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The First Report of CCR5 Delta 32 Mutant in Thai Injecting Drug Users

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SUMMARY **CCR5, a chemokine receptor, is the principal coreceptor for macrophage-tropic HIV-1 which is the most important variant for viral transmission. It has been demonstrated that a homozygous genotype of a 32-bp deletion in CCR5 gene (Δ32CCR5) shows a high degree of resistance to HIV-1 infection. To demonstrate that Δ32CCR5 does exist in Thai natives, the CCR5 genotypes and allelic frequencies in 860 Thai injecting drug users (IDUs) were determined by PCR and DNA sequencing. Of these, six (0.7%) were CCR5/Δ32CCR5 heterozygotes and no homozygote was found. The overall Δ32CCR5 allelic frequency was 0.0035 and in HIV-1 seronegative (n = 490) and seropositive (n = 370) IDUs were 0.0051 and 0.0004, respectively, which were not significantly different (p = 0.3776). Here we report that the Δ32CCR5 does exist in Thai IDUs as it is present in other human races. Such low allelic frequency may indicate that this mutation does not attribute a significant role in HIV-1 transmission in Thai IDUs.**

After the discovery of HIV-1 inhibition by selected chemokines,[1] distinct chemokine receptors are proved to serve as coreceptors for HIV entry into target cells[2-6] and have attracted attention for the study of their roles in HIV infection. Among them, CCR5[1-3,7-9] and CXCR4[10-14] are the principal coreceptors for macrophage-tropic (R5) and T-cell line tropic (X4) strains of HIV-1, respectively. Since the R5 strains are the chief virus type involved in transmission from person to person,[7] the CCR5 and its ligands were extensively studied particularly in high exposure, as yet uninfected groups. It has been demonstrated that a homozygote of a 32-bp deletion in CCR5 gene (Δ32CCR5) shows a high degree of, but not absolute, resistance to HIV-1 infection.[15-21] The heterozygote of this mutation did not have an agreement on transmission but may delay the progression to AIDS for infected individuals.[15-16,19,22-24] Studies of the Δ32CCR5 mutation alleles in various populations and risk groups have been reported with varying frequencies, which are quite high (5-15%) in Caucasians[15-20,25-27] but very low (<1%) or absent among Africans, Asians and South Americans.[15,17,19,28-30]

Thailand has a high prevalence of HIV-1 infection. The Thai Ministry of Public Health reported AIDS cases at about 130,000 from 1984 to the end of October 1999; meanwhile, the estimate of the total number of infected individuals may reach a million. There were no clear explanations in terms of virologic or genetic factors for a difference in infection rates among countries. The frequency of the Δ32CCR5 allele in Thai people is unknown; several researchers have tried to determine the Δ32CCR5 allele but failed as the sample sizes were too small to detect such a case. The only one case of Δ32CCR5 heterozygote

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was reported from the total of 101 Thai samples.[27] To demonstrate that Δ32CCR5 does exist in Thai natives and to illustrate the role of Δ32CCR5 in HIV-1 transmission, we determined the CCR5 genotype and allelic frequencies in injecting drug users (IDUs), a high risk group with a large number of samples.

MATERIALS AND METHODS

Clinical samples

The cross-sectional subjects consisted of 860 Thai IDUs who attended Thanyarak Hospital for methadone treatment during 1997-1998. After thorough counselling, all IDUs were willing to sign consent forms to have their blood checked. Intravenous blood specimens of 5 ml were collected in citrate-collecting tubes.

Serological tests

Plasma samples were tested for anti-HIV-1 antibody by a commercial ELISA kit (Genelavia Mixt®, Sanofi Pasteur Diagnostic Ltd., France). Positive samples were retested with gel particle agglutination (Serodia®, Fujirebio Inc., Japan). If the 2 tests gave discordant results, the plasma samples were further confirmed by Western blotting (HIV Blot 2.2®, Diagnostic Biotechnology Pte, Singapore).

HIV-1 subtyping

The plasma that gave a positive result in an anti-HIV antibody test were further identified for specific subtype by peptide EIA (PEIA) as previously described.[31]

DNA preparation

PBMCs were separated by Ficoll-Hypaque density gradient centrifugation, then, lysed with lysis buffer (0.32 M sucrose, 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1% Titron X-100). Nuclei were pelleted by centrifugation and digested with proteinase K (PK) (200 μg/ml PK in 1 x PCR buffer) at 37°C overnight. After that, PK was heat-inactivated by boiling for 15 minutes, and DNA lysates were clarified by centrifugation and stored frozen at -20°C.

CCR5 genotyping

Genomic DNA samples were determined for the presence of the Δ32CCR5 by PCR using GeneAmp PCR System 9600 (Perkin Elmer, Connecticut, USA). The PCR was performed in a total volume of 50 μl of 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0 at 25°C], 0.1% Triton X-100) containing 2.0 mM $MgCl_2$, 0.2 μM each primer, 100 μM each dNTPs and 1 unit of *Taq* DNA polymerase. Oligonucleotide 5'-GGTGGCTGTGTTTGCGTCTC-3' and 5'-ATGACAAGCAGCGGCAGGAC-3' (61944-61963 and 62111-62092, GenBank U95626) were used as the forward and reverse primers, respectively. The amplification cycles were 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and end-up with 72°C for 5 minutes to complete the extension step. These primers amplified 168-bp fragment of wild type and 136-bp of Δ32CCR5 mutation. DNA products were identified by the difference in sizes by direct viewing under UV light after electrophoresis on 2.8% agarose gel and staining with ethidium bromide. On each run, the DNA from CCR5 wild type whose PCR products were confirmed as CCR5 by DNA sequencing, was added as positive control for amplification checking. For DNA size estimation, the 100 bp DNA ladder (Promega Corp., New York, USA) was used.

The sample giving 2 bands of 168-bp and 136-bp would be interpreted as a heterozygote while the one that gave a band of 168-bp or 136-bp would be interpreted as a homozygote of wild type and mutant, respectively.

DNA sequencing

The DNA products of the Δ32CCR5 specific band (136-bp) and wild type DNA products were analyzed by sequencing. The 168-bp and 136-bp bands were cut and purified from the gel as described by the manufacturer (High Pure™ PCR Product Purification, Boehringer Mannheim, Germany). These purified products were then used as templates in the sequencing reactions (BigDye™ Terminator, PE Applied Biosystems, USA) by using the same forward and reverse primers, and then automatically sequenced by ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). The sequence data were analyzed using MacVector 4.5.1 software.

Statistical analysis

Significance in allelic frequency comparisons were calculated according to Fisher's exact test[32] because the expected values in a 2 x 2 contingency table are less than 5.

RESULTS

In this study, we determined the presence of Δ32CCR5 in 860 Thai IDU individuals living in suburban Bangkok. Of these, 490

were HIV-1 seronegative and 370 were HIV-1 seropositive. So the prevalence of HIV-1 infection in IDUs was 43.02%. For HIV-1 infected IDUs, 103 were subtype B, 240 were subtype E, and 27 were nontypable. The ratio of % subtype B:E was 30:70. The biological data of these IDUs are summarized in Table 1.

We screened for the presence of Δ32CCR5 mutation in a total of 860 IDUs and only six heterozygous of CCR5/Δ32CCR5 were found; five of these were HIV-1 seronegative and one was HIV-1 seropositive. The ethidium bromide stained gel of PCR products from wild type homozygotes and CCR5/Δ32CCR5 heterozygotes are shown in Fig. 1. The Δ32CCR5 allelic frequency in these IDUs, as shown in Table 2, was 0.0035. When these IDUs were classified into HIV-1 seronegative and seropositive groups, the Δ32CCR5 allele frequencies were 0.0051 and 0.0004, respectively. The allelic frequencies of HIV-1 seropositive and seronegative IDUs were not significantly different (Fisher's exact test, $p = 0.3776$).

The two bands of CCR5/Δ32CCR5 amplified products from the six heterozygous samples and 186-bp bands sampled from six wild type individuals were analyzed and the sequences were shown to be exactly identical to those of previously submitted data (GenBank U95626, AF009962) and the deletion was also present at the same nucleotide position (4486/4487, AF009962).

Table 1 Biological data of 860 Thai IDUs

	HIV-1 subtype n (%)	Male n (%)	Female n (%)	Median age (years)	Positive for Opiate test n (%)
HIV-1 positive (n = 370)	B = 103 (27.84)	98 (95.15)	5 (4.85)	34	97 (94.17)
	E = 240 (64.86)	224 (93.33)	16 (6.67)	24	218 (90.83)
	N/D* = 27 (7.3)	26 (96.30)	1 (3.70)	25	24 (88.89)
HIV-1 negative (n = 490)	-	468 (95.51)	22 (4.49)	23	450 (91.84)

N/D* = non-reactive or dual reactive with peptide EIA (nontypable)

Table 2 Genotype and allele frequencies of Δ32CCR5 in Thai IDUs (n = 860)

	HIV-1 Negative		HIV-1 Positive		*p* value*
	n	frequency	n	frequency	
Genotype					
Homologous wild type (wt/wt)	485	0.9898	369	0.9973	
Heterozygous mutant (wt/mt)	5	0.0102	1	0.0027	0.3766
Homozygous mutant (mt/mt)	0	0	0		
Total	490		370		
Allele					
CCR5	975	0.9949	739	0.9986	
Δ32CCR5	5	0.0051	1	0.0004	0.3776

*p values according to Fisher's exact test, two tailed.
wt = wild type mt = mutant

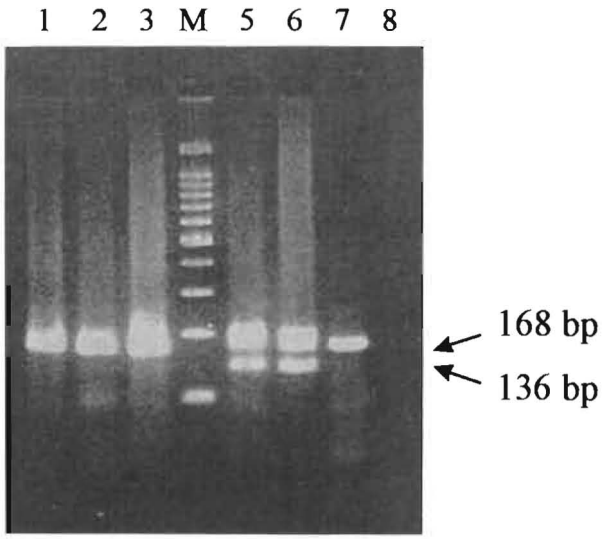

Fig. 1 PCR amplification products from IDUs' DNA samples. Lanes 1, 2 and 3 are CCR5 homozygous wild type which showed a single band of 168 bp. Lanes 5 and 6 are heterozygous of CCR5/Δ32CCR5 having 136-bp and 168-bp bands. Lane 7 is CCR5 wild type positive control and lane 8 is reagent (negative) control. M = 100 bp DNA size marker.

Of the six CCR5/Δ32CCR5 heterozygous subjects, we could follow up three persons for second blood samples. All of them declared their ancestors to be Thai natives without Caucasian admixture. One of them knew his first HIV-1 seropositive result on August 16, 1994 and remained asymptomatic until the second blood-taking on September 29, 1999. All second blood samples of these three persons gave the same two fragments of CCR5/Δ32CCR5, which confirmed our finding.

DISCUSSION

This study is the first report of Δ32CCR5 mutation found in Thai IDUs, a high risk group with HIV-1 prevalence of 43.02%. We evaluated 860 high risk Thai IDUs, and found the heterozygote of CCR5/Δ32CCR5 in six individuals. The overall Δ32CCR5 allelic frequency in these Thai IDUs was 0.0035, while it was 0.0052 in HIV-1 seronegatives and 0.003 in HIV-1 seropositives. A previous report[33] which studied 35 Thai female sex workers did not find a case of Δ32CCR5 mutation. Other investigators reported the absence[15,19,25] or very low number[17,23,26-27] of Δ32CCR5 allele frequency in Asians which are similar to our finding. Since the Δ32CCR5 allelic frequencies were not significantly different between HIV-1 positive and HIV-1 negative Thai IDUs and no homozygous Δ32CCR5/Δ32CCR5 was found, we can say that Δ32CCR5 mutation does not play a significant role in transmission of HIV-1 in Thai IDUs. Other researchers reported that Δ32CCR5 may partially protect against HIV-1 transmission by heterosexual intercourse, but minimally or not protect against homosexual intercourse or perinatal transmission.[34-36] This statement also supported by cell tropism of HIV-1 that the X4 (T-tropic) strains which are responsible for parenteral transmission prefer CXCR-4 as coreceptor to CCR5, while M-tropic HIV, which used CCR5 as coreceptor, are transmitted *via* sexual contact.

A report of the study among 108 HIV-1 infected Spanish IDUs[37] showed no effect of the Δ32CCR5 (allele frequency = 11.34%) on disease progression. In our study, we could not conclude any relation between these two events, since we found only one HIV-1 infected man who was heterozygote.

Because it is hard to find any reports of Δ32CCR5 in Thais, we confirmed our finding of Δ32CCR5 allele by following up persons who were heterozygote for the second blood samples and interviewing them about their ancestors. Only three individuals were available for the following up and all of them gave the same result of heterozygous genotype. They also informed that they had no ancestors such as Caucasian or other races, at least for three generations before them. Therefore, we can conclude that the Δ32CCR5 allele does exist in Thai natives. However, most Thai people have tendency to be an admixture of Thai and Chinese and the allele frequency of this mutation in Chinese is also very low.[19,30]

The CCR5 locus did not show a high degree of genetic variation as has been shown in our sequencing data. However, the 136/168-bp fragments may be too short to verify the variation since there were reports on point mutation at various other sites of the CCR5 gene.[38-40]

Other mutations of CCR5 gene that cause an unfunctional CCR5, inhibit cell surface expression, or limit the expression level of this chemokine receptor could interfere with the entry of the X4 strains.[4,45] Also, mutations and allelic variants of other HIV-1 coreceptors should affect HIV-1 pathogenesis. Even though they are rare, studies of these factors should be concerned to find out the genetic factors related to HIV-1 infection in Thais.

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