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Original article

Sequence variants of chemokine receptor genes and susceptibility to HIV-1 infection

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Abstract. Genetic susceptibility to HIV infection was previously proven to be influenced by some chemokine receptor polymorphisms clustering on chromosome 3p21. Here the influence of 5 genetic variants was studied: Δ32 *CCR5*, G(–2459)A *CCR5*, G190A *CCR2*, G744A *CX3CR1* and C838T *CX3CR1*. They were screened in a cohort of 168 HIV-1 positive adults [HIV(+) group] and 151 newborns [control group] from northwestern Poland. PCR-RFLP was performed to screen for the variants (except for Δ32 *CCR5* polymorphism, where PCR fragment size was sufficient to identify the alleles) and then electrophoresed on agarose gel to determine fragment size. Distribution of genotypes and alleles was not significantly different between the groups except for the *CCR5* polymorphisms, with the Δ32 allele and the (–2459)A *CCR5* allele more frequent among neonates than in the HIV(+) group. No Δ32/Δ32 homozygotes were found in the HIV(+) group, but 16.1% were Δ32/wt heterozygotes. In the control group, 1.3% were Δ32/Δ32 homozygotes and 26.0% were Δ32/wt heterozygotes. Linkage between the chemokine polymorphisms was calculated using the most informative loci for haplotype reconstruction. Haplotypes containing Δ32 *CCR5*, 190G *CCR2* and 744A *CX3CR1* were found to be significantly more common in the control group. This suggests an association between these haplotypes and resistance to HIV-1 infection.

Keywords: chemokine polymorphism, haplotype, HIV infection susceptibility.

Introduction

As HIV-1 entry into host cells is dependent on coreceptor binding, certain polymorphisms (and mutations) in the coreceptor genes strongly affect viral ability to infect susceptible cells, by impairing its fusion with the cell membrane. Thus infectivity is often decreased, or the rate of HIV progression to acquired immunodeficiency syndrome (AIDS) is significantly slower if a certain restricting allele is present. As it has become apparent that HIV fusion with a cell is dependent on chemokine receptors CCR5 and CXCR4, genes encoding for the chemokine receptors have been thoroughly investigated (Alkhatib et al. 1996; Dragic et al. 1996; Liang and Secombes 2004; Magierowska et al. 1999).

The CCR5 chemokine receptor is utilized by macrophage tropic HIV-1 strains, and is the main coreceptor in the early stages of infection. Allelic variations within its gene influence receptor expression at the cell surface, thus altering infectivity and restricting disease progression. The most widely studied variation is a 32-bp deletion (Δ32) within the open reading frame of the CCR5 gene, which encodes a non-functional protein that protects against infection with R-5 tropic HIV-1 strains (Stewart et al. 1997; Garred et al. 1997; Samson et al. 1996; O'Brien 1998; O'Brien and Moore 2000). Although Δ32/Δ32 homozygotes do

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not express a functional receptor, a single heterozygous Δ32 mutation in *CCR5* reduces the level of the receptor at the cell surface (Hladik et al. 2005). Prevalence of this mutation among Caucasian populations has risen up to 9.1%, probably due to a selective pressure exerted by plague and smallpox epidemics in the past (Lucotte and Mercier 1998; Duncan et al. 2004; Galvani and Slatkin 2003). Other genetic factors influencing *CCR5* expression are single nucleotide polymorphisms (SNPs) within the cis-regulatory promoter region, which modify transcriptional efficiency of the gene and alter CCR5 protein expression at the cell surface. Seven SNPs (relative positions –2733, –2554, –2459, –2135, –2132, –2086, and –1835) within *CCR5*, together with 190 *CCR2* alleles and the Δ32/wt *CCR5*, form 9 distinct human haplogroups (HHA, HHB, HHC, HHD, HHE, HHF*1, HHF*2, HHG*1, HHG*2), which are associated with disease-modifying effects (Mummidi et al. 2000; Gonzales et al. 1999; Nguyen et al. 2004). The other weak coreceptor for HIV-1 cell fusion, *CCR2,* remains in linkage with *CCR5*, being located at the same p21 locus of the third chromosome and has been included into the above haplogroups. It has been shown that the 190A (64I) *CCR2* allele delays progression to AIDS (Lee et al. 1998).

Polymorphisms in the *CX3CR1* (fractalkine receptor) have also been proposed to influence progression of HIV infection to AIDS (Liang and Secombes 2004; Garin et al. 2002). This receptor is expressed on monocytes, NK cells, and T lymphocytes, as well as on microglial cells and astrocytes in the brain (Combardiere et al. 1998; Nishimura et al. 2002). On chromosome 3p22, within the reading frame of the gene, 2 non-synonymous polymorphisms G744A (V249I) and C838T (T290M) have been shown to modify the amount of the receptors at the cell surface. Alleles 744A (249I) and 838T (290M) are associated with a reduced number of binding sites for fractalkine, and have been linked with HIV disease acceleration but with a reduced risk of coronary and cardiovascular complications (Garin et al. 2003; Faure et al. 2000; Umehara et al. 2004; Moatti et al. 2001; Rector et al. 2001).

The aim of this study was to investigate the association between genotypes or alleles in the chemokine receptor cluster and susceptibility to HIV infection within a sample cohort of Polish population. We genotyped the Δ32 mutation and A(-2459)G polymorphism in the *CCR5* gene. Additionally, the G190A polymorphism in *CCR2* and 2 polymorphisms in *CX3CR1* (G744A and C838T) were studied in HIV-1 infected individuals and newborns.

Materials and methods

Ethical issues

The study protocol was reviewed and approved by the local ethics committee. Permission to collect and analyse samples from both groups was obtained. Informed consent was given by HIV-infected participants and the mothers of the newborns in our sample.

Subjects

The studied HIV(+) group comprised 168 HIV-1 infected Caucasians aged 23–66 years (mean 36 ± 9 years; median 35 years) recruited randomly from the patients of the Department of Infectious Diseases and Hepatology, Pomeranian Medical University, Szczecin, Poland. Only adult subjects (28.0% women and 72.0% men), with confirmed HIV-1 infection by western-blot analysis, were recruited into the study. Sexual transmission of HIV-1 was the most prevalent route of infection (94 patients – 56.0%) with the reminder infected via injection drug use (74 individuals – 44.0%). The following HIV infection categories were represented (CDC/WHO 1993): A – 24 (14.3%); B – 97 (57.7%); and C – 47 (28.0%).

The control group consisted of 151 newborns (49.7% male and 50.3% female) delivered at the Department of Neonatology, Pomeranian Medical University Hospital. Umbilical blood samples were collected during delivery, and next DNA extraction was performed.

DNA extraction and genotyping

QIAamp DNA Blood Mini Kit (QIAgen, Hilden, Germany) was used to extract genomic DNA from whole blood samples, previously collected into tubes containing EDTA (anticoagulant). The extraction was performed following to the manufacturer's protocol, DNA was re-suspended in 200 μL of AE buffer (QIAgen, Hilden, Germany) and stored at 4°C for further analyses. To assess the distribution of single nucleotide polymorphisms, PCR-RFLP protocols were used except for the Δ32 *CCR5* variation, which was screened by a previously described PCR technique (Umehara et al. 2004; Kristiansen et al. 2001; Rector et al. 2001) .

All resulting reaction products were electrophoresed on a 3% agarose gel (SIGMA, Saint Louis, USA) stained with ethidium bromide. Results were visualized under UV light (Transilluminator 4000, Stratagene, La Jolla, USA) and recorded with DS-34 Polaroid Direct Screen Camera.

Statistics

Significance of allele and genotype differences between the groups was assessed using the chi-square test in Epi-Info 6 Statcalc software (CDC, USA), with 95% confidence interval. Odds ratio was calculated if the difference proved statistically significant. Hardy-Weinberg equilibrium assessment was performed by the chi-square goodness-of-fit test, for each locus in each group separately and in both groups jointly. Linkage disequilibrium was analysed by the "genetics 1.1.3" module, with value of standardised correlation coefficient (D') calculated, while haplotype analysis was performed with "haplo.stats 1.2.1" module (http://r.meteo.uni.wroc.pl),(http://cran.r-project.org), both created for the R 2.0.1 statistical package for Windows. Odds ratios and 95% confidence intervals were calculated by univariate analysis adjusted for sex, incorporating either dominant or recessive inheritance model to the variant allele. Parameters were assessed separately for these 2 models of inheritance. Linkage between the chemokine polymorphisms was calculated with subsequent selection of the most informative loci for haplotype reconstruction. Computations for each combination of 3 loci were performed, with subsequent selection of the following: wt/Δ32 *CCR5*, A190G *CCR2* and G744A *CX3CR1*, representing distinct chemokine receptor genes. $P < 0.05$ was considered statistically significant.

Sequence data

For the analysed sequence variants, the following gene IDs were used: 1234 for *CCR5*, 1231 for *CCR2*, and 1524 for *CX3CR1.* For the analysed SNPs, the following ref SNP IDs were applied: rs1799987 for G(–2459)A *CCR5*, rs1799864 for G190A *CCR*, rs3732379 for G744A *CX3CR1*, and rs3732378 for C838T *CX3CR1.*

Results

All the analysed genotypes were in Hardy-Weinberg equilibrium. No statistically significant differences in allele and genotype distribution in the investigated groups were found except for *CCR5* polymorphisms. The Δ32 *CCR5* genotypes and allele were significantly more prevalent in the group of neonates than in the HIV(+) group ($P < 0.01$, odds ratios: 0.51 (95% CI: 0.28–0.91) and 0.52 (95% CI: 0.30–0.89) for the Δ32 *CCR5* allele and genotypes respectively) (Table 1). Homozygotes for this polymorphism remained rare, with only 2 found in the control group, and no such a genotype present in the HIV(+) group. A significant difference for the A(-2459)G *CCR5* SNP was also observed between the groups ($P < 0.03$, odds ratio 1.44, 95% CI 1.02–2.01). No differences in the remaining allele and genotype distributions were detected.

Results of the analysis of the relationship between the investigated polymorphisms and susceptibility to HIV infection are presented in Table 2. Correlation between Δ32 *CCR5* and (–2459)A *CCR5* alleles with HIV infection susceptibility was found for both assessed *CCR5* loci. No other significant correlation was detected between the remaining polymorphisms and susceptibility to HIV infection. Tight linkage disequilibrium ($D` > 0.99$, $P < 0.01$) was observed between the alleles located within one gene, as well as G190A *CCR2* and both investigated *CCR5* polymorphisms (Figure 1). Of the possible 8 haplotypes, only 6 occurred (Table 3). Haplotype H6 (Δ32 *CCR5*, 190G *CCR2* and 744A *CX3CR1*) was significantly more prevalent in the control group ($P < 0.01$). No other significant difference between the calculated haplotype distributions was observed.

Haplotype analysis for the haplotype pairs revealed that out of the 21 possible combinations, only 13 were observed, with 6 being extremely rare (Table 4). Furthermore, the H2/H3 haplotype was significantly less common in the HIV(+) group than among neonates.

Discussion

Novel aspects of this study include investigation of the distribution of two *CX3CR1* polymorphisms in HIV-1 infected individuals, with subsequent reconstruction of haplotypes based on 3 selected chemokine receptors. The control group included into the study comprised newborns, i.e. a population not influenced by environmental pressure exerted in postnatal life. Analyses of differences in haplotype distribution between the groups for both single haplotype and combinations were per-

Table 1. Frequencies of chemokine receptor genotypes and alleles in the analysed groups (wt = wild type)

Locus	Genotype or allele	HIV infected, n (%)	Newborns,n (%)	*P* value
CCR5 wt/Δ32	wt/wt	141 (83.9)	109 (72.7)	
	wt/Δ32	27 (16.1)	39 (26.0)	
	Δ32/Δ32	0 (0.0)	2 (1.3)	0.01[1]
	Δ32	26 (8.0)	43 (14.3)	
	wt	310 (92.0)	259 (85.8)	0.01
CCR5 A(–2459)G	A/A	57 (33.9)	69 (46.0)	
	A/G	84 (50.0)	64 (42.7)	
	G/G	27 (16.1)	17 (11.3)	n.s.
	A	198 (58.9)	202 (67.3)	
	G	138 (41.1)	98 (32.5)	0.03
CCR2 G190A (V64I)	G/G	136 (81.0)	117 (78.0)	
	G/A	31 (18.5)	31 (42.8)	
	A/A	1 (0.6)	2 (1.3)	n.s.
	G	305 (90.2)	267 (88.3)	
	A	33 (9.8)	35 (11.7)	n.s.
CX3CR1 G744A (V249I)	G/G	90 (53.6)	75 (50.0)	
	G/A	61 (36.3)	60 (40.0)	
	A/A	17 (10.1)	15 (10.0)	n.s.
	G	241 (71.7)	210 (70)	
	A	95 (28.3)	90 (30.0)	n.s.
CX3CR1 C838T (T290M)	C/C	102 (60.7)	95 (63.3)	
	C/T	55 (36.3)	49 (32.7)	
	T/T	11 (6.5)	6 (4.0)	n.s.
	C	259 (77.1)	239 (79.7)	
	T	77 (22.9)	61 (20.3)	n.s.

[1]For estimation of this *P* value, wt/Δ32 and Δ32 /Δ32 were summed up, due to small sample size; n.s. = not significant

Table 2. Association of chemokine receptor polymorphisms and susceptibility to HIV-1 infection in dominant (d) and recessive (r) inheritance models (wt = wild type)

Polymorphism	Inheritance model	OR (95% CI)*	*P* value
CCR5 wt/Δ32	d: (wt/Δ32 + Δ32/Δ32) vs. wt/wt	0.50 (0.29?0.88)	0.02
	r: Δ32/Δ32 vs. (wt/wt+wt/Δ32)	–	–
CCR5 A(–2459)G	d: (G/G +A/G) vs. A/A	1.61 (1.01–2.57)	0.04
	r: G/G vs. (A/A+A/G)	1.51 (0.77–2.95)	n.s.
CCR2 G190A	d: (A/A + G/A) vs. G/G	0.79 (0.45–1.39)	n.s.
	r: A/A vs. (G/G+G/A)	0.57 (0.05–6.75)	n.s.
CX3CR1 G744A	d: (A/A + G/A) vs. G/G	0.78 (0.50–1.24)	n.s.
	r: A/A vs. (G/A+G/G)	0.91 (0.43–1.94)	n.s.
CX3CR1 C838T	d: (T/T +C/T) vs. C/C	1.10 (0.69–1.76)	n.s.
	r: T/T vs. (C/T+C/C)	1.71 (0.61–4.81)	n.s.

*Adjusted by sex; n.s. = not significant

formed to analyse its influence on susceptibility to HIV-1 infection.

In our study the frequency of Δ32 *CCR5* was significantly higher in the control group. This is in accordance with the described protective effect of the allele (Samson et al. 1996; O'Brien SJ 1998; O'Brien and Moore 2000). It must be also noted that the frequency of the Δ32 *CCR5* allele among the investigated neonates was notably higher in our study than reported by Jagodzinski et al. (2000), although the difference did not reach the threshold of statistical significance ($P = 0.06$). Mean frequency of the Δ32 allele in Poland among healthy adults was 10.9% , while in northwestern Poland it was even lower (9%) (Jagodzinski et al. 2000), which contrasts with the frequency of 14.3% found in healthy neonates in our study. Our results may reflect the distribution of the allele in

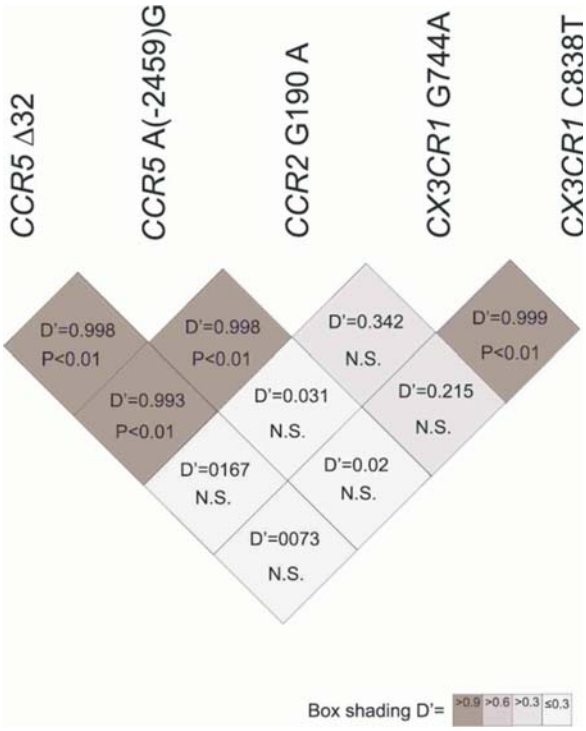

Figure 1. Linkage disequilibrium plot for pairs of loci

this area more accurately, as the sample size was bigger, or could be due to some unknown selective pressure eliminating the Δ32 *CCR5* carriers in the later life, which seems unlikely. Additionally, the population recruited into this study was a sample of the population of northwestern Poland, colonized mainly by immigrants from eastern regions of the country after the 2nd World War. This movement might be responsible for the higher Δ32 allele frequency in the region, as it is notably higher in the eastern and northern areas neighbouring the Baltic sea (Lucotte and Mercier 1998).

Also the number of (-2459)G *CCR5* allele carriers was significantly lower among newborns, in comparison to HIV-infected individuals. This allele is supposed to limit promoter activity and reduce the amount of receptor at the cell surface; thus it may extend the life expectancy of HIV-1 infected individuals (McDermott et al. 1998). Hladik et al. (2005), described a dominant protective effect of the (–2459)G allele among exposed, uninfected individuals, enhanced by the Δ32 *CCR5* mutation, while Tang et al. (2002), found a

Table 3. Haplotype frequencies for loci *CCR5*, *CCR2* and *CX3CR1*

Haplotype no.	*CCR5* wt/Δ32	*CCR2* G190A	*CX3CR1* G744A	Haplotype frequency		*P* value
				newborns	HIV (+)	
H1	wt	G	G	0.522	0.577	n.s.
H2	wt	G	A	0.218	0.251	n.s.
H3	wt	A	G	0.081	0.074	n.s.
H4	wt	A	A	0.036	0.017	n.s.
H5	Δ	G	G	0.097	0.065	n.s.
H6	Δ	G	A	0.047	0.012	0.01
H7	Δ	A	G		absent	
H8	Δ	A	A		absent	

Table 4. Distribution of haplotype combinations for chemokine receptor loci

Haplotype combination[1]	HIV infected n (%)	Newbornsn (%)	OR (CI)*	P value
H1/H1	41 (0.273)	56 (0.333)	1.49 (0.90–2.46)	n.s.
H1/H2	46 (0.274)	31 (0.207)	1.38 (0.81–2.36)	n.s.
H1/H3	18 (0.107)	12 (0.08)	1.44 (0.65–3.18)	n.s.
H1/H5	13 (0.077)	17 (0.113)	0.59 (0.27–1.29)	n.s.
H2/H2	12 (0.071)	6 (0.04)	1.52 (0.54–4.26)	n.s.
H2/H4	4 (0.024)	3 (0.02)	1.10 (0.23–5.20)	n.s.
H2/H3	4 (0.024)	14 (0.093)	0.21 (0.07–0.67)	0.01
H2/H5	9 (0.054)	14 (0.093)	0.62 (0.25–1.51)	n.s.
H2/H6	1 (0.006)	6 (0.04)	0.16 (0.02–1.39)	n.s.
H3/H3	1 (0.006)	2 (0.014)	0.57 (0.05–6.75)	n.s.
H3/H5	3 (0.018)	1 (0.007)	3.23 (0.31–33.3)	n.s.
H3/H6	1 (0.006)	1 (0.007)	0.62 (0.04–10.17)	n.s.
H5/H5	0 (0.000)	2 (0.013)	(–)	(–)

*Adjusted by sex. [1]haplotypes are numbered according to their order in Table 3

higher susceptibility to HIV infection among individuals bearing the HHE haplotype, which includes the (–2459)G SNP. Relatively high frequencies of the (–2459)A allele in our randomly selected group, with most of the individuals remaining in observation for several years, may be related to the higher mortality rates among HIV(+) individuals bearing the (–2459)A allele. The odds ratio calculated for the (–2459) *CCR5* locus implies that carrying the G allele may be related to a greater risk of contracting HIV-1 infection. This seems counterintuitive but remains in accordance to the findings by Shresta et al. (2006) that the A allele, in addition to being related to the faster progression, may also promote acquisition of HIV-1 infection.

CCR2 polymorphism is an 'additional AIDS restricting' genetic variant with no influence on HIV infection susceptibility found so far. Our results are also in agreement with this conclusion, as no significant differences in frequencies were found between the groups, and confirm earlier reports for genotype and allele distribution in Poland (Lewandowska et al. 2002). For both investigated *CX3CR1* loci, no statistically significant differences were found between the groups, proving that none of the polymorphisms influenced HIV-1 infectivity. This is in accordance with the previously reported findings (Vidal et al. 2005). However, the report by Faure et al. (2000), suggested that the *CX3CR1* 744A and 838T allele combination may be linked to increased susceptibility to infection. This contrasts with the research by Vidal et al. (2005), indicating that the number of 744A alleles was higher in a Spanish HIV-infected long-term non-progressing group. Additionally, it should be noted that 744 A/A and 838 T/T homozygotes may protect an individual from adverse cardiovascular action of the antiretroviral therapy, balancing the negative effects on progression with better prospects for lifelong therapy (Combardiere et al. 1998; Nishimura et al. 2002).

Haplotype analysis is a means to enhance the power of association studies where multiple disease-linked loci are analysed. We implemented this method in order to assess the predictive value of such an approach when investigating susceptibility to infection. Research on chemokine receptor haplotypes have been previously described by Li et al. (2005) and Clark and Dean (2004). In our study, 3 loci (wt/Δ32 *CCR5*, G190A *CCR2* and G744A *CX3CR1*) were selected to represent each of the investigated chemokine receptor genes based on the linkage disequilibrium between them. One locus for each gene was chosen. Among the investigated haplotypes the most common was haplotype H1, with no protective alleles within all 3 loci. As expected, haplotypes H7 and H8, which belong to the distinct *CCR5* haplogroups, did not occur at all. Haplotype H3 may be compared to the previously described HHF*2 *CCR* haplogroup, while haplotypes H5 and H6 can be compared to the HHG*2 haplogroup, which was the only haplotype from the group containing the Δ32 *CCR5* mutation, and was expected to reduce the susceptibility to HIV-1 infection. This is confirmed by our data, as haplotype H6 (containing Δ32 deletion) was significantly more prevalent in the control group than among HIV(+) adults. Both HHG*2 and HHF*2 are associated with inhibition of disease progression (Gonzales et al. 1999), which was not assessed in this study. Haplogroup HHE is also associated with enhancement of HIV transmission and faster progression (Gonzales et al. 1999, Mangano et al. 2001). A lack of both chemokine polymorphisms Δ32 *CCR5* and 190A *CCR2*, may be represented by haplotypes H1 and H2 from our study. No significant differences between the groups was noted in the distribution of these haplotypes. Having analysed haplotype combinations, the one containing the Δ32 variation seemed to confer protection (combination H2/H6). Although the size of the group was adequate to indicate such a result, further studies are needed.

Interestingly, the H2/H3 combination was significantly more prevalent in the control group than in the HIV(+) group, which may imply that heterozygosity within both *CX3CR1* and *CCR2* is related to reduced susceptibility to the infection in the absence of Δ32 mutation. This unique result may suggest that protective mechanisms may exist when heterozygosity is present within the protective 190A *CCR2* and 744A *CX3CR1* alleles. This is not unusual, as a similar protective effect provided by the Δ32 allele when combined with (–2429)G allele has already been reported (Hladik et al. 2005).

It must be noted that seroconversion data for most of the patients are unknown or unreliable, including time and route of infection, as it is a regular practice for individuals tested for HIV in Poland not to disclose the details (especially those related to sexual orientation and risky behaviour). As proper risk assessment was impossible, selection of a seronegative control group presenting similar risks was difficult. Therefore we tried to choose the unbiased control group, which pro-

vided baseline genetic data for analysis. The randomly selected newborns from the same region as the HIV infected individuals seem a control group free of any background influence.

Conclusions regarding susceptibility to HIV-1 in the studied populations require a further discussion. The risk of allele frequency stratification between the populations of neonates and HIV-1 infected adults enrolled into the study is high, but the neonates may better reflect allele distribution in the general population. However, the differences in *CCR2* and *CX3CR1* allele distribution were not significant, which may indicate that stratification was minimal.

In summary, our results indicate that the *CCR5* Δ32 allele and haplotype combinations containing this variation, but also combinations containing the wild-type *CCR5* and heterozygous within 2 remaining analysed loci (190A *CCR2* and 744A *CX3CR1*), may protect from acquisition of HIV-1. Further studies on larger cohorts are necessary to confirm host resistance and susceptibility to infection with potential beneficial effects based on the genetic background.

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