

Identification of Differentially Expressed Proteins in the Cervical Mucosa of HIV-1-Resistant Sex Workers

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Novel tools are necessary to understand mechanisms of altered susceptibility to HIV-1 infection in women of the Pumwani Sex Worker cohort, Kenya. In this cohort, more than 140 of the 2000 participants have been characterized to be relatively resistant to HIV-1 infection. Given that sexual transmission of HIV-1 occurs through mucosal surfaces such as that in the cervicovaginal environment, our hypothesis is that innate immune factors in the genital tract may play a role in HIV-1 infection resistance. Understanding this mechanism may help develop microbicides and/or vaccines against HIV-1. A quantitative proteomics technique (2D-DIGE: two-dimensional difference in-gel electrophoresis) was used to examine cervical mucosa of HIV-1 resistant women ($n = 10$) for biomarkers of HIV-1 resistance. Over 15 proteins were found to be differentially expressed between HIV-1-resistant women and control groups ($n = 29$), some which show a greater than 8-fold change. HIV-1-resistant women overexpressed several antiproteases, including those from the serpin B family, and also cystatin A, a known anti-HIV-1 factor. Immunoblotting for a selection of the identified proteins confirmed the DIGE volume differences. Validation of these results on a larger sample of individuals will provide further evidence these biomarkers are associated with HIV-1 resistance and could help aid in the development of effective microbicides against HIV-1.

Keywords: HIV-1 • innate immunity • HIV-1-resistance • mass spectrometry • two-dimensional gel electrophoresis • biomarkers • antiproteases

Introduction

The Human Immunodeficiency Virus type 1 (HIV-1) pandemic has continued unabated for 25 years. Currently, HIV-1 infects an estimated 40 million individuals worldwide.¹ Globally, the HIV-1 pandemic disproportionately affects women due to biological and social factors. This is particularly evident in sub-Saharan Africa and other developing areas of the world.² As a result of the inexorable spread of HIV-1, slow HIV-1 vaccine development and the need for female-controlled HIV-1-prevention technology, interest has grown in the development of mucosal HIV-1 microbicides. Thus, the identification of natural factors involved in protection against HIV-1 in the genital mucosa is important.

A subset of 140 women out of a total of over 2000 participants from the Pumwani Sex Worker cohort have been identified to be relatively resistant to HIV-1 infection.^{3,4} Previously described resistance mechanisms, such as Δ -32-CCR5 polymorphisms, have been discounted in this population as their cells are

readily infected in vitro and this genotype has not been detected in this group.⁵⁻⁹ Evidence suggests that the mucosal layer in the cervicovaginal compartment plays a role in mediating resistance to HIV-1 infection, which is the first site of contact for the HIV-1 virus. Studies have shown that protective, innate and adaptive factors exist in this environment against HIV-1. These include HIV-1-specific IgA, cellular immune responses,^{10,11} and a plethora of HIV-1 inhibitory factors such as RANTES, SLPI, alpha/beta-defensins, lysozyme, lactoferrin, calprotectin, cystatin, and histone H2A.¹²⁻²² This suggests that these, and potentially other undiscovered factors, may also be playing a role in HIV-1 resistance, and understanding this mechanism could aid in the development of microbicides and/or vaccines against HIV-1.

To more thoroughly characterize differences in genital secretions between HIV-1-resistant, -infected, and -uninfected individuals, this study employed a gel-based proteomics technique, two-dimensional difference in-gel electrophoresis (2D-DIGE). This technique allows the quantitation of differentially abundant proteins in complex biological samples and has been used previously to examine cervicovaginal fluid for biomarkers of pre-term birth.²³ Here, we report the results of this study which includes the identification of proteins present at differential levels in cervical lavage fluid between HIV-1-resistant,

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HIV-1-infected, and HIV-1-uninfected commercial sex workers (CSW). These proteins represent potential biomarkers of HIV-1 resistance in the Punwani Sex Worker cohort.

Materials and Methods

Study Population. The study was performed with the University of Manitoba and Nairobi human research ethics board approval. Samples were collected from consenting women enrolled in the Punwani Sex Worker cohort in Kenya. Thirty-nine women were included in the study, which were assigned to the following study groups based on HIV-1 status: HIV-1-resistant (HIV-R, $n = 10$), HIV-1-negative (HIV-N, $n = 10$), HIV-1-positive (infected) sex workers (HIV-P, $n = 10$), and HIV-1-uninfected women from the Mother to Child Transmission cohort (MCH, $n = 9$) which were included as an additional low risk of infection, HIV-1-uninfected control group. All of the women from the sex worker cohort were actively engaged in sex work at the time of sample collection. Selection criteria for HIV-1 resistance included enrollment in the cohort for at least 3 years and remained uninfected (PCR and HIV-1 antibody negative) for more than 3 years while engaged in sex work. Criteria for the HIV-1-negative group were women enrolled in the cohort for less than 3 years and were HIV-1 uninfected. The average age for the HIV-R group was 40.6 ± 5.2 years; HIV-P group 36.5 ± 5.3 years; HIV-N group 30 ± 6.83 years; and MCH 39 ± 4.5 years. Women included in the study did not have any other detectable STIs or bacterial infections of the genital tract at the time of sample collection.

Cervical Lavage (CVL) Sample Collection. The endocervix was washed with 2 mL of sterile $1 \times$ phosphate buffered saline (PBS) and the lavage was collected from the posterior fornix. Samples were placed into a 15 mL conical tube and centrifuged to remove cellular debris, and the supernatant was stored at -70°C until analysis. For DIGE analysis, the CVL fluid was pooled (500 μL from each sample) to form HIV-R, HIV-N, HIV-P, or MCH groups. The pooled samples were concentrated by Amicon ultracel 5 kDa MW filter, desalted, and precipitated using the 2D Clean-Up Kit (GE Healthcare).

Protein Labeling with CyDye Fluors. Cervical lavage samples were labeled with Cy2, Cy3 or Cy5 following the protocols in the Ettan DIGE System User Manual (18-1173-17 Edition AA, GE Healthcare). Briefly, 50 μg of protein (~ 5 mg/mL) was labeled with 400 pmol of dye. Labeling reactions were carried out for 30 min followed by a 10 min lysine quench (1 μL of 10 mM lysine). Samples from each cohort (Gel one consisted of a pooled sample, HIV-R and HIV-N sample, while gel two consisted of a pooled sample, HIV-P and MCH sample) were mixed and incubated in an equal volume of $2 \times$ 2D sample buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 130 M DTT, 1% (v/v) IPG buffer, GE Healthcare) for at least 10 min. After reduction, samples were brought to a volume of 450 μL with $1 \times$ 2D sample buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 65 mM DTT, 0.5% (v/v) IPG buffer). All reactions were carried out in the dark on ice. The labeled samples were then used for 2D-DIGE analysis. The Cy2-labeled pooled sample consisted of an equal amount of protein from all four populations and served as an internal control against which all protein volumes were normalized.

Two-Dimensional Gel Electrophoresis. Labeled proteins were separated on an IPGphor isoelectric focusing unit using 24 cm pH 4–7 IPG strips (GE Healthcare) in the dark using the following profile: 10 h passive rehydration, 4 h at 30 V, 0.5 kVh stepped to 500 V, 1.0 kVh gradient to 1000 V, 13.5 kVh

gradient to 8000 V, 45 kVh stepped to 8000 V. Prior to the second-dimension run, proteins in the IPG strips were reduced and alkylated in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, containing 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue) for 15 min in 1% (w/v) DTT followed by 15 min in equilibration buffer containing 2.5% (w/v) IAA. Samples were then processed simultaneously on 26 cm \times 20 cm \times 1 mm precast 10–20% gradient Optigels (Nextgensciences) using the Ettan Dalt 6 Electrophoresis System (GE Healthcare). Gels were run in the dark at 10°C at constant power (2 W/gel) overnight. The power was increased to 100 W, and gels were run for 20 min past the dye front migrating from the gel.

Image Analysis and Poststaining. Gels were imaged directly between low-fluorescence glass plates on the Typhoon 9400 variable mode imager (GE Healthcare). The DIGE images were previewed with ImageQuant software version 5.2 to ensure the absence of detector saturation. After image acquisition, gels were fixed overnight in a solution containing 40% methanol and 10% acetic acid. Gels were then stored at 4°C until further processing. Finally, gels were subjected to silver staining as described previously.²⁴

Spot Volume Determination. DeCyder v.5.02 (GE Healthcare) was used to analyze the DIGE images. Scanned images were cropped in ImageQuant software version 5.2 to remove gel artifacts and then each image underwent spot detection in the differential in-gel analysis (DIA) module without any restrictions set. Protein maps were then exported to the biological variance analysis module (BVA), where all gels were land marked with at least 20 protein spots by hand, followed by software alignment of the remaining proteins. Protein spots were deemed of interest and identified based on the following parameters: a volume ratio of at least ≥ 1.50 when dividing the protein spot volume of resistant women to the average of all others (i.e.: $V_{\text{HIV-R}}/V_{\text{AVG:HIV-P,HIV-N,MCH}}$) and clear separation from other protein spots on the gel to ensure accurate spot removal.

Protein Digest Preparation. Silver-stained gel spots were destained with 1:1 ratio of 30 mM potassium ferricyanide to 100 mM sodium thiosulfate as described previously.²⁵ Gel spots were washed and dried in 40% acetonitrile/100 mM ammonium bicarbonate, followed by 100% acetonitrile wash, and dried in a vacuum centrifuge. Gel spots were rehydrated with 200 μL of trypsin (Trypsin Gold, Promega) at a concentration of 5 ng/ μL and incubated overnight at 37°C .

LC-MS/MS Configuration. Nanoflow LC of tryptic peptide samples was performed with an Agilent 1100 nanoflow LC system equipped with a C_{18} precolumn (Zorbax 300SB-C18, 5 μm , 5 mm \times 0.3 mm, Agilent) and a C_{18} analytical column (Zorbax 300SB-C18, 3.5 μm , 15 cm \times 75 μm , Agilent). The aqueous mobile phase (solution A) contained 5% acetonitrile and 0.1% formic acid, and the organic mobile phase (solution B) contained 95% acetonitrile and 0.1% formic acid. Samples (5- μL injected) were loaded and washed on the precolumn for 5 min with solution A at 50 $\mu\text{L}/\text{min}$. Peptides were then eluted off the precolumn and through the analytical column with a 125-min gradient from 1% to 40% solution B, 5-min gradient from 40% to 95% solution B, and a 5-min rinse with 95% solution B at a flow rate of 250 nL/min. The columns were immediately re-equilibrated for 10 min at initial conditions (100% solution A for the precolumn and 1% solution B for the analytical column). Eluting peptides were injected via nano-spray source into a QStar XL Qq-TOF (Applied Biosystems). The

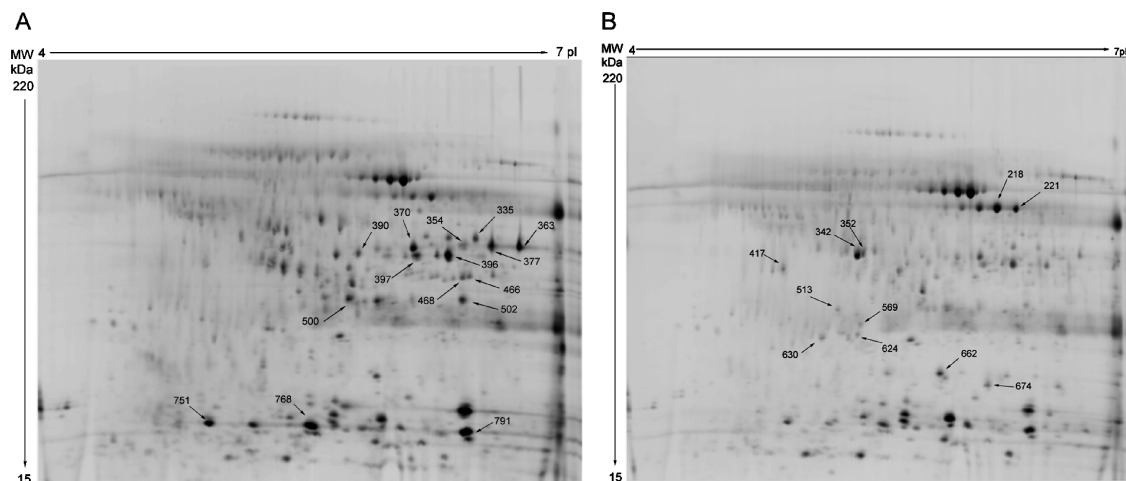


Figure 1. Two dimensional DIGE analysis of proteins in cervical lavage fluid. 2D-E was performed on 50 μg of fluor-labeled CVL protein using 24 cm isoelectric focusing strips with a pH range of 4–7 in the first dimension and SDS-PAGE (10–20% gradient) in the second. The images represent protein spots that are more abundant (A) or less abundant (B) in the HIV-1-resistant group compared to the control group. Protein spots that showed a >1.50 -fold change in abundance are numbered.

ion source was equipped with a 50- μm inner-diameter, fused-silica needle with a 15- μm tip (PicoTip Emitter, New Objective). Data-dependent acquisition was used with a 10 s cycle: 1-s interval for acquiring intact peptide signal (MS), and three 3-s intervals for collision-induced dissociation of the 3 most intense peptides signals in the initial 1-s interval (MS/MS). The MS m/z range was 350–1500, and the MS/MS m/z range was 70–2000. Collision energy was automatically determined by the data acquisition software (Analyst QS 1.1). MS/MS data was acquired for the entire LC run.

Data Analysis. The Mascot search engine (Matrix Science, London, U.K.; version 2.1.03) was used to search the NCBIInr (20070922) database with the MS/MS data. The search parameters were as follows: taxonomy was restricted to *Homo sapiens*, protein molecular weight was unrestricted, fixed modification was Carbamidomethyl (C), variable modification was Oxidation (M), peptide and fragment mass tolerance was ± 0.4 Da, and up to one missed cleavage was allowed.

Criteria for Protein Identification. Scaffold (version Scaffold-01_06_19, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Individual Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm,²⁶ while protein identifications were accepted if they could be established at greater than 80% probability and had at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.²⁷ Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped.

Western Immunoblotting. Protein (1 μg) from each sample was resolved by SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). The blots were blocked with 5% nonfat milk for 1 h at room temperature and incubated overnight at 4 $^{\circ}\text{C}$ with the appropriate dilution of antibody. The blots were then washed three times with TBST, and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. The bands were developed with Immobilon detection reagent (Millipore). The primary antibodies used and catalogue numbers were as follows: mouse monoclonal anti-Cystatin A (Cat. no. ab10442, Abcam), rabbit polyclonal anti-SerpinB13 (Cat. no. ab47743, Abcam), mouse monoclonal anti-SerpinB3

(Cat. no. ab55733, Abcam), and Heat-shock protein 70 mouse monoclonal (Cat. no. ab6535, Abcam).

Results

2D-DIGE Analysis of HIV-R, HIV-P, HIV-N, and MCH CVL Fluid. To define differentially expressed proteins in CVL fluid between HIV-1-resistant women and control groups, this study used the 2D-DIGE multiplex proteomic approach. CVL protein samples were differentially labeled with a fluorescent cyanine dye and co-resolved in the same gel to obtain relative quantitative information. Two independent 2D-SDS PAGE gels were run: gel one included CVL protein from HIV-R and HIV-P labeled with Cy5 and Cy3 fluorescent dyes, respectively; gel two included MCH and HIV-N labeled with Cy5 and Cy3, respectively. A Cy2-labeled internal standard control (pool of all four groups) was multiplexed with the samples and co-resolved in each of the gels. Gel images were imported into the DeCyder BVA package software where protein volumes were normalized to the internal control. Protein maps were matched and protein volume ratios were determined for the resistant group versus all three control groups both independently (e.g., HIV-R versus HIV-P) or together (HIV-R vs average of the 3 others). Figure 1 shows the 2D-PAGE gels images of the co-electrophoresed HIV-R sample (Figure 1A) and the HIV-P (Figure 1B) sample. Analysis of the protein maps revealed that 72 protein spots demonstrated a clear change in volume (at least ≥ 1.50) between the HIV-R and HIV-P, and HIV-R versus the average of the 3 other groups. These spots were excised and analyzed by tandem mass spectrometry. From these 72 picked protein spots, 26 of them (representing 17 unique proteins) were identified confidently by mass spectrometry and are numbered in Figure 1. Table 1 shows the identity of these protein spots along with the volume ratio change with respect to the HIV-R group.

Eight proteins were overexpressed and nine were underexpressed in the HIV-R group. The proteins that demonstrated the greatest overexpression included serpin B3 (4.65-fold, spot 363) and serpin B4 (3.17-fold, spot 500), followed by alpha-2 macroglobulin-like 1 protein (2.72-fold, spot 502), cystatin A (2.15-fold, spot 768), thioredoxin (2.23-fold, spot 751), S100A7 protein (1.88-fold, spot 791), serpin B13 (1.71-fold, spot 390), serpin B1 (1.53-fold, spot 370). Rho-dissociation inhibitor was

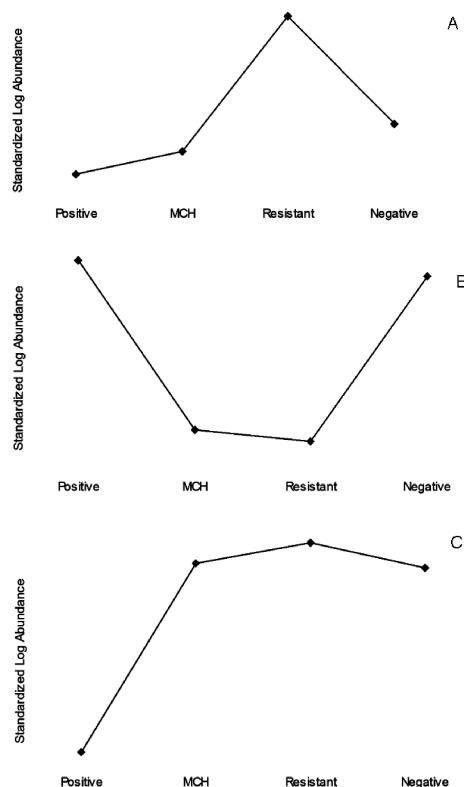


Figure 2. A representative diagram of protein abundance trends observed in cervical lavage fluid between study groups. Each of the proteins identified showed either an “A”, “B”, or “C” trend of abundance. “A” trend proteins were those that were clearly differentially abundant in the HIV-1-resistant group; the “B” trend proteins were similar in abundance between the HIV-1-resistant and one other group; and the “C” trend proteins were those that did not clearly segregate the HIV-1-resistant group from the other three.

the most underexpressed protein in the HIV-R group (8.43-fold, spot 674), followed by haptoglobin (5.74-fold, spot 662), IGHA1 protein (4.71-fold, spot 221), beta-actin (3.94-fold, spot 342), transglutaminase-3 (3.26-fold, spot 569), complement component 3 (2.93-fold, spot 417), chloride intracellular channel 1 (1.65-fold, spot 513), and apolipoprotein A1 (1.50-fold, spot 630).

To more directly assess the trend of protein differences, the standardized log abundance of each protein spot was calculated and compared across all groups. This was done by dividing the individual protein spot volume by the internal control. Thus, a 50% difference in spot volume would have a standardized log value of ± 1.5 , while no changes would have a value of ± 1.0 . When these values were plotted, different trends of abundance were observed, and a visual representation of this is shown in Figure 2. For simplicity, each protein spot was assigned a trend letter (A, B, or C) depending on which it was most closely matched. Proteins which were clearly segregated in the HIV-R group from the others, either overexpressed or underexpressed with at least 50% (standardized log ratio of $> \pm 1.5$) difference to the nearest group, such that all 3 controls clustered together, fell into the “A” trend. An example of this is serpin B3 (spot 363) where the HIV-R group was clearly overexpressed (2.6) compared to the HIV-P (−4.0), HIV-N (−1.3) and MCH groups (−1.5). Protein spots that were differentially expressed in the HIV-R group and had one other group within 50% abundance, but separated from the other groups by at least

50%, were assigned the “B” trend. S100A7 protein (spot 791) fell into this category, clustering the HIV-R group (1.6) with MCH (1.4), but overexpressed compared to HIV-P (−1.5) and HIV-N (−1.8). The “C” trend represents those protein spots which showed differential expression in the HIV-R group to at least one other group by 50%, but not in a clear pattern from the other two. This was observed with serpin B1 (spot 370) where HIV-R was overexpressed (1.3) compared to HIV-P (−2.0), but not overly so to HIV-N (−1.1) and MCH (1.1).

Visual inspection of the gel images shows that there is a clear cluster of overexpressed proteins in the right-hand portion of the gel (Figure 1A). The majority of these spots were identified as serpin B3. The separation of serpin B3 into distinct locations highlights the resolution capacity of 2DE and ability to distinguish different isoforms from one another. Shifts in the horizontal plane represents different *pI*s (i.e., protein spots 377 and 363) due to different post-translational modifications that alter the charge state. Alternatively, shifts in the vertical plane (i.e., protein spots 354 and 468) are most likely different cleavage products although other large post-translational modifications such as glycosylation and even phosphorylation have been shown to dramatically effect protein migration in certain cases. Serpin B3 is known to have multiple isoforms: a native form of 44.5 kDa and *pI* of 6.36, with shorter versions at 38.5 kDa and *pI* of 6.29, and 35.5 kDa and *pI* of 7.98, which would account for the presence of this protein in multiple spots at different molecular weights and/or isoelectric points.

Gene Ontology of Differentially Expressed Proteins in HIV-1-Resistant Women. Differentially expressed proteins in the HIV-R sample were grouped based on their primary biological function according to their gene ontology. Table 2 shows the list of overexpressed proteins (top) in HIV-R and underexpressed proteins (bottom) with their major biological function listed. A visual representation of these groups is shown in Figure 3. The majority of overexpressed proteins tended to be those with antiprotease activity. A small portion of these are involved in immune response and cellular proliferation. The underexpressed proteins in the HIV-R group did not show a clear trend in biological function clustering. Many are involved in the immune response, cell organization, with some having antiapoptotic activity, or involvement with ion transport/homeostasis.

Western Blots. To confirm the trends observed by 2D-DIGE, immunoblots using antibodies specific for cystatin A, serpin B3, and serpin B13 were performed on the same protein pools. The results confirm the trends seen in the DIGE analysis for these proteins and are shown in Figure 4. The top blot shows serpin B3 and there is clear overabundance of all isoforms in the HIV-R groups, at 45 kDa (native MW), 39, 36, and 32 kDa. This matches the literature values of 44.5, 39.5, and 35.5 kDa. The smaller 32 kDa isoform may represent a further cleavage product. Serpin B13 also showed a clear overabundance in the HIV-R sample, for all MWs of 46, 40, 38, and 29 kDa. Cystatin A also followed the trend observed in the 2D-DIGE analysis and is overabundant in the HIV-R group, with the other three groups at roughly the same levels. To exclude the possibility that the HIV-R sample was overloaded in the immunoblots, another protein known to be present in cervicovaginal fluid, HSP70, which was not found to be differentially abundant by 2D-DIGE, was used as a control. As of yet there is no defined protein in cervical lavage fluid that is known to be present in equal amounts across individuals, so the selection of HSP70 was simply to demonstrate a immunoblotting trend that

Table 1. Abundance Ratios of Protein Spots That Are Overexpressed (A) and Underexpressed (B) in Cervical Lavage Fluid between HIV-1-Resistant Women and Control Groups

Swiss-Prot accession number	protein	gel spot no.	no. of peptides	% coverage	volume ratio ^a	trend ^b	standardized log ratio ^c			
							HIV-P	MCH	HIV-R	HIV-N
(A) Overexpressed Proteins in HIV-1-Resistant Women										
Q86VF3	Serpine B3	363	14	38	4.65	A	-4.0	-1.5	2.6	-1.3
		377	6	20	3.74	A	-6.6	-1.4	2.3	1.0
		468	5	16	3.51	A	-4.3	1.0	2.3	-1.4
		354	3	7.7	3.46	A	-3.1	-1.7	1.7	-1.7
		335	10	27	3.0	A	-2.4	-1.8	2.0	-1.0
		466	6	21	2.61	A	-3.0	-1.0	2.0	1.0
		396	4	13	1.89	B	-1.5	1.2	1.6	-1.5
		397	6	17	1.64	B	-2.2	1.2	1.3	-1.5
Q86W03	Serpine B4	500	10	26	3.17	A	-2.8	-1.0	2.0	-1.7
Q6ZW52	Alpha 2-macroglobulin like-1 protein	502	6	6.5	2.72	A	-2.6	-1.4	2.0	1.1
P10599	Thioredoxin	751	3	34	2.23	A	-1.9	-1.2	1.8	1.1
P01040	Cystatin A	768	5	67	2.15	B	-2.3	1.3	1.8	-1.2
P31151	S100A7 protein	791	8	50	1.88	B	-1.5	1.4	1.6	-1.8
Q9UIV8	Serpine B13	390	6	18	1.71	B	-2.3	1.1	1.5	1.0
P30740	Serpine B1	370	11	33	1.53	C	-2.0	1.1	1.3	-1.1
(B) Underexpressed Proteins in HIV-1-Resistant Women										
P52566	Rho GDP dissociation inhibitor	674	2	25	-8.43	A	2.2	-2.0	-7.2	-1.2
P00738	Haptoglobin	662	2	7.6	-5.74	B	2.6	-1.7	-4.4	-1.4
P02647	Apolipoprotein A1	630	2	9	-4.90	B	1.4	-1.2	-4.3	1.2
		624	3	13	-1.50	B	1.4	1.3	-1.3	-1.3
Q6GMX2	IGHA1 protein	221	2	6.7	-4.71	A	2.5	-1.3	-3.3	1.0
		218	2	5.3	-2.98	A	1.1	-1.3	-2.6	1.5
P60709	Actin beta	342	8	28	-3.94	A	2.2	-2.3	-3.4	-1.1
		352	7	24	-2.82	A	1.4	-1.5	-2.3	1.5
Q08188	Transglutaminase 3	569	4	8.7	-3.26	A	1.2	-1.3	-2.9	1.4
P01024	Complement component 3	417	6	5.2	-2.93	A	1.4	1.0	-2.6	-1.0
O00299	Chloride intracellular channel 1	513	2	8.7	-1.65	B	1.4	-1.4	-1.6	1.1

^a Volume ratio: the standardized ratio between the HIV-1-resistant group versus the standardized average of the other three groups determined by DIGE. ^b Trend: according to Figure 2. ^c Standardized log ratio: the log value of the sample spot volume ratio over that of the internal control (pool of all four groups).

Table 2. Biological Function of Differentially Expressed Proteins in Cervical Lavage of HIV-1-Resistant Women

Swiss-Prot accession number	protein	protein biological function	protein MW (kDa)
Overexpressed in HIV-1-Resistant Women			
Q86VF3	Serpine B3	Cysteine protease inhibitor	44.6
Q86W03	Serpine B4	Serine protease inhibitor	45
Q6ZW52	Alpha 2-macroglobulin like-1 protein	Elastase inhibitor, broad-spectrum endopeptidase inhibitor	161
P10599	Thioredoxin	Cell proliferation	12
P01040	Cystatin A	Cysteine protease inhibitor	11
P31151	S100A7 protein	Innate immune response, epidermis development	11
Q9UIV8	Serpine B13	Cysteine protease inhibitor	44
P30740	Serpine B1	Elastase/Cathepsin G inhibitor	43
Underexpressed in HIV-1-Resistant Women			
P52566	Rho GDP dissociation inhibitor	Antiapoptosis, cell motility, cell adhesion	23
P00738	Haptoglobin	Ion homeostasis, defense response	45
Q6GMX2	IGHA1 protein	Immune response	51
P60709	Actin beta	Cell organization	42
P02647	Apolipoprotein A1	Lipid carrier	31
P01024	Complement component 3	Immune response	187
Q08188	Transglutaminase 3	Cell organization	77
O00299	Chloride intracellular channel 1	Chloride transport, signal transduction	27

differed from cystatin A, serpin B3, and serpin B13. The bottom immunoblot shows that the HIV-R sample had relatively the same amount of HSP70 as HIV-N, where the MCH and HIV-P samples had slightly higher amounts.

To address the possibility that the overexpression of these markers was due to an overabundance in a few samples, immunoblots of cystatin A were performed on each individual unpooled sample. The trends closely matched that seen with

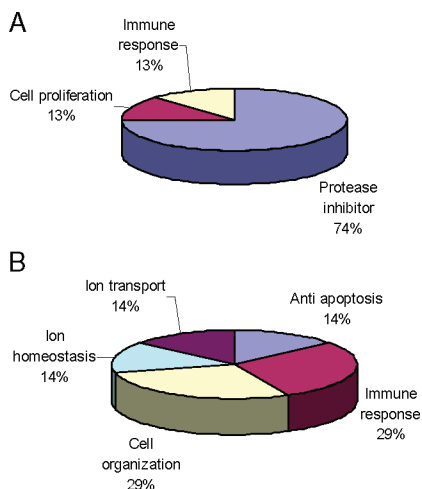


Figure 3. Biological functional clustering of overexpressed (A) and underexpressed (B) proteins in cervical lavage fluid of HIV-1-resistant women. Identified proteins were classified according to their major biological function according to their gene ontology.

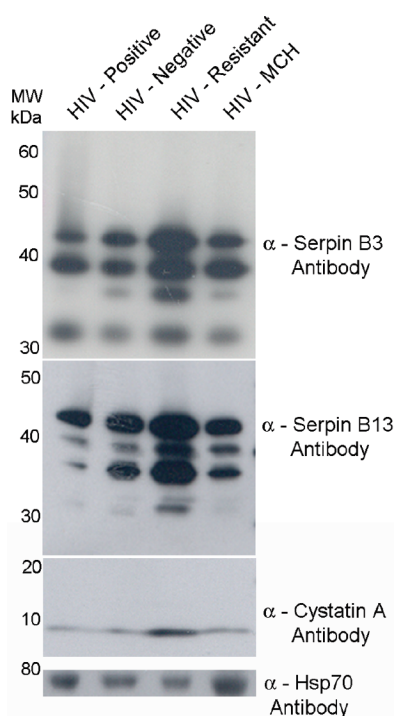


Figure 4. Immunoblots of potential biomarkers of HIV-1-resistance in cervical lavage fluid. Protein (1 μ g) from each sample group was resolved on SDS-PAGE gels and blotted with specific antibodies. Both Serpin B3 and Serpin B13 show multiple isoforms with molecular weights ranging from \sim 45 to \sim 30 kDa.

the pooled samples with the HIV-1-resistant women having medium to high levels in almost all individuals (9 of the 10) (data not shown).

Discussion

In humans, heterosexual transmission for HIV-1 occurs across the genital mucosa. The risk of transmission is mediated by the ability of the virus to gain access to susceptible target cells. This is dependent on many factors, including the number

of cells which bind HIV-1 and allow it to migrate through this barrier to infect CD4+ T-cells,^{28,29} damage to the integrity of the epithelial barrier due to mucosal trauma, inflammation, and ulceration gives HIV-1 direct access to activated macrophages and T-cells³⁰ and the presence of mucosal antiretroviral factors, including defensins, RANTES, elafin, SLPI, histatins, statherin, mucins, lysozyme, lactoferrin, and cystatins, affects this process.^{13–22,31} Therefore, factors that may influence the integrity of the epithelial barrier, reduce inflammation, aid in wound repair, or affect abundance and/or effectiveness of antiviral factors could contribute to altered susceptibility to HIV-1.

This study used a gel-based approach to examine the cervicovaginal proteome of HIV-1-resistant sex-workers. Steps were taken to minimize the possibility that these results were due to natural biological variation between study groups. This included having a high cutoff threshold of protein selection to those with only >1.5-fold change in volume despite the ability of DIGE to reliably detect changes as little as 20%.³² In addition, the protein expression profiles of three control groups were compared to HIV-1-resistant women to increase the stringency of biomarker selection. The possibility exists that differential expression of these proteins could be the result of significantly over/under abundance in a few individuals which could have skewed the pooled sample. Although not all biomarkers were validated on an individual basis, immunoblots of cystatin A confirmed the overexpression trend observed in the pooled samples. Ongoing studies on the entire commercial sex worker cohort, including over 100 HIV-1-resistant women, will be used to confirm these results.

Comparison of protein profiles revealed that a group of antiproteases were upregulated in HIV-1-resistant women. These included those in the serpin B family (B1, 3, 4, and 13), alpha-2 macroglobulin-like 1, and cystatin A. Of these, cystatin A is of particular interest given its known anti-HIV-1 properties.³³ The exact mechanism of how cystatin A acts against HIV-1 is not known, but it has been implicated in the interference of HIV-1 viral processing, especially during the late stages of the viral life cycle.³⁴ It also blocks epithelial-lymphocyte interactions which can contribute to HIV-1 infection.³⁴ Cystatin's cysteine antiprotease activity may directly affect the function of Vif, a viral protein essential for HIV-1 replication and, therefore, reduce HIV-1 infection of CD4+ T cells.³⁵ Although we found cystatin A to be overexpressed, we have not yet determined the physiological concentration in these women. Cystatin A has moderate antiviral activity at physiological concentrations in human saliva (30% inhibition at 2 μ g/mL) but increasing in effectiveness at higher concentrations (90% inhibition at >20 μ g/mL).³³ Therefore, determining the actual level of overexpression in HIV-1-resistant women will further support a role in defense against HIV-1 infection.

Serpins B1, B4, and B13 are all inhibitors of cathepsin G, an inflammatory protease.^{36–38} Cathepsin G is found in high amounts in cervicovaginal fluid and it is a chemoattractant for monocytes/neutrophils and also stimulates T-cells which are targets for HIV-1 infection.³⁹ Cathepsin G is known to enhance HIV infectivity in vitro,⁴⁰ and cleave anti-HIV-1 factors such as RANTES to render them less effective against HIV-1.⁴¹ Therefore, an overabundance of serpins to antagonize cathepsin G action may be protective by lowering its inflammatory capability and potentially lowering targets for HIV-1 infection in the genital tract. Indeed, specific studies have shown that serpin B1 is crucial to the innate mucosal immune system by

protecting against cathepsin G and elastase-mediated inflammatory damage during host infection by *Pseudomonas aeruginosa*.⁴² In addition, serpin B4 has been proposed to protect the epithelial barrier against chymase-induced inflammation and tissue degradation.³⁷ It is interesting to note that other serpins have anti-HIV-1-specific properties. A recent study showed that the C-terminal region of Serpin A1 has strong antiviral activity,⁴³ and that its receptor, CD91, is upregulated and overexpressed in individuals infected with HIV-1 who are true long-term nonprogressors.⁴⁴

Serpin B1 and A2ML1 have specific elastase inhibitory properties.^{36,45} Although not identified in this study, human neutrophil elastase (HNE), a serine protease, is known to be present in the cervicovaginal compartment.⁴⁶ It has been suggested that it can increase the risk of HIV-1 infection by enhancing myeloid-related protein (MRP-8) expression,⁴⁷ an inflammatory protein in cervicovaginal fluid known to stimulate HIV-1 production. HNE is also associated with increased risk of intrauterine transmission of HIV-1⁴⁸ and can impair wound healing.^{49–51} It is possible that overexpression of serpin B1 and A2ML1 may be protective by antagonizing these actions of HNE and/or maintaining the integrity of the epithelial barrier. However, whether the concentration differences have biological significance is unknown and it may be possible that a small increase has little effect on total anti-elastase activity in vivo.

Some of the downregulated proteins in HIV-1-resistant sex workers identified in this study have known roles in HIV-1 infection. Complement component 3 serves as a chemoattractant that contributes to the generation of a specific immune response. It is activated upon HIV-1 infection and can enhance infection by allowing opsonized virus to bind to complement receptor positive cells and infect them more efficiently.⁵² It also allows the fusion to nonpermissive cells, such as follicular dendritic cells. Rho dissociation inhibitor has been implicated in mediating HIV-1-infected cell migration through tight junctions.⁵³ Beta-actin, a normal cytoskeletal component in cells, is known to bind to HIV-1 reverse transcriptase and may be involved in HIV-1 secretion.⁵⁴ Apolipoprotein A1, surprisingly, is known to bind gp41 of HIV-1 and act as a competitive inhibitor for binding to the cell surface,^{55–57} although apolipoprotein levels are known to be affected upon HIV-1 infection, so this may be a result of HIV-1 infection rather than being related to HIV-1-resistance.

Conclusion

The identification of novel protein biomarkers that are associated with HIV-1 resistance is important to understanding what mediates protection against infection. This study found that HIV-1-resistant sex workers differentially express proteins in cervical mucosal fluid compared to HIV-1-infected and HIV-1-uninfected counterparts. The most biologically interesting of these include those from the serpin B family, alpha-1 macroglobulin-like 1, and cystatin A, which have antiprotease and/or anti-inflammatory properties. Cystatin A is known to have anti-HIV-1 activity in vitro and it may be contributing, by itself or in concert with other factors, to a protective environment against HIV-1 infection. The hope is to use this information to aid in the design or improvement of microbicides against HIV-1. This technique will be carried out on a larger sample of individuals to confirm their association with resistance to HIV-1 infection. The potential role these proteins play in HIV-1 infection is currently under investigation.

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