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ORIGINAL ARTICLE

THE LATITUDE WISE PREVALENCE OF THE CCR5-Δ32-HIV RESISTANCE ALLELE IN INDIA

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ABSTRACT

The chemokine receptor CCR5 plays a crucial role during CD4-mediated entry of HIV-1 in macrophages and a 32 bp deletion in the CCR5 gene (CCR5-Δ32) confers protection against HIV infection and AIDS progression. To evaluate the contribution of this host genetic factor in aggravating India's HIV/AIDS problem, we exclusively examined the frequency of CCR5-Δ32 in 43 different ethnic endogamous Indian populations comprising 1,882 individuals and its latitude-wise distribution in India. This is the first report of prevalence and latitude-wise distribution of CCR5-Δ32 in such large scale in India, which indicates that most of the Indian populations lack the CCR5-Δ32 mutation. This mutation was exhibited in only 13 out of the 43 ethnic populations of India studied with allelic frequency 0.62 - 5%. Southward decreasing cline was observed for frequencies of CCR5-Δ32 (0.79% to 5.0% in North vs. 0.62% to 1.4% South). These results are in accordance with HIV/AIDS prevalence in India, and suggest that absence of CCR5-Δ32 mutation may be one of the important factors for HIV/AIDS incidence in India.

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Key words: CCR5-Δ32, Chemokine coreceptors, HIV, AIDS, Polymorphism, Indian, Population

INTRODUCTION

The role of CCR5 as a primary coreceptor for HIV-1 during CD4-mediated entry in macrophages is well defined [1], although its role has also been implicated in many diseases like the West Nile virus infection [2], tick borne encephalitis [3], coronary artery disease [4], type-2 diabetes and renal insufficiency [5] and even atopic asthma [6] *etc.* CCR5 is expressed on macrophages, monocytes, memory T-cells, dendritic cells and microglia and activates cells by chemokine macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β) and RANTES-mediated signaling [7]. Human CCR5 consists of 352 amino acids and is a member of the serpentine G protein coupled receptors [8]. The CCR5 gene, CKMBR5, is present on the p21.3-p24 location of human chromosome-3 [9,10]. Several genetic polymorphisms have been identified within the CCR5 regulatory/promoter region and coding regions that affect HIV transmission and/or disease progression [11]. Studies have suggested that the distribution of the CCR5 polymorphisms and their possible role in the progression of the disease varies between individuals of different racial, ethnic and risk groups [12].

As reported earlier, a 32 bp deletion in the CCR5 gene (CCR5-Δ32), corresponding to the second extracellular loop of CCR5, in the homozygous con-

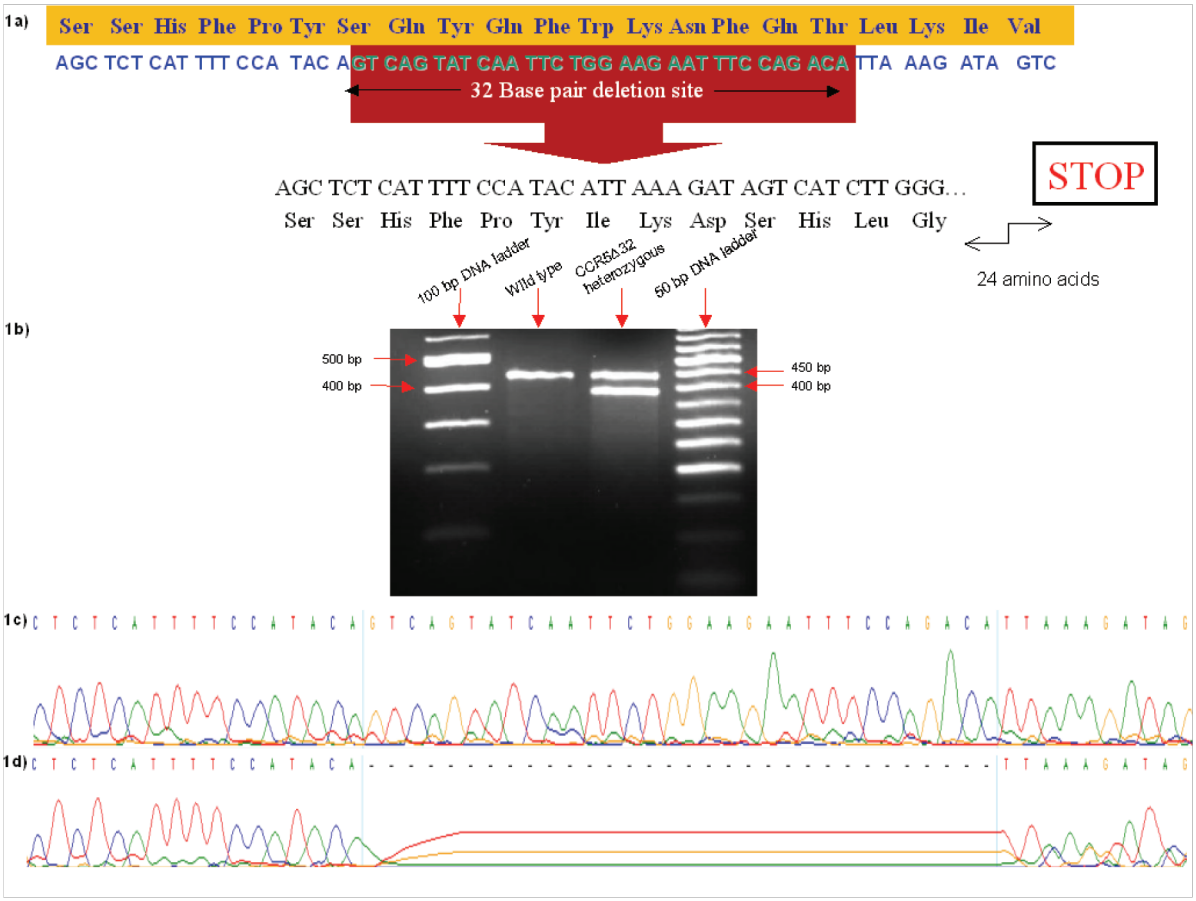

Figure 1. The CCR5 gene indicating a 32 bp deletion, which resulted in a frameshift mutation and electrophoresis and sequencing patterns. **1a)** represents a portion of the CCR5 gene indicating the 32 bp deletion site (CCR5-Δ32) in the coding region of the CCR5 gene and the frame shift caused in the reading frame by the same. **1b)** shows the agarose gel electrophoresis of PCR products. Lane 1: 100 bp DNA ladder, lane 2: PCR product from a representative CCR5 wild type individual, lane 3: PCR product from a representative individual heterozygous for the CCR5-Δ32 mutation, lane 4: 50 bp ladder. **1c and 1d)** represent the sequencing results of a CCR5 amplicon for a representative CCR5 wild type and CCR5-Δ32 heterozygous individual, respectively.

dition confers almost complete resistance to HIV infection [13]. The individuals homozygous for the CCR5-Δ32 allele do not express any of the CCR5 receptor on their cell surfaces because of a frameshift mutation in the reading frame of CCR5 leading to an early incorporation of a stop codon and premature termination of the protein (Figure 1a) [14]. Although role of CCR5-Δ32 as an HIV suppressive allele is already well defined [15], this protection is not absolute as a few individuals homozygous for this deletion were found infected with the X4 virus, which infects T-lymphocytes [16]. Individuals heterozygous for the CCR5-Δ32 mutation showed less susceptibility and delayed progression of HIV/AIDS towards an M-tropic HIV-1 infection [17] as they express only 20-30% of CCR5 on the cell surface and therefore have a reduced risk of HIV infection and, on average, a 2-4 year delay in the progression to AIDS [10,13]. The heterozygous condition of this mutation does not provide protection against vertical HIV-1 transmission, but it is associated with a delayed disease progression in HIV-infected children [18].

Geographical distribution of the CCR5-Δ32 allele has previously been reported by various investigators around the globe [19-23]. The allelic frequency of CCR5-Δ32 has been found to be highest among Caucasians (10-20%), particularly in those of Northern European descent (with a 1% homozygosity) [20], and a Southeast decrease in the fre-

Table 1. Statistical analysis of the CCR5-Δ32 distribution frequency in the various populations included in the current study

States	Population	Latitude	Total Number of Samples (number of heterozygotes)	Genotype Frequency			Allelic Frequency		χ2 Value
				wt/wt[a]	wt/mt[a]	mt/mt[a]	p	q	
Northern States									
Chattisgarh	Bhunjiya	21.15 N	41 (1)	0.97561	0.02439	0	0.9878	0.0122	0.0062
Chattisgarh	Bahelia	19.10 N	51	1	0	0	1	0	–
Jammu & Kashmir	Kashmiri Pandit	32.55 N	50 (2)	0.96	0.04	0	0.98	0.02	0.02
Jharkand	Birhor	23.23 N	36	1	0	0	1	0	–
Madhya Pradesh	Gond	22.43 N	80 (3)	0.9625	0.0375	0	0.98125	0.01875	0.0292
Punjab	Ramgarhia	31.37 N	24	1	0	0	1	0	–
Uttar Pradesh	Thakur	25.46 N	35 (1)	0.97143	0.02857	0	0.98571	0.01429	0.0074
Uttar Pradesh	Durgvanshi	25.46 N	49	1	0	0	1	0	–
Uttar Pradesh	Kshatriya	26.03 N	41	1	0	0	1	0	–
Uttar Pradesh	Ansari	25.46 N	46 (2)	0.95652	0.04348	0	0.97826	0.02174	0.0277
Uttar Pradesh	Lodh	27.30 N	42 (1)	0.97619	0.02381	0	0.9881	0.0119	0.0061
Uttar Pradesh	Brahmin	25.28 N	63 (1)	0.98413	0.01587	0	0.99206	0.00794	0.0086
Uttar Pradesh	Saryuparin Brahmin	25.46 N	38	1	0	0	1	0	–
Uttar Pradesh	Yadava	25.46 N	31 (2)	0.93548	0.06452	0	0.96774	0.03226	0.0344
Uttar Pradesh	Tharu	25.10 N	50 (5)	0.9	0.1	0	0.95	0.05	0.1385
Uttar Pradesh	Harizan	25.34 N	42	1	0	0	1	0	–
Uttaranchal	Unyal	29.23 N	60	1	0	0	1	0	–
Subtotal			**779 (18)**	**0.977**	**0.023**	**0**	**0.988**	**0.012**	**0.1124**
Western States									
Rajasthan	Meghwal	26.55 N	36	1	0	0	1	0	–
Gujarat	DungriBhils	22.35 N	50	1	0	0	1	0	–
Gujarat	Kathodi	21.41 N	13	1	0	0	1	0	–
Gujarat	Vasavas	22.00 N	48	1	0	0	1	0	–
Subtotal			**147**	**1**	**0**	**0**	**1**	**0**	**–**

Continue ➥

Table 1 continue

States	Population	Latitude	Total Number of Samples (number of heterozygotes)	Genotype Frequency			Allelic Frequency		$\chi 2$ Value
				wt/wt[a]	wt/mt[a]	mt/mt[a]	p	q	
Eastern States									
Mizoram	Mizo	23.36 N	45	1	0	0	1	0	–
Mizoram	Chakhesang Naga	26.00 N	34	1	0	0	1	0	–
Orissa	Oraon	21.28 N	32	1	0	0	1	0	–
Subtotal			**111**	**1**	**0**	**0**	**1**	**0**	**–**
Southern States									
Andhra Pradesh	Yanadi	18.03 N	20	1	0	0	1	0	–
Andhra Pradesh	Koyas	17.58 N	45	1	0	0	1	0	–
Andhra Pradesh	Chenchu	14.28 N	51	1	0	0	1	0	–
Andhra Pradesh	Peruka	14.10 N	39	1	0	0	1	0	–
Andhra Pradesh	Pradhan	13.30 N	28	1	0	0	1	0	–
Andhra Pradesh	Telega	14.27 N	33	1	0	0	1	0	–
Andhra Pradesh	Andha	15.50 N	40	1	0	0	1	0	–
Andhra Pradesh	Naikapod	17.03 N	45 (1)	0.97778	0.02222	0	0.98889	0.01111	0.0057
Andhra Pradesh	Goudak	17.58 N	35	1	0	0	1	0	–
Andhra Pradesh	Pattapu	17.03 N	30	1	0	0	1	0	–
Tamilnadu	Baduga	11.00 N	51	1	0	0	1	0	–
Tamilnadu	Thoti	10.34 N	37	1	0	0	1	0	–
Tamilnadu	Thoda	12.37 N	37	1	0	0	1	0	–
Tamilnadu	Adidravida	11.20 N	41	1	0	0	1	0	–
Tamilnadu	Lingayath	11.31 N	35 (1)	0.97143	0.02857	0	0.98571	0.01429	0.0074
Maharashtra	Mahadeo Koli	18.31 N	81 (1)	0.98765	0.01235	0	0.99383	0.00617	0.0031
Maharashtra	Thakar	18.60 N	79	1	0	0	1	0	0.0321
Kerala	Paniyas	11.28 N	34	1	0	0	1	0	–
Karanataka	Halakki	13.50 N	84 (2)	0.97619	0.02381	0	0.9881	0.0119	0.0322
Subtotal			**845 (5)**	**0.992**	**0.008**	**0**	**0.996**	**0.004**	**0.0076**
Grand Total			**1,882 (23)**	**0.989184**	**0.0108**	**0**	**0.9945919**	**0.0054081**	**0.067**

[a] wt/wt: wild type/wild type; wt/mt: wild type/mutant type; mt/mt: mutant type/mutant type.

quency has been observed towards the Mediterranean Sea. It has been seen at low frequencies, *i.e.,* 5% in the near east of Europe, 5.6% in Asia and 3.2% in Africa, and is absent elsewhere apart from isolated occurrences [19,21-23]. Variable distribution of this mutant allele throughout the globe may be attributed either to selection pressure of some pathogen, infectious diseases and/or admixture of the population leading to the diversity.

Host genetic makeup determines susceptibility/protection of any individual against HIV/AIDS [24]. India has the largest number of persons living with HIV/AIDS in the world, with the epidemic getting more intense as we move from North to South in India (higher to lower latitude). The highest rates of HIV prevalence are found in Andhra Pradesh, Maharashtra, Tamil Nadu and Karnataka in the South (comprising of ~63% of all people living with HIV in India) [25]. In this context, although distribution of the CCR5 promoter polymorphisms has been reported in a representative study on a limited number of individuals from Delhi and around (in North India) [26], no extensive information is available regarding the distribution pattern and frequency of the host chemokine receptor CCR5 gene polymorphism (CCR5-Δ32) with the corresponding latitude, in vast and different representative Indian populations. Therefore, it is imperative to study the diverse population of India, with special reference to the presence and absence of this protective mutation, which is known to be genetically diverse, due to their endogamous nature. This prompted us to examine the frequency and trend of CCR5-Δ32 in different ethnic Indian populations. To the best of our knowledge this is the first study that has taken such a large number of ethnic populations of India (n = 43) comprising such a large number of individuals (1,882) and worked **o**ut the prevalence and latitude-wise distribution of the CCR5-Δ32 allele to show the North to South cline of the same.

MATERIALS AND METHODS

Collection of Samples and DNA Isolation. Forty-three different ethnic, endogamous, Indian populations without any known history of HIV-1 infection were selected on a random basis from all over India (Table 1), which were then subjected to the present study. Ethnic and regional bias within the studied population was minimized by excluding subjects outside the castes and tribes of particular regions of India. Fresh blood samples from 1,882 healthy, unrelated individuals were collected from 15 different states of India after approval from institutional ethical and biosafety committees and written consent from the individuals concerned. Genomic DNA was isolated from 10 mL EDTA treated venous blood samples using the standard proteinase K digestion and phenol-chloroform extraction method [27].

Amplification of the CCR5-Δ 32 Regions. The primers to amplify the target segment for the 32 bp deletion of the CCR5 gene (spanning from position 3245 to 3685 with reference to NCBI sequence ID no. U95626) were designed using Gene Tool software (BioTools Inc., Edmonton, Alberta, Canada). The sequences of the primers were: 5'-GCT GTC GTC CAT GCT GTG TTT-3' (forward primer) and 5'-CAA CCT GTT AGA GCT ACT GCA ATT-3' (reverse primer), and these were commercially synthesized by Bioserve Biotechnologies India Pvt. Ltd., Hyderabad, Andhra Pradesh, India. The polymerase chain reaction (PCR) was carried out in 20 μL reaction volume using 10X PCR buffer (Applied Biosystems Corporation, Foster City, CA, USA), 25 mM $MgCl_2$ (Applied Biosystems Corporation), 5 mM dNTP mix (Eppendorf, Hamburg, Germany), 5 pM each of forward and reverse primers, 2 units of DFS (DNA free sensitive)-Taq DNA polymerase (Bioron GmbH, Ludwigshafen, Germany) and 40 ng of genomic DNA. Amplification was carried out for 35 cycles in an Eppendorf Mastercycler EP (Eppendorf), each consisting of denaturation at 95°C for 30 sec, annealing at 62.6°C for 30 sec and extension at 72°C for 30 sec. Pre denaturation and final extension were performed at 95°C for 10 min and 72°C for 10 min, respectively.

Amplified PCR products from wild type and homozygous mutant type individuals were expected as single bands of 441 and 409 bp, respectively. The PCR products from heterozygous individuals was expected to contain two fragments of 441 and 409 bp lengths. The PCR products were electrophoresed (Figure 1b) on a 4% Tris-acetate-EDTA agarose gel (Sigma-Aldrich Corporation, St. Louis, MO, USA) and visualized by staining with ethidium bromide along with 50 and 100 bp ladders (New England BioLabs, Ipswich, MA, USA) under an UV-transilluminator.

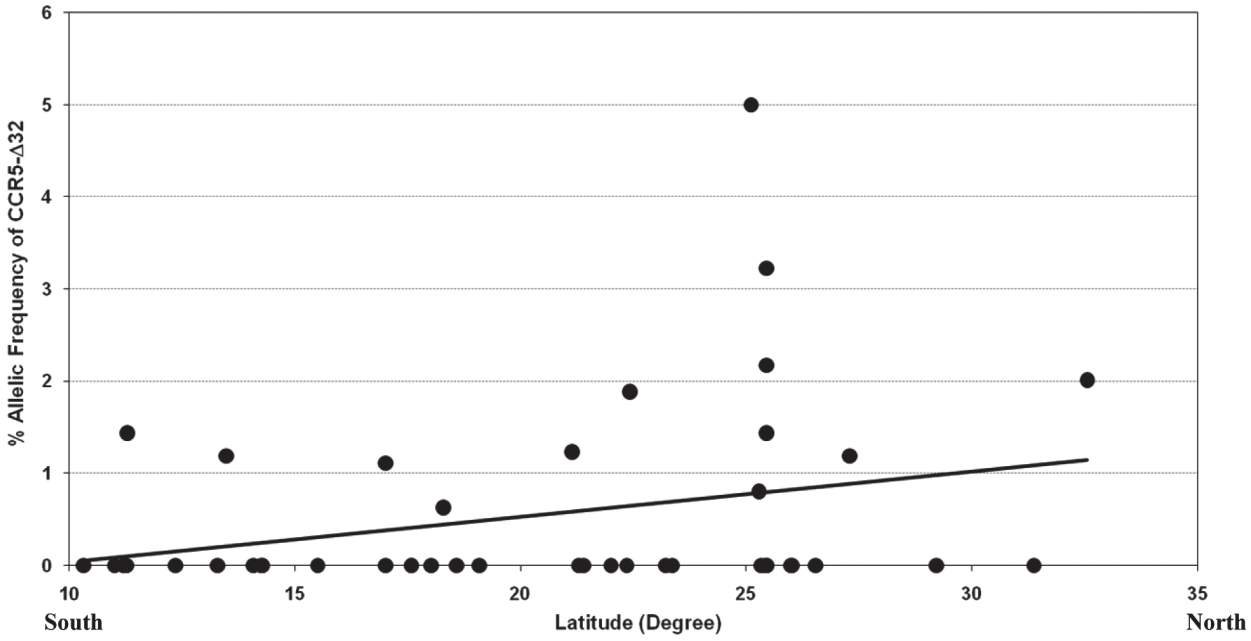

Figure 2. Latitude-wise distribution of CCR5-Δ32. The black dots represent the respective allelic frequency at that particular latitude and the black line indicates the trend while moving to higher latitudes, *i.e.,* from South to North India.

Genotyping for the CCR5-Δ32 Polymorphism. To reconfirm the results observed on gel for CCR5-Δ32 polymorphism, PCR products were sequenced directly on both strands as described elsewhere [27] using 25 ng of PCR product and 5 pM of forward primer, sequencing buffer and big dye (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems Corporation) and the volume adjusted to 5.0 µL/reaction. Cycle sequencing was carried out in an Eppendorf Mastercycler EP (Eppendorf) employing 30 cycles at 95°C for 10 sec, 55°C for 5 sec, and 60°C for 4 min. Extended products were purified by absolute alcohol precipitation followed by washing with 70% alcohol. Purified samples were dissolved in 50% Hi-Di™ formamide (Applied Biosystems Corporation) and analyzed in an ABI PRISM® 3700 automated DNA Analyzer (Applied Biosystems Corporation). Sequences were assembled using Auto Assembler sequence assembly software (Applied Biosystems Division, Perkin Elmer, Foster City, CA, USA) with a reference sequence and any type of heterogeneity was checked manually and was noted down (Figure 1c and 1d).

Latitude-Wise Distribution of the CCR5-Δ32 Allele in India. To examine the trend in variation of CCR5-Δ32 allele frequencies from North to South in India, we plotted (Figure 2) Δ32 allele frequencies (percentage) against latitude (in degrees), reported in Table 1, for each geographical site of origin of the study population. To further verify our results we also plotted graphs (Figure 3) between mean allelic frequencies of CCR5-Δ32 between North and South India and the significance was checked by the $\chi 2$ test.

Statistical Analyses. The data obtained was then subjected to statistical analyses. Genotypes observed for CCR5-Δ32 were categorized into wild type, and heterozygous or homozygous mutant for each population. Allele frequencies of CCR5 (p) and CCR5-Δ32 (q) were calculated using [(2H+h)/2N] formula where H, h and N represented the number of homozygous individuals, heterozygous individuals and the sample size of each population, respectively. The genotype distribution was tested for agreement with the Hardy-Weinberg equilibrium. The value of $\chi 2$ with associated *p* value for significance was calculated.

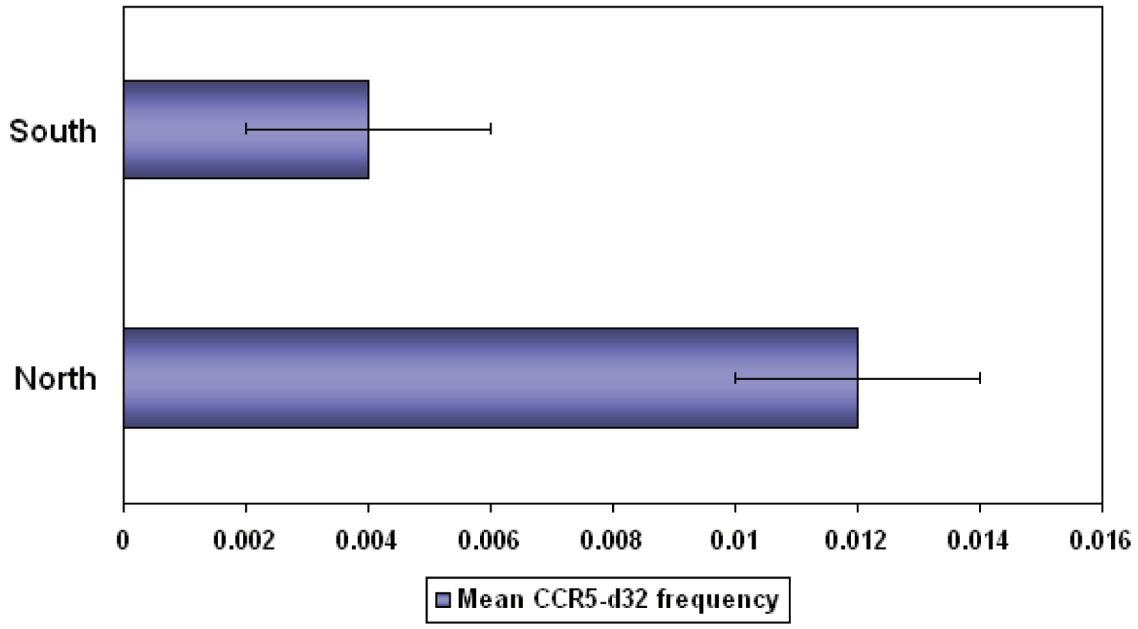

Figure 3. Mean allelic frequencies of the CCR5-Δ32 mutation in North and South India.

RESULTS

Prevalence of the CCR5-Δ32 Allelic Frequencies in 43 Populations of India. Our data for the distribution of CCR5-Δ32 suggests that it is either absent or present at low frequencies (0.62-5%) in most of the studied ethnic Indian populations, and the overall presence of CCR5-Δ32 in India is only 1.1% (Table 1). It was also observed that the homozygous mutation is absent in all the studied populations. The heterozygous mutation was present in 13 of the 43 studied ethnic populations of India. It was present in nine of the 17 populations of North India analyzed for CCR5-Δ32 mutations, with a frequency varying from 0.79 to 5.0%. Seven populations of the Eastern and Western Indian states do not have any such mutation. However, the mutation was found to be present in four of the 19 analyzed populations of South India with frequencies varying from 0.62 to 1.4%.

Latitude-Wise North-South Decreasing Cline For CCR5-Δ32 Frequencies in India. The allelic distribution data presented in Table 1 exhibits a low allelic frequency for CCR5-Δ32 as we move toward the lower latitude, which implies that North Indians have the highest frequency distribution (0.79-5.0%) decreasing to 0.62-1.4% in South Indians. Amid all the latitudinal values, the highest allelic frequency of CCR5-Δ32 was observed at latitude 25.10 N (5.0%, $\chi 2$ value 0.1385), which corresponds to the Northern region of India (Uttar Pradesh) as compared to the smallest at latitude 18.31 N (0.62% $\chi 2$ value 0.0031) corresponding to the Southern region of India (Maharashtra). The Δ32 allele frequencies *vs.* latitude plots (Figure 3) present a clearer picture of the southward decline of CCR5-Δ32. Thus, it is clear that there is a southward decline in the CCR5-Δ32 allelic frequency in India.

Enfeebled Δ32 Values in South Indian Populations. Our results, presented in Figure 3, suggest that the mean allelic frequency of CCR5-Δ32 was three-fold higher in North India in comparison to South India. The $\chi 2$ analyses further suggest that the prevalence of CCR5-Δ32 is significantly low ($\chi 2$= 4.032, p <0.05) in South India than North India.

DISCUSSION

The CCR5 gene acts as one of the coreceptors for the entry of M-tropic strains of HIV-1 [28]. The global distribution pattern of CCR5-Δ32 reveals that most susceptible populations such as African populations lack the protective CCR5-Δ32 allele [19,29]. In less susceptible populations, the majority of per-

sons carry the CCR5-Δ32 allele in the heterozygous condition [19]. For the first time, we have reported the approximate absence of the CCR5-Δ32 allele in such a large sample of the Indian population. Our results have exhibited that either the CCR5-Δ32 mutation is completely absent or found in very low frequencies in various ethnic populations of India. Lack of the homozygous CCR5-Δ32 mutation and low prevalence of heterozygous CCR5-Δ32 mutations suggest that Indians are highly susceptible to HIV/AIDS, and this correlates with the highest number of HIV/AIDS infected individuals in India. The presence of low frequencies of CCR5-Δ32 in some individuals of upper castes and Muslims of North India (2%, $\chi 2$ value 0.0277%) implies that these communities may have a better resistance toward HIV/AIDS. These results are in accordance with previous studies that reported a high allelic frequency of 5.36% in Muslims of North India and upper castes [30,31].

The spread of HIV in India has been variable, with the epidemic being most intense in Southern India [32]. Our results suggest that the distribution frequency of CCR5-Δ32 (Table 1) decreases significantly as we go down (North to South) along with the latitudes. The distribution of CCR5-Δ32 (Figure 2) and the significant variance in its mean frequency between South and North India (Figure 3) also propose that there is southward decreasing cline in CCR5-Δ32. These results suggest that South Indian populations might lack a genetic make-up, which is protective against HIV/AIDS. This is in accordance with an antenatal clinical HIV prevalence survey in 2005, which reports a higher frequency of HIV/AIDS in South Indian populations [32]. Therefore, our data reveal that the CCR5-Δ32 mutation may be one of the important factors for HIV/ AIDS prevalence in India.

A possible reason for the presence of CCR5-Δ32 in some populations is hypothesized to be the strong selective pressure for increased resistance against an infectious agent like small pox [33]. Smallpox was present in India as early as 1000 B.C. The presence of a low frequency of the CCR5-Δ32 mutation, in spite of having significantly higher cases of small pox in India than estimated, for unprotected populations in 18th century Europe, does not support the hypothesis of positive selection pressure of smallpox for CCR5-Δ32 [34]. But the presence of heterozygous mutations in some ethnic populations of North India, in our results, is consistent with the historical and Y chromosome marker studies that North Indian upper caste and middle caste are descendants of Europeans [9,35,36]. Indo-European languages (*i.e.,* Indian classical languages and most European languages) also suggest that contemporary Hindu Indians are descendants of primarily West Eurasians who migrated from Europe, the Near East, Anatolia, and the Caucasus 3000-8000 years ago [37]. Therefore, the possible cause of higher frequencies of CCR5-Δ32 among Muslims and North Indians may be because of admixture as the Eurasians migrated through Europe [38]. Our results also suggest a sporadic presence of CCR5-Δ32 mutations in some of the South Indian populations, which may be accredited to the presence of Eurasian Y chromosome haplotype groups in some of these populations [39,40]. Thus, the genetic admixture of Eurasians and Caucasians could possibly be one of the reasons for such distribution patterns and frequencies in India. The absence of the CCR5-Δ32 allele frequency in eastern states may be attributed to the common ancestry that they share with the Myanmar population [38].

Collectively, it is evident from our data that the genetic protection conferred by the CCR5-Δ32 mutation is absent in Indian populations and corresponds to the HIV/AIDS dynamics in India. It suggests that apart from behavioral factors, host genetics also play an important role in influencing the dynamics of HIV infection in India. Thus, determining the genetic make-up and understanding the role and consequence of variable distributions of host susceptibility factors in India, may help in identifying the correct drug to be administered and help patients survive longer. Our study may help in developing a therapeutic or preventive strategy based on targeting the CCR5 gene. As per international estimates, India could have 20-25 million HIV cases by 2010 [41]. With this new information of the absence of host resistance factors in Indians, the effort to control the HIV epidemic in India needs to be further strengthened to curb the epidemic menace.

Gene Accession Numbers. The sequencing results led to the identification of 441 nucleotide length (wild type; encoding 147 amino acids) and 409 nucleotide length (mutant type; encoding 87 amino acids), that were submitted to NCBI with

EF202087 and EF202088 accession numbers, respectively.

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