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CCR2, MCP-1, SDF-1α & DC-SIGN gene polymorphisms in HIV-1 infected patients with & without tuberculosis

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***Background & objectives*: Variability in the clinical outcome of persons exposed to and infected with HIV-1 and tuberculosis (TB) is determined by multiple factors including host genetic variations. The aim of the present study was to find out whether chemokine, chemokine receptor and DC-SIGN gene polymorphisms were associated with susceptibility or resistance to HIV and HIV-TB in south India.**

***Methods*: CCR2 V64I (G/A), monocyte chemoattractant protein-1 (MCP-1) -2518 A/G, stromal cell derived factor-1α (SDF-1α) 3'UTR G/A and DC-SIGN gene polymorphisms were studied by polymerase chain reaction based methods in HIV-1 infected patients without TB (n=151), with pulmonary TB (PTB) (n=81) and extrapulmonary TB (n=31), 155 PTB patients without HIV and 206 healthy controls.**

***Results*: The genotype frequencies of CCR2 V64I, MCP-1 -2518 and DC-SIGN polymorphisms did not differ significantly between the study groups. A significantly increased frequency of GG genotype of SDF-1α polymorphism was observed among HIV+PTB+ patients compared to healthy controls (*P*=0.009, Pc=0.027).**

***Interpretation & conclusions*: Our data suggest that GG genotype of SDF-1α 3'UTR polymorphism may be associated with susceptibility to PTB in HIV-1 infected patients. A better understanding of genetic factors that are associated with TB could help target preventive strategies to those HIV patients likely to develop tuberculosis.**

Key words Chemokine - chemokine receptor - gene polymorphisms - HIV - HIV-TB

The emergence of human immunodeficiency virus (HIV) and its synergistic association with tuberculosis (TB) pose a great challenge to the health systems in developing countries. TB is the most life threatening opportunistic infection among HIV patients accounting 40 per cent of acquired immunodeficiency syndrome (AIDS) deaths in Asia[1]. Interaction between chemokine and chemokine receptors and their expression patterns regulate migration of cells involved in immune response to tuberculosis (TB) as well as HIV and contribute to the pathogenesis of the disease[2-4]. Dendritic cell-specific intercellular adhesion molecule -3- grabbing non integrin (DC-SIGN), a C type lectin expressed on dendritic cells recognize pathogens through their carbohydrate structure and internalize the pathogen for antigen presentation. HIV and *Mycobacterium tuberculosis* target DC-SIGN by different mechanisms to promote their survival[5].

Variability in the clinical outcomes of persons exposed to and infected with HIV-1 and tuberculosis is determined by multiple factors including host genetic variations. Variants of CC chemokine receptor - CCR2, Monocyte chemoattractant protein-1 (MCP-1) and stromal cell derived factor-1α (SDF-1α) and DC-SIGN genes have been shown to be associated with susceptibility or resistance to HIV-1 manifestations and disease progression[3,6-8]. Polymorphisms in MCP-1 and DC-SIGN genes have also been shown to be associated with susceptibility to tuberculosis[9,10]. Although a few studies on chemokine, chemokine receptor and DC-SIGN exon 4 repeat number polymorphisms have been reported in north Indian (Aryan descent) HIV patients and healthy controls[11-14], there is a dearth of reports on HIV patients with and without TB of south Indian (Dravidian descent) origin. So far only one study has been carried out in healthy individuals of tribes and Muslim ethnic groups of Andhra Pradesh, south India[15]. The present study was therefore carried out to find out whether CCR2, MCP-1, SDF-1α and DC-SIGN gene polymorphisms are associated with susceptibility or resistance to HIV and HIV associated TB in south India.

Material & Methods

Study subjects: The study population comprised 151 HIV-1 seropositive subjects without TB (HIV+TB-) (64 male, mean age ± SD : 32.1 ± 6.9 yr; 87 female, 28.6 ± 6.7 yr), 81 HIV-1 seropositive subjects with pulmonary TB (HIV+PTB+) (60 male, 35.3 ± 6.7 yr; 21 female, 31.9 ± 6.5 yr), 31 HIV-1 seropositive subjects with extrapulmonary TB (HIV+ETB+) (23 male, 33.5 ± 4.5 yr; 8 female, 31.6 ± 9.0 yr), 155 HIV negative pulmonary TB positive (HIV-PTB+) (102 male, 37.8 ± 10.8 yr; 53 female, 30.2 ± 9.0 yr) recruited from the HIV and TB clinics of Tuberculosis Research Centre (TRC) and its subcentres *viz.*, Government General Hospital, Chennai, and Government Hospital of Thoracic Medicine, Tambaram Sanatorium, Chennai, and 206 healthy controls (118 male, mean age ± SD : 31.3 ± 8.4 yr; 88 female, 30.9 ± 8.7 yr). The diagnosis of tuberculosis was confirmed by clinical, radiographic and/or bacteriological observations (pulmonary TB) or by the presence of acid fast bacilli in the aspirated fluid/tissue (extrapulmonary TB). The diagnosis of HIV-1 was made using two rapid tests (HIV-1 tridot, J. Mitra, India; CombAids, Span diagnostics, India), and a positive result was confirmed by a third test (Western Blot, J. Mitra, India), according to the manufacturer's instruction.

Among the 81 HIV+PTB+ patients, 74 had smear positive PTB while 7 had smear negative PTB. Among the HIV patients with ETB, 11 had lymph node TB, 5 had miliary TB and 5 had pleural effusion. Seven patients had both pulmonary TB and one of the extrapulmonary forms of TB such as lymph node TB, pleural effusion, pneumothorax, tuberculoma and abdominal TB. Two patients had pericardial effusion while one patient had both lymph node TB and pleural effusion. The clinical characteristics of HIV patients with tuberculosis of the present study have been described earlier[16].

The healthy controls group included university students, laboratory personnel and volunteers of Tuberculosis Research Centre who were physically normal and asymptomatic for TB and willing to participate in the study. Healthy controls were not diagnosed for HIV since the prevalence of HIV is 0.34 per cent (less than 1%) among adults of general population according to the latest revised estimates released by National AIDS Control Organization (NACO)[17]. Patients and controls were recruited consecutively from January 2004 to December 2006. The present study was approved by the institutional ethical committee, and a written informed consent was obtained from all the participants of the study. Both the patients and controls were of Tamil speaking Dravidian descent of Tamil Nadu living in and around Chennai.

Genotyping of CCR2, MCP-1, SDF-1α and DC-SIGN exon4 repeat polymorphisms: Genomic DNA was isolated from peripheral blood mononuclear cells and granulocytes obtained from the blood of patients and healthy controls using salting out procedure[18]. CCR2 V64I (G/A), MCP-1 -2518 A/G, SDF-1α 3'UTR G/A genotypes were identified using polymerase chain reaction based restriction fragment length polymorphisms (PCR-RFLP) as described elsewhere[19-21]. The PCR reaction conditions for CCR2 V64I (G/A) were an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 2 min. For MCP-1 -2518 A/G and SDF-1α 3'UTR G/A polymorphisms, the annealing temperature for PCR was 60 and 58°C respectively and all other conditions were the same as that for CCR2 V64I (G/A). Number of 69 base pair repeats in the DC-SIGN exon4 was identified using PCR as described earlier[22]. The PCR reaction conditions for DC-SIGN polymorphism were same as that of CCR2 V64I (G/A) PCR, except that annealing was done at 65°C. PCR was set in a programmable

thermal cycler (PTC-225, MJ Research Inc, MA, USA). The sequences of primers (Sigma Genosys, Bangalore, India) and restriction digestion enzymes (New England BioLabs, USA) used for genotyping as well as the size of PCR and restriction digestion products are given in Table I.

Statistical analysis: Allele and genotype frequencies of individual polymorphisms were determined by direct counting. Confirmation to Hardy-Weinberg equilibrium was tested using χ^2 test with one degree of freedom. Frequencies of genotypes, of patients and healthy controls were compared by χ^2 test or Fisher's exact test wherever applicable, *P* values with Yates correction and odds ratio (OR) with 95 percent confidence intervals were calculated using Statcalc program (Epi info version 6.0.4, CDC, Atlanta, GA, July 1996). HIV+TB+ patients with different clinical features were grouped as HIV+PTB+ and HIV+ETB+ since the number of patients in each extrapulmonary form of TB (ETB) group was very low[16]. $P<0.05$ was considered significant. Power calculations were performed using the software GraphPad StatMate version 2.

Results

Allele and genotype frequencies of CCR2 V64I (G/A), MCP-1 -2518 A/G, SDF-1α 3'UTR G/A and DC-SIGN exon 4 repeat polymorphisms among different study groups are presented in Tables II and III. Genotype frequencies of all the polymorphisms were in Hardy-Weinberg equilibrium among healthy controls.

The allele and genotype frequencies of CCR2 V64I (G/A), MCP-1 -2518 A/G, SDF-1α 3'UTR G/A and DC-SIGN exon 4 repeat polymorphisms were not different between total HIV patients (includes both HIV patients with and without TB) and healthy controls (data not shown). When the HIV patients were stratified as HIV patients without TB (HIV+TB-) and with TB (HIV+TB+), HIV patients with PTB (HIV+PTB+) and HIV patients with ETB (HIV+ETB+), the allele and genotype frequencies of CCR2 V64I (G/A) and MCP-1 -2518 A/G polymorphisms did not differ significantly between the HIV patients groups with and without TB as well as HIV negative TB patients compared to healthy controls.

A trend towards an increased frequency of GG genotype of SDF-1α 3'UTR G/A polymorphism was seen among HIV-PTB+ patients compared to healthy controls (*P*=0.053, OR 1.57). Significantly increased frequency of G allele and decreased frequency of A allele of SDF-1α 3'UTR G/A polymorphism was observed in HIV+TB+ (*P*=0.02) and HIV+PTB+ (*P*=0.004) patients as compared to healthy controls. An increased frequency of GG genotype was noticed in HIV+TB+ patients (*P*=0.037) and HIV+PTB+ patients in comparison to healthy controls (*P*=0.009; Pc=0.027) and no such difference was observed in HIV+ETB+ patients. The AA genotype of SDF-1α 3'UTR G/A polymorphism was not observed in HIV+PTB+ patients as compared to healthy controls (*P*=0.037) and HIV+TB- patients (*P*=0.028). A significantly decreased frequency of GA genotype was observed among HIV-PTB+ patients as compared to healthy controls (*P*=0.006; Pc=0.018) and a decreased trend was observed in HIV+PTB+ patients (*P*=0.08).

The most frequent DC-SIGN gene exon 4 repeat number observed was 7.5 repeat with frequencies more than 0.97 in all study groups. The other alleles

Table I. Primers sequences, restriction enzymes used and restriction digestion patterns for genotyping of CCR2 V64I (G/A), MCP-1 -2518 A/G, SDF-1α 3'UTR G/A and DC-SIGN exon 4 repeat polymorphisms

SNPs studied	Sequence of the primers	Product size	Restriction enzyme used	Restriction digestion patterns for different alleles
CCR2 V64I (G/A)	5′- GGATTGAACAAGGACGCATTTCCCC -3′ 5′- TTGCACATTGCATTCCCAAAGACCC -3′	380 bp	*Fok* I	G – 380 bp A – 215 + 165 bp bands
MCP-1 -2518 A/G	5′- CCGAGATGTTCCCAGCACAG -3′ 5′- CTGCTTTGCTTGTGCCTCTT- 3′	930 bp	*Pvu* II	A – 930 bp G – 708 + 222 bp bands
SDF-1α 3'UTR G/A	5′- CAGTCAACCTGGGCAAAGCC-3′ 5′- CCTGAGAGTCCTTTTGCGGG-3′	293 bp	*Msp* I	G – 193 + 100 bp A – 293 bp
DC-SIGN exon 4 repeat (rpt)	5′- CACCACACAACGACCATCTC -3′ 5′- AGGTCCCCAGCTCCATAAGT - 3′	*686/616/ 547/478/ 409 bps	None	None

SNPs, single nucleotide polymorphisms

***** For DC-SIGN, the alleles are assigned on the basis of PCR product size; 7.5 repeat – 616 bp, 686 bp – 8.5 rpt, 547 bp – 6.5 rpt, 478 bp - 5.5 rpt; 409 bp - 4.5 rpt

Table II. Allele frequencies of CCR2 V64I (G/A), MCP-1 -2518 A/G, SDF-1α 3'UTR G/A and DC-SIGN exon 4 repeat polymorphisms among different study groups

Alleles	Controls (n=206)	HIV+TB- (n=151)	HIV+TB+ (n=112)	HIV+PTB+ (n=81)	HIV+ETB+ (n=31)	HIV-PTB+ (n=155)
CCR2 V64I (G/A)						
G	0.913	0.924	0.901	0.894	0.919	0.880
A	0.087	0.076	0.099	0.106	0.081	0.120
MCP-1 -2518 A/G						
A	0.658	0.626	0.687	0.685	0.694	0.686
G	0.342	0.374	0.313	0.315	0.306	0.314
SDF-1α 3'UTR G/A						
G	0.749*,†	0.768	0.830*	0.864†	0.742	0.784
A	0.251	0.232	0.170	0.136	0.258	0.216
DC-SIGN exon 4 repeats						
8.5	0.003	0.004	0.005	0.006	0	0.003
7.5	0.997	0.990	0.990	0.994	0.970	0.994
6.5	0	0.006	0.005	0	0.030	0.003

n, Number of subjects studied
For CCR2, n = 111 in HIV+TB+, 80 in HIV+PTB+ and 154 in HIV-PTB+
For MCP-1, n = 203 in controls, and 153 in HIV-PTB+
For SDF-1 α, n = 205 in controls
For DC SIGN, n = 201 in controls, 150 in HIV+TB-, 108 in HIV+TB+, 78 in HIV+PTB+, 30 in HIV+ETB+ and 153 in HIV-PTB+
With regard to DC-SIGN, the numbers 8.5, 7.5 and 6.5 represent the number of 69 base pair repeats
*****Controls versus HIV+TB+ *P*= 0.024, Odds ratio (OR) 1.64; 95 per cent Confidence intervals (CI) 1.07-2.56
† Controls versus HIV+PTB+ *P* = 0.004, OR 2.14; 95 per cent CI 1.27-3.17

Table III. Genotype frequencies of CCR2 V64I (G/A), MCP-1 -2518 A/G, SDF-1α 3'UTR G/A and DC-SIGN exon 4 repeat polymorphisms among different study groups

Genotypes	Controls (n=206) % GF	HIV+TB- (n=151) % GF	HIV+TB+ (n= 112) %GF	HIV+PTB+ (n=81) % GF	HIV+ETB+ (n=31) % GF	HIV-PTB+ (n=155) % GF
CCR2 V64I (G/A)						
GG	84.0 (173)	84.8 (128)	81.1 (90)	80.0 (64)	83.9 (26)	78.6 (121)
GA	14.6 (30)	15.2 (23)	18.0 (20)	18.8 (15)	16.1 (5)	18.8 (29)
AA	1.4 (3)	0	0.9 (1)	1.2 (1)	0	2.6 (4)
MCP-1 -2518 A/G						
AA	45.8 (93)	41.1 (62)	48.2 (54)	45.7 (37)	54.84 (17)	51.0 (78)
AG	39.9 (81)	43.0 (65)	41.1 (46)	45.7 (37)	29.03 (9)	35.3 (54)
GG	14.3 (29)	15.9 (24)	10.7 (12)	8.6 (7)	16.13 (5)	13.7 (21)
SDF-1α 3'UTR G/A						
GG	55.1(113)* #,†	59.6 (90)	67.8 (76)†	72.8 (59)*	54.8 (17)	65.8 (102)#
GA	39.5 (81)@	34.4 (52)	30.4 (34)	27.2 (22)	38.7 (12)	25.2 (39)@
AA	5.4 (11) δ	6.0 (9) Ω	1.8 (2)	0 δ, Ω	6.5 (2)	9.0 (14)
DC-SIGN exon 4 repeat						
8.5/7.5	0.5 (1)	0.7 (1)	0.9 (1)	1.3 (1)	0	0.65 (1)
7.5/7.5	99.5 (200)	98.0 (147)	98.2 (106)	98.7 (77)	96.7 (29)	98.7 (151)
7.5/6.5	0	1.3 (2)	0.9 (1)	0	3.3 (1)	0.65 (1)

n, Number of subjects studied, numbers in the parentheses represent individuals positive for that genotype, %GF = per cent genotype frequency
For CCR2, n = 111 in HIV+TB+, 80 in HIV+PTB+ and 154 in HIV-PTB+; For MCP-1, n = 203 in controls, and 153 in HIV-PTB+; For SDF-1, 205 in controls; For DC SIGN, n = 201 in controls, 150 in HIV+TB-, 108 in HIV+TB+, 78 in HIV+PTB+, 30 in HIV+ETB+ and 153 in HIV-PTB+; With regard to DC-SIGN, the numbers 8.5/7.5, 7.5/7.5 and 7.5/6.5 represents the number of 69 base pair repeat genotypes
***** Controls versus HIV+PTB+ *P* = 0.009, Pc = 0.027 Odds ratio (OR) 2.18; 95% Confidence intervals (CI) 1.21 - 4.03
# Controls versus HIV-PTB+ *P* = 0.053, OR 1.57 95% CI 1.00 – 2.47
@Controls versus HIV-PTB+ *P* = 0.006 OR 0.51 95% CI 0.32 - 0.83
†Controls versus HIV+TB+ *P* = 0.037 OR 1.72 95% CI 1.03 -2.88
δ Controls versus HIV+PTB+ *P* = 0.037 OR 0 95% CI 0.00 -0.98
Ω HIV+TB- versus HIV+PTB+ *P* = 0.028 OR 0 95% CI 0.00 -0.92

observed at very low frequencies included 8.5 and 6.5 number repeats. The allele and genotype frequencies did not differ significantly between the study groups.

Discussion

The allele frequencies of CCR2 V64I, SDF-1α 3'UTR and DC- SIGN exon 4 repeat polymorphisms in the present study were similar to that reported earlier in south and north Indian populations[11-15]. A significantly increased frequency of GG genotype of SDF-1α 3'UTR G/A polymorphism was observed among HIV patients with PTB suggesting that GG genotype may be associated with susceptibility to PTB in HIV infected individuals. SDF-1α 3'UTR AA genotype was not observed among HIV+PTB+ patients suggesting its possible association with resistance to PTB in HIV infected individuals. SDF-1α 3'UTR GA genotype was observed in low frequency among HIV-PTB+ patients which suggests that GA genotype may be associated with resistance to PTB in HIV negative individuals. Due to the low number (n = 31) and heterogeneity in the forms of TB of HIV+ETB+ patients, no association was observed with extrapulmonary forms of TB in HIV patients. Since this is a cross sectional study with limited sample size, further follow up study in HIV patients is warranted. However, based on the frequencies of the allele associated with susceptibility (G allele of SDF-1α 3'UTR G/A polymorphism) to PTB in HIV patients, the present study had power of 80 per cent to detect an odds ratio above 1.2.

The association of GG genotype with susceptibility to PTB in HIV patients in our study may be due to linkage of G/A alleles with alleles of other polymorphisms found elsewhere in the SDF-1α gene as described earlier[23]. Since SDF-1α is a potent lymphocyte chemoattractant and is involved in haematopoiesis[24], the G allele along with the other linked alleles might alter SDF-1α levels and may influence recruitment of T lymphocyte and other immune cells to the site of infection as well as replenishment of lymphocytes and hence may be associated with susceptibility to PTB in HIV patients. Decreased serum SDF-1α levels in individuals with 3'A/3'A genotype compared to 3'G/3'G have been reported in Spanish HIV-1 infected patients[25]. However, it has been proposed that 3'A/3'A genotype might be associated with overproduction of SDF-1 α[7]. No association between SDF-1α 3'UTR genotypes and HIV-1 infection was observed in the present study. This may be due to the observation that R5 strains that use CCR5 as co-receptor rather than CXCR4, receptor for SDF-1α, are preferentially transmitted due to the co-receptor expression patterns in mucosal sites associated with sexual transmission[26]. Lack of association between HIV-1 infection and SDF-1α 3'UTR genotypes has also been reported in north Indian population [11].

Our results suggested that CCR2 V64I (G/A) polymorphism was not associated with susceptibility or resistance to HIV and TB, and similar finding has also been shown in north Indian population[12]. In contrary, CCR2 64I homozygosity has been reported to be associated with reduced risk of acquiring HIV-1 infection from a study on HIV-1 discordant couples in Thailand[27]. Association of GG genotype of MCP-1 -2518 A/G polymorphism with susceptibility to PTB has been shown in Korean and Mexican PTB patients [9] and another study on mixed American HIV patients revealed that GG genotype is associated with accelerated progression towards AIDS and increased risk of HIV associated dementia[3]. In the present study, no association between MCP-1 -2518 polymorphism and HIV/TB was observed and this may be due to ethnic differences.

The number of DC-SIGN exon 4 repeat was highly conserved, with 7.5/7.5 genotype being the most common repeat genotype in all study groups. No association with susceptibility or resistance to HIV and TB was observed. Studies from north India, South Africa and Thailand also revealed no association between DC SIGN exon 4 repeat polymorphism and TB or HIV[10,14,28,]. Further studies on the other single nucleotide polymorphisms in the promoter region of DC-SIGN gene that influence its expression may identify the variants that are associated with HIV/TB in south Indian population.

In the present study, it is also possible that certain proportion of the HIV patients might have progressed to full blown AIDS cases and tuberculosis among HIV patients might also be due to the result of disease progression. However, the influence of chemokine and chemokine receptor gene variants on HIV-1 disease progression could not be assessed due to the non availability of data on CD4+ T cell count and viral load. Since HIV+TB+ patients were older than HIV+TB- patients, it is also conceivable that HIV+TB- patients would become HIV+TB+ gradually. Moreover, the period of HIV exposure is also not known among the patients, a lead-time bias in our sample recruitment cannot be excluded. To the best of our knowledge, this is the first study to explore the role of chemokine and chemokine receptor gene variants in susceptibility to

tuberculosis in the context of HIV infection. Genetic factors might play a role in determining the progression from latent to active disease in TB endemic countries. A better understanding of these factors could help target preventive strategies to those HIV infected individuals most likely to develop tuberculosis.

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