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Role of chemokine and cytokine polymorphisms in the progression of HIV-1 disease

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Abstract

Allelic variants of the genes for chemokine receptors and their natural ligands, the chemokines, and cytokines can affect HIV-1 disease progression. This study investigates the level of expression of the CCR5-Δ32, CCR2b-641, RANTES In1.1C, SDF-1 3'A, IL-10-5'-592A and IL-4-589T alleles in two unique HIV-1 infected patient cohorts that represent the two distinct stages of disease progression, namely rapid progressors (RPs) and long term non-progressors (LTNPs) ($n = 12/\text{group}$) were recruited. Quantitation of the gene expression of CCR5-Δ32, CCR2b-641, RANTES In1.1C, SDF-1 3'A, IL-10-5'-592A and IL-4-589T in peripheral blood mononuclear leukocytes (PBML) isolated from patients was performed by real time, quantitative (Q)-PCR using DNA was isolated from PBML. We observed that expression of these HIV-protective alleles was generally greater in the LTNP cohort than the RP cohort. LTNPs expressed more of the protective chemokine, SDF-1 α than RPs, and no statistically significant difference was observed in RANTES production between the LTNPs and RPs. The LTNPs expressed significantly less amounts of cytokines IL-10 and IL-4 as compared to the RPs. Our results demonstrate that gene polymorphisms for CCR5-Δ32, CCR2b-641, RANTES In1.1C, SDF-1 3'A, IL-10-5'-592A and IL-4-589T may be used as clinical markers to predict progression of HIV-1 infections.

Keywords

HIV-1 disease progression; Chemokines; Cytokines; Single nucleotide polymorphisms (SNPs); Long term non-progressors (LTNPs); Rapid progressors (RPs)

1. Introduction

HIV-infections remain the deadliest epidemic in human history, killing more than 25 million people worldwide, including more than 500,000 Americans (<http://www.unaids.org>). In general, clinical progression of HIV-1 disease is relatively slow, taking several years from

initial infection to the development of severe immune suppression. The rate of clinical disease progression varies between individuals and factors such as host susceptibility, genetics and immune function as well as viral genetic variability may affect the rate of progression to AIDS. A subset of individuals who are persistently infected with HIV-1, but show no signs of disease progression for over 12 years and remain asymptomatic are classified as long term non-progressors (LTNPs). LTNP appear to be related to host genetic factors and/or effective immunological factors and this group may have unique host genetic factors which may control rates of HIV disease progression. HIV-infected individuals that rapidly progress to AIDS within four years after primary HIV-infection and are termed rapid progressors (RPs). The goal of the current study is to evaluate the expression levels of certain key host gene polymorphisms in the LTNP and RP patient group as these groups represent two distinct and contrasting stages of HIV-1 disease and that such a comparison may highlight the role of host genetics in HIV-1 disease progression.

Genetic susceptibility to HIV-1 infections and the subsequent rate of disease progression to AIDS is influenced by allelic polymorphisms of several key chemokine and cytokine genes [1–6]. Genomic analyses have shown that allelic variants of the genes for chemokine receptors, that are also HIV-1 entry co-receptors, and their natural ligands, the chemokines, can affect the transmission and progression of HIV-1 infections [7–13]. Delayed HIV-1 disease progression has been attributed to mutations in genes for CCR2 (CCR2-64I) and CCR5 (CCR5-D32). The chemokine, RANTES (regulated on activation normal T cell expressed and secreted) In1.1C single nucleotide polymorphism (SNP) of the RANTES gene does not protect against HIV-1 infections, and is also associated with rapid progression of HIV-1 infections [14,15]. By contrast the defective chemokine receptors/HIV-1 entry co-receptors alleles, CCR2b-64I and the CCR5-Δ32, are associated with delayed progression of HIV-1 disease due to poor binding of HIV-1 to target cells.

The increased expression of β -chemokines is associated with resistance to infection with R5 strains of HIV-1 in vitro and slow disease progression in vivo. After initial infection of macrophages with R5 HIV-1 strains there is a subsequent switch in prevalence to T lymphocytotropic HIV-1 strains that use a different chemokine receptor, CXCR4. The chemokine, stromal cell-derived factor-1 (SDF-1), is the natural ligand for CXCR4 and it prevents infection of susceptible target cells in vitro by X4 strains of HIV-1 that use this receptor, subsequently preventing the emergence of predominant X4 strains. The X4 strain of HIV-1 is associated with accelerated disease progression. Selective blockade of the CXCR4 receptor may prevent the switch from the less pathogenic R5 strain of HIV-1 to the more pathogenic X4 H-1 strain which, in turn, may inhibit disease progression. SDF-1 not only is the natural ligand of CXCR4 but it also downregulates the expression of CXCR4 on the surface of T lymphocytes [16]. Cytokines such as IL-4 and IL-10 can modulate viral expression and thereby influence HIV-1 disease progression.

The current focuses on evaluating the expression of specific gene polymorphisms in the chemokine receptors (CCR5-Δ32, CCR2b-64I, chemokines (RANTES In1.1C, SDF-1 3'A); and cytokines (IL-10-5'-592A, IL-4-589T) in two very unique HIV-1 patient cohorts namely the long term non-progressors (LTNPs) and the rapid progressors (RPs) that represent two distinct and variable stages of HIV-1 infection. This study will provide insight on the role of host genetics in determining the rate of progression of HIV-1 infections. Our results may lead to the development of targeted immunotherapeutic strategies that could slow down disease progression by modulating the levels of these specific chemokines and cytokines.

2. Materials and methods

2.1. Patient population

The Immunodeficiency Services Clinic at the Erie County Medical Center is the designated AIDS center of western NY, providing clinical care to approximately 1200 active patients through more than 5000 clinic visits per year. We have an on-site medication adherence program administered by the University at Buffalo, School of Pharmacy and GYN/women's health, drug counseling, and nutritional services to ensure that all HIV-infected patients receive state of the art, comprehensive care. Patients were recruited to this study after obtaining informed consent according to NIH and our Institutional IRB guidelines. Based on their clinical histories, patients were separated in two study groups, namely RPs and LTNP. RPs are defined as patients that develop AIDS between 1 and 4 year post-infection whose CD4 counts rapidly decline to $<500/\text{mm}^3$. Additionally, it is important to note that these RP patients were all anti-retroviral (ARV) therapy naïve and were enrolled in the study on their first visit to the clinic. LTNP are HIV-1 infected patients with stable CD4⁺ T lymphocyte counts $>500/\text{mm}^3$ who remain symptom free for 12 year post-infection. Subjects in both groups are age and sex matched.

2.2. HIV-1 serological and immunological assays

Detailed histories of the subjects were taken and patients were enrolled in the study based on CDC criteria for diagnosis of HIV-1 infection, which included either detectable serum p24 antigen levels or ≥ 2 positive tests for HIV-1 antibody from at least two different specimens and with at least one confirmatory Western blot analysis. Baseline and follow-up CD4⁺ T cell counts were evaluated by flow cytometry as part of the routine clinical evaluation and the results were recorded as number of cells/ mm^3 . Baseline and follow-up plasma HIV-1 viral load was measured using the AMPLICOR™ HIV-1 Monitor UltraSensitive Test (Roche Diagnostics Corporation, Indianapolis, IN). Viral load was measured as the number of HIV-1 RNA copies/ml. The AMPLICOR HIV-1 Monitor UltraSensitive Test has a linear range of 50–75,000 copies of HIV-1 RNA per milliliter of plasma.

2.3. PBML isolation

PBML were isolated from 10 ml of heparinized whole blood from HIV-1 infected patients using the Ficoll-Paque gradient. Whole blood from patients was diluted 1:1 with Hank's balanced salt solution and was carefully overlaid on 15 ml of Ficoll-Paque® Plus (Amersham-Pharmacia, Piscataway, NJ) in a 50 ml culture tube. Samples were centrifuged for 20 min at 700g at 20 °C without applying a brake. The PBML interface was carefully removed by pipetting, washed twice with PBS/EDTA, and resuspended in 2 ml of complete RPMI medium. Cell numbers were determined with a hemocytometer.

2.4. Isolation of genomic DNA

Genomic DNA was isolated from PBML using the high salt precipitation method [17]. DNA purity was assessed with a Nano-Drop ND-1000 spectrophotometer (Nano-Drop™, Wilmington, DE) to measure the A260/A280 ratio. Isolated DNA was stored at 20 °C until used.

2.5. Real time quantitative PCR (Q-PCR)

Q-PCR was used to quantitate the expression of CCR5-Δ32 CCR2b-641, RANTES In1.1C, SDF-1 3'A IL-10-5'–592A and IL-4–589T gene polymorphic variants using well validated allele specific primers [3,18–20]. Relative abundance of each allelic variant in the patients samples was determined by Q-PCR (MX 3005P; Stratagene Inc., La Jolla, CA) and the Q-PCR conditions were standardized for each allelic variant. Relative expression was calculated using

the comparative C_T method and results are expressed as transcript accumulation index (TAI) [21]. This calculation assumes that all PCRs are working with 100% efficiency. All data were controlled for quantity of DNA input by performing measurements on an endogenous reference gene, β -actin.

2.6. ELISA

SDF-1 α , RANTES, IL-10 and IL-4 protein expression in the serum of RP and LTNP patients was quantitated using commercially available ELISA kits (R&D Systems, Minneapolis, MN) per the manufacturer's instructions.

2.7. Statistical analysis

To compare gene and protein expression levels between the study groups, a one-way ANOVA (analysis of variance) on the data was performed followed by a Bonferroni post hoc test for multiple comparisons to determine significance between the groups. The statistical software package used was GraphPad Prism software (GraphPad Prism Software, Inc., San Diego, CA).

3. Results

Host genetic variations can influence susceptibility to HIV-1 infection and disease progression. In this study, we investigated the allelic frequency and potential mechanisms of action for polymorphisms in the genes for chemokines and cytokines that have been associated with HIV-1 disease progression comparing two unique cohorts of HIV-infected patients, LTNPs and RPs. These studies seek to evaluate the usefulness of these host genetic factors as unique biomarkers to predict progression (or the lack thereof) of HIV-1 infections.

3.1. Characterization of the study population

Our LTNP cohort ($n = 12$) showed an average CD4⁺ lymphocyte counts of $13,524 \pm 591$ cells/mm³ while the CD4⁺ counts in the RP group ($n = 12$) was 313 ± 190 cells/mm³ ($p < 0.001$). The average viral load in our RP cohort was $130,422 \pm 7326$ and LTNP subjects had an average viral load of <50 copies at the lower end of the sensitivity of the assay ($p < 0.000001$). The subjects in both groups were HAART treatment naïve and were age and sex matched. The ethnic distribution in the LTNP cohort was 27.2% Caucasians, 58.3% African Americans and 7.6% Hispanics, while the ethnic distribution in the RP cohort was 36.3% Caucasians, 54.5% African Americans and 9.0% Hispanics. The sex ratio was 81% male to 19% female in the LTNP group, and 72% male to 28% female in the RP group. The mean ages were 43.3 ± 12.9 and 48.4 ± 8.2 year in LTNP and RP groups, respectively. None of the patients enrolled had any other risk factors such as intra venous drug use (IVDU), additionally the transmission of HIV-1 in these subjects was through heterosexual contact.

3.2. Allelic distribution of CCR5-Δ32, CCR2b-641, RANTES In1.1C, SDF-1 3'A, IL-10-5'–592A, and IL-4–589T in LTNP and RP subject cohorts

The expression of CCR5-Δ32, CCR2b-641, RANTES In1.1C, SDF-1 3'A, IL-10-5'–592A, and IL-4–589T alleles in the RP and LTNP patient cohorts was determined by Q-PCR using primers specific for these alleles (Figs. 1 and 2). Statistical analysis showed that the expression levels of CCR5-Δ32 ($p < 0.0001$) and CCR2b-641 ($p < 0.003$) variants were significantly higher ($p < 0.0001$ and $p < 0.003$, respectively) in the LTNP compared to RP patient cohorts (Fig. 1A and B). No differences in the allelic distribution of RANTES In1.1C was observed between the LTNP and RP groups however, a significantly ($p = 0.023$) higher allelic distribution of the SDF-1 3'A allele in the LTNP group as compared to the RP group was observed (Fig. 1C and D). The cytokine allelic variants IL-10-5'–592A and IL-4–589T showed significantly higher

($p < 0.003$ and 0.0001 , respectively) expression levels in LTNP as compared to the RP subject cohorts (Fig. 2A and B).

3.3. Protein expression levels of chemokines and cytokines in LTNP and RP cohorts

SDF-1 α , RANTES, IL-10 and IL-4 protein expression in the plasma of RP and LTNP patients was quantitated using commercially available ELISA kits. Data in Fig. 3 show the SDF-1 α , RANTES, IL-10 and IL-4 levels in the LTNP, RP and normal (HIV-1 negative age and sex matched) control samples. Statistical comparisons were made between levels of SDF-1 α , RANTES, IL-10 and IL-4 in the LTNP and RP cohorts. Our data show a significantly increased SDF-1 α levels in LTNP (1547.23 ± 102.5 ; $p < 0.0001$) as compared to the RP (1053.69 ± 93.1) patient cohort. On the other hand, a significant decrease was observed in the IL-10 (489.12 ± 25.5 (RP) vs 129.31 ± 7.86 (LTNP) $p < 0.0001$, respectively) and IL-4 (647.2 ± 66.9 (RP) vs 56.43 ± 3.35 (LTNP) $p < 0.0001$, respectively) levels in the LTNP cohort as compared to the RP cohort. The RANTES (1108 ± 145.9 (RP) vs 1202 ± 123.5 (LTNP) $p = 0.103$ (NS), respectively) protein expression levels in the LTNP and RP groups, they were not statistically significant. These data confirm that gene expression levels of the specific allelic variants SDF-1 3'A, RANTES In1.1C, IL-10-5'-592A, and IL-4-589T correlate with the protein expression data of these chemokines and cytokines, and the changes in the expression of these allelic variants can result in significant modulation of the levels of the respective chemokines and cytokines which in turn affect HIV-1 disease progression.

4. Discussion

Genetic factors play an important role in susceptibility to HIV-1 infections and disease progression however, no single gene or polymorphism is likely to be responsible for these effects. Although, LTNPs are typically <2% of HIV-1 seropositive patients they are characterized by a prolonged course infection that does not progress to active disease even in the absence of anti-retroviral therapy. Rapid progressors on the other hand rapidly progress to AIDS within four years after primary HIV-infection and some individuals have been known to progress to AIDS and death within a year after primary infection. Host genetic factors may be one of the factors responsible for the rate of disease progression in both these patients cohorts that represent opposite spectrums in HIV-1 disease. In the current study our goal was to investigate the constitutive expression of important polymorphic alleles such as CCR5- $\Delta 32$, CCR2b-64I, RANTES In1.1C, SDF-1 3'A, IL-10-5'-592A and IL-4-589T of the chemokine and cytokine family that are critical to progression of HIV-1 infection. Herein, we compare the differential gene expression and the expression of these important genetic polymorphisms in 2 unique age and sex matched LTNPs and RPs cohorts who are at the opposite end of the HIV-1 disease progression scale. Genetic markers thus may be useful in predicting prognosis of HIV-1 infections and may identify unique targets for therapy.

Polymorphisms in CCR5 and CCR2 have been reported to be protective against HIV-1 infections [7,22]. A 32 bp deletion in the coding region of the CCR5 gene termed CCR5 $\Delta 32$ is protective in both heterozygous and homozygous individuals against HIV-1 infections. In the CCR2-64I polymorphism there is a valine to isoleucine substitution at position 64 in the trans-membrane domain of CCR2 that is protective in heterozygous individuals against progression of HIV-1 infections to AIDS. We observed a significantly higher allelic expression of CCR5- $\Delta 32$ and CCR2b-64I in the LTNP cohort as compared to the RP cohort suggesting that the expression of these alleles contribute, at least in part, to the mechanism underlying the LTNP phenotype. Unlike the frequency of the CCR5- $\Delta 32$ allele that widely fluctuates between different populations, the allelic frequency of CCR2b-64I does not vary much between populations and is between 10% and 20%. CCR2b-64I has no influence on the incidence of HIV-1 infection, but homozygotes for this allele have a slower rate of HIV-1 disease

progression; additionally the effect of CCR2b-64I polymorphism on disease progression may differ according to the stage of disease progression and interaction with other genetic variants [18]. The CCR2 HIV-1 co-receptor is used by a few HIV-1 strains and the CCR2b-64I mutation does not alter the functional properties of this chemokine receptor in terms of its binding and co-receptor function [23].

Several SNPs in the RANTES gene have been reported to influence the course of HIV-1 infection by modulating the expression of RANTES. These SNPs include RANTES -28C/G and -403A/G in the promoter region and In1.1 T/C in the first intron region of the RANTES gene. The RANTES -28C/G and -403A/G variants up-regulate RANTES transcription, while the In1.1 T/C variant decreases RANTES gene expression [12,13]. The frequency of the RANTES In1.1C allele in the US is about 13.8% in Caucasians and is lower in African American populations [12]. In the current study, no differences in the allelic distribution of RANTES In1.1C were observed between the LTNP and RP groups. We did not observe a significant difference in the total RANTES protein expression in the LTNP cohort as compared to the RP cohort. The RANTES In1.1C allele may not contribute to the slower disease progression observed in our LTNP subjects.

The frequency of the SDF-1 3'A allele also varies among different patient populations. In the US, the SDF-1 3'A has an allele frequency of 21% in Caucasians and 6% in African American populations [8,24]. The HIV-1 protective effect of SDF-1 3'A, was reported to be twice as effective as the effects conferred by CCR5 and CCR2 allelic variants, however it is reported to be decreased with longer duration of infection [8].

Conflicting reports exist regarding the role of SDF-1 3'A homozygosity in HIV-1 disease progression. Some studies have shown that subjects both heterozygous and homozygous for the SDF-1 3A allele have higher rates of decline of CD4⁺ T cells compared to patients who did not express the allele [25]. Our results demonstrate a significantly higher allelic distribution of the SDF-1 3'A allele in the LTNP cohort as compared to the RP cohort. Additionally significantly higher SDF-1a protein levels were also observed in the sera of LTNP patients as compared to the RP cohort.

Our results demonstrate a significant decrease in the allelic expression of IL-10-5'-592A and IL-4-589T and consequently a decreased production of cytokines IL-10 and IL-4. The presence of the IL-10-5'-592A (IL-10-5'A) allele is associated with rapid HIV-1 disease progression likely through facilitation of viral replication in infected patients. Interleukin (IL)-10 may also promote viral persistence by inactivation of effector immune mechanisms. Studies have also shown that IL-10 directly inhibits HIV-1 replication in human macrophages [26] and that a decrease in IL-10 or a blockade of the IL-10 pathway may enhance T cell immune responses, resulting in the rapid elimination of virus and the development of antiviral memory T cell responses, given the lower IL-10 levels in our LTNP cohort it is likely that this is the likely mechanism that contributes to delayed progression of HIV-1 disease in this cohort.

IL-4 can modulate the expression of the HIV-1 co-receptors, CCR5 and CXCR4, although it is not clear whether modulation of the HIV-1 co-receptors is sufficient to influence viral entry [27]. IL-4-589T allele protects against HIV-1 disease progression in part due to a reduction in viral load as a consequence of decreased production of IL-4.

The current study is novel in that two highly, unique HIV-1 cohorts, LTNPs and RPs, were investigated for the expression of polymorphic alleles that could serve as clinical biomarkers for the progression or lack thereof of HIV-1 infections. We provide evidence that the polymorphic alleles, CCR5-Δ32, CCR2b-64I, RANTES In1.1C, SDF-1 3'A, IL-10-5'-592A and IL-4-589T can be used to predict clinical outcomes of HIV-1 infections. While the current study is novel in that these two unique HIV-1 cohorts, LTNPs and RPs who were HAART

therapy naïve were investigated, additional multi-center, studies with an increased number of patients from similar cohorts are warranted to determine the detailed mechanisms underlying the functional effects of these genetic polymorphisms. Larger scale studies also will allow us to stratify our results on the basis of ethnicity. Nevertheless our results support the monitoring of at risk subjects for HIV-1 infections for these biomarkers to facilitate rational, prospective decisions on treatment regimens. Moreover, our findings may identify targets for new strategies for the prevention and treatment of HIV-1 infections.

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Abbreviations

SNPs	single nucleotide polymorphisms
LTNPs	long term non-progressors
RP	rapid progressors
Q-PCR	real time, quantitative polymerase chain reaction
RFLP	restriction fragment length polymorphism
ARV	anti-retroviral
HAART	highly active anti-retroviral therapy
TAI	transcript accumulation index
IVDU	intra venous drug use

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Fig. 1. CCR5- Δ 32, CCR2b-641, SDF-1 3'A and RANTES In1.1C in LTNP and RP patient groups. Genomic DNA was extracted from the patient PBML and Q-PCR was used to determine the expression of the CCR5- Δ 32, CCR2b-641, SDF-1 and RANTES In1.1C alleles in the LTNP, and RP patient cohorts. Relative gene expression was calculated using the comparative C_T method. Distribution of the (A) CCR5- Δ 32, (B) CCR2b-641, (C) RANTES In1.1C and (D) SDF-1 3'A allelic variants in the RP and LTNP groups. Data are the means $\pm$ SD of three separate experiments done in duplicate. Statistical significance was determined using ANOVA based on comparisons between the LTNP and RP groups ($n = 12/\text{group}$).

Fig. 2. IL-10-5'-592A and IL-4-589T in LTNP and RP patient groups. Genomic DNA was extracted from the patient PBML and Q-PCR was used to determine the expression of the IL-10-5'-592A and IL-4-589T alleles in the LTNP, and RP patient cohorts. Relative gene expression was calculated using the comparative C_T method. Distribution of the (A) IL-10-5'-592A and (B) IL-4-589T allelic variants in the RP and LTNP groups. Data are the means $\pm$ SD of three separate experiments done in duplicate. Statistical significance was determined using ANOVA based on comparisons between the LTNP and RP groups ($n = 12/\text{group}$).

Fig. 3.

Protein expression of SDF-1α, RANTES, IL-10 and IL-4 in RP and LTNP patients. The total protein levels of SDF-1α, RANTES, IL-10 and IL-4 were quantitated using commercially available ELISA kits. Data are the means $\pm$ SD of three separate experiments done in duplicate. Statistical significance was determined using ANOVA based on comparisons between the LTNP and RP groups ($n = 12/\text{group}$).