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A Genetic Polymorphism of *FREM1* Is Associated with Resistance against HIV Infection in the Pumwani Sex Worker Cohort

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A subgroup of women enrolled in the Pumwani sex worker cohort remain seronegative and PCR negative for human immunodeficiency virus type 1 despite repeated exposure through high-risk sex work. Studies have shown that polymorphisms of genes involved in antigen presentation and viral restriction factors are associated with resistance to HIV infection. To discover other possible genetic factors underlying this HIV-resistant phenotype, we conducted an exploratory nonbiased, low-resolution, genome-wide single-nucleotide polymorphism (SNP) analysis comparing 60 HIV-resistant women to 48 HIV-infected controls. The SNP minor allele rs1552896, in an intron of *FREM1*, was significantly associated with the resistant phenotype ($P = 1.68 \times 10^{-5}$; adjusted $P = 2.37 \times 10^{-4}$; odds ratio [OR], 9.51; 95% confidence interval [CI], 2.82 to 32.05). We expanded the sample size by genotyping rs1552896 in the Pumwani cohort and comparing 114 HIV-resistant women to 609 HIV-infected controls and confirmed the association ($P = 1.7 \times 10^{-4}$; OR, 2.67; 95% CI, 1.47 to 4.84). To validate the association in a second cohort, we genotyped 783 women enrolled in a mother-child health study and observed the minor allele of rs1552896 enriched in HIV-uninfected women ($n = 488$) compared to HIV-infected enrollees ($n = 295$) ($P = 0.036$; OR, 1.69; 95% CI, 0.98 to 2.93). Quantitative reverse transcription-PCR showed that *FREM1* mRNA was highly expressed in tissues relevant for HIV-1 infection, and immunohistochemical analysis revealed that FREM1 protein is expressed in the ectocervical mucosa of HIV-resistant women. The significant association of rs1552896 with an HIV-resistant phenotype, together with the expression profile of FREM1 in tissues relevant to HIV infection, suggests that *FREM1* is a potentially novel candidate gene for resistance to HIV infection.

An estimated 34 million people were living with HIV-1, with 2.7 million new infections in 2010 (www.unaids.org). There is currently no effective vaccine or cure for HIV. HIV-exposed seronegative (HESN) individuals provide an opportunity to study natural protection against HIV infection and to develop novel interventions against the virus. The Pumwani sex worker cohort was established in Nairobi, Kenya, in 1985 (27, 48) and has become well known for a small group of women who remain seronegative and PCR negative to HIV despite repeated exposure through active high-risk sex work. These women can be defined epidemiologically as resistant to HIV infection (15).

Over the last decade, our studies have shown that resistance to HIV infection observed in these women does not correlate with altered sex practices or behavioral differences. Instead, the protective phenotype is associated with adaptive cellular and mucosal responses to HIV, reduced systemic immune activation, and an elevated frequency of regulatory T cells compared to HIV-uninfected controls (1, 2, 12, 23–25, 50). These HESN women have elevated expression of HIV inhibitory factors at the genital tract, including serpins, other antiproteases, SLPI, and RANTES, as well as several undefined factors (11, 21, 22, 50). In addition, endogenous antiretroviral elements, such as APOBECs and TRIM-5α, may also be important in resistance to HIV infection. Together, these data demonstrate that natural resistance to HIV is multifactorial and the result of a combination of host genetics and innate and adaptive immune responses. Many immunological (1, 2, 25, 30, 46), proteomic (11, 22), and genetic correlates (5, 16, 17, 28, 32, 41) to the resistant phenotype have been identified but have not been sufficient to fully explain this phenomena. Genome-wide SNP (single-nucleotide polymorphism) analysis allows for an unbiased identification of genetic factors that influence complex traits, and high-throughput technologies have allowed for tremendous progress in this field (3, 8, 18, 31). To comprehensively analyze genetic factors underlying the resistant phenotype in the Pumwani sex worker cohort, we conducted an exploratory study with low-resolution genome-wide SNP analysis. An association with SNP rs1552896, located in an intron of *FREM1*, was observed and confirmed by genotyping the SNP in the entire cohort. We also genotyped a second cohort and identified a similar association. Further study showed that *FREM1* is highly expressed in tissues relevant for HIV-1 infection, implicating *FREM1* as a novel candidate gene for determining HIV resistance, warranting further investigation.

MATERIALS AND METHODS

Ethics statement. The Ethics Committee of the University of Manitoba, the Ethics and Research Committee of Kenyatta National Hospital, and the Regional Ethical Review Board of Karolinska Institutet have approved this study, and informed consent was obtained from all women enrolled in the study.

Study population. (i) Pumwani sex worker cohort. The Pumwani sex worker cohort was established in Nairobi, Kenya, in 1985 as an observational cohort study of the immunobiology and epidemiology of sexually

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transmitted infections (STI) (34–40, 44, 45). It is an open prospective cohort located in the heart of the Pumwani slum. The patients enrolled in the cohort have been monitored biannually since cohort establishment. In addition to research, the cohort provides services related to STI and HIV prevention and care, including consultation, provision of free condoms, and treatment of other infections. The overall HIV prevalence of the Pumwani cohort is around 70%. With effective intervention programs, the annual incidence of HIV infection among initially seronegative women is currently 4 per 100 person years (PY), a dramatic decrease from the initial annual incidence of 45%. Due to the introduction of the PEPFAR program (U.S. President's Emergency Plan for AIDS Relief), after 2003 antiretroviral drug treatment was made available for HIV-infected women whose CD4 counts were below 200 cells/mm^3. There was no program for antiretroviral drug treatment from 1985 to 2003. From 1985 to 2010, more than 3,000 sex workers have been enrolled in the cohort. Through more than 20 years of biannual biological and clinical follow ups, a subgroup of women who are resistant to HIV infection has been identified. These women remain seronegative and PCR negative for HIV for prolonged periods despite heavy exposure to the virus through active sex work (15). In contrast, many individuals from the same regions seroconverted shortly after enrolment. Women are classified as resistant if they have been monitored for more than 7 years and remained HIV seronegative and PCR negative at the time of this study. All 114 HIV-resistant women in this study were enrolled in the cohort before 1999, with a median follow-up time of 12.52 years. The control group for the genetic association studies comprised women who were HIV infected and were from the Pumwani sex worker cohort.

(ii) MCH cohort. A mother-child health (MCH) clinic operates in the same area of Pumwani as the sex worker cohort (9, 15). The University of Nairobi HIV Perinatal Transmission and Pediatric AIDS study began at this clinic in January 1986, and the enrollment protocol is described elsewhere (9, 15). The non-sex worker enrollees of this cohort are of similar socioeconomic status and ethnicity to the members of the Pumwani sex worker cohort but with lower exposure to HIV due to a smaller number of sexual partners. Demographic, social, and clinical data are collected from these women at every visit.

A low-resolution pilot genome-wide SNP analysis. Selected study participants were genotyped with Affymetrix 50K GeneChip Xba240 arrays. The Affymetrix GeneChip analysis was carried out by the Microarray Facility at the Hospital for Sick Children in Toronto, Canada. The average call rate per array was 99.2%. RLMM (Robust Linear Model with Mahalanobis Distance Classifier) algorithm was used for genotype calls (42). HelixTree 6.4.3 (Golden Helix Inc.) was used for the interactive tree-based SNP analysis using formal inference recursive modeling technology. After removing invariant and low-frequency SNPs, 37,871 SNPs with a call rate of >95% were used for the interactive tree analysis using HelixTree 6.4.3 (Golden Helix).

Genotype of rs1552896. To confirm the association of the SNP identified by the pilot low-resolution SNP analysis, 723 women (114 resistant women, 609 HIV$^+$ women) from the Pumwani sex worker cohort were genotyped for the SNP rs1552896 by PCR and sequencing. Forward (5′-GGGAAACTCATGCAACCTAACT-3′) and reverse (5′-TGTCCATGGC TTCCTTCAGAG-3′) primers were designed based on GenBank submissions (NG_017005). Amplification was performed with a Peltier Thermal Cycler-200 (MJ Research) with the following steps: initial denaturation for 5 min at 96°C; 1 min at 96°C, 1 min at 53.4°C, and 1 min at 72°C, repeated 44 times; and a final extension of 10 min at 72°C. Reaction mixtures were purified using Montage PCR96 plates (Millipore) and sequenced in both directions (primers were 5′-AATGTTTTCTTGAGTGTCG-3′ [forward] and 5′-TCATTTATTATCGTCATTTTGGTG-3′ [reverse]) using the ABI Prism BigDye Terminator version 3.0 cycle sequencing system (Applied Biosystems). Data were analyzed using SPSS 13.0.

To validate the association identified in the Pumwani sex worker cohort, we also genotyped rs1552896 of 783 women (295 HIV$^+$, 488 HIV$^-$) enrolled in a mother-child health clinic by PCR and sequencing.

***FREM1* RNA tissue-specific expression analysis.** Quantitative PCR (qPCR) analysis was conducted to investigate *FREM1* expression in various tissues using the FirstChoice Human Total RNA Survey Panel (Ambion). The QuantiTect reverse transcription kit (Qiagen) was used to generate cDNA, and the *FREM1* QuantiTect primer assay (exon36/37; Qiagen) was used in conjunction with the QuantiTect SYBR green PCR kit (Qiagen) to quantify the known full-length *FREM1* expression levels by following the manufacturer's instructions. All reactions were performed in triplicate, and expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and to total input RNA.

Immunohistochemical detection of FREM1 in ectocervical tissue. Ectocervical biopsy specimens (3 by 3 mm) from the superior portion of the ectocervix were collected according to standard procedures. The biopsy specimens were processed as previously described (19, 29). Cryopreserved biopsy specimens were cut to 8 μm thick, fixed in 2% formaldehyde, and blocked for endogenous biotin (biotin/avidin blocking kit; Vector Laboratories, Burlingame, CA). The FREM1-specific monoclonal antibody F170 was generated in mice against the peptide NH2-CRFDYD RMASLECTVSLDTARTRLPAHG-COOH, which is specific to the full-length isoform. Peptide specificity was searched for homology to known proteins by BLAST and confirmed as unique to FREM1. Antibody specificity was validated by ELISA (enzyme-linked immunosorbent assay) and Western blotting. Negative-control staining consisted of irrelevant mouse or goat antibody (Dako). Staining reactions were developed by using diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA), and nuclear counterstaining was performed with hematoxylin. Acquired computerized image analysis was performed on the labeled sections, generating digital images that were transferred from a DMR-B microscope (Leica, Wetslar, Germany) into a computerized image analysis system (Quantimet Q 550 IW; Leica Imaging Systems, Cambridge, United Kingdom).

Statistical analysis. Analyses were performed with the SPSS13.0 for Windows statistical analysis software package. Standard univariate methods, such as Fisher's exact test (*P* value), odds ratio (OR), and 95% confidence intervals (CI) were utilized to determine the relationship between binary outcomes and explanatory variables. All 114 HIV-resistant women in this study were enrolled in the cohort before 1999 with a median follow-up time of 12.52 years. The HIV-1-infected women have a median follow-up time of 3.3 years. The HIV-1-infected women were used as the control group in the association analysis because they are known to be susceptible to HIV-1.

RESULTS

rs1552896 was identified by an exploratory, low-resolution, genome-wide SNP analysis. A low-resolution, genome-wide analysis using Affymetrix 50K GeneChip Xba arrays was used to identify SNPs that correlate with the HIV-resistant phenotype. Sixty resistant women were compared to 48 HIV-infected controls from the Pumwani sex worker cohort. The study participants were assessed for 37,871 SNPs, and the highest ranking association was for SNP rs1552896 ($P = 1.68 \times 10^{-5}$; adjusted $P = 2.37 \times 10^{-4}$; OR, 9.51; 95% CI, 2.82 to 32.05) (Fig. 1). This is a guanine-cytosine polymorphism in an intron of the *FREM1* gene on chromosome 9p22.3, and the minor allele was detected only in the HIV-resistant women.

Minor allele frequencies of rs1552896 in the Pumwani sex worker cohort and MCH cohort. Because of the small sample size in the pilot exploratory study, we expanded our analysis to a larger proportion of the Pumwani cohort by genotyping rs1552896 of 114 HIV-resistant women and 609 HIV-infected women by PCR amplification of the gene segment containing the rs1552896 locus, followed by Sanger sequencing. This analysis includes the 60 cases and 48 controls from the pilot study. The minor allele frequency of

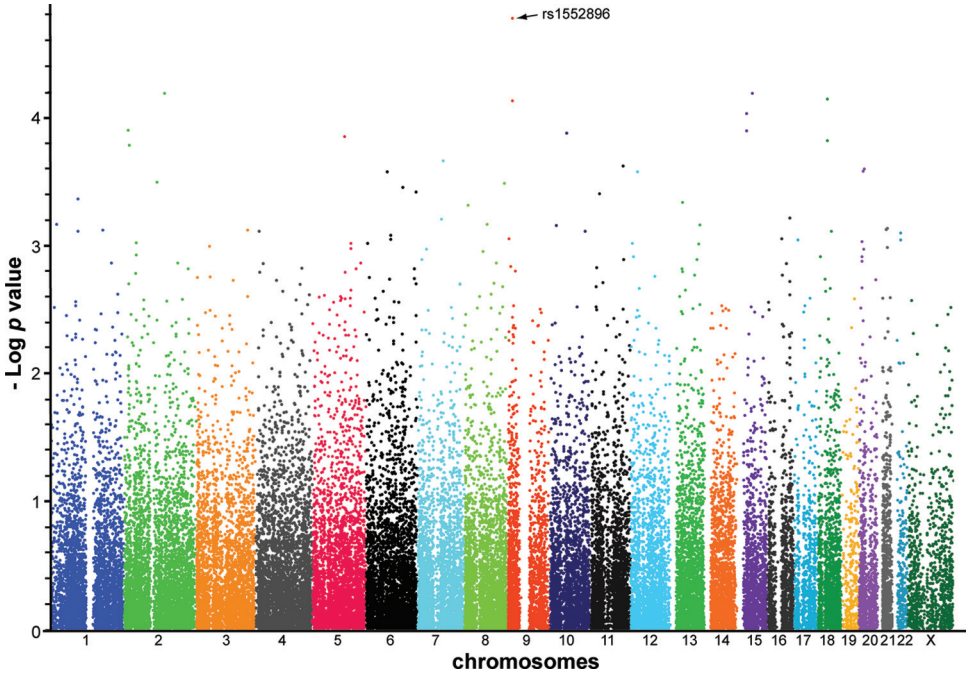

FIG 1 Manhattan plot of −log *P* values of case-control association analysis of SNPs using Affymetrix Xba240 GeneChip arrays of 108 samples in the exploratory study.

rs1552896 in the Pumwani sex worker cohort is higher than in available genotyped populations elsewhere in the world, with the exception of the Utah Residents with Northern and Western European Ancestry (CEU) population (Table 1). When we compared the minor allele frequency of rs1552896 in HIV-resistant women to that of the HIV-positive women in the cohort, the difference was statistically significant ($P = 0.00017$; OR, 2.67; 95% CI, 1.47 to 4.84) (Table 2).

To validate this finding, we also genotyped 783 (295 HIV infected and 488 HIV uninfected) women enrolled in a second cohort at the mother-child health (MCH) clinic. Women in this cohort are of similar socioeconomic status and ethnicity to women in the Pumwani cohort but with lower exposure to HIV due to a lower number of sexual partners. The results showed that the minor allele of rs1552896 is enriched in the HIV-uninfected women compared to HIV-positive women ($P = 0.036$; OR, 1.69; 95% CI, 0.98 to 2.93) (Table 2). Although the HIV phenotypes of the members of the MCH and Pumwani cohorts cannot be directly compared due to differences in sexual behavior, because the national HIV prevalence in the Kenyan general population is relatively high (from 5.1 to 13.4%), especially for women of reproductive age, we expected to see a lower minor allele frequency of rs1552896 in the HIV-positive mothers than in HIV uninfected mothers. The enrichment of the minor allele of rs1552896 in the HIV uninfected mothers compared to that of the HIV-positive mothers is consistent with the observation in the Pumwani sex worker cohort that women with the minor allele of rs1552896 are less likely to be infected by HIV-1.

FREM1 is highly expressed in kidney, small intestine, and cervix. The SNP rs1552896 is mapped to intron 11 near (57 bp) the 3′ end of exon 11 of the *FREM1* gene on chromosome 9 (9p22.3) (Fig. 2A). The direct involvement of two nearby genes, *CER1* (cerberus 1, cysteine knot superfamily) and *LDHAL4* (lactate dehydrogenase A-like 4) was ruled out through linkage disequilibrium analysis (Fig. 2B). This polymorphism does not occur in a known splice site.

TABLE 1 Population frequencies of rs1552896[a]

Population	No. of chromosomes	Individual no.	C/C	C/G	G/G	C	G
Pumwani cohort (Kenyan) (HIV^+ and HIV-resistant)	1,446	723	0.920	0.075	0.007	0.957	0.043
MCH cohort (Kenyan) (HIV^+ and HIV^-)	1,566	783	0.911	0.089		0.955	0.045
HapMap-CEU	120	60	0.667	0.300	0.033	0.817	0.183
HapMap-CHB	90	45	0.956	0.044		0.978	0.022
HapMap-JPT	88	44	0.955	0.045		0.977	0.023
HapMap-YRI	120	60	0.967	0.033		0.983	0.017
JP-ML	142	71	0.944	0.056		0.972	0.028
KR-KR	180	90	0.922	0.078		0.961	0.039

[a] Cytosine (C) and guanine (G) are the alleles detected at SNP rs1552896. C/C, C/G, and G/G are the three genotype combinations observed. Allele and genotype frequencies are given.

TABLE 2 Minor allele of rs1552896 is highly enriched in the HIV-resistant women in the Pumwani sex worker cohort

	No. (%) of women in which rs1552896 is detected[a]								
	HIV resistant (Pumwani; $n = 114$) or uninfected (MCH; $n = 488$)			HIV infected[b] ($n = 609$)					
Cohort	C/C	C/G	G/G	C/C	C/G	G/G	*P* value	OR	95% CI
Pumwani	96 (84.2)	15 (13.2)	3 (2.6)	569(93.4)	39 (6.4)	1 (0.16)	0.00017	2.67	1.47–4.84
MCH	437 (89.5)	51 (10.5)	0 (0)	276 (93.6)	19 (6.4)	0 (0)	0.036	1.69	0.98–2.93

[a] Cytosine (C) and Guanine (G) are the alleles detected at SNP rs1552896. C/C, C/G, and G/G are the three genotype combinations observed.
[b] For the Pumwani cohort, $n = 609$ HIV-infected women; for the MCH cohort, $n = 295$ HIV-infected women.

To explore the expression of *FREM1* in humans, we carried out *FREM1* mRNA-specific quantitative reverse transcription-PCR (qRT-PCR) on 20 different human tissues. *FREM1* mRNA was expressed in all tissues examined, with the kidney, cervix, and small intestine showing the highest levels of expression (Fig. 3). To qualify FREM1 protein expression in the female genital tract, ectocervical biopsy specimens from 15 women from the Pumwani sex worker cohort were assessed by immunohistochemistry. FREM1 was detected in the tissue samples of all 15 individuals. This set of samples included 9 HIV-resistant women (with a follow up of >9 years) and 6 HIV-negative new enrollees (with a follow up of <4 years) with protective and wild-type *FREM1* genotypes in both groups. When a median area of 1.9×10^5 μm^2 (range, 1.02×10^5 to 2.96×10^5 μm^2) was analyzed, a median value of 0.7% (range, <0.1 to 2.96%) of the total cellular area was found to be positive for FREM1 (data not shown). The protein was localized to the epithelial layer and the lamina propria just below the basal membrane (Fig. 4).

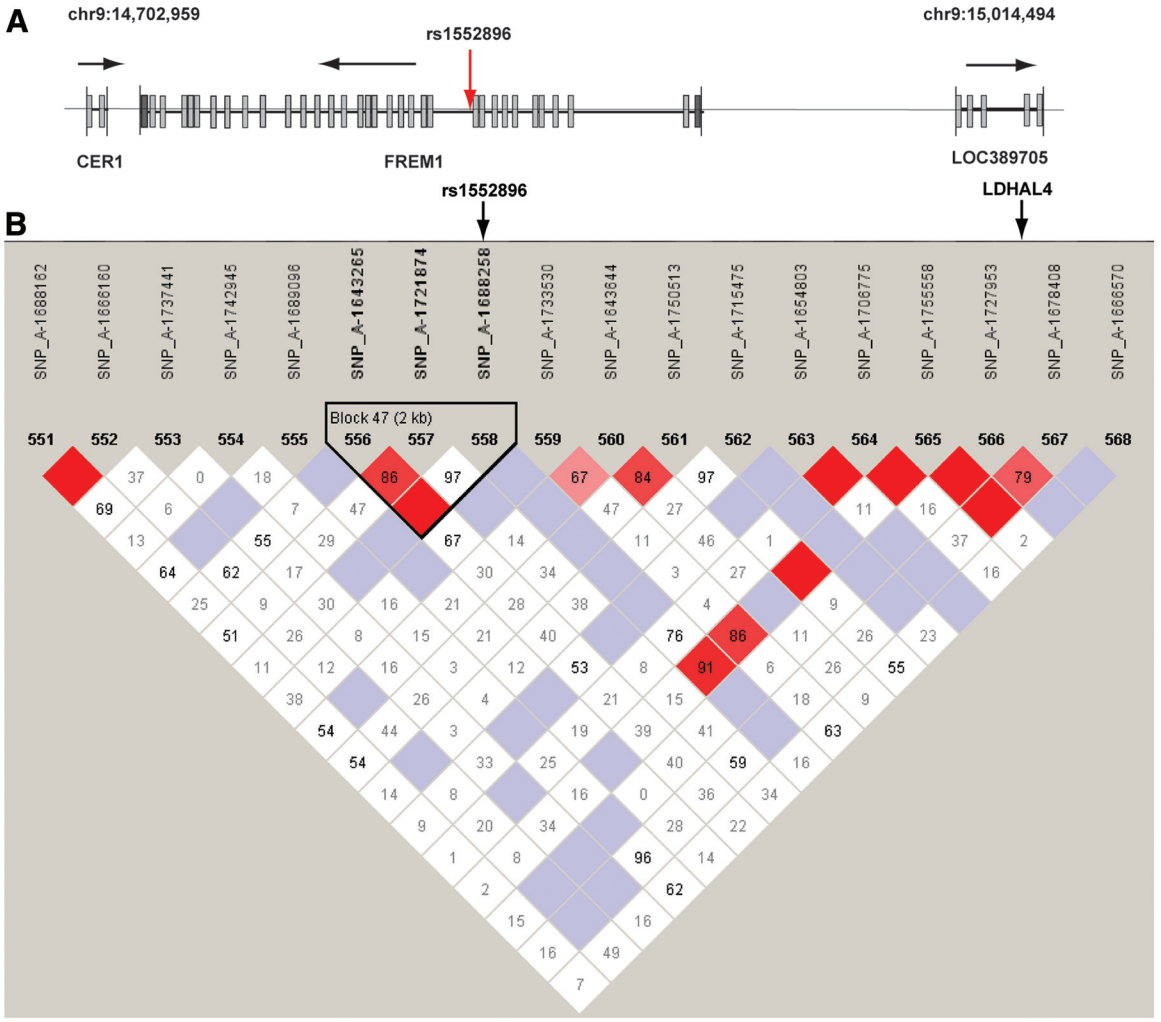

FIG 2 Location of FREM1 and linkage disequilibrium map. (A) Location of rs1552896 in intron 11 of the FREM1 gene on chromosome 9, Entrez Gene cytogenetic band 9p22.3. (B) Linkage disequilibrium map of SNPs around rs1552896.

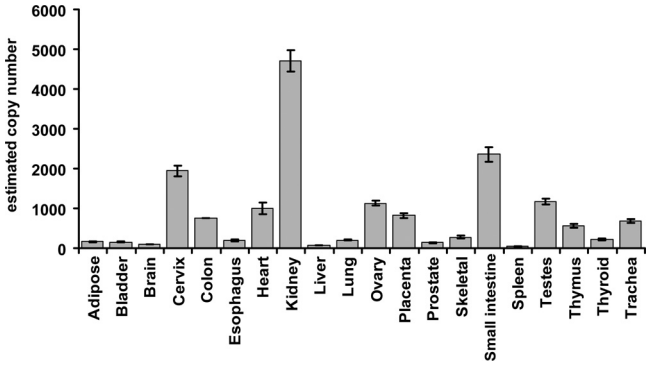

FIG 3 *FREM1* is highly expressed in mucosal tissues. qPCR specific for *FREM1* was conducted on a panel of RNA from various human tissues. High levels of *FREM1* transcript were identified in mucosal tissues, especially the kidney, cervix, and small intestine. In this figure, *FREM1* expression is normalized to total input RNA. When normalized to the housekeeping gene GAPDH, identical trends are observed.

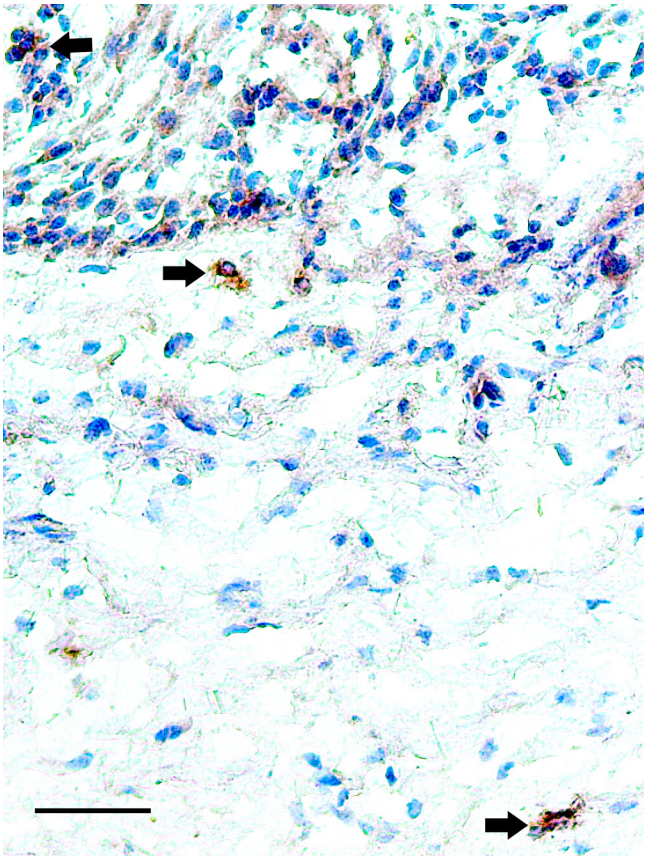

FIG 4 Immunohistochemical staining of ectocervical tissue of a selected HIV-resistant woman from the Pumwani sex worker cohort. FREM1 proteins (brown staining) were located in both the epithelial layer and upper lamina propria. Scale bar, 100 μm. Arrows indicate positive staining in epithelium and submucosa.

DISCUSSION

In an exploratory, nonbiased, low-resolution, genome-wide association study, we observed that the minor allele of SNP rs1552896, located in an intron of *FREM1*, was associated with the HIV-resistant phenotype in the Pumwani sex worker cohort, implicating *FREM1* as a candidate gene in HIV-1 infection. We verified the association by PCR amplification and sequencing of the gene segment containing the SNP rs1552896 and comparing the rs1552896 minor allele frequencies of 114 HIV-resistant women to those of 609 HIV-resistant controls ($P < 0.00017$). Further genotyping of 783 women of a second cohort of women enrolled at a mother-child health clinic showed that the minor allele was also enriched in the HIV uninfected women compared to HIV-1-infected women ($P < 0.036$). These results showed that the minor allele of rs1552896 is associated with a phenotype that is less susceptible to HIV infection. We also showed that *FREM1* mRNA is highly expressed in tissues that are relevant to sexual transmission and establishment of HIV infection, such as the cervix, small intestine, and colon. *FREM1* expression was further assessed at the protein level by immunohistochemistry in tissue biopsy specimens from the ectocervix of women from the Pumwani cohort. FREM1 was detected in both the epithelium and in the submucosa below the basal membrane of women with both the protective and wild-type *FREM1* genotypes. Expression at this location suggests that the protein has direct contact with virus particles in the early stages of sexual HIV transmission. It was not possible to correlate FREM1 expression levels or alternative splicing profiles with the *FREM1* genotype in this assay, because the specific antibody available was only able to detect the full-length isoform and cannot differentiate the extended repertoire of over 15 FREM1 isoforms.

Although little is known about the biological role of FREM1 in HIV infection and how the rs1552986 polymorphism in an *FREM1* intron affects the functionality of the protein, several molecular features of FREM1 suggest that it is plausible as a candidate gene influencing HIV-1 transmission. As an extracellular matrix protein, it is in a relevant location with respect to the early stages of HIV-1 exposure through heterosexual sex. With two RGD (arginine-glycine-aspartic acid) and 12 CSPG (chondroitin sulfate proteoglycan) domains, FREM1 variants can potentially exert different functions by differentially interacting with integrin, collagen, and fibronectin through variations in functional domains (26). These are important components of the genital mucosal barrier and play an important role in HIV-1 infection (4, 6, 7, 10, 13, 14, 33, 43, 51). The C-type lectin and Calx-beta domain of FREM1 suggest the ability of this protein to bind sugar and calcium and modify biological function. FREM1 has been characterized as an essential component of the extracellular matrix maintaining dermal/epidermal cohesion with mutations in FREM1 resulting in a general breakdown of epidermal integrity in Fraser syndrome patients (49). In the context of epithelial integrity in the female genital tract, it is believed that breaches in the wall of the vaginal vault increase the risk of HIV transmission, and it also has been demonstrated that HIV is capable of penetrating into the superficial gaps between squamous genital epithelium as reviewed by Hladik and Hope (20). Whether or not FREM1 has the ability to influence the integrity of the female genital tract mucosa is currently unknown. A splice variant of FREM1, TILRR, has been shown to influence the immune response via two GAG (glycosaminoglycan) binding sites that bind to interleukin-1 receptor 1 (IL-1-R1). The binding increased MyD88 recruitment to IL-1-R1 with subsequent NF-κB activation and enhanced inflammatory response (52). This isoform of FREM1 is a particularly attractive candidate for protection, as these are immunological correlates (1, 2, 25, 30, 46) to the HESN phenotype in the Pumwani cohort. Further in-

vestigation is required to assess FREM1 splicing variants, including TILRR, in the context of the observed association of FREM1 in this study.

The potential for turning naturally occurring protection against HIV-1 infection into an effective intervention has been demonstrated by the CCR5-Δ32 deletion. Having two copies of this polymorphism results in strong protection against HIV-1 infection (47). Informed by this observation, several commercial drugs (e.g., Maraviroc; Pfizer) that interfere with the CCR5–HIV-1 interaction have been developed. A thorough understanding of the role of CCR5 in HIV-1 transmission facilitated understanding of the CCR5-Δ32 deletion and the subsequent development of drugs that can be used to treat HIV infections worldwide, including populations without CCR5-Δ32 deletions, such as African populations. Although the frequency of the minor allele of rs1552896 is low, understanding the role of FREM1 in heterosexual HIV-1 transmission represents an opportunity to identify novel targets and develop new approaches to prevent heterosexual HIV-1 transmission, the route of the majority of global HIV-1 transmission events.

In summary, a pilot low-resolution, nonbiased, genome-wide SNPs analysis identified the minor allele of the SNP rs1552896 as being associated with the HIV-resistant phenotype in the Pumwani sex worker cohort. The association was verified by PCR and sequencing of the gene segment containing the SNP in a large population of sex workers with a defined HIV-resistant phenotype in the Pumwani sex worker cohort. Furthermore, it was also associated with HIV uninfected status in a cohort of 783 women enrolled in a mother-child health clinic. The SNP rs1552896 was mapped to intron 11 near exon 11 of the *FREM1* gene on chromosome 9 (9p22.3). Quantitative RT-PCR showed that *FREM1* mRNA is highly expressed in cervix, small intestine, and colon, which are tissues relevant to sexual HIV-1 transmission. Immunohistochemical staining showed that FREM1 protein is expressed both in the epithelial and submucosal compartment of ectocervix tissue. FREM1 is an extracellular protein with multiple annotated functional domains and expressing multiple splicing variants. These functional domains may interact with integrin, collagen, fibronectin, and IL-1-R1 and influence transendothelial migration, epithelial integrity, and inflammatory responses. These are important factors for HIV-1 infection, establishment, and dissemination. Thus, *FREM1* is a novel candidate gene involved in HIV-1 infection and warrants further investigation.

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Monoclonal antibodies against FREM1 have been developed and listed for licensure by the Office of Intellectual Property Management and Business Development, Public Health Agency of Canada.

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