.1C allele was associated with reduced RANTES expression thus contributing to the rapid progression of AIDS in European-Americans and African-Americans [An et al., 2002], however, the SNP was not found to influence disease progression in Japanese Hemophiliacs [Koizumi et al., 2007]. RANTES In1.1 C without RANTES -28G had an accelerating effect on HIV disease progression [Wichukchinda et al., 2006]. In contrast to previous studies, authors have recently reported that homozygosity for the In1.1 C allele was associated with protection from death in HIV-1 positive individuals in Ugandans [Cooke et al., 2006].

HIV-1 infected individuals have widely different rates of disease progression and several SNPs are reported to be effective in different clinical stages of HIV/AIDS patients. Among North Indians, we recently reported that homozygous DC-SIGNR 5/5 showed significant reduced risk of HIV-1 infection [Rathore et al., 2008a] while repeat region polymorphism in the DC-SIGN neck domain does not effect risk of HIV-1 transmission [Rathore et al., 2008b]. Till date, only single study was conducted in which effect of RANTES genetic variants was assessed for HIV/AIDS transmission among North Indians [Suresh et al., 2006]. The global, ethnic and regional distribution of this HIV/AIDS protective mutations frequency varies significantly giving each population a different genetic resistance profile to the HIV infection and AIDS progression. Therefore, it is important to investigate factors modulating rates of disease progression for designing novel therapies and vaccines study.

MATERIALS AND METHODS

Patient Selection

One hundred and ninety-six HIV-1 seropositive patients (HSPs) were enrolled from the outpatients attending the clinics of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India from January 2004 to December 2006. The HIV-1 seropositive subjects were in different stages of disease progression and had no history or a less than 6-week prior history of antiretroviral therapy. Depending on their CD4 counts and based on clinical symptoms, HIV-1 patients were divided into three subgroups, on the basis of disease severity, as per the Centers for disease control and prevention (CDC) criteria. Three groups were as follows: Stage I (n = 86; asymptomatic HIV-1 patients in CDC category A1 to A3, Stage II (n = 61; symptomatic HIV-1 patients without AIDS in CDC category B1 to B3), Stage III (n = 49; symptomatic HIV-1 patients with AIDS in CDC category C1 to C3) [McNicholl et al., 2003; Vajpayee et al., 2005]. Only individuals who had a minimum follow-up time of at least 6 months were included in the study. 315 age-matched normal healthy controls of similar ethnicity with HIV-1 seronegative status (HSN) were recruited for the present study. 47 HIV-1 seronegative individuals with history of repeated sexual intercourse (twice a week) without any protection (e.g. condoms) with HIV-1 infected partners for at least 1-year 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 3 months till 1 year. Demographic profiles of the study groups are given in Table I. After an informed consent, 5-ml blood sample was taken in EDTA for analysis of DNA.

Determination of HIV-1 Status and CD4 Cell Count

All individuals were screened for their HIV-1 status by primarily screening with ELISA (Vironostika, HIV Uni-FormII Ag/Ab, Biomerieux, Netherlands) and sub-

TABLE I. Demographic and Clinical Characteristics of the Study Groups

HIV-1 seronegative (HSN; n = 315)	
Age (median, range)	58, 35–60
Sex (M/F ratio)	6/1
HIV-1 exposed seronegative (HES; n = 47)	
Age (median, range)	59, 35–65
Sex (M/F ratio)	7/1
HIV-1 seropositive (HSP; n = 196)	
Age (median, range)	57, 30–67
Sex (M/F ratio)	8/1
CD4 counts/ μ l (median, range)	145, 30–1304
Stage I ^a (n = 86)	
Age (median, range)	58, 30–65
Sex (M/F ratio)	7/1
CD4 counts/ μ l (median, range)	315, 65–1304
Stage II ^a (n = 61)	
Age (median, range)	55, 35–60
Sex (M/F ratio)	9/1
CD4 counts/ μ l (median, range)	175, 60–512
Stage III ^a (n = 49)	
Age (median, range)	52, 38–67
Sex (M/F ratio)	8/1
CD4 counts/ μ l (median, range)	120, 30–450

n: number of individuals.

^aThese groups were subgroups of the HIV-1 seropositive patient and were classified according to CDC (Centers for disease control and prevention) (23,24).

sequently confirmed with Western blot (LAV Blot I, BioRad, France). CD4 cell count was measured by flow cytometer FACScan (Becton Dickinson Immunocytometry systems, San Jose, CA) using fresh EDTA-treated whole blood at the time of recruitment.

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, CA) according to the protocols supplied by the manufacturer. Usually ~0.1 μ g genomic DNA was used for the genotyping studies.

Polymerase Chain Reaction and Sequencing

RANTES -403G/A genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described previously [Liu et al., 1999b]. Amplification of a 124 bp fragment spanning position -403 was done by PCR using forward primer 5'-taa cat cct tcc atg gat cag gg-3' and reverse primer 5'-tgg cag tta gga cag gat-3'. PCR was carried out in 25 μ l reaction volume containing 0.1 μ M each primer, 100 μ M dNTPs, 2.5 mM MgCl₂, and 0.5 unit of Taq DNA polymerase (Roche, Germany) in 1 \times buffer supplied with enzyme. The reaction mixture was subjected to 40 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 3 sec. Digestion with MnlI (Fermentas) yielded a 103 bp and a 21 bp fragment only when G was present at position -403 and was determined by electrophoresis in 20% polyacrylamide gel.

RANTES promoter -28C/G was genotyped by PCR-RFLP as described previously [Liu et al., 1999b]. Briefly, amplification of 136 bp fragment spanning position -28 was amplified from extracted genomic DNA using forward primer 5'-ccg gag get att tca gtt tt-3' and reverse

primer 5'-gca gct cag gct ggc cct t-3' with same PCR conditions as mentioned above. PCR was carried out in 25 μ l reaction volume containing 0.1 μ M each primer, 100 μ M dNTPs, 2.5 mM MgCl₂, and 0.5 unit of Taq DNA polymerase (Roche, Germany) in 1 \times buffer supplied with enzyme. The presence of a MnlI site yielded an 111 bp and 25 bp fragment only when C was present at position -28, and was determined by electrophoresis in 20% polyacrylamide gel.

Genotyping of RANTES In1.1C was carried out by previously described protocol [Ahlenstiel et al., 2005] using forward primer: 5'-CCT GGT CTT GAC CAC CAC A-3' and reverse primer: 5'-GCT GAC AGG CAT GAG TCA GA-3'. A 343-bp fragment spanning position In1.1C was amplified from extracted genomic DNA. Amplification was done for 40 cycles at 94°C for 1 sec, 55°C for 40 sec, and 72°C for 1 min. PCR was carried out in 25 μ l reaction volume containing 0.1 μ M each primer, 100 μ M dNTPs, 2.5 mM MgCl₂, and 0.5 unit of Taq DNA polymerase (Roche, Germany) in 1 \times buffer supplied with enzyme. The presence of MboII (Fermentas) site yielded a 234 and 109 bp corresponding to the presence of a C at position RANTES In1.1, and was determined by electrophoresis in 2.5% agarose gel. All the PCR-amplified products were randomly selected to be cloned and sequenced to verify the PCR-RFLP results according to the manufacturers protocols (Applied Biosystems/Perkin Elmer Foster City, CA) using the Dye Terminator cycle sequencing kit in ABI PRISM 310 genetic Analyzer.

Statistical Analysis

Statistical analysis was done by SPSS software version 11.5 (SPSS, Chicago, IL). Direct gene counting method was used to determine the frequency of genotypes and alleles. The Fisher's exact or χ^2 test was used to determine differences in allele/genotype frequencies of RANTES genetic variants. Odds ratios (OR) and its 95% confidence interval (C.I.) were obtained to describe the strength of association. Observed and expected genotype frequencies were compared by χ^2 test to check deviation from Hardy Weinberg Equilibrium. A *P* value <0.05 was considered to be statistically significant. The haplotype frequencies were calculated from the observed genotypes by using the Arlequin software vers. 2.000 [Schneider et al., 2000].

RESULTS

We genotyped 315 HIV-1 seronegative (HSN), 47 HIV-1 exposed seronegative (HES) and 196 HIV-1 seropositive (HSP) patient for RANTES -403A/G, -28C/G and In1.1T/C polymorphism. Genotype distribution and allelic frequency were compared between HSN, HES and HSP and also between three different subgroups (Stages I, II, III) of HSP group. The distributions of the genotypic frequencies of the RANTES polymorphism were in Hardy-Weinberg equilibrium. The percent allele/genotype frequencies corresponding to three SNPs are shown in Tables II and III.

TABLE II. Genotype and Allele Frequencies of RANTES -403 G/A, -28 C/G, In1.1 T/C Polymorphism Among HIV-1 Seropositive, HIV-1 Seronegative and HIV-1 Exposed Seronegative Individuals

Genotype	HSP (n = 196)	HSN (n = 315)	Odds ratio	P value	Confidence interval	HES (n = 47)	Odds ratio ^a	P value ^a	Confidence interval ^b
RANTES -403 A/G									
G	319(81.37)	534(84.76)	0.786	0.166	0.562-1.098	72(76.59)	1.335	0.311	0.777-2.293
A	73(18.623)	96(15.23)	1.273	0.166	0.911-1.779	22(23.40)	0.749	0.311	0.436-1.286
GG	132(67.34)	230(73.01)	0.762	0.193	0.517-1.124	27(57.44)	1.528	0.233	0.797-2.929
GA	55(28.06)	74(23.49)	1.270	0.251	0.846-1.907	18(38.25)	0.628	0.214	0.323-1.223
AA	9(4.59)	11(3.49)	1.330	0.640	0.541-3.270	2(4.25)	1.083	1.000	0.226-5.186
RANTES -28 C/G									
C	358(98.97)	617(97.93)	2.044	0.314	0.662-6.313	91(96.80)	3.198	0.135	0.703-14.537
G	4(1.02)	13(2.06)	0.489	0.314	0.158-1.511	3(3.19)	0.313	0.135	0.069-1.422
CC	192(97.95)	302(95.87)	2.066	0.310	0.664-6.429	44(93.61)	3.273	0.134	0.707-15.150
CG	4(2.04)	13(4.12)	0.484	0.310	0.156-1.506	3(6.38)	0.306	0.134	0.066-1.414
GG	0	0	—	—	—	0	—	—	—
RANTES In1.1 T/C									
T	358(91.32)	543(86.19)	1.687	0.013	1.110-2.563	74(78.72)	2.846	0.001	1.552-5.219
C	34(8.67)	87(13.80)	0.593	0.013	0.390-0.901	20(21.27)	0.351	0.001	0.192-0.644
TT	166(84.69)	230(73.01)	2.045	0.002	1.289-3.245	38(59.5)	3.755	<0.001	1.864-7.564
TC	26(13.26)	83(26.34)	0.427	<0.001	0.264-0.693	18(38.2)	0.246	<0.001	0.120-0.505
CC	4(2.04)	2(0.63)	3.260	0.210	0.592-17.970	1(2.12)	0.958	1.000	0.105-8.778

HSP, HIV-1 seropositive; HSN, HIV-1 seronegative; HES, HIV-1 exposed seronegative.

Number in parentheses gives the data in percentage.

^aHSP vs. HES.

TABLE III. Genotype and Allele Frequencies of RANTES -403 G/A, -28 C/G, In1.1 T/C Polymorphism Among HIV-1 Seropositive Patients Classified into Stages I, II and III

Genotype	Stage III (n = 49)	Stage I (n = 86)	Odds ratio	P value	Confidence interval	Stage II (n = 61)	Odds ratio ^a	P value ^a	Confidence interval ^b
RANTES -403 A/G									
G	75(76.53)	148(86.04)	0.529	0.065	0.280-0.999	96(78.68)	0.883	0.746	0.467-1.670
A	23(23.46)	24(13.95)	1.891	0.065	1.001-3.571	26(21.31)	1.132	0.746	0.599-2.141
GG	30(61.22)	63(73.25)	0.576	0.177	0.273-1.217	39(63.93)	0.891	0.844	0.410-1.937
GA	15(30.61)	22(25.58)	1.283	0.552	0.590-2.791	18(29.50)	1.054	1.000	0.464-2.392
AA	4(8.16)	1(1.16)	7.556	0.058	0.820-69.629	4(6.55)	1.267	1.000	0.300-5.346
RANTES -28 C/G									
C	97(98.97)	171(99.41)	0.567	1.000	0.035-9.171	120(98.36)	1.617	1.000	0.144-18.096
G	1(1.02)	1(0.58)	1.763	1.000	0.109-28.501	2(1.63)	0.619	1.000	0.055-6.924
CC	48(97.95)	85(98.83)	0.565	1.000	0.035-9.234	59(96.72)	1.627	1.000	0.143-18.491
CG	1(2.04)	1(1.16)	1.771	1.000	0.108-28.955	2(3.27)	0.615	1.000	0.054-6.984
GG	0	0	—	—	—	0	—	—	—
RANTES In1.1 T/C									
T	84(85.71)	161(93.60)	0.410	0.047	0.178-0.943	113(92.62)	0.478	0.121	0.197-1.156
C	14(14.28)	11(6.39)	2.439	0.047	1.061-5.609	9(7.37)	2.093	0.121	0.865-5.064
TT	36(73.46)	76(88.37)	0.364	0.033	0.146-0.910	54(88.52)	0.359	0.050	0.131-0.987
TC	12(24.48)	9(10.46)	2.775	0.047	1.074-7.168	5(8.19)	3.632	0.032	1.182-11.165
CC	1(2.04)	1(1.16)	1.771	1.000	0.108-28.955	2(3.27)	0.615	1.000	0.054-6.984

Number in parentheses gives the data in percentage.

^aStage III vs. Stage II.

RANTES Allele/Genotype Distribution Analysis

The frequency of RANTES In1.1 T allele was significantly higher in HSP compared with HSN (91.32% vs. 86.19%) and HES (91.32% vs. 78.72%) and associated with high risk ($P=0.013$, OR=1.687, C.I.=1.110–2.563 and $P=0.001$, OR=2.846, C.I.=1.552–5.219), respectively. The frequency of RANTES 1.1 TT genotype was significantly higher in HSP compared with HSN (84.69% vs. 73.01%) and HES (84.69% vs. 59.57%) and associated with high risk ($P=0.002$, OR=2.045, C.I.=1.289–3.245 and $P<0.001$, OR=3.755, C.I.=1.864–7.564), respectively (Table II).

A further comparison in between different stages of HIV-1 infected patients revealed higher frequency of RANTES 1.1 C allele in Stage III compared with Stage I (14.28% vs. 6.39%) and was significantly associated with high risk ($P=0.047$, OR=2.439, C.I.=1.061–5.609). The frequency of RANTES 1.1 TC genotype was higher in Stage III compared with Stage I (24.48% vs. 10.46%) and II (24.48% vs. 8.19%) and was significantly associated with high risk ($P=0.047$, OR=2.775, C.I.=1.074–7.168) and ($P=0.032$, OR=3.632, C.I.=1.182–11.165), respectively. RANTES -403A/G and -28G/C allele or genotype frequencies were similar in all the compared groups (Table III).

RANTES Haplotype Analysis

Four haplotype I (GCT), II (ACT), III (ACC) and IV (AGC) were detected in North Indian individual (Tables IV and V). Frequency of haplotype I (GCT) was found to be predominant and fairly similar in all the studied groups. Haplotype II (ACT) was found at significantly higher frequency in HSP compared with HSN (9.69% vs. 1.58%) and was associated with high risk ($P<0.001$, OR=6.655, C.I.=2.443–18.132). Though, frequency of haplotype II (ACT) was higher in HSP compared with HES (9.69% vs. 2.12%) but was not significantly associated with high risk ($P=0.136$, OR=4.938, C.I.=0.644–37.857) (Table IV). Frequency of haplotype III (ACC) was though higher in Stage III compared with Stage I (12.24% vs. 4.65%; $P=0.169$, OR=2.86, C.I.=0.766–10.685) and Stage II (12.24% vs. 6.55%; $P=0.336$, OR=1.988, C.I.=0.528–7.485), but was not significant (Table V). The minor haplotype observed in the study was haplotype IV (AGC), but there was no significant difference in its frequency in the healthy controls versus HIV-1-infected study subjects.

Further stratification of the groups by age and gender did not provide evidence of an association between RANTES alleles or genotypes and HIV infection (data not shown).

DISCUSSION

Several single nucleotide polymorphisms in the RANTES gene have been reported to influence the natural course of HIV infection by up or down-regulating RANTES gene activity. The most frequent of those polymorphic sites comprise -28 C/G and -403 G/A in the promoter region and In1.1 T/C in the first intron

TABLE IV. Haplotype Frequencies of RANTES 403/28/In.1.1 Polymorphism Among HIV-1 Seropositive, HIV-1 Seronegative and HIV-1 Exposed Seronegative Individuals

Haplotype (403/28/In.1.1)	HSP (n = 196)	HSN (n = 315)	Odds ratio	P value	Confidence interval	HES (n = 47)	Odds ratio ^a	P value ^a	Confidence interval ^a
Haplotype I GCT	160(81.63)	267(84.76)	0.799	0.391	0.497–1.284	36(76.59)	1.358	0.418	0.631–2.921
Haplotype II ACT	19(9.69)	5(1.58)	6.655	<0.001	2.443–18.132	1(2.12)	4.938	0.136	0.644–37.857
Haplotype III ACC	15(7.65)	37(11.74)	0.623	0.175	0.332–1.167	8(17.02)	0.404	0.091	0.160–1.019
Haplotype IV AGC	2(1.02)	6(1.90)	0.531	0.717	0.106–2.657	2(4.25)	0.232	0.169	0.032–1.691

HSP, HIV-1 seropositive; HSN, HIV-1 seronegative; HES, HIV-1 exposed seronegative. Number in parentheses gives the data in percentage.
^aHSP vs. HES.

TABLE V. Haplotype Frequencies of RANTES 403/28/In1.1 Polymorphism Among HIV-1 Seropositive Patients Classified into Stages I, II and III

Haplotype (403/28/In1.1)	Stage III (n = 49)	Stage I (n = 86)	Odds ratio	P value	Confidence interval	Stage II (n = 61)	Odds ratio ^a	P value ^a	Confidence interval ^a
Haplotype I GCT	37(75.51)	74(86.04)	0.500	0.160	0.205-1.220	48(78.68)	0.835	0.820	0.342-2.042
Haplotype II ACT	5(10.20)	7(8.13)	1.282	0.757	0.384-4.281	8(13.11)	0.753	0.770	0.230-2.466
Haplotype III ACC	6(12.24)	4(4.65)	2.860	0.169	0.766-10.685	4(6.55)	1.988	0.336	0.528-7.485
Haplotype IV AGC	1(2.04)	1(1.16)	1.771	1.000	0.108-28.955	1(1.637)	1.250	1.000	0.076-20.508

Number in parentheses gives the data in percentage.

^aStage III vs. Stage II.

region of RANTES gene. The -28G and -403A variant were reported to up-regulate RANTES transcription [Liu et al., 1999a; Nickel et al., 2000], while In1.1C allele was reported to decrease RANTES transcriptional activity thus reducing the gene expression [An et al., 2002].

This is the first host-genetic polymorphism study with a substantial sample size in the North Indians, addressing an effect of RANTES genetic variants on HIV-1 disease progression. The frequency of the RANTES -403 A and -28 G allele at the promoter region was 15.23% and 2.06% respectively in the North Indian general population. The frequency was the lowest compared with those in other populations studied (Table VI). The frequency of In1.1 C allele was 13.80% similar to Caucasians and European American, but lower than Han Chinese Asians, Ugandans and African American suggesting the ethnic variability in the allelic frequencies of this gene. Because of this variation in gene frequencies between different ethnic groups it is crucial to repeat genetic association studies in each group rather than to generalize these findings when we try to establish the relationship between a polymorphic marker and HIV/AIDS susceptibility or progression. The most common haplotype observed in this study was haplotype I (GCT), which is in concordant with previous studies [An et al., 2002; Wichukchinda et al., 2006].

A relative comparison between HSN, HES and HSP subjects was carried out to assess the effect of RANTES variability on risk of HIV transmission/susceptibility. The results obtained in this study are consistent with previous studies on HIV-1 exposed but seronegative North Indian individuals [Suresh et al., 2006] and Whites individuals [Liu et al., 2004], that showed no correlation of RANTES -403A/G and -28G/C polymorphism with susceptibility against HIV-1 infection. Cross-sectional genetic association case-control study on White Spaniards population [Vidal et al., 2006] and Japanese [Liu et al., 1999a] also revealed no association of -403 A/G and -28G/C polymorphism with HIV-1 susceptibility. In contrast, 403A allele and wild-type 403 G allele increases susceptibility to HIV-1 infection in white Americans [McDermott et al., 2000; An et al., 2002], Han Chinese [Liu et al., 2003] and in Chinese subjects [Zhao et al., 2004], respectively. Furthermore, RANTES In.1.1 C allele was reported to be associated with increased susceptibility to HIV-1 infection in US cohorts of HIV-1 positive patients [An et al., 2002]. In contrast, present study reports that RANTES In1.1 T allele was found at a higher frequency in the HSP group compared with HSN and HES suggesting that the RANTES 1.1 T allele may be associated with increased susceptibility to HIV-1 transmission. To approve these results, we use the haplotype analysis to test association of this polymorphism with HIV-1 susceptibility. Haplotype II (ACT) that corresponds to the haplogroup R2 in other studies [An et al., 2002] was found to be over-represented in HIV-1 seropositive individuals compared to HIV-1 seronegative though statistical significant association was not observed when compared with

TABLE VI. Frequency of RANTES -403 G/A, -28 C/G, and In.1.1 T/C Alleles in North Indian and Other Ethnic Population

Populations	Samples	RANTES -403G (%)	RANTES -403A (%)	RANTES -28C (%)	RANTES -28G (%)	RANTES In.1.1 T (%)	RANTES In.1.1 C (%)	Reference
North Indians	315	84.76	15.23	97.93	2.06	86.19	13.80	Present study
African American	1,574	57.00	43.00	Very high	Very low	80.00	20.00	Paxton et al. [2001]
Caucasians	109	84.86	15.13	97.20	2.70	88.99	11.00	Liu et al. [1999]
Chinese	439	62.30	37.70	87.70	12.30	N.D.	N.D.	Zhao et al. [2004]
European Americans	2,594	82.00	18.00	Very high	Very low	86.00	14.00	Paxton et al. [2001]
Han Chinese Asian	129	64.00	36.00	87.00	13.00	66.00	34.00	Paxton et al. [2001]
Japanese	193	62.40	37.56	83.40	16.50	N.D.	N.D.	Liu et al. [1999]
North Indians	75	88.00	12.00	99.00	1.00	N.D.	N.D.	Suresh et al. [2006]
Thai Females	119	73.10	26.90	92.40	7.60	76.50	23.50	Wichukchinda et al. [2006]
Ugandans	94	53.00	47.00	N.D.	N.D.	80.00	20.00	Cooke et al. [2006]
White Spaniards	100	82.00	18.00	97.00	3.00	N.D.	N.D.	Vidal et al. [2006]

N.D.: not detected.

HIV-1 exposed seronegative. But, when HIV-1 seropositive group was compared with HIV-1 exposed seronegative and HIV-1 seronegative taken together significant association was found suggesting its possible role in HIV-1 acquisition. Similarly, another RANTES haplotype III (ACC) in this study can be compared with the haplotype R4 that has been shown to be associated with increased risk of infection in European Americans [An et al., 2002], but not in presently studied Indian individuals. Possible explanation for discrepancy between previous and present study may be because influence of the allelic variants of RANTES gene on the risk of HIV-1 susceptibility varied widely and depended on the origin of the subjects analyzed.

HIV-1 seropositive patients classified into different stages of HIV/AIDS disease (Stages I, II and III) were analyzed to evaluate their effect on development of AIDS. However, one limitation of this study is that the HIV-1 positive patients could not be classified on the basis of number of years of known seroprevalence without antiretroviral therapy and viral load. Nonetheless, in the absence of such a longitudinal follow up data, the patients were categorized on the basis of their CD4 counts and clinical CDC classification. RANTES In.1.1 C allele was found at a higher frequency in the patients classified as CDC Stage III compared with Stage I, suggesting its possible role in development of AIDS. Our results were in agreement with the previous studies that reported, RANTES In.1.1 C contribute to the rapid progression and had an accelerating effect on HIV disease progression in European Americans, African Americans [An et al., 2002] and HIV-infected Thais [Wichukchinda et al., 2006]. None of the haplotypes were found to be associated with disease progression among North Indian HIV-1 infected patients, though previous studies have showed haplotype III (ACC) which correspond to haplogroup R3, accelerated AIDS progression in both European American and African American [An et al., 2002]. The lack or the presence of association in HIV-1 susceptibility/progression between previous and present study may reflect genetic heterogeneity in the pathogenesis of HIV/AIDS. Different risk alleles may be important in different populations. Discrepancies between our results and the previous studies may be due to differences in ethnicity, environmental and social conditions of the population. Moreover, differences in design and recruitment of subjects between this study and previous genetic studies cannot be ignored.

In conclusion, we have studied genetic polymorphism known to be involved in the risk of HIV infection and the rate of HIV disease progression. We have shown that, with respect to HIV-1 susceptibility RANTES In.1.1 TT genotype/T allele and haplotype ACT are major risk factors for HIV-1 transmission while RANTES In.1.1 C allele is a risk factor for accelerating disease progression among North Indians. Further genetic studies covering more number of SNPs in the RANTES and neighboring region tested in a larger number of well-categorized seropositive subjects are warranted.

ACKNOWLEDGMENTS

We gratefully thank Mr. S. Pandey, V. K. Mishra, Department of Microbiology, SGPGIMS, Lucknow for assistance in coordinating the clinical samples and all of the individuals who participated in the study. The Senior Research Fellowship provided by Indian Council of Medical Research (ICMR, New Delhi) to Mr. Anurag Rathore (first author) is greatly acknowledged.

REFERENCES

- Ahlenstiel G, Iwan A, Nattermann J, Bueren K, Rockstroh JK, Brackmann HH, Kupfer B, Landt O, Peled A, Sauerbruch T, Spengler U, Weitas RP. 2005. Distribution and effects of polymorphic RANTES gene alleles in HIV/HCV coinfection—a prospective cross-sectional study. *World J Gastroenterol* 11:7631–7638.
- An P, Nelson GW, Wang L, Donfield S, Goedert JJ, Phair J, Vlahov D, Buchbinder S, Farrar WL, Modi W, O'Brien SJ, Winkler CA. 2002. Modulating influence on HIV/AIDS by interacting RANTES gene variants. *Proc Natl Acad Sci USA* 99:10002–10007.
- Clegg AO, Ashton LJ, Biti RA, Badhwar P, Williamson P, Kaldor JM, Stewart GJ. 2000. CCR5 promoter polymorphisms, CCR5 59029A and CCR5 59353C, are under represented in HIV-1-infected long-term non-progressors. The Australian Long-Term Non-Progressor Study Group. *AIDS* 14:103–108.
- Cooke GS, Tosh K, Ramaley PA, Kaleebu P, Zhuang J, Nakiyingi JS, Wattera C, Gilks CF, French N, Whitworth JA, Hill AV. 2006. A polymorphism that reduces RANTES expression is associated with protection from death in HIV-seropositive Ugandans with advanced disease. *J Infect Dis* 194:666–669.
- Furci L, Scarlatti G, Burastero S, Tambussi G, Colognesi C, Quillent C, Longhi R, Lloverro P, Borgonovo B, Gaffi D, Carrow E, Malnati M, Lusso P, Siccardi AG, Lazzarin A, Beretta A. 1997. Antigen-driven C-C chemokine-mediated HIV-1 suppression by CD4 (+) T cells from exposed uninfected individuals expressing the wild-type CCR-5 allele. *J Exp Med* 186:455–460.
- Gonzalez E, Dhanda R, Bamshad M, Mummid S, Geevarghese R, Catano G, Anderson SA, Walter EA, Stephan KT, Hammer MF, Mangano A, Sen L, Clark RA, Ahuja SS, Dolan MJ, Ahuja SK. 2001. Global survey of genetic variation in CCR5, RANTES, and MIP-1alpha: impact on the epidemiology of the HIV-1 pandemic. *Proc Natl Acad Sci USA* 98:5199–5204.
- Kelly MD, Naif HM, Adams SL, Cunningham AL, Lloyd AR. 1998. Dichotomous effects of beta-chemokines on HIV replication in monocytes and monocyte-derived macrophages. *J Immunol* 160:3091–3095.
- Kinter A, Catanzaro A, Monaco J, et al. 1998. CC-chemokine enhance the replication of T-tropic strains of HIV-1 in CD4 (+) T cells: role of signal transduction. *Proc Natl Acad Sci USA* 95:11880–11885.
- Koizumi Y, Kageyama S, Fujiyama Y, Miyashita M, Lwembe R, Ogino K, Shioda T, Ichimura H. 2007. RANTES –28G delays and DC-SIGN –139C enhances AIDS progression in HIV type 1-infected Japanese hemophiliacs. *AIDS Res Hum Retroviruses* 23:713–719.
- Koning FA, Kwa D, Boeser-Nunnick B., et al. 2003. Decreasing sensitivity to RANTES neutralization of CC chemokine receptor-5 using non-synctium-inducing virus variants in the course of human immunodeficiency virus type 1 infection. *J Infect Dis* 188:864–872.
- Liu H, Chao D, Nakayama EE, Taguchi H, Goto M, Xin X, Takamatsu JK, Saito H, Ishikawa Y, Akaza T, Juji T, Takebe Y, Ohishi T, Fukutake K, Maruyama Y, Yashiki S, Sonoda S, Nakamura T, Nagai Y, Iwamoto A, Shioda T. 1999a. Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. *Proc Natl Acad Sci USA* 96:4581–4585.
- Liu H, Hwangbo Y, Holte S, Lee J, Wang C, Kaupp N, Zhu H, Celum C, Corey L, McElrath MJ, Zhu T. 2004. Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1. *JID* 190:1055–1058.
- Liu H, Shioda T, Nagai Y, Iwamoto A, Wasi C, Ma J, Liang W, Takebe Y, Theodorou I, Magierowska M, Krishnamoorthy R, Chaventre A, Debre P. 1999b. Distribution of HIV-1 disease modifying regulated on activation normal T cell expressed and secreted haplotypes in Asian, African and Caucasian individuals. French ALT and IMMUNOCO Study Group. *AIDS* 13:2602–2603.
- Liu XL, Wang FS, Jin L, Liu MX, Xu DZ. 2003. Preliminary study on the association of chemokine RANTES gene polymorphisms with HIV-1 infection in Chinese Han population. *Zhonghua Liu Xing Bing Xue Za Zhi* 24:971–975. Chinese.
- Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, Signoret N, Marsh M, Stangassinger M, Borlat F, Wells TN, Schlondorff D, Proudfoot AE. 1998. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med* 187:1215–1224.
- McDermott DH, Beecroft MJ, Kleeberger CA, Al-Sharif FM, Ollier WE, Zimmerman PA, Boatman BA, Leitman SF, Detels R, Hajeer AH, Murphy PM. 2000. Chemokine RANTES promoter polymorphism affects risk of both HIV infection and disease progression in the Multicenter AIDS Cohort Study. *AIDS* 14:2671–2678.
- McNicholl JM, Lal RB, Kaslow R. 2003. Human immunodeficiency virus (HIV) infection. In Cooper DN, (ed.): *Encyclopedia of the Human Genome*. Nature Publishing Group. www.ehgonline.net.
- Nickel RG, Casolaro V, Wahn U, Beyer K, Barnes KC, Plunkett BS, Freidhoff LR, Sengler C, Plitt JR, Schleimer RP, Caraballo L, Naidu RP, Levett PN, Beaty TH, Huang SK. 2000. Atopic dermatitis is associated with a functional mutation in the promoter of the C-C chemokine RANTES. *J Immunol* 164:1612–1616.
- Paxton WA, Kang S, Liu R, Landau NR, Gingeras TR, Wu L, Mackay CR, Koup RA. 1999. HIV-1 infectability of CD4+ lymphocytes with relation to beta-chemokines and the CCR5 coreceptor. *Immunol Lett* 66:71–75.
- Paxton WA, Martin SR, Tse D, O'Brien TR, Skurnick J, VanDevanter NL, Padian N, Braun JF, Kotler DP, Wolinsky SM, Koup RA. 1996. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. *Nat Med* 2:412–417.
- Paxton WA, Neumann AU, Kang S, Deutch L, Brown RC, Koup RA, Wolinsky SM. 2001. RANTES production from CD4+ lymphocytes correlates with host genotype and rates of human immunodeficiency virus type 1 disease progression. *J Infect Dis* 183:1678–1681.
- Rathore A, Chatterjee A, Sivarama P, Yamamoto N, Dhole TN. 2008a. Role of homozygous DC-SIGNR 5 tandem repeat polymorphism in HIV-1 exposed seronegative North Indian individuals. *J Clin Immunol* 28:50–57.
- Rathore A, Chatterjee A, Sood V, Khan SZ, Banerjee AC, Yamamoto N, Dhole TN. 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. *Clin Chim Acta* 2008b. Jan 12; [Epub ahead of print].
- Saha K, Bentsman G, Chess L, Volsky DJ. 1998. Endogenous production of beta-chemokines by CD4+, but not CD8+, T-cell clones correlates with the clinical state of human immunodeficiency virus type 1 (HIV-1)-infected individuals and may be responsible for blocking infection with non-synctium-inducing HIV-1 in vitro. *J Virol* 72:876–881.
- Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382:722–725.
- Schneider S, Roessli D, Excoffier L. Arlequin ver 2.00. A software for population genetics data analysis. Switzerland: Genetics and Biometry Laboratory, University of Geneva. 2000.
- Suresh P, Wanchu A, Bhatnagar A, Sachdeva RK, Sharma M. 2007. Spontaneous and antigen-induced chemokine production in exposed but uninfected partners of HIV type 1-infected individuals in North India. *AIDS Res Hum Retroviruses* 23:261–268.
- Suresh P, Wanchu A, Sachdeva RK, Bhatnagar A. 2006. Gene polymorphisms in CCR5, CCR2, CX3CR1, SDF-1 and RANTES in exposed but uninfected partners of HIV-1 infected individuals in North India. *J Clin Immunol* 26:476–484.
- Vajpayee M, Kaushik S, Sreenivas V, Wig N, Seth P. 2005. CDC staging based on absolute CD4 count and CD4 percentage in an HIV-1 infected Indian population: treatment implication. *Clin Exp Immunol* 141:485.
- Vidal F, Péraire J, Domingo P, Broch M, Cairo M, Pedrol E, Montero M, Vilades C, Gutierrez C, Sambaat MA, Fontanet A, Dalmat D, Deig E, Knobel H, Sirvent JJ, Richart C, Veloso S, Saumoy M,

- Lopez-Dupla M, Olona M, Cadafalch J, Fuster M, Ochoa A, Soler A, Guelar A, Gonzalez J. 2006. Chemokines and Long-Term Nonprogressive HIV01 Infection Study Group. Polymorphism of RANTES chemokine gene promoter is not associated with long-term nonprogressive HIV-1 infection of more than 16 years. *J Acquir Immune Defic Syndr* 41:17-22.
- Wichukchinda N, Nakayama EE, Rojanawiwat A, Pathipvanich P, Auwanit W, Vongsheree S, Ariyoshi K, Sawanpanyalert P, Shioda T. 2006. Protective effects of IL4-589T and RANTES -28G on HIV-1 disease progression in infected Thai females. *AIDS* 20:189-196.
- Zhao XY, Lee SS, Wong KH, Chan KC, Ma S, Yam WC, Yuen KY, Ng MH, Zheng BJ. 2004. Effects of single nucleotide polymorphisms in the RANTES promoter region in healthy and HIV-infected indigenous Chinese. *Eur J Immunogenet* 31:179-183.

cause this technology is available in relatively few clinical laboratories, cases of infection with *M. massiliense* may be mistakenly attributed to *M. abscessus*. Although infections with *M. massiliense* may be underrecognized, reports of these infections are raising concern. The capacity of this bacteria to infect different body sites is further evidence for the pathogenic potential of a rapidly growing mycobacteria in human infections (10).

Enrico Tortoli,* Rita Gabini,†
Irene Galanti,†
and Alessandro Mariottini*

*Careggi Hospital, Florence, Italy; and †San Donato Hospital, Arezzo, Italy

References

1. Böddinghaus B, Rogall T, Flohr T, Blocker H, Böttger EC. Detection and identification of mycobacteria by amplification of rRNA. *J Clin Microbiol.* 1990;28:1751-9.
2. Adékambi T, Colson P, Drancourt M. *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. *J Clin Microbiol.* 2003;41:5699-708.
3. Delaney V, Sumrani N, Hong JH, Sommer B. Mycobacterial infections in renal allograft recipients. *Transplant Proc.* 1993;25:2288-9.
4. Branger B, Gouby A, Oulès R, Barducci JP, Mourad G, Fourcade J, et al. *Mycobacterium haemophilum* and *Mycobacterium xenopi* associated infection in a renal transplant patient. *Clin Nephrol.* 1985;23:46-9.
5. Adékambi T, Reynaud-Gaubert M, Greub G, Gevaudan MJ, Lu Seola B, Raoult D, et al. Amoebal coculture of "*Mycobacterium massiliense*" sp. nov. from the sputum of a patient with hemoptoic pneumonia. *J Clin Microbiol.* 2004;42:5493-501.
6. Simmon KE, Pounder JI, Greene JN, Walsh F, Anderson CM, Cohen S, et al. Identification of an emerging pathogen, *Mycobacterium massiliense*, by *rpoB* sequencing of clinical isolates collected in the United States. *J Clin Microbiol.* 2007;45:1978-80.
7. Kim HY, Yun YJ, Park CG, Lee BH, Cho YK, Park BJ, et al. Outbreak of *Mycobacterium massiliense* infection associated with intramuscular injections. *J Clin Microbiol.* 2007;45:3127-30.
8. Viana-Niero C, Lima KV, Lopes ML, da Silva Rabello MC, Marsola LR, Brilhante VC, et al. Molecular characteriza-

tion of *Mycobacterium massiliense* and *Mycobacterium bolletii* in outbreaks of infections after laparoscopic surgeries and cosmetic procedures. *J Clin Microbiol.* 2008;46:850-5.

9. Clinical and Laboratory Standards Institute (CLSI). Susceptibility testing of *Mycobacteria*, *Nocardia*, and other aerobic actinomycetes: approved standard M24-A. Wayne (PA): The Institute; 2003.
10. Brown-Elliott BA, Wallace RJ Jr. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin Microbiol Rev.* 2002;15:716-46.

Address for correspondence: Enrico Tortoli, Regional Reference Center for Mycobacteria, Microbiology Laboratory, Careggi Hospital, Viale Morgagni 85, 50134 Florence, Italy; email: enrico.tortoli@unifi.it

Bovine Kobuviruses from Cattle with Diarrhea

To the Editor: A new species of kobuvirus, named U-1 strain, was first recognized in 2003 as a cytopathic contaminant in a culture medium of HeLa cells that had been used for >30 years in the laboratory (1). The RNA genome of the U-1 strain comprises 8,374 nucleotides; the genome organization is analogous to that of picornaviruses. Morphologically, the U-1 strain resembles the Aichi virus, but genetically it is distinct (1). Therefore, the U-1 strain is classified as a new species of genus *Kobuvirus* in the family *Picornaviridae*, and it is called *bovine kobuvirus* (1). To date, the genus *Kobuvirus* consists of 2 species, *Aichi virus* and *bovine kobuvirus* (2). The Aichi virus is associated with acute gastroenteritis in humans (3-5); bovine kobuvirus infection has been detected only in cattle (1).

Only 1 report has described the discovery and epidemiologic features

of bovine kobuvirus (1). Of serum samples from 72 healthy cattle, 43 (59.7%) were positive for neutralizing antibody against bovine kobuvirus U-1 standard strain at a titer of ≥ 16 . In addition, 12 (16.7%) of 72 stool samples collected from the cattle were positive for the bovine kobuvirus genome by reverse transcription-PCR (RT-PCR) (1). This finding suggested that bovine kobuvirus is common and that the virus particles could be detected in the stool samples of infected cattle. We therefore conducted an epidemiologic survey of bovine kobuvirus and report detection of this virus in stool samples from calves with diarrhea during 2001-2004 in Chiang Mai Province, Thailand.

From November 2001 to July 2004, a total of 72 fecal specimens were collected. The age of the calves ranged from 7 to 49 days. The presence of bovine kobuvirus in fecal specimens was detected by using RT-PCR with a protocol modified from the method described by Yamashita et al. (1). All the bovine kobuvirus strains detected in our study were analyzed further by direct sequencing of their PCR amplicons with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI 3100, Applied Biosystems). The nucleotide sequences of these portions were compared with those of reference strains available in the GenBank database by using BLAST (6). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 3.1 (7). The nucleotide sequences of bovine kobuvirus strains described in this study were deposited in GenBank under accession nos. EF659450-EF659455.

The bovine kobuvirus was detected by the RT-PCR screening method in 6 (8.3%) of the 72 fecal specimens collected. The partial 3D regions of all 6 bovine kobuviruses exhibited highly conserved sequences of 99.3%-100% nucleotide and 100% amino acid