CONCISE COMMUNICATION

Polymorphisms in the CCR5 Promoter Region Influence Disease Progression in Perinatally Human Immunodeficiency Virus Type 1–Infected Children

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The effect of CC–chemokine receptor 5 (CCR5) promoter polymorphisms on the natural history of human immunodeficiency virus (HIV) disease was studied in 73 HIV-1–infected children. The $CCR5_{59338–59537}$ promoter haplotype, CCR5-59029A/G polymorphism, and CCR5Δ32 and CCR2-64I alterations were investigated. After exclusion of carriers of CCR5Δ32 or CCR2-64I, Kaplan-Meier analysis disclosed that children with the $P1/P1_{59353C,59356C,59402A}$ genotype progressed faster to disease than did children with other haplotypes ($P = .016$). When CCR2-64I carriers were included, this effect had borderline significance ($P = .065$) and was lost when CCR5Δ32 carriers were also considered ($P = .387$). The P1/P1 effect was strongest early after infection, when progression to disease was mainly associated with CCR5 coreceptor–using viruses. These results indicate that the P1/P1 genotype is predictive of rapid progression in HIV-1–infected children lacking CCR5Δ32 or CCR5-64I alleles. The observation of a linkage disequilibrium between P1 and 59029A might explain the previously reported association between 59029A homozygosity and rapid disease progression.

Human immunodeficiency virus (HIV) type 1–infected children have accelerated disease progression, compared with HIV-1–infected adults, and, in the absence of highly active antiretroviral therapy (HAART), about one-third develop severe symptoms of disease within the first 2 years of life [1]. This suggests that factors that influence disease progression may have different roles in children than in adults.

Several genetic polymorphisms in the HIV-1 coreceptor CC–chemokine receptor 5 (CCR5) are important host genetic factors that are capable of influencing susceptibility to HIV-1 infection or affecting the rate of disease progression. A 32-nucleotide deletion (Δ32) in the CCR5 gene–coding region protects against infection by CCR5 coreceptor–using HIV-1 variants in CCR5Δ32 homozygotes and partially protects against disease progression in CCR5Δ32 heterozygotes [2]. The point mutation at nucleotide position 303 (m303), a rare genetic alteration also located in the CCR5 coding region, determines a functional inactivation of the CCR5 coreceptor [3].

The observation that the CCR5 expression level on blood T lymphocytes varies substantially among persons with 1 or 2 wild-type CCR5-coding alleles [2] suggests that polymorphisms in the CCR5-untranslated regulatory region might influence CCR5 expression and, thus, HIV-1 transmission and disease progression. Several genetic variations within the CCR5 regulatory region have been identified, some of which affect the rate of disease progression in adults, such as 59029A/G [4]; 59029G homozygotes lacking the CCR5Δ32 mutation and have a slower progression to AIDS than do the 59029A homozygotes [4].

To date, 10 polymorphic nucleotide positions in CCR5 promoter region 58934–59537 have been identified. Combinations of the 10 polymorphic sites specify 10 CCR5 promoter haplotypes: 4 are common (CCR5-P1–CCR5-P4) and 6 are rare (CCR5-P5–CCR5-P10) [5]. Homozygosity for the P1 haplotype is associated with accelerated HIV-1 disease progression [5]. Another polymorphism in the CCR5 regulatory region, CCR5-59653T, is genetically associated with a mutation in the coding region of the CCR2 gene (CCR2-64I) [6]. HIV-1–infected adults who possess the CCR2-64I allele progress less rapidly to disease [2, 6], although the mutation does not affect CCR2 protein function [2] or CCR5 expression or function [7]. In some studies, this protective effect was observed in black, but not white, subjects [8, 9]. In the present study, we investigated the role of CCR5 promoter polymorphisms in the natural history of disease progression in perinatally HIV-1–infected children.

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Patients and Methods

Patients. We studied 73 HIV-1–perinatally infected infants who attended the University of Padova Pediatric Department (Padova, Italy). All were born to HIV-1–seropositive mothers and were enrolled at birth. The median follow-up time was 79 months (range, 3–173 months). Because HAART had a substantial impact on the natural course of HIV-1 infection in 33 children who started HAART during the study period, follow-up was stopped before its initiation (median, 88 months; range, 13–173 months). The end of follow-up corresponded to death in 22 children (median, 64 months; range, 3–135 months) and to the last visit for 12 children (median, 102 months; range, 44–158 months). Only 6 children were lost to follow-up. Clinical status (categories N, A, B, C) and immunologic status (classes 1–3) were defined by Centers for Disease Control and Prevention (CDC) AIDS criteria [10].

CCR5 genotyping. The CCR5Δ32 deletion and the CCR5m303 point mutation were detected, as reported elsewhere [11]. We characterized the CCR2-V64I polymorphism by using the polymerase chain reaction (PCR) sequence-specific primers method [12]. To characterize the CCR5-59029A/G polymorphism, PCR was done by using primers designated by McDermott et al. [4] and subsequent *Bsp*1286 I digestion of the amplified product.

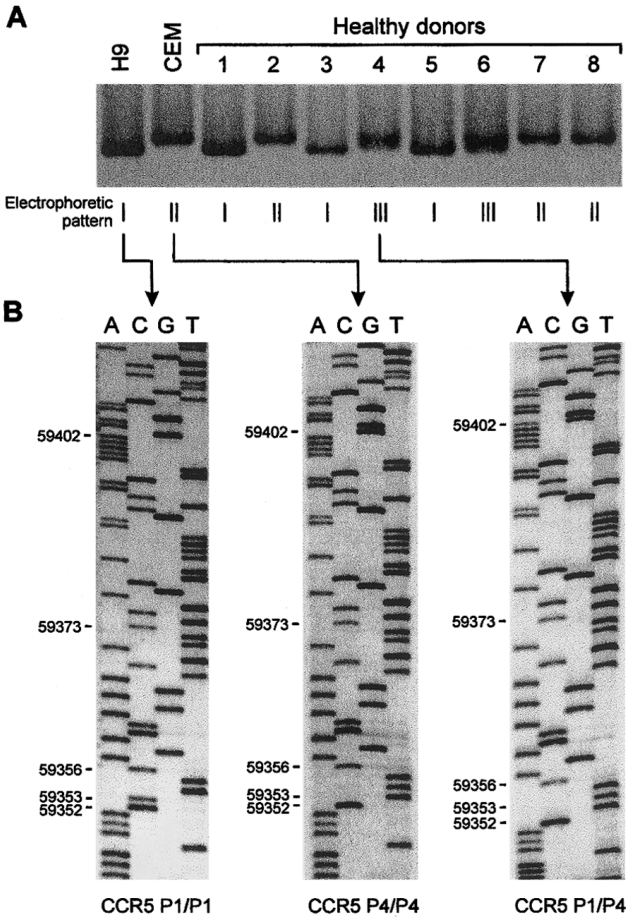
Heteroduplex analysis and sequence analysis were performed to analyze the region between the 59338 and 59537 positions of the CCR5 promoter (GenBank sequence U95626; figure 1*A,* 1*B*). The promoter region was amplified by using the F9 and R9 CCR5-specific primers [5]; after 30 amplification cycles, 10 μL of the mixture was mixed with 1 μCi of α-^{33}P-dATP and 0.5 U of AmpliTaq DNA polymerase and was subjected to 5 additional amplification cycles. The samples then were denatured and run on a 6% polyacrylamide gel. Double-strand homoduplex and heteroduplex DNA were thus retained on the gel and were visualized by exposure to x-ray film. PCR products were sequenced by using the T7 Sequenase PCR product sequencing kit (Amersham Life Sciences).

Viral phenotype analyses. Primary isolates were obtained by culturing peripheral blood mononuclear cells (PBMC) from each subject with phytohemagglutinin-stimulated PBMC from healthy donors, as described elsewhere [11]. Coreceptor use of primary isolates was determined by viral infection in U87.CD4 cells that stably expressed CCR5 or CXCR4 coreceptors, as reported elsewhere [11].

Statistical analysis. The influence of CCR5 promoter genotype on disease progression was examined by the Kaplan-Meier method with statistical software (SAS Institute). Between-group analyses were accomplished by log rank, likelihood ratio, and Wilcoxon rank sum tests. We also used Fisher's exact test (2-tailed) where appropriate.

Results

Of 73 HIV-1–infected children, 5 were CCR5Δ32 heterozygous; none was CCR5Δ32 homozygous. No children carried the CCR5m303 mutation. Eighteen children were CCR2-64I heterozygous, and 1 child was homozygous. $CCR5_{59338–59537}$ and $CCR5_{59029}$ promoter genotype distribution is summarized in fig-



C

CCR5$_{59338-59537}$ promoter genotype	No. of children	CCR5 59029 promoter genotype		
		59029 A/A	59029 G/G	59029 A/G
P1/P1	18	18	—	—
P1/P2	2	—	—	2
P2/P2	1	—	1	—
P1/P4 or P2/P4	38	—	5	33
P4/P4	14	—	14	—
Total	73	18	20	35

Figure 1. Analysis of CC–chemokine receptor 5 (CCR5) promoter region in H9 and CEM cell lines and in peripheral blood mononuclear cells (PBMC) of healthy donors, to assess relationship between electrophoretic patterns in heteroduplex analysis (HA) and sequence analysis. *A,* HA disclosed 3 electrophoretic patterns (I–III) of CCR5 promoter amplicon. By sequence analysis, pattern I corresponded to the CCR5 P1/P1, P2/P2, or P1/P2 genotypes. CCR5 promoter genotype of samples with pattern I was resolved by sequencing. Pattern II corresponded to the CCR5 P4/P4 genotype, and pattern III to the CCR5 P1/P4 or P2/P4 genotypes. *B,* Sequence analysis of 3 representative samples: H9 cells homozygous for P1 haplotype, CEM cells homozygous for P4 haplotype, and healthy donor PBMC with P1/P4 genotype. Crucial nucleotide positions for defining different CCR5 haplotypes are shown. *C,* CCR5 promoter genotype distribution in human immunodeficiency virus type 1–infected children.

ure 1*C*. We did not find the rare P5–P10 haplotypes or the P3 haplotype that was recently reported to be largely restricted to ethnic Africans [12]. Of interest, all 18 P1/P1 children had a 59029A/A genotype and the P1/P1 genotype. None of the 20 children who were homozygous for the 59029G allele carried the P1 haplotype (figure 1*C*). These observations suggested a linkage disequilibrium between the P1 haplotype and the 59029A allele. Conversely, the 59029G allele was associated with both the P2 and P4 haplotypes, and all children with a P4/P4 (14 children) or P2/P2 (1 child) genotype had a 59029G/G genotype (figure 1*C*).

Disease progression rates among children with specific CCR5 genotypes were compared by Kaplan-Meier analysis by considering 2 different end points: progression to CDC stage C and progression to the first CDC stage C or class 3 event. Because of the dominant protective effect of the CCR5Δ32 and CCR2-64I alleles [4, 5], although the latter's effect seems restricted to blacks [8, 9], the analysis was conducted either excluding or including children who carried these genetic alterations. Children lacking the CCR5Δ32 or CCR2-64I alleles and carrying a P1/P1 genotype showed a more rapid disease progression to CDC stage C ($P = .016$; figure 2*A*) and to CDC stage C or class 3 ($P = .006$; figure 2*B*) than did non-P1/P1 children. When children with CCR2-64I alleles were included, the difference between P1/P1 homozygotes and non-P1/P1 infants was less significant, although the negative role of P1/P1 genotype on disease progression was still weakly evident for both end points considered (figure 2*C,* 2*D*). When children with the CCR5Δ32 allele were also included, no significant differences were found between P1/P1 homozygotes and non-P1/P1 children for both end points (figure 2*E,* 2*F*).

Because the pattern of P1/P1 disease acceleration observed in figure 2 suggested that the strength of the promoter genotype effect is strongest early after infection, we investigated the frequency of progressors within the first 2 years of life (i.e., rapid progressors) in children with different CCR5 promoter genotypes. These analyses excluded children carrying the CCR5Δ32 allele, whereas CCR2-64I allele carriers were either excluded or included. When CCR2-64I carriers were excluded, the frequency of rapid progressors to CDC stage C was significantly higher in P1/P1 children (5 of 8) than in non-P1/P1 children (6 of 43; $P = .008$). This was confirmed when we also considered progression to either CDC stage C or class 3 (5 of 8 vs. 9 of 43; $P = .028$). Furthermore, even when CCR2-64I carriers were included, the frequency of rapid progressors to CDC stage C was significantly higher in P1/P1 children (6 of 13) than in non-P1/P1 children (6 of 55; $P = .008$). This finding was confirmed when we also considered progression to either CDC stage C or class 3 (6 of 13 vs. 9 of 55; $P = .030$). Further supporting the association between P1/P1 genotype and rapid disease progression was the finding that the frequency of P1/P1 children, even including CCR2-64I carriers, was significantly higher in rapid progressors to CDC stage C (6 of 12), compared with nonprogressors, by age 8 years, to CDC stage C (2 of 16; $P = .040$). Results were similar for rapid progressors to either CDC stage C or class 3 versus nonprogressors, by age 8 years, to neither CDC stage C nor to CDC class 3 (6 of 15 vs. 1 of 13; $P = .060$).

The finding that acceleration to AIDS mediated by the P1/P1 genotype is strongest early after infection is consistent with the knowledge that infection mainly occurs with CCR5 coreceptor–using HIV-1 [2]. Thus, we investigated the coreceptor usage of virus isolates obtained at the time of progression to CDC stage C in 12 rapid progressor and in 16 nonrapid progressors. We found that the frequency of CCR5 coreceptor–using viruses was higher in rapid progressors (10 of 12), compared with nonrapid progressors (10 of 16).

Discussion

We found that HIV-1–infected children without the CCR5Δ32 or CCR2-64I allele who carried the P1/P1 genotype progressed more quickly to an advanced clinical or immunologic disease stage than did non-P1/P1 children. Because HIV-1–transmitting mothers are genetically related to their children, we cannot exclude that the transmission of a viral variant adapted to growth in a P1 carrier might also contribute to the rapid progression observed in P1/P1 children. The accelerating effect of the P1/P1 genotype on disease progression was reduced when the analysis included CCR2-64I carriers, which suggests that a protective effect of this genetic alteration in our cohort of children cannot be excluded. Of note, although the CCR2-64I protective effect seemed to be restricted to blacks in some studies [8, 9], a delay in progression to AIDS associated with the CCR2-64I allele was recently observed in a cohort of HIV-1–infected children composed mainly of Spanish and Italian descendants [13].

The P1/P1 effect on disease progression was strongest early after infection, an observation that is consistent with the knowledge that infection occurs mainly with CCR5 coreceptor–using HIV-1 [2]. Accordingly, at the time of progression to stage C, most rapid progressors had a CCR5 coreceptor–using virus, whereas a large proportion of nonrapid progressors had a CCR5/CXCR4 coreceptor–using virus. Our findings agree with those of some previous studies in adults and might explain some of the reported results. McDermott et al. [4] found that the P1 haplotype was associated with accelerated disease progression in HIV-1–infected adults but did not investigate the 59029A/G polymorphism. Martin et al. [5] reported that persons homozygous for the 59029A allele progressed to AIDS more rapidly than did persons with the 59029G/G genotype. Moreover, Clegg et al. [14] reported a lower frequency of 59353C (definitive of the P1, P6, and P7 haplotypes) and 59029A alleles in long-term nonprogressors, compared with progressors [14]. Conversely, Easterbrook et al. [15] found that the 59353C allele was associated with delayed disease progression, albeit with borderline

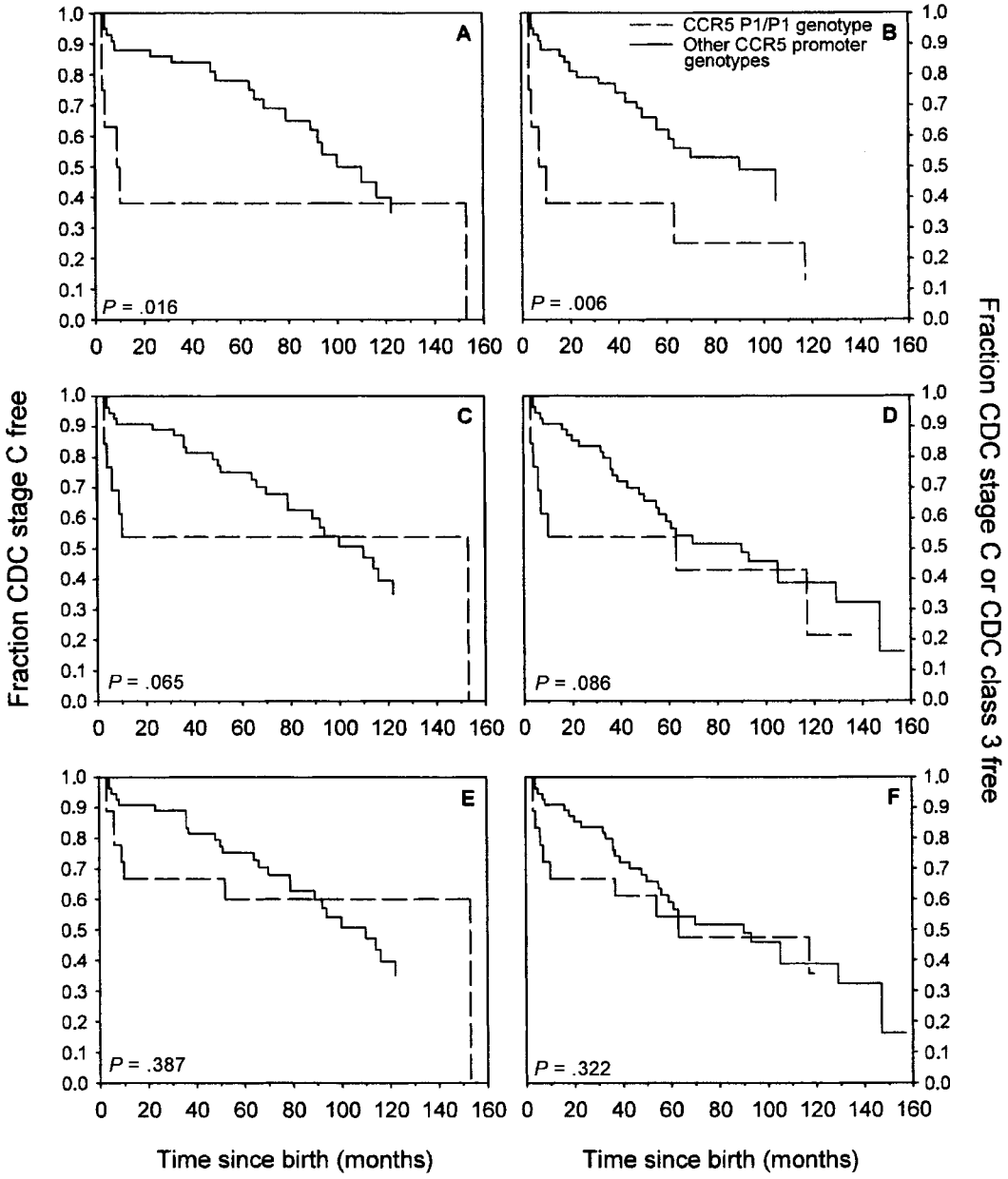


Figure 2. Disease progression in human immunodeficiency virus type 1–infected children with different CC–chemokine receptor 5 (CCR5) promoter genotypes. Time to Centers for Disease Control and Prevention (CDC) stage C and to first CDC stage C or CDC class 3 event were examined by the Kaplan-Meier method. *A* and *B,* Analyses excluded children with CCR5Δ32 and/or CCR2-64I alleles. *C* and *D,* Analyses included children with the CCR2-64I allele. *E* and *F,* Analyses included children with CCR2-64I and/or CCR5Δ32 alleles. *P* values were determined by use of the Wilcoxon rank sum test.

significance; however, no role for the 59029A/G polymorphism in HIV-1 disease progression was observed.

Our results suggest a disease-accelerating effect of the P1 haplotype and the 59029A allele and document a linkage disequilibrium between these 2 polymorphisms. In agreement with our findings, a linkage disequilibrium between P1, associated with an accelerated disease progression, and 59029A was recently reported [7, 9]; however, which, if either, of the 2 polymorphisms is key remains to be elucidated. Our findings are consistent with those of Gonzalez et al. [9] and indicate that the effect of the P1/P1 genotype on HIV-1 disease progression may differ, depending on either the combination with CCR5Δ32 and CCR2-64I alleles or time since infection. This finding is evidence of the complexity of the relationship between the CCR5 genotype and the disease-

modifying phenotype and underlines the need to investigate the role of CCR5 polymorphisms on the whole. Identification of the functionally active polymorphisms and the mechanisms by which they mediate their effects will be an important step in identifying potential treatment targets and for devising new strategies for slowing disease progression.

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