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Validation of Genetic Case-Control Studies in AIDS and Application to the CX3CR1 Polymorphism

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Summary: TOP

New polymorphisms have been recently identified in CX3CR1, a coreceptor for some HIV-1 strains, one of which was associated with a strong acceleration of HIV disease progression. This effect was observed both by a case-control study involving 63 nonprogressors (NP) from the asymptomatic long-term (ALT) cohort and Kaplan-Meier analysis of 426 French seroconverters (SEROCO cohort).

These results prompted us to analyze these polymorphisms in 244 nonprogressors (NPs) and 80 rapid progressors (RPs) from the largest case-control cohort known to date, the GRIV cohort. Surprisingly, the genetic frequencies found were identical for both groups under all genetic models ($p > .8$). The discrepancy with the previous work stemmed only from the difference between GRIV NPs versus ALT NPs. We hypothesized this might be due to the limited number of NPs in ALT ($n = 63$) and in this line we reanalyzed the data previously collected on GRIV for over 100 different genetic polymorphisms: we effectively observed that the genetic frequencies of some polymorphisms could vary by as much as 10% (absolute percentage) when computing them on the first 50 NP subjects enrolled, on the first 100, or on all the NPs tested (240 study subjects). This observation emphasizes the need for caution in case-control studies involving small numbers of subjects:*p* values should be low or other control groups should be used.

However, the association of the CX3CR1 polymorphism with progression seems quite significant in the Kaplan-Meier analysis of the SEROCO cohort (426 individuals), and the difference observed with GRIV might be explained by a delayed effect of the polymorphism on disease. Further studies on other seroconverter cohorts are needed to confirm the reported association with disease progression.

Since the discovery of chemokines as inhibitors of HIV-1 infection (1) and the identification of their receptors as the coreceptor with CD4 for virus entry in cells (2), the chemokine system has been an object of intense scrutiny (3). This involved experimental functional studies as well as genetic epidemiology studies based on natural history AIDS cohort analysis or on case-control groups with marked progression patterns. These studies have pointed to the roles of CCR5 as the key coreceptor for infection by primary R5 HIV-1 strains (4,5), of CCR2, although the mechanism for this effect remains unclear (6), and of CXCR4 through its ligand SDF-1 (7). At least 13 additional chemokine and orphan receptors have been identified that are able to bind HIV Env or support HIV replication in vitro, but the in vivo role of these minor chemokine receptors remains largely unknown (8).

CX3CR1, the leukocyte chemotactic and adhesion receptor for fractalkine, was recently identified as an HIV coreceptor for a limited number of HIV-1 strains (9-13). CX3CR1 is located on human chromosome 3p21.3 about 10 Mb from a major chemokine receptor cluster consisting of CCR1, CCR3, CCR2, and CCR5 (14). Faure et al. (15) reported two nonsynonymous single nucleotide polymorphisms (SNPs) in CX3CR1, corresponding to the mutations V249I (V into I) and T280M (T into M) localized in the transmembrane domain of the receptor, and commonly observed only in Caucasians. The effect of the 280M mutation was striking because the relative risk for disease progression was 2.1, which was much higher than all those previously described in the chemokines system. This detrimental effect of the 280M/280M genotype was confirmed by both an survival analysis of a cohort of all-stage patients and a case-control study involving 63 nonprogressors (NPs) and 73 standard progressors (15). Moreover, the proportion of homozygotes for the 280M mutation among infected subjects was much higher than expected from Hardy-Weinberg equilibrium suggesting an effect on infection.

These data prompted us to analyze these polymorphisms in the Genetics of the Resistance to Infection by the Immunodeficiency Virus (GRIV) study, a large case-control cohort of NPs/rapid progressors (RPs), who represent the extremes of disease outcome from a pool of 30,000 patients (16,17). This cohort has been previously used to demonstrate influential effects of variants such as CCR5-Δ32, CCR2-64I, SDF1-3`A (18,19), the CCR5 promoter 59353 T/C (unpublished data), as well as some human leukocyte antigens (HLA) alleles and tends to identify polymorphisms with an early effect on progression (17). Because more than 100 polymorphisms have now been genotyped in the patients of GRIV, we also provide experimental evidence showing that important fluctuations in genetic frequencies may occur when dealing with small cohorts (typically <100 patients), which may explain discrepant results observed on various case-control studies.

MATERIALS AND METHODS TOP

Patients TOP

The GRIV Cohort was established in 1995 in France to generate a large collection of DNA samples for genetic studies of candidate human polymorphisms associated with rapid and slow progression to AIDS (16). To avoid confounding effects associated with racial/ethnic differences in the genetic analyzes, only Caucasians of European descent were recruited from hospital-based AIDS units throughout France. Slow progressors were defined as asymptomatic individuals who had tested seropositive for ≥8 years with a $CD4^+$ cell count >500/mm^3 in the absence of antiretroviral therapy. A seropositive test result >8 years was necessary for inclusion in the study. RPs were defined by CD4 count < 300/mm^3 <3 years after the last seronegative testing. On enrollment, each patient signed an informed consent form and donated 40 ml of blood. Blood was shipped overnight from the collection centers and immediately processed in the laboratory. Peripheral blood mononuclear cells (PBMCs) were collected and Epstein-Barr Virus (EBV)-transformed B-cell lines were generated as a renewable source of genetic material.

Genotyping TOP

The polymorphisms have been genotyped by polymerase chain reaction-reverse transcription/restriction fragment length polymorphism (PCR-RFLP) as previously described (15).

Statistics TOP

These *p* values were computed using the 2 × 2 Fisher exact tests comparing the numbers associated with a given allele (allelic frequency, recessive model, dominant model) among NPs versus RPs (Table 1) or the numbers associated with an haplotype among GRIV NPs versus ALT NPs (Table 2). The *p* value for comparing the genotypic distribution with the Hardy-Weinberg prediction was obtained from χ^2 analysis.

V249I		NP (n = 244)	RP (n = 80)	OR	p value
Allele frequency, no. (%)	1	335 (68, 6)	120 (75)	1.37	.14
	2	153 (31, 4)	40 (25)	0.73	.14
Dominant model, no. (%)	1	214 (87, 7)	76 (95)	2.66	.09
	2	123 (50, 4)	36 (45)	0.8	.44
Recessive model, no. (%)	1	121 (49, 6)	44 (55)	1.24	.44
	2	30 (12, 3)	4 (5)	0.37	.09
T280M		NP (n = 240)	RP (n = 79)	OR	p value
Allele frequency, no. (%)	1	400 (83, 3)	133 (84, 1)	1.06	.9
	2	80 (16, 7)	25 (15, 8)	0.94	.9
Dominant model, no. (%)	1	233 (97)	77 (97, 5)	1.15	1.00
	2	73 (30, 4)	23 (29, 1)	0.95	.88
Recessive model, no. (%)	1	167 (69, 6)	56 (70, 9)	1.06	.88
	2	7 (2, 9)	2 (2, 5)	0.86	1.00

NP, nonprogressors; RP, rapid progressors; OR, odds ratio.

The WT alleles have been named 1 (V249 and T280) and the variants 2 (I249 and M280). The table gives the number of alleles, the number of patients carrying a given allele under the dominant model (subjects carrying the allele) or under the recessive model (subjects homozygous for the allele) in each group. In parentheses are the corresponding percentages. Odds ratio (OR) and *p* values (Fisher exact test) were computed from the contingency tables comparing NPs versus RPs for each allele.

TABLE 1. *Genetic frequencies of the two CX3CR1 single nucleotide polymorphisms in the genetics of the resistance to infection by the immunodeficiency virus cohort*NP, nonprogressors; RP, rapid progressors; OR, odds ratio.The WT alleles have been named 1 (V249 and T280) and the variants 2 (I249 and M280). The table gives the number of alleles, the number of patients carrying a given allele under the dominant model (subjects carrying the allele) or under the recessive model (subjects homozygous for the allele) in each group. In parentheses are the corresponding percentages. Odds ratio (OR) and *p* values (Fisher exact test) were computed from the contingency tables comparing NPs versus RPs for each allele.

Genotype	GRIV RP (n = 78)[a]	GRIV NP (n = 237)[a]	ALT NP (n = 63)	IMMUNOCO (n = 73)	SEROCO (n = 426)	p1 ALT/IMMUNOCO	GRI
1: V249 T280/V249 T280	42 (53.8)	120 (50.6)	35 (55.5)	33 (45.2)	222 (52.1)	0.23	
2: I249 T280/I249 T280	1 (1.2)	6 (2.5)	1 (1.6)	2 (2.7)	4 (0.9)	1	
3: I249 M280/I249 M280	2 (2.5)	7 (2.9)	2 (3.2)	3 (4.1)	16 (3.8)	1	
4: I249 T280/I249 M280	1 (1.3)	15 (6.3)	3 (4.8)	2 (2.7)	17 (4)	0.65	
5: V249 T280/I249 T280	12 (15.3)	39 (16.4)	13 (20.6)	12 (6.4)	80 (18.8)	0.06	
6: V249 T280/I249 M280	20 (25.6)	51 (21.1)	9 (14.3)	21 (28.8)	87 (20.4)	0.66	
1 + 2 + 5	55 (70.5)	165 (69.6)	49 (77.7)	47 (64)	306 (71.8)	0.07	
3 + 4 + 6[b]	23 (29.5)	72 (30.4)	14 (22.3)	26 (35.6)	120 (28.2)	0.07	

[a] Numbers are slightly smaller than in Table 1 since not all individuals were genotyped for both single nucleotide polymorphisı
[b] Genotypes carrying the M280 mutant.
RP, rapid progressors; NP, nonprogressors.
This table takes data from Faure et al. (15) and compares the GRIV 240 NPs and 80 RPs with the 63 NPs from ALT, the 73 sta patients from IMMUNOCO, and the 426 seroconverter patients exhibiting all profiles of progression from SEROCO. The 2 SNPs V are in linkage disequilibrium and only 3 haplotypes are observed: V249 T280, I249 T280, I249 M280. The 2 closest groups are, in GRIV NPs and SEROCO (p = .75 from the χ^2 of the genotypic distribution). The different p values are obtained with the Fisher × 2 contingency tables comparing IMMUNOCO with GRIV or ALT cohorts for a given genotype versus the other genotypes con

TABLE 2. *Comparison of asymptomatic long-term (ALT) and genetics of the resistance to infection by the immunodeficiency study (GRIV) nonprogressors with IMMUNOCO a* Numbers are slightly smaller than in Table 1 si genotyped for both single nucleotide polymorphisms.*b* Genotypes carrying the M280 mutant.RP, rapid progressors; NP, nonprogressors.This table takes data from Faure et al. (15) and compares the GRIV 240 NPs and 80 ALT, the 73 standard progressor patients from IMMUNOCO, and the 426 seroconverter patients exhibiting all profiles of progression from SEROCO. The 2 SNPs V249I and T280M are in linkage disequilibrium and only 3 h V249 T280, I249 T280, I249 M280. The 2 closest groups are, in fact, the largest GRIV NPs and SEROCO (p = .75 from the χ^2 of the genotypic distribution). The different p values are obtained with the Fisher exact test fro tables comparing IMMUNOCO with GRIV or ALT cohorts for a given genotype versus the other genotypes combined.

RESULTS TOP

We genotyped 244 NPs and 80 RPs from the GRIV cohort for the two single nucleotide polymorphisms (SNPs) V249I and T280M. For each group of NPs and RPs, the genotypic distribution conformed to the Hardy-Weinberg equilibrium (p > .3 for V249I and p = 1 for T280M), and we did not observe a distortion for M280 homozygotes indicating a higher infection rate. In fact, V249I exhibited a slight difference between NPs and RPs with a trend to significance (31.3% of the 249I allele among NPs versus 25% among RPs, p = .14), but for the T280M polymorphism the allele frequencies between the two groups were almost identical (16.7% of the M280 allele among NPs, versus 15.8% among RPs, p = .9) and identical to the SEROCO control group previously published (16% of the M280 allele [15]). No significant differences were observed in either the recessive or dominant genetic models (Table 1). Since an interaction with the CCR5-Δ32 protective allele had been suggested (15), we also performed the analysis comparing the 181 CCR5-$^{+/+}$ NPs and the 74 CCR5-$^{+/+}$ RPs and found p values and odds ratios exactly identical to those given in Table 1. Finally, we investigated the two SNPs in subcategories by gender (male/female), and risk group (homosexual/intravenous drug user/HIV-contaminated blood transfusion recipients) that have been previously suggested to modify the influence of genetic polymorphisms (20). Again, we could not observe any significant difference between NPs and RPs for each category, nor between these categories among NPs or among RPs (data not shown). When comparing our results with those previously published (15), it appears that the most divergent groups in their genotypic distribution are the smallest (p = .17), the 63 ALT NPs and the 73 IMMUNOCO patients (Table 2), whereas GRIV NPs, GRIV RPs, and the 426 SEROCO patients have close genotypic distributions (p > .7). The major difference observed between the ALT NPs and IMMUNOCO (p = .07) does not hold when comparing GRIV NPs and IMMUNOCO (p = .47) (Table 2). Criteria for nonprogression are nearly identical in GRIV and ALT: the patients at inclusion must have been asymptomatic for >8 years, their CD4 cells counts >600/mm^3 in ALT and >500 in GRIV. Because both groups come from the same French Caucasian population, we postulated that this difference was due to statistical fluctuations resulting from the smaller number of study subjects involved in ALT (63 versus 244 in GRIV). Genotypic data have been collected for 100 genetic polymorphisms in the GRIV study and we thus evaluated the fluctuations of the genetic frequencies among GRIV NPs when taking only the 50 first NP subjects enrolled, then the first 100, and so on. Figure 1 presents the percentages of study subjects' homozygotes for a given allele (recessive model) for some genetic polymorphisms according to the number of study subjects genotyped. For simplicity of presentation, the percentage obtained for the total of 240 subjects genotyped has been subtracted to see these fluctuations more readily (see Fig. 1 legend). For most polymorphisms, fluctuations of the genetic frequencies among 50, 100, and 240 GRIV NPs were minor; however, in some instances, (about 15 polymorphisms out of 100 tested), it could vary by as much as 10%. For instance, in the MIP polymorphism (Fig. 1), the frequency of homozygotes for allele 1 in the recessive model (MIP-1rec) is 54.7% for the first 50 study subjects enrolled, 67.8% for the first 100, and 57.6% for 240 tested, respectively. Incidentally, we observed an important variation for the V249I CX3CR1 polymorphism, not for T280M (Fig. 1). Similar results are found when computing the allelic frequencies or the percentage of subjects carrying a given allele (dominant model) (data not shown). These observations emphasize the need for caution when analyzing genetic data on a limited number of patients (<100), especially when the p value of the effect is borderline (p = .07 in the study of Faure et al. [15]).

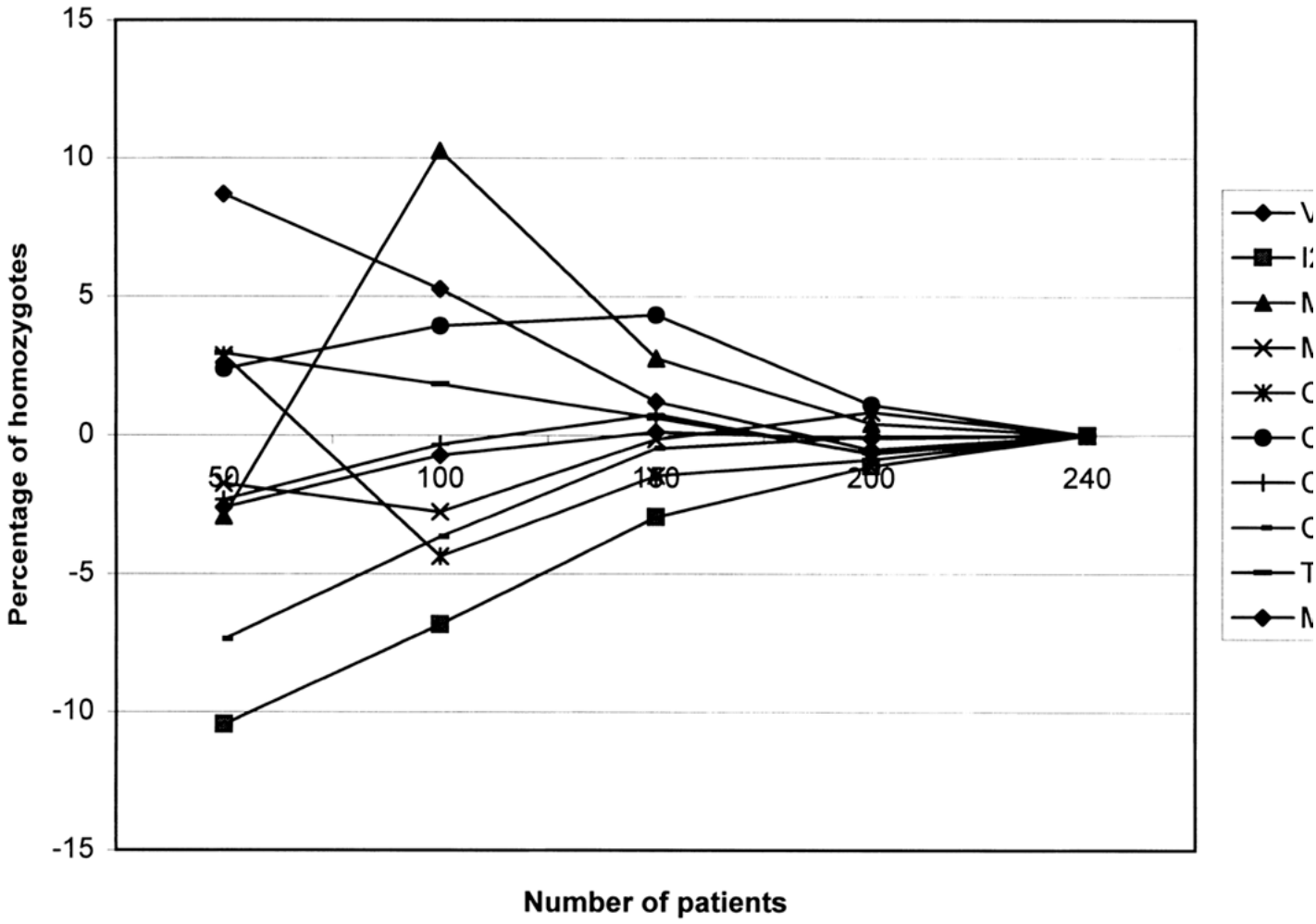

FIG. 1. Examples of fluctuations of genetic frequencies for various polymorphisms. The figure shows the percentage of patients homozygous (recessive model) for various biallelic genetic polymorphisms. Except for V249r M280rec (Table 1), the two alleles are numbered 1 and 2. To see the variations according to the number of patients taken into consideration (the first 50 enrolled, the first 100, ... up to the total 240 genotyped) clearly , percer by subtracting the percentages obtained with the 240 nonprogressor patients genotyped. The percentages for 240 patients were: V249rec = 49.6%, I249rec = 12.3%, MIP-1rec = 57.6%, MIP-2rec = 5.5%, CDK9-1rec = 38.(CCR5P-1rec = 23.7%, CCR5P-2rec = 21.6%, T280rec = 70.13%, and M280rec = 2.6%.

DISCUSSION AND CONCLUSIONS TOP

We have analyzed the polymorphisms of CX3CR1 and found no difference in allele frequencies between NPs and RPs in the GRIV cohort for the T280M polymorphism, and a trend for V249I (p = .09). This result is in contradiction with the data initially published on the case-study of the ALT cohort (15) and may be explained by the smaller size of the ALT cohort (63 patients). In fact, a study performed on our entire genetic database (over 100 gene polymorphisms) shows that large variations in a case-control analysis can be obtained whether 50, 100, or 240 individuals are analyzed. Because the NP subjects for ALT were recruited in France, like GRIV, it is unlikely that there is any bias of inclusion linked to ethnic or geographic factors to explain the discrepancy between these 2 cohorts. However, the study of Faure et al. (15) also presented a survival analysis for the SEROCO cohort that supports an association between accelerated progression to AIDS and the CX3CR1-280M/280M genotype. An explanation for this difference observed with GRIV could be that the genetic effect of the mutation acts late in disease, given that we have previously observed that GRIV was prone to show early genetic effects (17). In fact, the survival curves of the SEROCO patients homozygous for the 280M allele and of the 280M/280$^+$ and 280$^+$/280$^+$ bearing patients diverge after 30 months as shown in the publication by Faure et al. (15). An alternative explanation could be that the 16 SEROCO homozygotes for 280M are not sufficient to get robust statistics. This point should be clarified by other cohort studies.

In conclusion, our results have not confirmed the effect of the CX3CR1 T280M polymorphism on progression; however, we cannot exclude that the effect of this polymorphism occurs late in disease. The example of CX3CR1 emphasizes the need for having large enough patient numbers to detect robust genetic effects, and for joining research efforts as much as possible to prevent fluctuations, especially when the cohorts, such as ALT and GRIV, come from the same country.

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