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Research Article

Alpha-1 antitrypsin variants in plasma from HIV-infected patients revealed by proteomic and glycoproteomic analysis

Novel tools are necessary to explore proteins related to human immunodeficiency virus (HIV) infection. In this work, proteomic and glycoproteomic technology were employed to examine plasma samples from HIV-positive patients. Through comparative proteome analysis of normal and HIV-positive plasma samples, 19 differentially expressed protein spots related to 12 non-redundant proteins were identified by ESI-ion trap MS. Among these, the 130-kDa isoform of α-1-antitrypsin was found to be decreased in HIV-positive patients while another variant with a molecular weight of 40 kDa was increased. SWISS-2-D-PAGE reference gel and protein sequence comparisons of the 40-kDa protein showed homology with α-1-antitrypsin minus the *N*-terminus, and its identity was further confirmed by 1-D Western blotting and glycoproteomic analysis. In all, our results showed that proteomics and glycoproteomics are powerful tools for discovering proteins related to HIV infection. Furthermore, this 40-kDa variant of α-1-antitrypsin found in the plasma of HIV-positive individuals may prove to be a potentially useful biomarker for anti-HIV research according to bioinformatics analysis.

Keywords:
α-1-antitrypsin variant / HIV-positive patients / Plasma / Proteomics
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1 Introduction

The human immunodeficiency virus type 1 (HIV-1) pandemic has continued unabated. Currently, HIV-1 infects an estimated 40 million individuals worldwide. There is an urgent need to understand the mechanism of HIV infection and to find new drug targets. At present, most research and anti-HIV drugs focus primarily on virus-encoded proteins. However, due to the combination of a short retroviral life cycle and the high mutation rate of HIV, progress in anti-virus treatment has thus far been limited. Other investigations have instead focused on the host cells [1, 2], including a large-scale small interfering RNA screen to identify HIV-dependency factors [1], and functional genomics to elucidate the cellular responses to the infections of HIV-1 and simian immunodeficiency virus [2]. However, proteins are ultimately responsible for controlling most aspects of cellular function. The consistency between genes and proteins is low. One gene can produce many unique proteins and proteins can display a broad range of PTM. So further study to explore changes in host proteins is very necessary.

We first sought to look for any potential biomarkers among plasma proteins but the dramatic variability of their relative concentrations requires methodology, which is both high throughput and highly sensitive. Proteomics can meet this need by enabling comparison of protein profiles of samples from different conditions. It has been widely used to study therapeutic targets for HIV-1 infection [3], to select diagnostic biomarkers of HIV-associated cognitive impairment [4, 5] and to understand the mechanism of HIV infection [6]. However, none of these proteomic studies to date have looked past the protein level to PTMs and/or degradation in relation to HIV, although the proteomic study by Sung-Soon Kim *et al.* [6] found different isoforms of apolipoprotein AI upregulated in HIV and predicted that these isoforms may be due to PTM.

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Abbreviations: GPMAW, General Protein/Mass Analysis for Windows; **HIV,** human immunodeficiency virus; **MRM,** multiple reaction monitoring; **MW,** molecular weight; **PBMCs,** peripheral blood mononuclear cells; **WB,** Western blotting

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In this work, we used a proteomic and glycoproteomic method to explore differences in plasma proteins between controls and HIV-infected individuals. We found that in HIV-positive patients, the amount of a high molecular weight (MW) isoform (MW of 130 kDa) of α-1-antitrypsin was decreased, and a lower MW variant (MW of 40 kDa) of α-1-anti-trypsin, minus the *N*-terminal sequence, was increased. According the reference map in SWISS-PRORT (http://www.expasy.org/swiss-2dpage/viewer&map = CSF_HUMAN&ac = P01009) α-1-anti-trypsin has multiple naturally occurring MW isoforms between 55 and 130 kDa. So we infer this lower MW variant of 1-anti-trypsin increase in HIV-positive patients might be a new variant related to HIV infection. Furthermore, α-1 antitrypsin [7] and its *C*-terminal peptide – virus-inhibitory peptide [8, 9] were reported to inhibit HIV infection *in vitro*. This lower MW variant might be a potentially useful target for HIV research. In all, our results show that proteomics and glycoproteomics are useful tools for HIV/acquired immune deficiency syndrome research and might offer a new way to identify biomarkers in HIV infection.

2 Materials and methods

2.1 Materials and chemicals

All IPG strips (3–10 NL, 18 cm) were obtained from GE Company (Piscataway, NJ, USA). Tris, glycine, IPG buffer, urea, SDS, DTT, thiourea and Coomassie Blue were from Bio-Rad or GE Company. Pro-Q Emerald 488 was purchased from Molecular Probes (Eugene, OR, USA). Trypsin with sequencing grade was from Promega (Madison, WI, USA).

2.2 Plasma sample preparation

Blood samples were collected from 40 HIV-positive patients and 42 healthy human, with EDTA as anticoagulant. All HIV-positive patients were hospitalized during 2007–2008 in Shanghai Public Health Clinical Center affiliated to Fudan University (Shanghai, China). All HIV-positive patients enrolled in this study did not have tuberculosis, HBV and hepatitis C virus infection, were not taking antiviral medicine, and had given their consent. Control plasma from 42 healthy volunteers (without HIV, tuberculosis, hepatitis B virus and hepatitis C virus infection) was obtained from samples taken during routine staff medical check-ups at Shanghai Public Health Clinical Center during 2007–2008. The male to female ratio of both controls and patients groups were 5:1. The mean age was 41.9 ± 5.6 and 40.8 ± 10.2 for healthy controls and HIV-positive patients, respectively. The plasma and peripheral blood mononuclear cells (PBMCs) were separated from blood within 2 h and stored to $-80^{\circ}C$ until used for further study. Each sample was used beyond 6 months after it was stored.

To remove the highly abundant proteins (albumin and IgG) in plasma, 60 μL of plasma sample was treated with Aurum™ Serum Protein Mini Kit as described by the manufacturer (Bio-Rad) [10]. Briefly, plasma was diluted fourfold with the binding buffer (20 mM phosphate buffer, pH 7.0) provided in the kit, and applied to the 1-mL column and vortexed. The unbound protein fraction was collected, precipitated with acetone, dried and lysed by 2-DE lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 1% Nonidet P 40, 65 mM DTT, 0.5% Pharmalyte 3–10), and stored at $-80^{\circ}C$ prior to further 2-DE analysis. The albumin and IgG bound to the column was eluted with SDS-lysis buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 100 mM DTT). Protein concentrations were determined using the Bradford assay. The untreated plasma samples (control), unbound proteins and bound-eluted samples were analyzed by SDS-PAGE to verify the efficiency of albumin and IgG removal. All samples were stored at $-80^{\circ}C$ prior to electrophoresis.

2.3 2-DE

The plasma after removing IgG and albumin from ten HIV-positive patients and ten healthy controls was used for 2-DE. 2-DE was performed on an IPGphor iso-electronic focusing system (GE Company) as previously described [11]. Briefly, frozen samples were briefly thawed and diluted into IEF sample buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 0.1 M DTT, 1.6% pH 3–10 linear carrier ampholyte buffer to yield 200 μg of protein in 350 μL sample buffer. The first dimension was performed on IPG strips (18 cm) at $20^{\circ}C$ under the following conditions: 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V gradient for 30 min and 8000 V for 6 h up to 52.1 kVh. The second-dimensional gel electrophoresis was carried out on 11.5% SDS-PAGE gels with Bio-Rad Protein II electrophoresis apparatus. After completion of the second-dimensional electrophoresis, gels were stained with silver nitrate, Coomassie brilliant blue G-250 or Pro-Q Emerald 488.

2.4 Image acquisition and data analysis

The 2-DE gels were scanned by Imagescaner (GE Company) in transmission mode and the image analysis was conducted with ImageMaster software (GE Company). To correct for variability due to staining, and to reflect the quantitative variations of the protein spots, the individual spot volumes were normalized by dividing their OD values by the total OD values of all the spots present in the gel. The difference in protein expression between HIV-positive patients and the controls was estimated by two-sample *t*-test ($p < 0.05$). Protein spots with more than twofold difference between patients and controls were cut for MS analysis.

2.5 Nanoflow LC-MS/MS analysis

The differentially expressed proteins from plasma after removing IgG and albumin in Coomassie brilliant blue

G-250 stained 2-D gels were analyzed by nanoLC-ESI-MS-MS (Esquire HCT, Bruker, Germany). Protein spots were cut out and digested by trypsin as published [11]. The lyophilized peptides were diluted with 2% ACN in water with 0.1% TFA and analyzed by high capacity ion trap MS as previously reported [12–14]. Briefly, the peptide mixture (5 μL) was directly subjected to a nanoLC-ESI-MS/MS system. Chromatography was performed by an Ultimate 3000 instrument (LC Packings, Dionex, Sunnyvale, CA, USA). Peptides were separated on a nanoflow column (15 cm × 75 μm id, C18 PepMap, Dionex) at a flow rate of 0.3 μL/min, employing a gradient from 4 to 60% mobile phase B (mobile phase A: 0.10% formic acid in water, mobile phase B: 0.10% formic acid in 80% acetonitrile) over a period of 40 min. Peptides were eluted into the MS system *via* a distal coated fused-silica needle (20 μm tube id, 10 μm tip id, PicoTip emitter; NewObjective, Woburn, MA, USA). The MS conditions were set as follows: capillary voltage, 1000–1800 V; dry gas (nitrogen) flow = 4.0 L/min; dry gas temperature = 150°C. Data-dependent acquisition was controlled by chromeleon software (Dionex). The acquisition cycle consisted of a survey scan covering the range of m/z 100–1800 followed by MS/MS fragmentation of the four most intense precursor ions with a scan range of 100–2800. The MS/MS data was input to MASCOT 2.0 program (MatrixScience, Boston, MA, USA) against IPI database v3.26. Searching parameters were set as follows: taxonomy, homo sapiens; enzyme, trypsin; allow up to one missed cleavages; the peptide mass tolerance, 1.2 Da and the fragment ion mass tolerance, 0.6 Da; peptide charge, 1+, 2+ and 3+; modification parameters, carbamidomethyl (C) (fixed modifications), oxidation (M) (variable modifications), 20 hits allowed. In our output result, only peptides with their individual ion score above 15 in the mascot report contributed to protein identification [15, 16]. Proteins were identified on the basis of mascot scores exceeded the threshold ($p < 0.05$), which indicates the protein identification at the 95% confidence level with all these matched peptides. Further, the peptides were manually checked to make sure at least one peptide with three or more continuous y- or b-series ions (*e.g.* y5, y6, y7) in a protein.

For multiple reaction monitoring (MRM) analysis of the differential protein – α-1-antitrypsin – the high capacity ion trap MS was operated in MRM mode. The MS conditions were set as follows: Scan mode, Ultra scan; MS(n) mode, MRM; precursor/fragment ions: 555.8/797.4, 508.3/829.51, 821.5/685.37. 555.8/797.4($y16^{+2}$) represents the *C*-terminal peptide LSITGTYDLK; 508.3/829.51($y8^{+1}$) represents the *C*-terminal peptide SVLGQLGITK; 821.5/685.37($y5^{+1}$) represents the *N*-terminal peptide ITPNLAEFAFSLYR.

2.6 Bioinformatics analysis

To select functional proteins for further research, a protein–protein network was done through String software (version 8.0, http://string.embl.de). The identified proteins were input to ''multiple names'' columns to draw the network. The node proteins were selected for further research. The locations of α-1-antitrypsin in this study were compared with the results reported in SWISS-2-D-PAGE. (http://www.expasy.org/swiss-2dpage/viewer&map = CSF_HUMAN&ac = P01009). The peptides of α-1-antitrypsin variants detected by MS in this work were compared with the protein sequence. For the undetected peptides, General Protein/Mass Analysis for Windows (GPMAW) software was used to analyze their characters [17, 18]. GPMAW can provide information on the concentration of acetonitrile for elution of the peptide from a typical C18 column. Peptides with HPLC indices < 5 will fail to bind to the column tightly enough under most experimental conditions (too hydrophilic). Conversely, peptides with HPLC indices > 30 often give unsatisfactory results due to interference with other hydrophobic polymeric ''chemical noise'' (too hydrophobic).

2.7 Real-time RT-PCR

Total RNA from PBMCs of 20 HIV-positive patients and 22 healthy controls was extracted using Trizol reagent (Invitrogen Life Technologies) following the manufacturer's instructions and our previous description [19].

2.8 Western blotting (WB)

Plasma from another ten HIV-positive patients and ten healthy controls was used. Twenty-five microgram of total protein extracts were separated by 1-DE and transferred to PVDF membrane (GE Company). Blots were incubated overnight at 4°C with the primary antibody (mouse monoclonal to α-1-antitrypsin (1:5000, Santa Cruz, CA, USA). After three washes with TBS-Tween, blots were incubated for 1 h at 20°C with secondary antibody (goat anti-mouse IgG-HRP (1:2000, Santa Cruz)). After further washes, the immune complexes were revealed by enhanced chemiluminescence and detected by X-ray films. Finally, the X-ray films were scanned. Each reaction was performed in triplicate.

2.9 Glycoproteomics

Proteins were separated by 2-DE, and stained by Pro-Q Emerald 488 glycoprotein gel stain kit (P21875) according to the manufacturer's instruction (http://probes.invitrogen.com/media/pis/mp21875.pdf). Briefly, gels were fixed overnight in 50% methanol and then incubated in oxidizing solution for 30 min. After washing, the gels were incubated in Pro-Q Emerald 488 dye solution overnight in the dark. The gels were then washed with 3% acetic acid before scanning. The glycoprotein spots were visualized by a laser scanner, Taphoon 9400 (Amersham Bioscience) with the excitation wave length of 488- and 520-nm band pass for emission.

Glycoprotein identification was carried according to the previous described protocol [20] with minor modifications. Briefly, the lyophilized peptides digested as described above were redissolved in 50 mM NH_4HCO_3 (pH 8.0) containing 0.1 U/mL PNGase F (Sigma, America). Deglycosylation was performed at 37°C overnight. Proteins were identified by MALDI-TOF-TOF (ABI 4700, USA) according to the previously described method [21]. Variable modifications, deamidation (NQ) were chosen.

3 Results

3.1 Improvement of the sample preparation for plasma proteins

As shown in Fig. 1, albumin and IgG were efficiently removed by the antibody affinity method. Compared with total plasma gel, more protein bands were visible in SDS-PAGE after albumin and IgG removal treatment.

3.2 2-DE separation of plasma protein and image analysis

As shown in Fig. 2A and B, protein profiles with good resolution were obtained. Using Image master software analysis of six gels stained by silver nitrate (three from HIV-positive patients and three from the controls), an average of 481 and 486 protein spots were detected in HIV-positive samples and healthy controls, respectively. Of these, 19 protein spots were found to be differentially expressed with a more than twofold difference between control and patient samples. A representative differential spot (U1) was highlighted in magnified 2-DE as shown in Fig. 2C and the difference, marked as column chart, was exported from Image master software analysis (shown in Fig. 2D). Figure 2A–C and D demonstrates that these differences are substantial and repeatable.

3.3 Identification of differentially expressed proteins

To further check the differential proteins shown in the gels stained by silver nitrate and improve the probability of successfully identified by MS, the 2-DE gels stained by Coomassie brilliant blue G-250 were used for protein identification. The digested peptides were analyzed by ESI-MS-MS. Twelve non-redundant proteins from 19 gel spots were identified as shown in Table 1. Of these 12 proteins, 10 were identified by more than 2 peptides, and only two were identified by one peptide with score of 57 and 59 (Supporting Information Table S1). For proteins identified by one peptide, the MS/MS spectra were checked manually and shown in Supporting Information Fig. S1. For proteins identified by more than two peptides, their MS score was higher than 65, and at least one peptide was identified by more than three continuous b or y ions through manual checking. For example, in the α-1-antitrypsin precursor with MW of 40 and 130 kDa, 58% and 38% amino acids were accurately detected by MS, and 21 and 14 peptides were matched, respectively (Fig. 3A, highlighted by underlines). Seeing from their representative MS/MS spectra of peptides (VFSNGADLSGVTEEAPLK from 40-kDa variant and ITPN-LAEFAFSLYR from 130 kDa), we found almost all b or y ions were detected by MS (Fig. 3B and D). MRM analysis of digested peptides from 130 and 40 kDa α-1-antitrypsin shown that the 40-kDa α-1-antitrypsin variant misses the *N*-terminal peptide (Fig. 4). Table 1 lists all 19 differentially expressed protein spots, including 3 downregulated (shown in Fig. 2A, marked with D) and 16 upregulated (shown in Fig. 2B, marked with U) in HIV-positive patients compared with the healthy controls ($p < 0.05$, *t*-test). These differentially expressed proteins include α-1-antitrypsin precursor, isoform 1 of fibrinogen chain precursor, α-1B-glycoprotein precursor, *etc*.

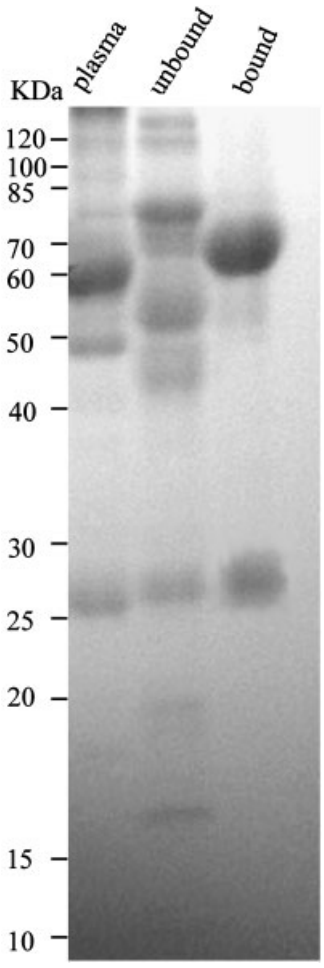

Figure 1. 1-DE gel with or without albumin and IgG protein removal treatment. The left lane ''plasma'' represents untreated plasma samples. The middle lane ''unbound'' represents sample after albumin and IgG removal. The right lane is albumin and IgG bound and eluted from the affinity column.

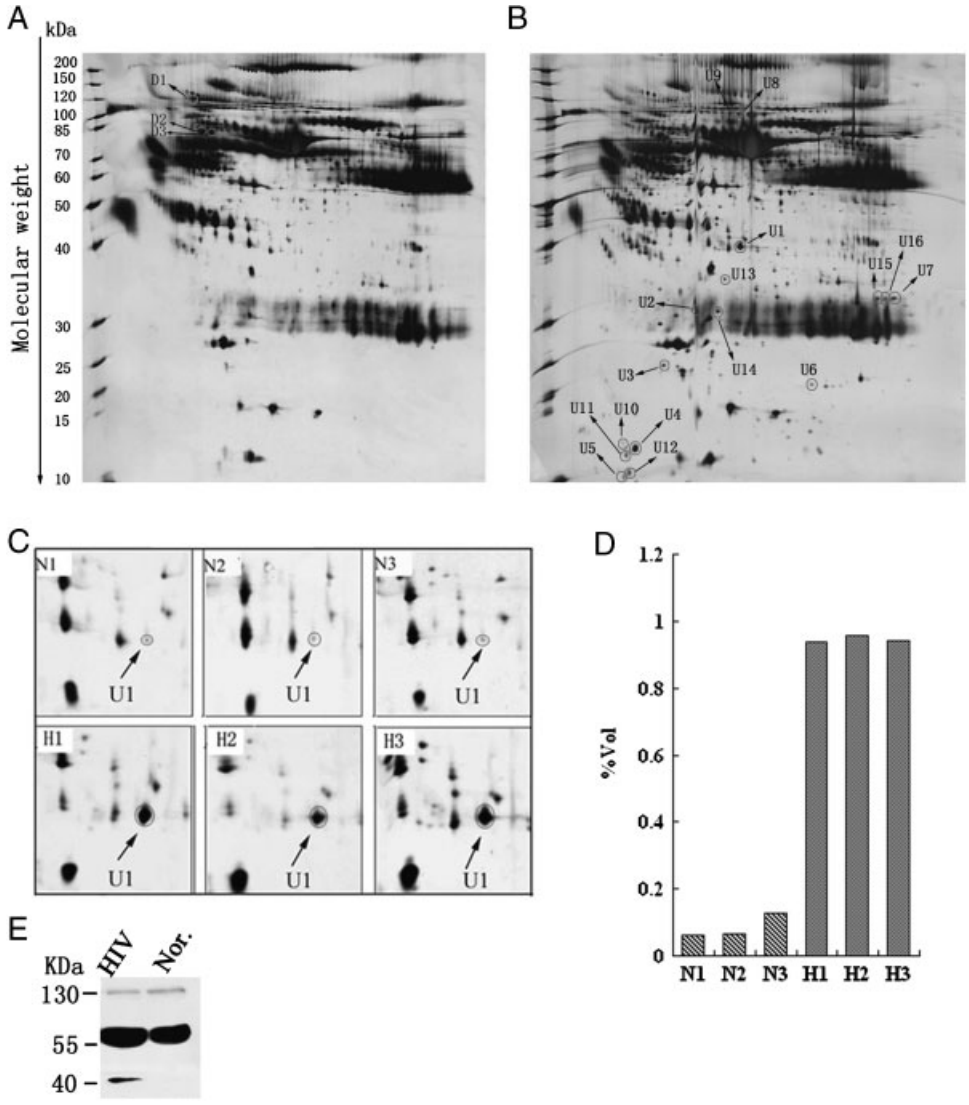

Figure 2. Identification and verification of differential proteins. (A and B) Silver nitrate stained 2-D-PAGE gels of depleted plasma samples. (A) normal; (B) HIV-positive patients. Molecular weight of markers is shown on the left. U indicates proteins upregulated in HIV-positive patients, $p<0.05$; D indicates proteins downregulated in HIV-positive patients. (C) Magnified regions of 2-D images showing spot U1 upregulated in HIV-positive patients (below) compared with normal controls (upper). N1, 2 and 3 show replicate gels from healthy controls; H1, 2 and 3 stand for samples from HIV-positive patients. (D) Each column of the graph represents the quantitation of spot U1 in the replicate gels from the controls (N1, N2 and N3) and the gels from HIV-positive patients (H1, H2 and H3). (E) WB of α-1-antitrypsin. Molecular weight was shown on the left. HIV, depleted HIV-positive plasma; Nor, depleted plasma of the healthy controls.

3.4 Bioinformatic analysis

Using STRING software, a protein–protein interaction for our identified proteins was obtained (Supporting Information Fig. S2). In this protein–protein network, α-1-antitrypsin precursor was an important node protein interacting with transthyretin precursor. Furthermore, we found increased amounts of 40-kDa α-1-antitrypsin variant and decreased amounts of the 130-kDa α-1-antitrypsin isoform in HIV-positive patients. To understand these contrasting results, we checked the 2-DE gel shown in SWISS-PROT and found the gel spots of this protein appeared with MW of about 55–60 and 130-kDa (http://www.expasy.org/swiss-2dpage/viewer&map = CSF_HUMAN&ac = P01009). The protein spot with MW of 40 kDa has not been previously reported. Among other possibilities, this novel spot may be due to the degradation and/or deglycosylation of α-1-antitrypsin since three glycosylation sites were reported in SWISS-PORT in HIV-positive patients. By checking the protein sequence and the matched peptides (Fig. 3A and C), we found that for 40-kDa protein, 124 amino acids from N-terminus were not be detected by MS (Fig. 3A); however, for the 130-kDa protein spots, the whole sequence including not only the *C*-terminus but also the *N*-terminus (Fig. 3C) was detected. To check whether the protein was genuinely truncated or whether the peptides had simply failed to be detected by MS, we used GPMAW software to analyze the sequence of α-1-antitrypsin. The results showed that three of five peptides for the 124 amino acids from *N*-terminus were not too hydrophilic or too hydrophobic, and could be detected by MS, *i.e.* those peptides with amino acids from 35 to 49 (35–49), 50–63 and 64–93 (shown in Supporting Information Table S2 and Supporting Information Fig. S3). In addition, the peptide with amino acids from 50 to 63 was detected in the 130-kDa isoform. Further MRM analysis also verified that the *N*-terminal peptide – ITPNLAEFAFSLYR – was only in the 130-kDa variant. It therefore seemed likely that our detection methods were accurate and the 40-kDa

Table 1. List of the differentially expressed protein spots in 2-DE of HIV-positive and healthy donors identified by HCT

Spot	Protein name	Accession number	MW	p*I*	Score	Matched[a)]	Expression in HIV	CV%[b)]	Abbreviations[c)]
U1	α-1-Antitrypsin precursor[d)]	IPI00553177	46 878	5.37	997	21	↑	58	SERPINA1
U2	Complement C4-A precursor	IPI00032258	194 247	6.65	108	3	Only	2	
U3	Apolipoprotein A-IV precursor	IPI00304273	45 371	5.28	375	9	Only	14	APOA4
U4	Transthyretin precursor	IPI00022432	15 991	5.52	59	1	Only	8	TTR
U5	Apolipoprotein A-I precursor	IPI00021841	30 759	5.56	411	6	Only	24	APOA1
U6	Isoform 1 of Fibrinogen α chain precursor	IPI00021885	95 656	5.7	119	4	Only	4	
U7	Isoform 2 of Fibrinogen α chain precursor	IPI00029717	70 227	8.23	443	8	Only	12	
U8	Isoform 1 of Serum albumin precursor	IPI00745872	71 317	5.92	2363	45	↑	45	
U9	Isoform 1 of Serum albumin precursor	IPI00745872	71 317	5.92	2006	37	↑	36	
U10	Apolipoprotein A-IV precursor	IPI00304273	45 371	5.28	170	3	only	2	
U11	Apolipoprotein A-IV precursor	IPI00304273	45 371	5.28	231	6	only	3	
U12	Apolipoprotein A-I precursor	IPI00021841	30 759	5.56	510	9	Only	5	APOA1
U13	Apolipoprotein E precursor	IPI00021842	36 246	5.65	73	3	Only	3	
U14	IGKV1-5 protein	IPI00419424	26 503	6.3	68	1	only	2	
U15	Isoform 2 of Fibrinogen α chain precursor	IPI00029717	69 714	8.23	125	6	Only	8	
U16	Isoform 2 of Fibrinogen α chain precursor	IPI00029717	69 714	8.23	349	6	Only	10	
D1	α-1-Antitrypsin precursor[e)]	IPI00553177	46 878	5.37	654	14	↓	38	SERPINA1
D2	α-1B-glycoprotein precursor	IPI00022895	54 809	5.58	607	12	↓	39	A1BG
D3	α-1B-glycoprotein precursor	IPI00022895	54 809	5.58	388	8	↓	24	A1BG

a) Number of matched non-redundant peptides.
b) CV% stands for the sequence coverage value.
c) The abbreviations of differential proteins shown in protein–protein interaction.
d) 40-kDa variant.
e) 130-kDa variant.

variant lacked the final *N*-terminal sequence present in the native 55-kDa α-1-antitrypsin molecule.

3.5 Real time RT-PCR

To determine if transcription contributed to the change in protein expression, we measured the transcriptional alterations of α-1-antitrypsin in PBMCs from 20 HIV-infected patients and 22 healthy donors by quantitative real-time RT-PCR using the mRNA transcript of GAPDH gene as a control housekeeping gene. No substantial difference ($p = 0.687$) was found between HIV-positive patients and the donors through Mann–Whitney *U* analysis (Supporting Information Fig. S4).

3.6 Validation of the variant of antitrypsin in plasma from HIV infected patient by WB analysis

We sought to validate the expression of antitrypsin variants in plasma from HIV infected patients by 1-DE WB analysis of plasma. As shown in Fig. 2E, one more band with MW of about 40 kDa in HIV-positive samples compared with the donors was detected.

3.7 Glycosylation analysis of antitrypsin

To exclude the possibility that these different MW forms of α-1-antitrypsin were generated solely by changes in glycosylation, a glycoproteomic analysis was carried out. Using Pro-Q Emerald 488 glycoprotein staining, the glycoprotein profiles of HIV-positive patients and healthy controls were obtained. As shown in Fig. 5, the fragment of α-1-antitrypsin with MW of 40 kDa was found to be strongly increased; however, the fragment with MW of 130 kDa was decreased in HIV-positive patients.

To further verify that these two proteins were glycosylated, we digested the two α-1-antitrypsin spots with *N*-glycosidase F and analyzed the peptides with MALDI-TOF-MS. Figure 3E and F shows the PMF spectra of 130- and 40-kDa α-1-antitrypsin variants before and after PNGase F treatment. The glycopeptide (YLGNATAIFFLPDEGK) was detected in both protein spots after PNGase F treatment and is highlighted with an asterisk in Fig. 3E and F. However, we did not detect this peptide in protein spots before PNGase F treatment.

4 Discussion

This report outlines the use of proteomic and glycoproteomic analysis to study acquired immune deficiency syndrome plasma, and selects and verifies the patients' plasma proteins related to HIV infection.

Kim *et al*. [6] had previously reported a plasma proteomic study identifying many overexpressed proteins such as isoforms of apolipoprotein AI. However, in Sung-Soon's research, highly abundant proteins were not removed from plasma and fewer protein spots displayed in 2-DE. In this

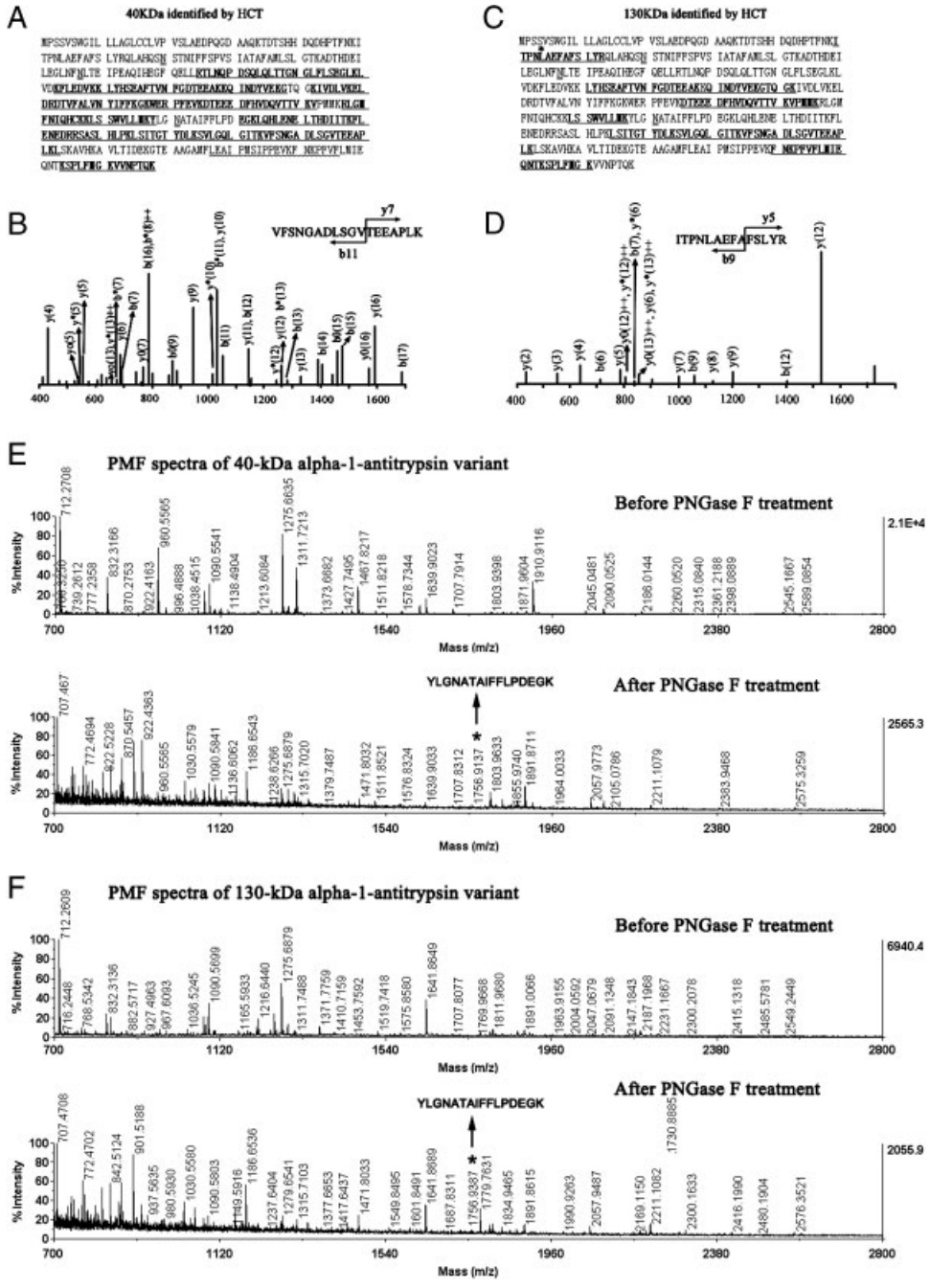

Figure 3. Sequence coverage and spectra of 40- and 130-kDa α-1-antitrypsin identified by MS. A, B and E for 40-kDa variant of α-1-antitrypsin; C, D and F for 130-kDa variant. (A and C) Protein sequence. Amino acids detected by high capacity ion trap MS were shown in bold and highlighted by underline. Virus-inhibitory peptides were highlighted by wave line. The glycosylated asparagines were marked with double underline and shown in A. The peptide detected by MS in the 124 amino acids from *N*-terminal of α-1-antitrypsin were marked with an asterisk (*) and shown in C. (B and D) Represent MS/MS from 40-kDa (B) and 130-kDa variant (D). (E and F) PMF spectra of 130- and 40-kDa α-1-antitrypsin variants before and after PNGase F treatment; the glycopeptide was shown and highlighted by an asterisk (*).

work, higher abundance proteins (albumin and IgG) were removed to separate more protein spots in 2-DE so that more detailed profile about the HIV-infection-related protein could be seen in our study. Because sometimes the host factors during virus infection were at very low abundance, the removing of highly abundant proteins give us a chance to focus on the lower abundance proteins. Among the 12 differentially expressed proteins we identified, the amount of 40-kDa α-1-antitrypsin fragment increased about twofolds in HIV-positive patients than in healthy controls. Similarly, Sung-Soon Kim *et al.* reported that α-1-antitrypsin was upregulated in HIV-positive patients [6]. These results showed that the 40-kDa α-1-antitrypsin could be a valuable indicator for HIV infection. While in Kim *et al.* [6] study, they found that a protein named apolipoprotein AI, which is the major protein component of high density lipoprotein in plasma, was downregulated in HIV-infected patients. But in our study, two variants of this protein were only expressed in HIV-positive patients. Through carefully comparing the 2-DE image, we found the two variants detected in this work were shown outside the gel run by Kim *et al.*, with MW

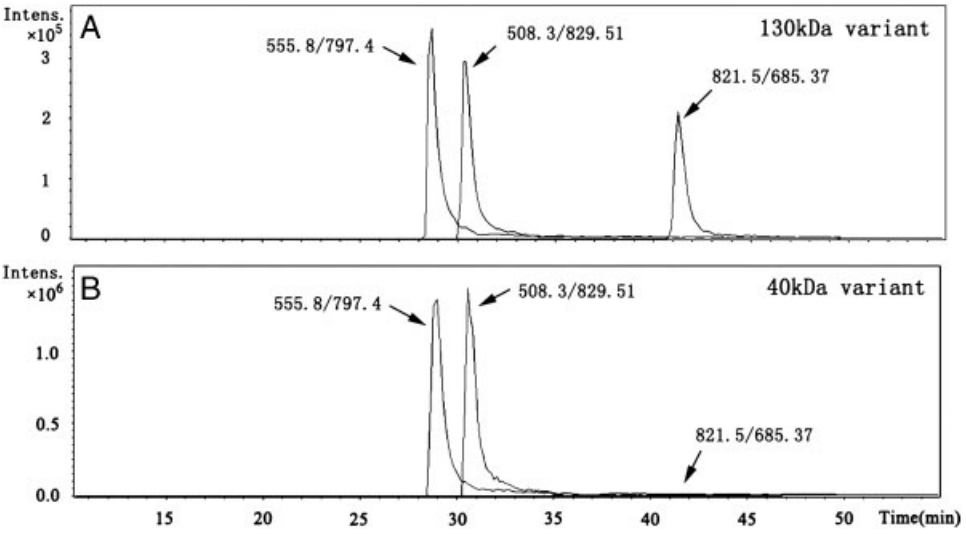

Figure 4. MRM chromatograms from the nanoflow LC/MS/MS analysis of digested peptides from 130- and 40-kDa α-1-antitrypsin 2-D gels protein spots. MRM mode was performed to study precursor/product ion (*m/z*) transitions for three peptides. 555.8/797.4($y16^{+2}$) represents the *C*-terminal peptide LSITGTYDLK; 508.3/829.51($y8^{+1}$) represents the *C*-terminal peptide SVLGQLGITK; 821.5/685.37($y5^{+1}$) represents the *N*-terminal peptide ITPNLAEFAFSLYR. It shows that the 40-Da α-1-antitrypsin variant misses the *N*-terminal peptide.

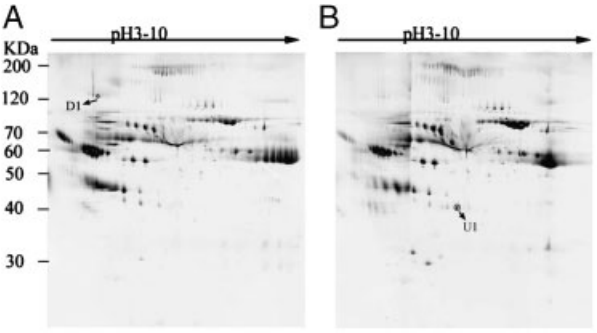

Figure 5. Comparative glycoproteome of depleted plasma from HIV-positive patients (B) and healthy controls (A) using 2-DE followed by Pro-Q Emerald 488 glycoprotein staining. Total cellular proteins (500 μg) were loaded. The 130-kDa variant was marked as D1 and shown in A. The 40-kDa variant was marked as U1 in B.

below 14 kDa. Probably, Apolipoprotein AI has the same truncated phenomenon as that in antitrypsin.

α-1-antitrypsin is an important circulating serine inhibitor, the whole protein is a 394 amino acids, 55-kDa glycoprotein primarily synthesized in the liver and secreted into the circulation. It has recently been shown that α-1–antitrypsin [7] and its fragment – virus-inhibitory peptide [8, 9] highlighted by wave line in Fig. 3A, can inhibit HIV-1 infection *in vitro*. Meanwhile Met/Arg358 variant of α1-antitrypsin [7] was shown to inhibit the convertase-catalyzed formation of either gp120/gp41 or gp70/50, and HIV-1-infected patient with α-1-antitrypsin deficiency displayed a very rapid loss of CD4 T cells according to the report by Potthoff *et al.* [22].

In this work, we found a unique 40-kDa variant of α-1-antitrypsin to be increased in HIV-positive patients. Through sequence matching, gel spot location checking, MRM detection of a *N*-terminal peptide and bioinformatics analysis, we hypothesized that this variant might be a shortened fragment of the α-1-antitrypsin protein. Real-time RT-PCR, 1-D-WB and glycoproteomic research further suggests that this novel form of α-1-antitrypsin identified in HIV positive patients might be a truncated version of the protein lacking up to the last 124 *N*-terminal amino acids. However, due to the limit of glycosylation analysis, it is very difficult to identify all the glycosylation sites by MS. So in this work, we can not totally exclude the possibility of partial deglycosylation of α-1-antitrypsin through experiment. Further studies are necessary to study the glycopeptide's structure of this variant through enrichment of glycopeptides, confirm our findings in a large number of individuals and to understand the relationship between HIV and this fragment.

In conclusion, we used proteomics combined with glycoproteomics to analyze plasma proteins in HIV-positive patients for the first time. This approach identified and verified a new variant of α-1-antitrypsin in HIV-positive individuals, which might be a new anti-viral agent or target for HIV research. Although we have done some experiments to verify this new variant of α-1-antitrypsin, further studies should still be needed, for example, studying the structure of this variant, researching the relationship between HIV and this variant, *etc.*

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