Impact of CCR2 and SDF1 Polymorphisms on Disease Progression in HIV-Infected Subjects in Thailand

Palanee Ammaranond,^{1,2*} Sayompoo Sanguansitthianan,¹

Poonlaph Phaengchomduan,¹ Chanachai Sae-Lee,¹ and Sirimarn Mardkhumchan¹

¹Department of Transfusion Medicine, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand

²Innovation Center for Research and Development of Medical Diagnostic Technology Project, Department of Transfusion Medicine, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand

Background: The genotypic polymorphisms of CCR5, CCR2, and SDF1 were analyzed to determine their impact as potential confounders with regard to disease progression because of the role that host genetic factors appear to be involved in determining rates of disease progression. **Methods:** Genomic DNA was extracted from Ethylenediaminetetraacetate whole blood using Qiagen DNA extraction kit. The amplification of CCR5, CCR2, and SDF1 genes was performed by PCR. **Results:** Two hundred and twenty-one samples were genotyped for the CCR5, CCR2, and SDF1 mutation. Among these, all (100%) were identified as wild type for CCR5. All were then investigated considering the impact on CD4+ T-cell counts. Samples were divided into two groups based on the CD4+ T-cell numbers. It revealed that in the group of

CD4+ T-cell counts ≥ 200 cells/ μ l, 15 were found for the homozygous for SDF1 gene (3'A/3'A) whereas one was found in the group of CD4+ T-cell counts < 200 cells/ μ l. Homozygosity for the CCR2 polymorphisms (64I/64I) were five in the group of CD4+ T-cell counts ≥ 200 cells/ μ l and none were found in the group of CD4+ T-cell counts < 200 cells/ μ l. These results demonstrated that there was a significant association between CD4+ T-cell numbers and CCR2 and SDF1 polymorphisms ($P < 0.001$). **Conclusions:** The mutation of CCR2 and SDF1 genes showed a significant difference in the distribution of CD4+ T-cell numbers ($P < 0.001$) whereas mutation of chemokine coreceptor CCR5 was not appeared to be associated with the impact of CD4+ T-cell counts. J. Clin. Lab. Anal. 27:38–44, 2013. © 2012 Wiley Periodicals, Inc.

Key words: HIV; SDF1; CCR5; CCR2; disease progression

INTRODUCTION

Host genetic factors appear to be involved in determining rates of disease progression. Polymorphism in the genes for HIV coreceptors CCR5, CCR2, and natural ligand of CXCR4, SDF1 has been suggested to play an important role in the HIV pathogenesis and transmission (1–9). The discovery of chemokine coreceptor polymorphisms in the gene coding for the CCR5 receptor, generated interest in exploring the associations of these polymorphisms with HIV pathogenesis (10). The most important CCR5 coreceptor polymorphism is an inactivating 32 bp deletion in CCR5 gene (CCR5 Δ 32) that results in truncation of the CCR5 protein that fails to express on the cell surface (11). Individuals who are heterozygous for this mutation have a slower disease progression up to

onset of AIDS (12, 13) whereas homozygotes have been reported to be highly resistant to HIV-1 infection (14). CCR2 is another putative HIV-1 chemokine coreceptor but its role in vivo is unclear (15). A CCR2 polymorphism results in substitution of an isoleucine for a valine (V $\rightarrow$ I) at position 64, is located in a transmembrane domain of

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*Correspondence to: Palanee Ammaranond, Faculty of Allied Health Sciences, Chulalongkorn University, 154 Chulapat1 Bldg, Rama I Rd., Pathumwan, Bangkok 10330, Thailand. E-mail: palanee@gmail.com

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CCR2 (1, 16). The variant, CCR2-64I, is associated with a delay in the onset of AIDS (1, 13, 16, 17). CCR2-64I is genetically independent and is not selected by linkage disequilibrium with CCR5 Δ 32 (1). Both CCR5 Δ 32 and CCR2-64I are independent and potent additive effects on delaying progression to AIDS (10). Homozygosity for CCR2-64I has been associated with a 2- to 4-year delay in the progression to AIDS (1). Stroma cell-derived factor 1 (SDF1) is the natural chemokine ligand of CXCR4, the major coreceptor for T-tropic HIV strains, an important coreceptor for HIV-1 late variants found in the disease course (18). The SDF1 polymorphism, SDF1-3'A, is a single nucleotide polymorphism G to A change at codon 801 located in the 3' untranslated region of an alternatively spliced mRNA transcript. HIV-infected individuals homozygous for the SDF1-3'A/3'A variant showed a remarkable level of protection against AIDS in cohort studies (8). Studies have demonstrated that SDF1-3'A is associated with delayed progression to AIDS in homozygous individuals (8). The mechanism of SDF1-3'A polymorphism is still unclear (19). However, studies have proposed that this mutation in the 3' untranslated region could involve up regulation of the quantity of SDF1 protein available to bind CXCR4, which competes with HIV-1 to bind the CXCR4 receptor (8).

The aim of this study was to demonstrate whether the presence of genetic polymorphisms in host factors associated with disease progression. We hypothesized that polymorphisms in CCR5, CCR2, and SDF1 genes would be a significant determinate of disease progression. We used HIV-1 positive individuals as a model to investigate the impact of genetic polymorphisms of CCR5, CCR2, and SDF1 genes on the outcome of the progression to AIDS.

MATERIALS AND METHODS

Study Subjects

Two hundred and twenty-one samples from HIV-infected individuals were included in this study. All patients were identified as HIV positive at the Chulalongkorn Hospital. All those who had a sterile plasma sample stored at -70°C with a confirmed diagnosis of HIV-1 infection and all had received an antiretroviral therapy.

Amplification of CCR5, CCR2, and SDF1 Polymorphisms

Genomic DNA was extracted from Ethylenediaminetetraacetate (EDTA) whole blood by using Qiagen DNA extraction kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. Characterization of CCR5 genotype was performed

by PCR using primers: CCR5F (5'TCACAGCCTGTGCCTCTTC3') and CCR5R (5'TTCATTACACCTGCAGCTCTC3'). PCR was performed in a volume of 25 μ l. Final reaction mix being 10 mM 10 $\times$ buffer, 2.0 mM dNTP, 0.5 mM primers, 500 U of Taq DNA polymerase (Fermentas International Inc., Burlington, Canada), and 3 μ l of DNA. The thermocycling conditions were 95°C for 15 min, 94°C for 20 sec, 61°C for 30 sec, and 72°C for 45 sec for 40 cycles, and during the last cycle an extension at 72°C for 7 min was added (MJ mini Thermal Cycler, Bio-Rad Laboratories Inc., CA). In individuals who are homologous for CCR5 Δ 32 genes, PCR produces a product of 151 bp whereas in individuals who are heterozygous for CCR5 gene, PCR produces products of 183 bp and 151 bp. For the wild type (wt) of CCR5 alleles, PCR produces a product of 183 bp. The primers CCR2F (5'CTCCCTGTGCATAAATTGACGTGAAG3') and CCR2R (5'GACCAGCCCCAAATGTTCTCT3') were used to amplify a 548 bp fragment of the CCR2 gene, containing the site of a single nucleotide polymorphism, the CCR2-64I mutation. The presence of SDF1-3'A polymorphisms was detected by using primers SDF1F (5' CCAC3') and SDF1R (5' ACATGAAAGCTTTG3'). Amplification with the SDF1 specific primer yielded a 367 bp PCR product. For the detection of CCR2-64I and SDF1-3'A mutations, the amplified fragments were subsequently submitted to restriction enzyme digestion by either *BtsCI* (NewEngland Biolab, Beverly, MA) for the CCR2 derived fragment or *MspI* (NewEngland Biolab) for the SDF1-derived fragment. Both digestion reactions were carried for 24 hr at 50°C for CCR2 and 24 hr at 37°C in the supplied buffer and then the size of products determined by 2% agarose gel electrophoresis. *BtsCI* digestion of the 548 bp fragment of the CCR2 gene yielded three fragments (304, 203, and 41 bp) for a wt allele. In the presence of a G $\rightarrow$ A mutation at position 190, the *BtsCI* enzyme cut the mutated sequence, resulting in five fragments (304, 203, 197, 107, and 41 bp) for the heterozygous CCR2-64I allele whereas four bands (203, 197, 107, and 41 bp) were obtained for the homozygous CCR2-64I polymorphism. For SDF1 gene, after digestion with *MspI* restriction enzyme, the wt allele is represented by bands at 220 and 147 bp while heterozygote carries three bands, 367, 220, and 147 bp. Homozygosity for SDF1-3A' polymorphism showed only 1 band, 367 bp.

CD4+ T-Cell Counts

A total of 50 μ l of EDTA whole blood were stained using a panel of fluorescein isothiocyanate, peridinin chlorophyll protein complex, or phycoerythrin, conjugated antibodies by incubation at room temperature for 20 min. Cells were then lysed in FAClysing solution for

TABLE 1. Characteristics of HIV-1 Positive Individuals in Study Population

Characteristics	
Age, median (range)	39 (21–76)
Sex, No. of (%) male	111 (50.2%)
Female	110 (49.8%)
Median CD4+ T-cell counts, cell per microliter (range)	429 (4–1,380)

10 min. After lysing, cells were washed in washing buffer (PBS with 1% BSA) and fixed in 0.5% PF at 4°C. Flow cytometry analysis was performed using a Becton Dickinson FACScalibur (Becton Dickinson, San Jose, CA). Data from at least 100,000 cells gated on the lymphocyte population were acquired.

Statistical Analysis

We performed analyses using the Kruskal–Wallis test and chi-square tests to analyze the genetic polymorphisms of host factors based on CD4+ T-cell numbers in the HIV-1 positive population. For statistical tests, the *P* values of < 0.05 were considered statistically significant. Subjects were compared for the cross-section characteristics using the Kruskal–Wallis test, for categorical variables and the nonparametric chi-square test for continuous variables. Kruskal–Wallis test and chi-square tests were performed using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL).

RESULTS

Demographic Characteristics of HIV-Infected Individuals

The criteria for entry to this study are defined as having CD4+ T-cell counts ranging from 4 to 1,380 cells/μl and were on antiretroviral therapy. Two hundred and twenty-one were identified as HIV-1 positive individuals. The characteristics of two hundred and twenty-one HIV-1 positive subjects included in this study are shown in Table 1. A hundred and eleven were men and a hundred and ten were women, with a median age of 39 years old (range 21–76). Median CD4+ T-cell counts at entry were 429 cells/μl. They all were infected with HIV-1 subtype A/E virus.

Characterization of CCR5, CCR2, and SDF1 Polymorphisms

CCR5

Host polymorphisms in CCR5 coding region were assessed. CCR5 wt and Δ32 alleles could be easily distin-

Fig. 1. Determination of CCR5 polymorphisms; this is a representative gel showing all wt (wt/wt) in lanes 1–4.**TABLE 2. Distribution of Polymorphisms of Chemokine Coreceptors CCR5, CCR2 and Natural Ligand, SDF1 in HIV-1 Positive Individuals**

Host factors	Wild type (%)	Mutation	
		Heterozygous (%)	Homozygous (%)
CCR5	221 (100%)		
CCR5Δ32		0 (0%)	0 (0%)
CCR2	152 (68.8%)		
CCR2–64I		64 (29%)	5 (2.2%)
SDF1	125 (56.6%)		
SDF1–3'A		80 (36.2%)	16 (7.2%)

guished on the basis of a 32 bp difference in the length of their PCR products as shown in Figure 1. In our study of 221 individuals, all (100%) were homozygous for the CCR5 wt alleles (wt/wt). None were heterozygous (Δ32/wt) and homozygous (Δ32/Δ32) for the mutated alleles (Table 2).

CCR2

PCR amplification using CCR2F and CCR2R primers resulted in a 548 bp product of the CCR2 gene. *Bts*CI digestion of the 548 bp yielded three fragments (304, 203, and 41 bp) for a wt allele (Fig. 2). In the presence of a G → A point mutation at position 190, the *Bts*CI enzyme cut the mutated sequence, resulting in five fragments (304, 203, 197, 107, and 41 bp) for the heterozygous CCR2–64I allele whereas four bands (203, 197, 107, and 41 bp) were obtained for the homozygous CCR2–64I polymorphism. We observed the CCR2 polymorphism 155 were homozygous for the CCR2 wt alleles (70.1%). Sixty-one (27.6%) were heterozygous for the CCR2–64I polymorphism (wt/64I). Five (2.2%) were homozygous for the CCR2–64I polymorphism (64I/64I; Table 2).

Fig. 2. Determination of CCR2 polymorphisms; this is a representation of gel showing homozygous (64I/64I) in lane 1, heterozygous (wt/64I) in lane 2, and wt (wt/wt) in lanes 3 and 4.

Fig. 3. Determination of SDF1 polymorphisms; this is a representation of gel showing homozygous (3'A/3'A) in lane 1 and 4, wt (wt/wt) in lane 2, and heterozygous (wt/3'A) in lane 3.

SDF1

Two hundred and twenty-one samples were genotyped for the SDF1-3A' mutation by PCR-RFLP. After digestion with *MspI* restriction enzyme, the wt allele is represented by bands at 220 and 147 bp while heterozygote carries three bands, 367, 220, and 147 bp (Fig. 3). Homozygosity for SDF1-3A' polymorphism showed only one band, 367 bp. The frequency of the SDF1-3A' allele in this population was 0.43. Among 221 individuals, 125 (56.5%) were identified as wt, 80 (36.1%) were identified as heterozygous (wt/3'A) for SDF1 gene, and 16 (7.2%) were homozygous for the SDF1-3'A mutation (3'/3'A; Table 2).

Since the CCR5 and CCR2 genes are located on chromosome 3, which is separated by approximately 15 kb, it is possible that linkage disequilibrium between the CCR5-Δ32 and CCR2-64I alleles might occur. The combination of CCR5 and CCR2 polymorphisms was investigated. In our study population, none of the subjects were ho-

TABLE 3. Combination of CCR5Δ32, CCR2-64I, and SDF1-3A' Genotype Frequencies in This Study

Genotype	CCR2			SDF1		
	wt/wt	wt/64I	64I/64I	wt/wt	wt/3A'	3A'/3A'
CCR5						
wt/wt	155	61	5	125	80	16
wt/Δ32	0	0	0	0	0	0
Δ32/Δ32	0	0	0	0	0	0
Total	155	61	5	125	80	16
CCR2						
wt/wt				3	2	0
wt/64I				40	18	6
64I/64I				82	60	10
Total				125	80	16

TABLE 4. Impact of Host Genetic Factors on CD4+ T-cell Numbers in HIV-Positive Individuals

Host factors	Median CD4	CD4 < 200 cells/μl	CD4 ≥ 200 cells/μl	P value
CCR5				
wt/wt	429	51	170	0.001
wt/Δ32	—	0	0	—
Δ32/Δ32	—	0	0	—
CCR2				
wt/wt	426	36	119	0.001
wt/64I	432	15	46	0.001
64I/64I	546	0	5	—
SDF1				
wt/wt	430	26	99	0.001
wt/3A	407	24	56	0.001
3A/3A	495	1	15	0.001

mozygous for both CCR5Δ32 and CCR2-64I polymorphisms. Furthermore, the combinations of heterozygous CCR5Δ32 and heterozygous CCR2-64I have not been observed (Table 3).

Impact of Host Factors on CD4+ T-cell Numbers

It has been proposed that host factors may play an important role in progression to AIDS. A comparative analysis of host genetic factors and CD4+ T-cell numbers was performed. These two groups were divided according to the CD4+ T-cell numbers and the variation of host genes. On comparison of host factors, none carried CCR5Δ32 homozygous alleles (Δ32/Δ32) whereas 5 and 16 were homozygous for CCR2 (64I/64I) and SDF1 (3'A/3'A) mutations, respectively. Fifteen individuals had homozygous on SDF1-3'A alleles (3'A/3'A) in the group of CD4+ T-cell counts ≥200 cells/μl and one were found in the group of CD4+ T-cell counts <200 cells/μl (Table 4).

There was statistical significantly different association between the number of CD4+ T-cell numbers and the development of polymorphisms of host genetic factors. The variation of CCR2 and SDF1 genes were statistical significantly difference ($P < 0.001$) when compared between CD4+ T-cell numbers. The variation of these two genes, CCR2 and SDF1, fell into the group of CD4+ T-cell counts ≥ 200 cells/ μ l. It was statistical significantly difference ($P < 0.001$). However, this difference was not statistically significant between CD4+ T-cell numbers and CCR5 gene (Table 4).

Disease Progression Associated with CCR2, SDF1 Polymorphisms, and CD4+ T-cell Numbers

We investigate whether there was potential confounding factor on host gene that may influence disease progression among individuals carrying wt or variants of CCR5, CCR2, and SDF1 gene. To address our hypothesis, a univariate analysis was performed for associations of host genetic polymorphisms and disease progression. Host factor including CCR5, CCR2, and SDF1 genes were considered for the statistical analysis. Sixty-nine individuals had variation on SDF1-3'A allele in the group of CD4+ T-cell counts ≥ 200 cells/ μ l and 25 were found in the group of CD4+ T-cell counts < 200 cells/ μ l. Similar result was observed for CCR2 gene. Fifty-one subjects had mutation on CCR2 gene in the group of CD4+ T-cell counts ≥ 200 cells/ μ l and 15 were found in the group of CD4+ T-cell counts < 200 cells/ μ l. A chi-square test for independence showed that both CCR2 and SDF1 polymorphism had a significant difference in the distribution of CD4+ T-cell numbers ($P < 0.001$). A univariate analysis showed that the presence of the CCR2-64I and SDF1-3'A polymorphisms were found to be correlated with the group of CD4+ T-cell counts ≥ 200 cells/ μ l ($P < 0.001$; Table 4).

DISCUSSION

In our study, we investigated host genetic factors for their associations with HIV-1 disease progression. It has been documented that there is an association between host genetic factors and CD4+ T-cell levels established in HIV-1 infection (5–7, 20). Because of the role of host, genetic factors may influence in disease progression the chemokine receptors, CCR5, CCR2, and natural ligand, SDF1 genotypes were analyzed to determine the impact as potential confounders of disease progression. In 221 HIV-1-infected individuals, none were observed for the frequencies of the CCR5 Δ 32 mutation. It is supported in other studies that CCR5 Δ 32 allele frequency varies among races and geographic re-

gions (5–7, 20–22). Previous studies demonstrated that CCR5 Δ 32 polymorphism was most commonly found in Caucasian (23, 24). Heterozygosity for CCR5 Δ 32 allele is commonly found among European Caucasians with the prevalence of 5–15%, but it is lacking in Africans and Asians (21, 22, 24–27). Although those who are homozygous for CCR5 Δ 32 is highly resistant to HIV, it is not fully protective from HIV infection through infections occurring with CXCR4 using virus (28). HIV-1 infected women who are heterozygous for CCR5 Δ 32 have decreased transmission to infants compared to mothers who carried wt for CCR5 genes (29). In other studies, CCR5 Δ 32 polymorphism is shown to be associated with slow rates of disease progression (24, 30, 31) and CCR2-64I allele is associated with a delay in the onset of AIDS (1, 2, 13, 16). We found no difference in the distribution of CCR5 Δ 32 and/or CCR2-64I genes with respect to the rate of progression to AIDS. The CCR2-64I has been reported across all races with a frequency of 10–26% (1, 7, 24, 32–35). However, unlike CCR5, CCR2 is not an essential HIV-1 coreceptor and conservative substitution of V for I in the protein would not be predicted to any serious defect in function, therefore CCR2-64I may influence the rate of progression to AIDS through indirect mechanisms rather than via the mutation itself or may be as a marker for another undescribed gene polymorphism that is in linkage disequilibrium (16).

SDF1-3'A gene frequency is distributed widely across ethnic groups with range of 3–71% (4, 5, 8, 25, 34, 36–39). In our study, the frequency of the CCR2-64I and SDF1-3'A' polymorphisms were observed at rates of 0.29 and 0.43, respectively. It was slightly higher than the data observed in other studies (3, 5, 36–39). Previous studies demonstrated that SDF1-3'A/3'A genotype had a strong protective effect against rapid progression to AIDS and was more present in late stages of HIV-1 infection (8, 9). In contrast to other studies, SDF1-3'A' genotypes were significantly associated with a more rapid disease progression (40). There are conflicting reports on the association of SDF1-3'A/3'A genotype on HIV disease progression. The role of the SDF1-3'A' polymorphism in HIV infection remains to be definitely elucidated.

Interestingly, we found that the variation of SDF1-3'A and CCR2-64I alleles was statistically significant and was associated with disease progression ($P < 0.001$). Patients who carried CCR2 and SDF1 variants were more likely to have a significantly higher CD4+ T-cell numbers than those with wt ($P < 0.001$). A univariate analysis was performed to determine an association between the polymorphism of host genetic factor and CD4+ T-cell numbers. Analysis showed that, CD4+ T-cell numbers and CCR2 and SDF1 polymorphisms, remain significant indicating they are independent predictors of HIV disease progression.

In conclusion, we found evidence that the host factor, CCR2-64I, and SDF1-3'A polymorphisms had a significant influence in the rate of progression of HIV-1 disease.

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