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iTRAQ based investigation of plasma proteins in HIV infected and HIV/HBV coinfected patients — C9 and KLK are related to HIV/HBV coinfection

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ABSTRACT

Objectives: Human immunodeficiency virus (HIV) and hepatitis B virus (HBV) share similar routes of transmission, and rapid progression of hepatic and immunodeficiency diseases has been observed in coinfected individuals. Our main objective was to investigate the molecular mechanism of HIV/HBV coinfections.

Methods: We selected HIV infected and HIV/HBV coinfected patients with and without Highly Active Antiretroviral Therapy (HAART). Low abundance proteins enriched using a multiple affinity removal system (MARS) were labeled with isobaric tags for relative and absolute quantitation (iTRAQ) kits and analyzed using liquid chromatography–mass spectrometry (LC–MS). The differential proteins were analyzed by Gene Ontology (GO) database.

Results: A total of 41 differential proteins were found in HIV/HBV coinfected patients as compared to HIV mono-infected patients with or without HAART treatment, including 7 common HBV-regulated proteins. The proteins involved in complement and coagulation pathways were significantly enriched, including plasma kallikrein (KLK) and complement component C9 (C9). C9 and KLK were verified to be down-regulated in HIV/HBV coinfected patients through ELISA analysis.

Conclusion: The present iTRAQ based proteomic analyses identified 7 proteins that are related to HIV/HBV coinfection. HBV might influence hepatic and immune functions by deregulating complement and coagulation pathways. C9 and KLK could potentially be used as targets for the treatment of HIV/HBV coinfections.

Introduction

Acquired immunodeficiency syndrome (AIDs) due to HIV infection has become a chronic disease, with a lower morbidity due to Highly Active Antiretroviral Therapy (HAART). However, HIV patients who are co-infected with HBV are at an increased risk (Lewden et al., 2005; Wu et al., 2015). Due to the fact that HIV and HBV share similar transmission routes, including sexual and blood–blood contacts, co-infections with human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) are common and have become a serious public health problem that leads to accelerated progression of the incidence of severe liver disease (Phung et al., 2014). In previous studies of HIV-infected patients, 5.5–13.2% were coinfected with both HIV and HBV (Thio et al., 2013; Zhang et al., 2014). In HIV/HBV coinfected patients, alanine aminotransferase and aspartate aminotransferase levels were higher and $CD4^+$ T-lymphocyte counts (CD4) cell counts were much lower than in HIV mono-infected patients (Thio et al., 2013; Zhang et al., 2014; Huang et al., 2016). Moreover, the prevalence of liver-related mortality was 17 times greater in HIV/HBV coinfected individuals than in those infected with HBV alone (Sun et al., 2014; Parvez, 2015). The progression of hepatic complications from HBV infection is accelerated in patients that are coinfected with HIV (Chun et al., 2014). Furthermore, patients who had HBV infection in addition to an HIV infection had about 4 times more risk of developing severe hepatic toxicity after administration of HAART (Hoffmann and Thio, 2007; Drake et al., 2004). However, our understanding of HIV/HBV coinfection is still very limited. The proteins that are related to HIV/HBV co-infection could be used as potential treatment targets in the clinical settings and might offer

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new clues for understanding the mechanism of HIV/HBV coinfection. However, few proteins are specifically related to HIV/HBV co-infections.

So as to find proteins that are related to HBV coinfection in HIV infected patients, we selected the patients that had not received HAART, and the patients that were treated with the same drugs to exclude drug effects. Patients with HIV and HBV coinfections have impaired immunity and impaired liver function. In addition to this, drug–drug interactions require a careful balance of HIV and HBV treatments to avoid adverse drug reactions and liver damage. To this end, drugs that are effective against both HIV and HBV and at the same time exhibit limited hepatoxicity are recommended, and these include tenofovir disoproxil fumarate (TDF) and lamivudine (3TC). Moreover, combination antiretroviral therapy (ART) with TDF, 3TC, and efavirenz (EFV) has been shown to be safe and effective (Wu et al., 2016). However, despite these patient selection criteria, it remained difficult to identify proteins that are related to HIV/HBV coinfection, thus warranting the use of novel technologies with high throughout and sensitivity.

Proteomics has been used to identify biomarkers for HIV (Zhang et al., 2010; Luo and Muesing, 2014) and HBV infections (Branza-Nichita et al., 2014; Xie et al., 2014), while some proteomic studies have focused on HIV/HCV coinfections (Shetty et al., 2011). Among identified proteins, Apolipoprotein A-II (APOA2), Apolipoprotein C-II (APOC2), Apolipoprotein E (APOE), Complement C3 (C3), and Histidine-rich glycoprotein (HRG) were found to be associated with HIV/HCV coinfection (Shetty et al., 2011). Hence, comparative proteomic technologies for differential proteins that are associated with HIV or HBV infection may facilitate identification of diagnostic biomarkers and drug targets (Luo and Muesing, 2014; Sun et al., 2009). However, comparative proteomic technologies have not yet been used to identify proteins with HIV/HBV mediated alterations that can be used as biomarkers. Therefore, in this study we investigated differential proteins in plasma samples from HIV/HBV coinfected patients compared to HIV mono-infected patients using iTRAQ based proteomic technologies. These analyses identified 7 proteins that were down-regulated in HIV/HBV coinfected patients as compared to HIV mono-infected patients, and these were highly enriched in complement and coagulation signaling pathways. Finally, the proteins C9 and KLK were verified using enzyme linked immunosorbent assay (ELISA).

Methods

Reagents

Hu-14 Multiple Affinity Removal Spin Cartridges (MARS Human-14) were purchased from Agilent technologies (Santa Clara, CA, USA). Strong cation exchange chromatography columns (SCX; 5 μm, 2.1 × 100 mm Polysulfethyl) were purchased from The Nest Group Company (Southborough, MA, USA) and Zorbax300SB-C18 columns (0.3 × 5 mm) were purchased from Agilent Technologies (Santa Clara, CA, USA). 4-plex iTRAQ kit was obtained from Applied Biosystems (Foster City, CA, USA). Formic acid (HPLC grade), ammonium bicarbonate, iodoacetamide, dithiothreitol (DTT), sequencing grade modified trypsin, and the protease inhibitor PMSF were obtained from Sigma (St. Louis, MO, USA). Deionized water from a MilliQ RG ultrapure water system (Millipore, MA, USA) was used in all procedures.

Ethics statement

All procedures involving human plasma were approved by the Shanghai Public Health Clinical Center Ethics committee and written informed consent was obtained from all participants prior to blood collection.

Clinical samples

Recruited patients included 10 HIV mono-infected and 10 HIV/HBV coinfected patients who had not been treated with HAART. Another 10 HIV infected and 10 HIV/HBV coinfected patients who had been treated with HAART for more than 6 months were recruited. HIV and HIV/HBV patient groups were age and gender matched, and 5-mL samples of EDTA anti-coagulated blood were drawn and the plasma was separated. The patients' clinical information is presented in Table 1.

Immunoaffinity depletion of high abundance proteins from human plasma

Low abundance proteins were enriched using a MARS Hu-14 column (Agilent, Santa Clara, California, USA), which removed 14 higher abundance proteins with 94–99% of total plasma proteins, including albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha 2-macroglobulin, alpha 1-macroglobulin, IgM, apolipoprotein A1, apolipoprotin A2, complement C3, and transthyretin, as described previously (Pan et al., 2011; Yadav et al., 2011). In these experiments, 10 μL human plasma samples were diluted 20-fold with buffer A and were filtered through a 0.22-μm spin filter (1.0 min, 14,000 × *g*). Non-bound protein fractions were collected and columns were washed twice with buffer A and were then centrifuged (2.5 min, 100 × *g*). Bound fractions were washed from cartridges with 2.5 mL of buffer B, and non-bound protein fractions were pooled and concentrated using a Nanosep 3K Omega Centrifugal Device (Pall Life Sciences, Port Washington, USA). Collected solutions were acetone precipitated and were lysed in lysis buffer containing 7 M urea, 2 M Thiourea, 50 mM Tris, 50 mM DTT, and 1 mM PMSF, and protein contents were determined using

Table 1
Biochemical data and drug treatments of the four patient groups.

	Without HAART		With HAART		p value[a]
	HIV mono	HIV/HBV	HIV mono	HIV/HBV	
Number	10	10	10	10	–
Age	35.2 ± 6.0	35.5 ± 6.3	39.6 ± 6.9	36.2 ± 9.4	0.914/0.371
Sex	Male	Male	Male	Male(9) Female(1)	1.000/0.343
CD4	282.3 ± 101.8	241.8 ± 149.2	232.3 ± 132.2[b] (489.2 ± 100.1)[c]	234.4 ± 104.3[b] (573.9 ± 115.3)[c]	0.487/0.969
Treatment time	NO	NO	19.3 ± 4.8 months	23.0 ± 3.9 months	0.248

HAART, TDF + 3TC + EFV.
[a] *p* values indicate significant differences between HIV and HIV/HBV patient groups without and with HAART treatments.
[b] Baseline data before treatment.
[c] Data are from within one week of sample collection after treatment for 6 months.

the Bradford assay (Bio-Rad, USA). Samples were then stored at −80 °C for proteomic studies.

iTRAQ labeling and fractionation of labeled peptides

iTRAQ labeling was performed according to the kit protocol (Applied Biosystems Inc., Foster City, CA) and as previously described (Wang et al., 2011; Zhang et al., 2011). Pooled protein samples (100 μg) from each condition were then reduced alkylated and digested with trypsin at 37 °C overnight. Digested samples were then labeled using a 4-plex iTRAQ kit (Figure 1). Samples from HIV and HIV/HBV patient groups with and without histories of HAART treatment for more than 6 months were labeled with iTRAQ tags 114, 113, 121, and 119, respectively. Then the mixed labeled peptides were separated into 10 compounds using SCX liquid chromatography at a constant flow rate of 0.2 mL/min with a 60-min gradient according to previously reported methods (Jiang et al., 2014). SCX solvent A contained 25% acetonitrile and 10-mM KH_2PO_4 (pH 2.6), and SCX solvent B contained 10-mM KH_2PO_4, 25% acetonitrile, and 350-mM KCl (pH 2.6). The 60-min gradient was started with 100% A for 5 min, and was followed by a linear gradient from 5% to 25% B over 35 min, further change to 80% B over 5 min and maintenance at 80% B for 5 min, and then a final return to 100% A over 10 min. The 10 collected SCX fractions were then desalted using a C18 cartridge (Sep-Pak C18 1 cc Vac Cartridge, Waters, Milford, USA) and were dried in a vacuum concentrator for subsequent nano-liquid chromatography-mass spectrometry (LC–MS/MS) analysis.

LC–ESI MS/MS analysis using 4600 Q-TOF or Q Exactive mass spectrometry

Liquid chromatography-electrospray ionization (LC–ESI) MS/MS analyses of iTRAQ-labeled peptides were performed as described previously (Jones et al., 2013). Briefly, dried peptide fractions (4 μL) were resolved in solvent A (0.1% formic acid in H_2O) and were analyzed on a nano-HPLC system (Eksigent Technologies, Dublin, California, USA) using tandem AB SCIEX Triple TOF 4600 (SCIEX, USA) or QExactive (Thermo Electron Corporation, Waltham, MA, USA) mass spectrometry. Elusion solvents included 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Peptides were loaded onto a trap column (Zorbax 300SB-C18, 0.3 × 5 mm; Agilent Technologies, Wilmington, DE, USA) at a flow rate of 20 μL/min, and were separated on a 10-cm resolving analytical BioBasic C18 PicoFrit column (inner diameter, 75 μm, New Objective, Woburn, MA). Peptides were then eluted at a flow rate of 250 nL/min using gradients of solvent B from 3% to 30% over 62 min, from 30% to 45% over 70 min, and then from 45% to 97% over 75 min. Peptides were detected using Triple TOF 4600 (Applied Biosystems, FosterCity, CA, USA) or QExactive mass spectrometry (Thermo Fisher, San Jose, CA, USA).

Quantification of relative protein abundance

Protein identification and quantification were performed according to previously reported methods (Shilov et al., 2007; Lu et al., 2008). Briefly, MS and MS/MS tolerances were set at 0.2 Da and proteins were searched using the Swiss-Prot database. Cysteins were modified with methyl methanethiosulphonate as a fixed modification, and one missed tryptic cleavage was allowed. Peptide reporter channel intensities were individually summed for each protein using ProteinPilot version 2.0.1 (ABI company, USA). False discovery rates (FDRs) were determined at spectrum, peptide, and protein levels. Critical FDRs of less than 1% were used for quantification and ratios were built based on isobaric tags 114 or 121 for all identified proteins in each iTRAQ experiment. Ratios of 113 (HIV/HBV without HAART) to 114 (HIV without HAART), and 119 (HIV/HBV with HAART) to 121 (HIV with HAART) were calculated. All identified proteins had 95% confidence and the protein confidence threshold cutoff was set at 1.3 (unused) with at least one peptide above the 95% confidence level ($p < 0.05$). Error factor (EF) and P value are calculated using ProteinPilot software which gave an indication of the deviation and significance in the protein quantification. True values for average ratios were expressed as error factors (EF = 10; 95% confidence interval). Protein quantification required an EF of less than 2 and a p value of <0.05, and only fold-changes of >1.5 or <0.66 were considered significant. Data were exported and saved as Excel files.

Bioinformatic analyses

Functions and locations of identified differential proteins were analyzed using uniprotkb (http://www.uniprot.org), and Gene

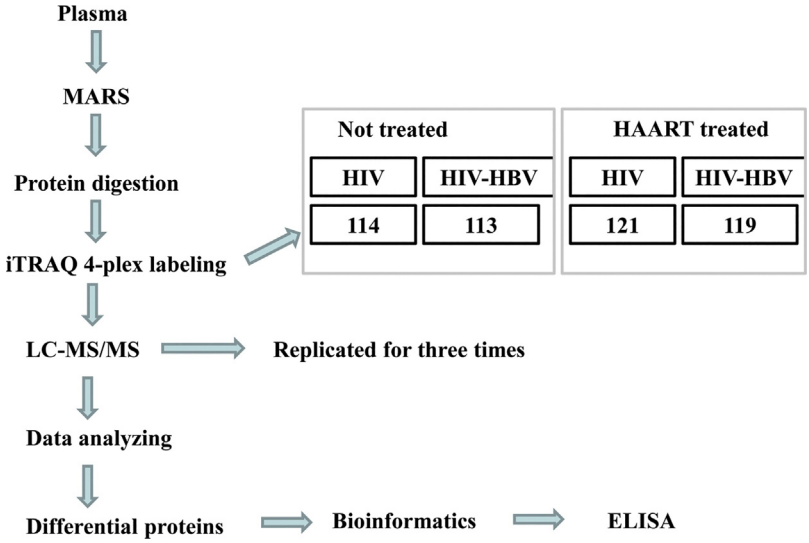

Figure 1. Workflow of iTRAQ based proteomic study. Samples from HIV, HIV/HBV infected patients without HAART treatment were labeled with 114 and 113, respectively. Samples from HIV, HIV/HBV patients with HAART treatment were labeled with 121 and 119, respectively.

Ontology (GO) database annotations were collected. Biological process (BP), molecular component (CC), and molecular function (MF) analyses of differential proteins were performed using a web-accessible tool of the DAVID 6.7 program (https://david.ncifcrf.gov/). BP-2, CC-ALL, and MF-ALL in the Gene Ontology from Functional Annotation Chart were selected. Only items with $p < 0.0001$ were collected for GO analysis. Protein–protein interaction analyses were performed using STRING 10 software (http://string-db.org/) (Szklarczyk et al., 2015). Relevant items were selected based on statistical significance ($p < 0.001$).

Detection of C9 and KLK proteins using ELISA

ELISA was performed using commercially available ELISA kits according to the manufacturer's instructions (Shanghai Boyan Biological Technological Company, Shanghai, China). Ranges of C9 and KLK were 0.4–20 μg/mL, and 1.5–50 ng/mL, respectively, and 10-μL plasma samples were diluted to 50 μL using sample dilution solution. The enzyme reaction was stopped after a 30-min incubation at 37 °C in 96-well plates, and reaction plates were then washed 5 times. Subsequently, plates were incubated with enzyme labeled reagent for 30 min, and with color-substrate solution for another 10 min at 37 °C. Finally, optical densities (OD) were read at 450 nm within 15 min using an enzyme-labeled instrument (Gene Company Limited, Shanghai, China).

Results

Experimental design

To identify proteins that are related to HIV/HBV coinfection, HIV/HBV coinfected patients with and without histories of HAART treatment were enrolled. Enriched low abundance proteins from a total of 40 plasma samples were representative of the four clinical conditions. As shown in Figure 1, iTRAQ based proteomic studies were performed using isobaric reporter ions, 113 is for HIV/HBV without HAART, 114 is for HIV without HAART, 119 is for HIV/HBV with HAART, 121 is for HIV with HAART. Bioinformatic analyses revealed a number of potential biomarkers and two were verified by ELISA.

Clinical information

Demographic data and clinical characteristics of all HIV mono-infected and HBV co-infected subjects are presented in Table 1. No differences ($p > 0.05$) in age, sex and CD4 were detected between HIV/HBV and HIV infected patients with or without HAART treatment. Average HAART treatment times for HIV/HBV and HIV groups were 23 and 19.3 months ($p = 0.248$), and HAART regimens in the two groups included TDF, 3TC, and EFV.

Enrichment of low abundance proteins

Due to the overlay of higher abundance proteins, it is difficult to define lower abundance proteins with potential as disease biomarkers. Thus, we enriched low abundance proteins from total plasma samples using MARS kits. Plasma samples from the four groups were treated using MARS cartridges, and were then mixed and separated using SDS-PAGE. As shown in Figure 2, higher abundance proteins including albumin and IgG were significantly decreased after this procedure, resulting in enrichment of some novel protein bands in lower abundance fractions compared with those in total plasma. Furthermore, band patterns of protein enrichment or removal in low, high, and total plasma samples from HIV/HBV and HIV infected patients were similar (Figure 2).

Identification and quantification of proteins related to HIV/HBV co-infection

As shown in Figure 1, plasma samples of HIV infected and HBV/HIV coinfected patients were analyzed using iTRAQ 4-plex techniques. We compared differences in protein abundance between plasma samples from patients with HIV/HBV coinfection or HIV infection alone with or without HAART treatment. Using iTRAQ labeling and LC–MS/MS analysis, 269, 299, and 272 proteins were identified in 3 replicated experiments (see Supplementary material Table S1 in the online version at DOI: 10.1016/j.ijid.2017.08.006). Significantly changed proteins ($p < 0.05$ and $EF < 2$) were selected for further evaluations. 33 and 15 differential proteins were found in un-treated (113/114) or HARRT-treated (119/121) HIV/HBV samples compared to HIV mono-infected samples (see Supplementary material Table S2 in the online version at DOI: 10.1016/j.ijid.2017.08.006), in which 7 of them were consistently up- or down-regulated in both un-treated (113/114) or HARRT-treated (119/121) groups.

One protein with opposite differential levels was excluded. A total of 41 differential proteins between HIV/HBV and HIV groups were identified. Among these combined differential proteins, 7 proteins were found to be differential in both HAART treated (119/121) groups and no treated (113/114) groups (Table 2 and Figure 3A).

Bioinformatic analysis of differential proteins

To investigate HIV regulatory mechanisms of HBV, a total of 41 differential proteins from the experiments with and without

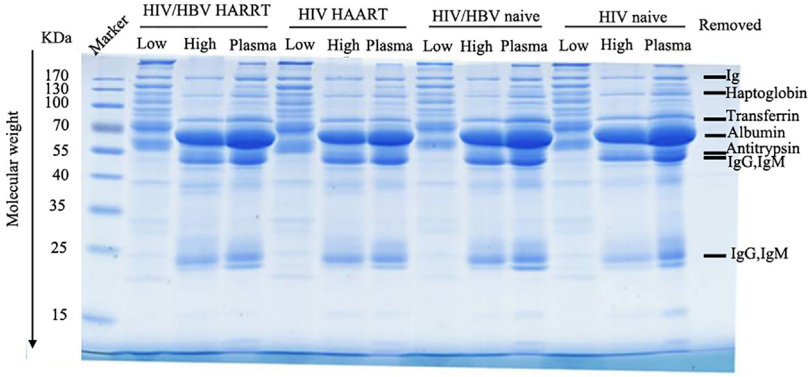

Figure 2. Immunoaffinity depletion of high abundant proteins from plasma samples. Fractions were subjected to SDS-PAGE and stained by Coomassie brilliant blue. Low, high and plasma represent fraction of lower abundant proteins, removed higher abundant proteins, and untreated plasma respectively.

Table 2
Details of 7 differential proteins related to HIV/HBV coinfection.

Protein name	Accession number	Ratio-B (113/114)	Ratio-A (119/121)	Peptides (95%)	% Cov (95%)	Subcellular location	Function	Gene name in interaction network
Haptoglobin	P00738	0.5	0.62	23	36.45	Secreted	Antioxidant activity; hemoglobin binding	HP
Alpha-1-antitrypsin	P01009	0.62	0.65	7	19.62	Secreted; ER	Glycoprotein binding; identical protein binding protease binding; serine-type endopeptidase inhibitor activity	SERPINA1
Alpha-1-antichymotrypsin	P01011	0.57	0.65	150	60.76	Secreted	DNA binding; serine-type endopeptidase inhibitor activity	SERPINA3
Complement component C9	P02748	0.50	0.59	37	27.19	Secreted; cell membrane; multi-pass membrane protein	Complement activation; alternative pathway	C9
Serum albumin	P02768	0. 60	0.61	27	33.66	Secreted	Antioxidant activity; chaperone binding; copper ion binding	ALB
Coagulation factor XI	P03951	6.27	1.72	11	15.68	Secreted	Heparin binding; serine-type endopeptidase activity	F11
Plasma kallikrein	P03952	0.60	0.57	45	38.71	Secreted	Serine-type endopeptidase activity	KLKB1

HAART treatment (see Supplementary material Table S2 in the online version at DOI: 10.1016/j.ijid.2017.08.006,Figure 3A) were analyzed using DAVID software (https://david.ncifcrf.gov/). As shown in Figure 3B, proteins that were mainly involved in response to external stimulus (n = 19), responses to stress (n = 21), and regulation of biological qualities (n = 17), had *p*-values of 7.1E − 12, 2.8E − 9, and 7.6E − 7, respectively. In analyses of the cellular components of these proteins (Figure 3C), almost all were localized in extracellular spaces (n = 33, $p = 8.7E-23$), extracellular regions (n = 22, $p = 4.2E-16$) and extracellular spaces (n = 19, $p = 5.5E-15$). Furthermore, according to GO annotations, only 4 proteins had collagen binding activity and all other proteins had various enzyme activities ($p < 0.0001$; Figure 3D). Protein–protein interaction analyses were performed using STRING 10 software

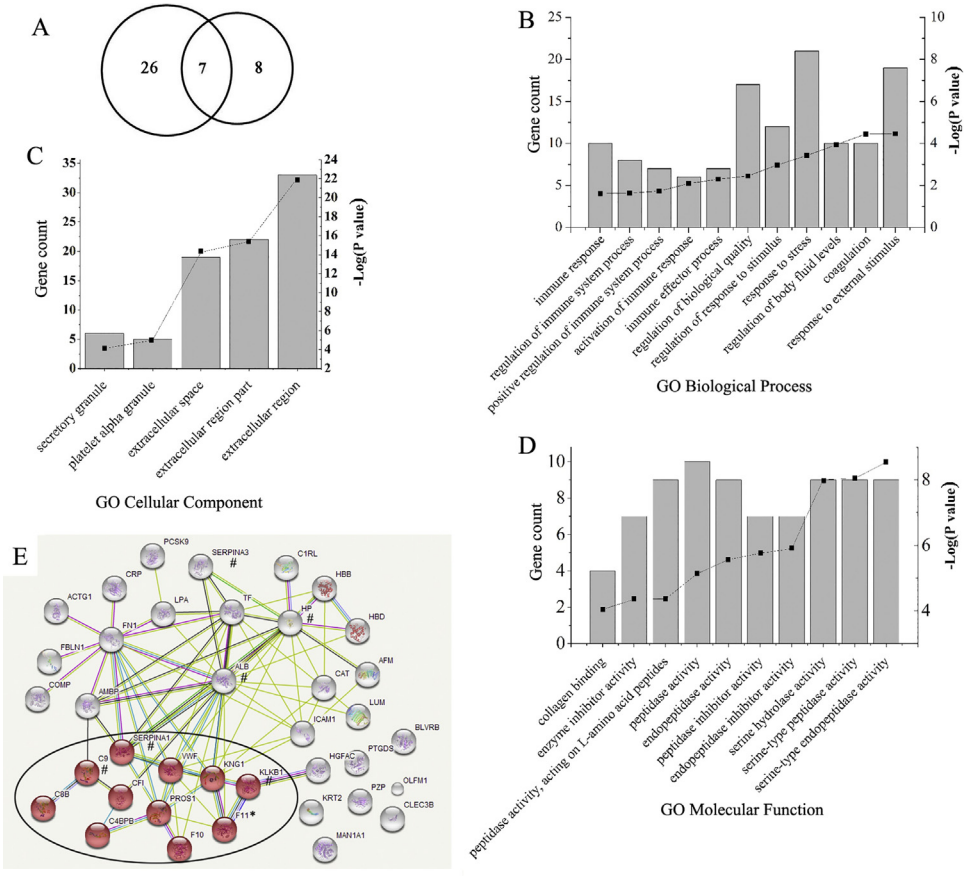

Figure 3. Bioinformatic analyses of differential proteins. (A) Venn diagram of differential proteins found in un-treated (HIV/HBV co-infection compared to HIV mono-infection) and HARRT treated (HIV/HBV co-infection compared to HIV mono-infection) patient plasma samples; (B) GO biological processes enrichment analysis; (C) GO cellular component enrichment analysis; (D) GO molecular function enrichment analysis; (E) protein–protein interaction network of differential proteins. Proteins highlighted in red were involved in complement and coagulation signaling pathways. 7 proteins consistently regulated in both HARRT treated and un-treated HIV/HBV co-infection samples are indicated with # or *. #, down-regulated proteins; *, up-regulated proteins.

(http://string-db.org/), and 4 pathways were significantly enriched. Among these, 11 proteins (shown in red) were involved in complement and coagulation pathways, and 10 of these were downregulated (Figure 3E and see Supplementary material Table S2 in the online version at DOI: 10.1016/j.ijid.2017.08.006).

In addition to brief analyses of the 41 differential proteins, we performed comprehensive analyses of 7 proteins that were consistently up- or down-regulated in HIV/HBV co-infection plasma samples with or without HARRT treatment. According to GO annotations, all 7 were secreted proteins with antioxidant (n = 2) or enzyme activating or inhibiting (n = 5) activities (Table 1). Moreover, 4 of these 7 proteins were involved in complement and coagulation pathways (Figure 3E).

Verification of identified differential proteins

ELISA analyses were performed to verify the differential abundance of the proteins C9 and KLK, which were located in complement and coagulation pathways. C9 levels in HIV/HBV co-infected naive patients (n = 10, replicate once) were significantly lower (1.5 fold) than in those with HIV infections alone ($p = 0.004$; n = 20, Figure 4A). A similar decrease (1.3 fold) was found between HIV/HBV and HIV patients that were treated with HAART, although this difference was not significant ($p = 0.512$; n = 20, Figure 4A). Among HIV/HBV co-infected patients without HAART treatments, KLK levels were decreased by 1.4-fold ($p = 0.006$, n = 20) compared to HIV infections alone. However, no differences between HAART treatment statuses were observed among patients with HIV infections alone ($p = 0.214$, n = 20; Figure 4B). These data suggest that decreased levels of C9, and KLK are indicative of HIV/HBV co-infection.

Discussion

In this study, we detected low abundance proteins using a multiple affinity removal system to reduce the complexity of the plasma proteome from HIV/HBV coinfected and HIV mono-infected patients. Subsequently, 14-high-abundant proteins from human biological fluids were greatly decreased by MARS Hu-14, which improved the resolution and dynamic range for proteomic analyses. This technology has been applied successfully for investigations of low abundance members of plasma/serum proteomes and has led to the discovery of biomarkers (Silbiger et al., 2011; Chromy et al., 2014; Johansen et al., 2009; Darde et al., 2007). The present data showed that some lower abundance protein bands were enriched after removing higher abundance proteins. However, fewer than 300 proteins were detected in each experiment, which was similar to a previously reported paper in which only 284 proteins were detected in MARS treated plasma (Tu et al., 2010). This might be due to two reasons: (1) There are potential solvent effects in the MARS kit. Therefore, in future studies, solvents may be removed by pre-dissociation of protein extracts using SDS-PAGE. (2) Some lower-abundant proteins were removed along with immunoaffinity depleted proteins (Yadav et al., 2011). It will be better to study both enriched and depleted fractions from MARS depleted plasma samples.

To further investigate the mechanisms of HIV/HBV co-infection, we characterized the 41 identified proteins in terms of subcellular locations, molecular functions, and biological processes. According to GO database annotations, with the exception of 5 proteins having the location of alpha granule proteins from platelets, all proteins had extracellular or secretory locations. In particular, all 7 differential proteins had subcellular secretory locations, which indicated that these identified proteins were from plasma samples and were not from blood cell contaminants. Most of these proteins have the function of enzymatic activities and were categorized into biological processes such as external stimuli. All 7 proteins had binding, enzyme, and antioxidant activities, indicating that HBV may affect liver function by regulating enzyme activities.

In further analyses of HIV/HBV coinfected patients, alanine aminotransferase and aspartate aminotransferase levels were elevated and CD4 cell counts were much lower than in HIV mono-infected patients (Thio et al., 2013; Zhang et al., 2014; Huang et al., 2016). These data suggest that pathways or biological processes were activated or inhibited by HBV in co-infected patients. We therefore performed analyses of the 41 differentially regulated proteins between HIV/HBV coinfected and HIV mono-infected patients using KEGG pathway software. These experiments identified 11 proteins (including C8B, C9, SERPIAN1, PROS1, KNG1, KLKB1 etc.) that are involved in complement and coagulation signaling pathways (see Supplementary material Figure S1 in the online version at DOI: 10.1016/j.ijid.2017.08.006). The complement system is a proteolytic cascade in blood plasma and acts as a mediator of non-specific innate defenses against pathogens. The main consequences of complement activation

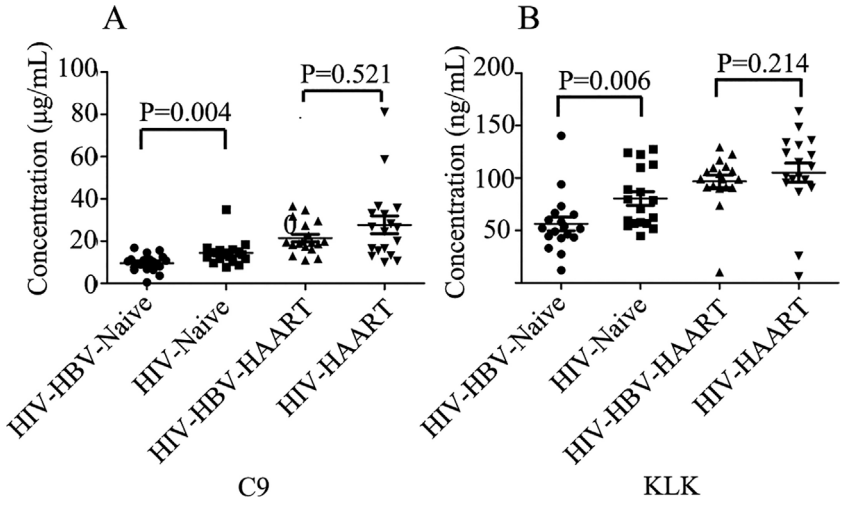

Figure 4. Verification the abundance of C9 and KLK in plasma by ELISA. C9 and KLK levels in HIV/HBV co-infected patients were lower than in those with HIV infections alone in both HARRT treated and un-treated circumstances.

include opsonization of pathogens, recruitment of inflammatory and immunocompetent cells, and direct killing of pathogens. Thus, HBV might inhibit activation of the complement system via a pathway that involves all of these proteins during HBV coinfections. Accordingly, the node proteins in the protein-protein interaction network such as the complement component C9 (C9), and plasma kallikrein (KLKB1) were significantly decreased in patients with HIV/HBV coinfections. Among these, C9 is a membrane protein and is a major host defense against pathogens. It is also involved in the pathogenesis of a variety of liver disorders, and in liver injury and repair (Wagner and Frank, 2010; Joller et al., 2011; Kim et al., 2013). C9 is a component of membrane attack complexes (MAC) and forms pores on bacterial cell surfaces (Lumry et al., 2013). In addition to this, KLKB1 was down-regulated and has many important roles in complement activation, inflammatory responses and modulation of blood pressure (Tang et al., 2005). Due to its important functions, plasma kallikrein has become an important drug target (Chyung et al., 2014), and its inhibitors (Lumry et al., 2013; Aygoren-Pursun et al., 2016) are being developed into drugs. Kallikrein is also a biomarker for hereditary angioedema and septic shock (Walport, 2001; Shariat-Madar and Schmaier, 2004). HBV infection targets host hepatocytes. We assume it is possible that liver might be the source organ of these differential proteins we found in plasma. Further research should be done to investigate the expression of these proteins in liver cells and to illustrate the role of complement and coagulation signaling pathways in HBV co-infection.

To confirm that complement and coagulation cascades are down-regulated by HBV in HIV/HBV co-infected patients, we verified down regulation of the two main node proteins C9 and KLKB1 in HIV/HBV coinfected naive patients compared to HIV mono-infected patients. However, no differences were found in the abundance of these proteins in HAART treated patients. Furthermore, although we removed high-abundant proteins, antitrypsin levels were detected to be less in HIV/HBV co-infected patients than in HIV mono-infected patients. These observations may reflect incomplete removal of higher abundant proteins including antitrypsin. Hence, further studies are required to optimize the enrichment method for lower abundant proteins in plasma and verify the relationship between these complement proteins and HBV.

Conclusion

In conclusion, the present iTRAQ based proteomic analyses discovered several proteins related to HIV/HBV coinfection. Specifically, 41 proteins were up- or down-regulated between patients with HIV/HBV coinfections and those with HIV mono-infections. Of these, 7 proteins were consistently differential in the patients with HIV/HBV co-infection compared to HIV mono-infection with or without HAART treatment. Subsequent bioinformatic analyses indicated that complement and coagulation signaling pathways are regulated by HBV in HIV/HBV coinfected patients. In particular, C9 and KLK were identified as potential biomarkers that are regulated by HBV in HIV/HBV coinfected patients. However, further experiments are required to validate these proteomic alterations in larger numbers of patient samples.

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Ethical approval

Written consent was obtained from each participant. The study design was approved by Shanghai Public Health Clinical Center ethics committee.

Conflict of interest

The authors declare no conflicts of interest.

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