Functional analysis of novel SLC11A1 (NRAMP1) promoter variants in susceptibility to HIV-1

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J. Med. Genet. 2004;41:49-
doi:10.1136/jmg.2003.013318

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The divalent cation transporter is the natural resistance associated macrophage protein 1 (formerly NRAMP1 and now named SLC11A1) for solute carrier 11A1 (OMIM accession number 600266). The gene that codes for this transporter has been studied intensively for its role in conferring susceptibility to infectious diseases such as tuberculosis, leprosy, meningococcal meningitis, visceral leishmaniasis, and HIV infection, as well as to autoimmune diseases such as rheumatoid arthritis, diabetes, sarcoidosis, inflammatory bowel disease, and, more recently, Kawasaki disease. Most studies have investigated a functional GT repeat sequence in the promoter region of this gene and have identified two commonly occurring repeat alleles and four rare repeat alleles. The common alleles are T(GT)5 and T(GT)5AC(GT)10 (allele 2) and T(GT)5AC(GT)5AC(GT)9 (allele 3; GenBank accession number AF229163, 5768 to 5808). Allele 2, which decreases gene expression, has been associated with susceptibility to infectious diseases; the common allele 3 enhances gene expression to protect against infectious diseases while enhancing susceptibility to autoimmune diseases. Although HIV is classified as an infectious disease, it affects the autoimmune system, which may explain why allele 3 is associated with susceptibility to HIV-1. This study aimed to screen the promoter region of SLC11A1 for novel sequence variations in people from sub-Saharan Africa infected with HIV-1 compared with uninfected people and to determine the effect of novel variants on normal promoter function.

MATERIALS AND METHODS

Participants
We studied 84 HIV-1 seropositive people (60 African and 24 of mixed African–European descent) and 133 HIV-1 seronegative people (64 African, 62 of mixed African–European descent, and seven Asian) who lived in the Western Cape of South Africa and who attended one of the HIV-1 clinics in Tygerberg or Langa or the blood transfusion services of the Western Cape. In this study, we use “African” to define people of predominantly Xhosa descent, while the mixed African–European population (known as “Coloured” in South Africa) consists of a well defined population with origins from an initial population mixture about 300 years ago. This study population is defined in a recent publication. The ethics review committee of the University of Stellenbosch gave ethical approval, and informed consent was obtained from all study participants. We took blood from the patients and volunteers, and we extracted DNA with the Qiagen Extraction Kit (Qiagen, Valencia, CA, USA), as per the manufacturer’s instructions.

Mutation detection
Two sets of denaturing gradient gel electrophoresis primers were designed to cover the upstream promoter region from the GT repeat sequence (from nucleotide –715 to nucleotide –488 from translation start site) and downstream promoter region (from nucleotide –415 to nucleotide –132). The upstream primer pairs were forward 5’-AACAATCTCCTGA GAAGGGACA-3’ and reverse 5’-TCTTTGATCTGGAGTT CCAA-3’, and the downstream primers were forward 5’-GGGTTGCTATGGGTTATT and reverse 5’-TGCCCTGCTCTTACATCAA-3’. Both forward primers were designed with a 40 base pair (bp) GC-clamp (CGCCGCGCGCGCGCG CGCCGCGCGCGCGCGCGCGCGG) attached to the 5’-end to prevent total strand dissociation during electrophoresis through a denaturing gradient. A 7 bp (GCGCGCG) GC stretch and a 10 bp (GCCGCCGCCG) GC stretch were added to the 5’-end of the upstream and downstream reverse primers, respectively; the latter created a single melting domain for optimal mutation detection, as previously described. The repeat allele configuration was determined by direct sequencing of the amplified product created with forward primer 5’-AAGACTGGATAGGCCCAAC-3’ and reverse primer 5’-GCCCTCAGTGGTATCTGTA-3’.

We performed DNA amplification in a 50 µl reaction mixture that contained 100 ng genomic DNA, 0.1 mmol/l of each deoxyribonucleoside triphosphate, 20 pmol of each...
primer, 2.5 mmol/l 10× magnesium ion reaction buffer, and 0.5 units DNA Taq polymerase (Boehringer Mannheim, Mannheim, Germany). We performed amplification under the following conditions: initial denaturation at 96°C for 3 minutes, 35 cycles of denaturation at 96°C for 45 seconds, annealing at 60°C for upstream amplicon and repeat region or 65°C for downstream amplicon for 1 minute, and elongation at 72°C for 1 minute 15 seconds. After cycling, we performed an additional elongation step for 10 minutes, at 72°C, followed by denaturation at 96°C for 10 minutes, and heteroduplexing for 45 minutes at the optimal annealing temperature (the latter two steps were applicable only for the denaturing gradient gel electrophoresis primers). We electrophoresed upstream and downstream amplicons through a 9% polyacrylamide gel that contained a 40–80% urea and formamide denaturing gradient (100% urea and formamide contained 7 M urea and 40% deionised formamide) at 59°C and 110 V overnight (phorU2 system; Ingeny, Leiden, Netherlands). We subjected the repeat region amplicon and samples that showed novel denaturing gradient gel electrophoresis banding patterns to automated sequencing after product purification with a high purity polymerase chain reaction (PCR) product purification kit (Roche Diagnostics, Mannheim, Germany).

We determined allele frequencies by allele counting. We used Fisher’s exact test, which included calculation of odds ratios by InStat software, to assess significance of association between HIV-1 status (seropositive vs seronegative) and genotype for the previously reported repeat alleles, as well as the g.332C>T (Genbank accession number AY363243) promoter single nucleotide polymorphism.10

Gene expression studies
We analysed variant sequences with MatInspector (version 2.2; German Research Center for Biotechnology, Braunschweig, Germany) to establish possible loss or gain of transcription factor binding sites to determine the effect of novel promoter variants on promoter function (fig 1). To determine whether these novel mutations had any true effect on promoter activity, we cloned the various mutant promoters, together with the wild type promoter, upstream of the luciferase gene, and we transiently transfected the resulting constructs into 293 cells (ATCC: CRL-1573). We measured transactivation activity relative to Renilla luciferase in three independent experiments and determined means and standard deviations. All clones were sequenced to confirm the configuration of the repeat allele and promoter single nucleotide polymorphism.

RESULTS
Analysis of the 217 participants identified three of the previously reported repeat alleles (alleles 1, 2, and 3) and four single nucleotide variants (the previously reported g.332C>T polymorphism and three novel rare variants). All polymorphic alleles were in Hardy-Weinberg equilibrium in the HIV seropositive and seronegative participants for both populations. The repeat allele 3 was most common in the African and African–European admixed populations, with allele frequencies of 0.84 (101/120) and 0.73 (35/48) in participants infected with HIV and 0.81 (103/128) and 0.80 (99/124) in uninfected participants, respectively. We saw no significant associations between the presence of allele 3 in infected compared with uninfected African participants (p = 0.5073; odds ratio 1.290) and the mixed African–European population (p = 0.4124; odds ratio 0.6799) or in the homozygous states (Africans: p = 0.3406; African–European: p = 0.4715) or heterozygous states (African: p = 0.2426; African–European: p = 0.8079). Allele 2, previously associated with protection from HIV-1 infection in the homozygous state, was not associated with altered risk of HIV infection in our study. Three of the seven “slow progressors” (three African and four African–European participants who were asymptomatic 10 years after becoming infected with HIV-1 and had not taken antiretroviral treatment) were heterozygous for allele 2, while all five “fast progressors” (three African and two African–European participants who progressed to AIDS within five years of infection with HIV-1) were homozygous for allele 3. Of the three slow progressors who were heterozygous for allele 2 (all African–European admixed), one was heterozygous for the CCR5-A32 bp deletion, which has been shown to be associated with slower progression of disease. Allele frequencies for the g.332C>T single nucleotide polymorphism in the African and African-European admixed populations infected with HIV-1 were 0.075 (9/120) and 0.02 (1/48), respectively; the frequencies for the uninfected participants were 0.05 (6/128) and 0.072 (9/124), respectively. The promoter single nucleotide polymorphism was associated with repeat allele 3 in both populations. We saw no significant associations between HIV-1 infected and non-infected African participants (p = 0.4136; odds ratio 1.706) and admixed African–European participants (p = 0.2560, odds ratio 0.2560).

The three novel sequence variants included a G to A base substitution at position 561 (g.561G>A), a T to C substitution at position 75 (g.75T>C), and a G to C transition at position 43 (g.43G>C) (GenBank accession number AY363243) at 156, −642 and −674 nucleotides from the initiator codon, respectively. The first two mutations were identified in HIV-1 seronegative participants of African and Asian descent, respectively; the g.43G>C mutation occurred in two unrelated HIV-1 seropositive patients of African descent. Promoter analysis showed that the G to C mutation at position 43 created a TCF11 transcription factor binding site. This mutation also interfered with a Nkx2–5 binding site, although it did not disrupt the site entirely. The single nucleotide polymorphism at position 332 introduced a FoxD3 binding site. The g.75T>C mutation additionally affected a FoxD3 binding site, and the transition at 561 altered, but did not destroy, a FoxD3 binding site. The 75 mutation did not create or alter any transcription factor binding sites.

We cloned these variants in a luciferase reporter system to determine their effect on promoter activity. Sequencing of the clones showed that all had the allele 3 repeat configuration and that all contained the C allele configuration for the g.332C>T polymorphism. Promoter analysis showed that the g.43G>C and g.75T>C mutations enhanced the activity of the SLC11A1 promoter 1.4 (SD 0.14)-fold and 1.6 (0.25)-fold, respectively, compared with the wild type sequence. The increase in activity for the g.75T>C mutation was significant (p<0.05), whereas the increase in activity for the g.43G>C mutation was not. The g.332C>T polymorphism also enhanced significantly the activity of the SLC11A1 promoter 1.6 (0.19)-fold (p<0.05), whereas the g.561G>A mutation

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from HIV-stricken sub-Saharan Africa. Predicting susceptibility to HIV-1, particularly in populations affecting protein expression, may play a pivotal role in unknown at present, the identification of novel population mechanism of \textit{SLC11A1} African population needs further investigation. As the exact enhanced susceptibility to pathogen infection within the possibility that this functional mutation, which creates an \textit{SLC11A1} Africans, was shown to increase the promoter activity of seropositive Africans but was absent in the 63 uninfected with susceptibility to HIV-1; it occurred in 1/8 HIV-1 progression, numbers were small, and this observation was not significant (p = 0.2045). In contrast with the literature, no associations were found with respect to the repeat polymorphism and risk of HIV infection within our population. In addition, the previously reported g.332C>T single nucleotide polymorphism was not associated with susceptibility to HIV. We used a comprehensive approach to screen the promoter region of \textit{SLC11A1} and identified three novel sequence variants in combination with allele 3 of the polymorphic repeat. Although the g.75T>C mutation showed an increase in promoter activity, it was not associated directly with the introduction of a transcription factor binding site or with susceptibility to HIV-1, it occurred in 1/8 HIV-1 seronegative Asian participants. The lack of HIV-1 seropositive Asians in this study, however, warrants further investigation of this mutation in the Asian population. The g.43G>C novel mutation, which presented in 2/64 HIV-1 seropositive Africans but was absent in the 63 uninfected Africans, was shown to increase the promoter activity of \textit{SLC11A1}, although this increase was not significant. The possibility that this functional mutation, which creates an additional transcription factor binding site, may lead to enhanced susceptibility to pathogen infection within the African population needs further investigation. As the exact mechanism of \textit{SLC11A1} function in HIV-1 infection is unknown at present, the identification of novel population specific variants in the promoter region of this gene, which affects protein expression, may play a pivotal role in predicting susceptibility to HIV-1, particularly in populations from HIV-stricken sub-Saharan Africa.

**DISCUSSION**

The \textit{z}-DNA forming polymorphic repeat in the \textit{SLC11A1} promoter acts as a functional polymorphism and influences gene expression, with allele 3 having the strongest promoter activity. Allele 3 has been associated with hyperactivation of macrophages and may be functionally linked to susceptibility to HIV-1.\textsuperscript{11} In this study, although we did find a possible association with the presence of allele 2 and delayed disease progression, numbers were small, and this observation was not significant (p = 0.2045). In contrast with the literature, no associations were found with respect to the repeat polymorphism and risk of HIV infection within our population. In addition, the previously reported g.332C>T single nucleotide polymorphism was not associated with susceptibility to HIV. We used a comprehensive approach to screen the promoter region of \textit{SLC11A1} and identified three novel sequence variants in combination with allele 3 of the polymorphic repeat. Although the g.75T>C mutation showed an increase in promoter activity, it was not associated directly with the introduction of a transcription factor binding site or with susceptibility to HIV-1; it occurred in 1/8 HIV-1 seronegative Asian participants. The lack of HIV-1 seropositive Asians in this study, however, warrants further investigation of this mutation in the Asian population. The g.43G>C novel mutation, which presented in 2/64 HIV-1 seropositive Africans but was absent in the 63 uninfected Africans, was shown to increase the promoter activity of \textit{SLC11A1}, although this increase was not significant. The possibility that this functional mutation, which creates an additional transcription factor binding site, may lead to enhanced susceptibility to pathogen infection within the African population needs further investigation. As the exact mechanism of \textit{SLC11A1} function in HIV-1 infection is unknown at present, the identification of novel population specific variants in the promoter region of this gene, which affects protein expression, may play a pivotal role in predicting susceptibility to HIV-1, particularly in populations from HIV-stricken sub-Saharan Africa.

**ACKNOWLEDGEMENTS**

We thank Ms A Laten (Department of Medical Virology, University of Stellenbosch) for help with DNA extractions and sequencing, Ms E Carr (Cancer Research Programme, Garvan Institute of Medical Research) for help with sequencing, Dr L Bryenbach (Department of Medical Virology, University of Stellenbosch) for sample collection and maintenance of the HIV database, and all clinicians and nursing staff at the HIV clinics and blood transfusion services of the Western Cape, as well as all study participants.

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Conflicts of interest: none declared.

Funding: The study was funded by the Poliomyelitis Research Foundation, South Africa.

Received: 10 August 2003

Accepted: 2 November 2003

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