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Proteomic analysis in peritoneal dialysis patients with different peritoneal transport characteristics

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ABSTRACT

Peritoneal membranes can be categorized as high, high average, low average, and low transporters, based on the removal or transport rate of solutes. In this study, we used proteomic analysis to determine the differences in proteins removed by different types of peritoneal membranes. Peritoneal transport characteristics in patients who received peritoneal dialysis therapy were assessed by a peritoneal equilibration test. Two-dimensional differential gel electrophoresis technology followed by quantitative analysis was performed to study the variation in protein expression from peritoneal dialysis effluents (PDE) among different groups. Proteins were identified by MALDI-TOF-MS/MS analyses. Further validation in PDE or serum was performed utilizing ELISA analysis. Proteomics analysis revealed ten protein spots with significant differences in intensity levels among different groups, including vitamin D-binding protein, complement C3, apolipoprotein-A1, complement factor C4A, haptoglobin, alpha-1 antitrypsin, immunoglobulin kappa light chain, alpha-2-microglobulin, retinol-binding protein 4 and transthyretin. The levels of vitamin D-binding protein, complement C3, and apolipoprotein-A1 in PDE derived from different groups were greatly varied ($P < 0.05$). However, no significant difference was found in the serum levels of these proteins among different groups ($P > 0.05$ for all groups). This study provides a novel overview of the differences in PDE proteomes of four types of peritoneal membranes. Vitamin D-binding protein, complement C3, and apolipoprotein-A1 showed enhanced expression in PDE of patients with high transporter.

1. Introduction

Peritoneal dialysis (PD) is a highly effective, convenient and reasonably safe treatment modality for patients with end-stage renal disease (ESRD) [1,2]. The therapy is based on the ability of the peritoneal membrane to function as a dialyzing membrane, allowing exchange of solutes and waste products between the peritoneal dialysis effluents (PDE) and the circulation. PDE is installed in the peritoneal cavity via a permanent catheter and contains an osmotic agent, mostly glucose, which facilitates fluid movement from the bloodstream to the peritoneal cavity, leading to removal of metabolic waste products and water [3]. PD also offers the unique opportunity for access to and analysis of PDE in patients. Emerging studies have taken on this challenge and produced preliminary proteomic analysis on PDE [4–9].

ESRD patients who received chronic PD treatment have largely different solute transport and ultrafiltration rates, suggesting varied removal or transport rate of solutes in peritoneal membranes [10]. According to peritoneal equilibration test (PET) results, patients are classified into four groups each representing a different peritoneal transport characteristic: high (H), high average (HA), low average (LA), and low (L) transporters, [10]. In an initial study of the proteome profile of PDE obtained from ESRD patients, the levels of five proteins, including serum albumin in a complex with myristic acid and triiodobenzoic acid, alpha1-antitrypsin, complement component C4A, immunoglobulin kappa light chain, and apolipoprotein A1, were found to be greatly differed among patients with different types of peritoneal membranes [7].

In this study, we performed protein profiling of PDE obtained from patients who received PD therapy using a sensitive and accurate two-dimensional differential gel electrophoresis (2D DIGE) technology [11]. Differentially-expressed proteins were analyzed and identified. Our results provide valuable insights into understanding the differences in proteins removed by different types of peritoneal membranes.

2. Materials and methods

2.1. Patients

The study included a total of 76 patients who received initial insertion of PD catheters at the Peritoneal Dialysis Center,

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Department of Nephrology, The First Affiliated Hospital of Sun Yat-sen University. Patients with the following characteristics were included in the study: (1) primary chronic glomerulo-nephritis; (2) age 18–65; (3) had undergone regular PD therapy for one month; (4) serum albumin level $\geqslant$ 35 g/L; (5) residual renal function (RRF) $\geqslant$ 2 mL/min/1.73 m^2; (6) 500 mL/d $\leqslant$ urine output $\leqslant$ 1000 mL/d. Patients with the following characteristics were excluded: (1) secondary renal diseases; (2) PD-associated peritonitis during therapy; (3) congestive heart failure, angina pectoris, myocardial infarction, severe valvular heart disease, malignant hypertension, hypertensive encephalopathy, cerebrovascular disorders, chronic liver diseases, cachexia or mental diseases; (4) abuse of alcohol or drugs. Written informed consent was obtained from each participant. The First Affiliated Hospital of Sun Yat-sen University approved the experimental protocols. The demographic and clinical characteristics of patients, including age, gender, weight, calculated BMI, height, dialysis duration, and 4 h ultrafiltration volume were recorded.

2.2. Peritoneal equilibration test (PET)

Peritoneal transport characteristics in patients were assessed by standard PET as described previously [10]. Serum creatinine, serum albumin, blood urea nitrogen and glucose levels were determined in subjects within one month after PD therapy. All patients were classified into four transport categories based on dialysate-to-plasma ratio of creatinine (D/Pcr), including high (*H*) (>0.80), high-average (HA) (0.65–0.80), low-average (LA), (0.50–0.65) and low (*L*) ($\leqslant$0.50).

2.3. Measurements

RRF, Kt/V, creatinine clearance (CCl), normalized protein catabolic rate (nPCR) and D/Pcr values were determined using PD adequest 2.0 software (Baxter Healthcare, Norfolk, UK).

2.4. Sample preparation for proteomic analysis

PDE samples were collected from patients at the end of the PET study and were prepared by precipitation with 75% acetonitrile (ACN) and removal of high abundance proteins as described previously [10]. All the samples were centrifuged within 30 min of collection at 10,000*g* for 20 min to remove insoluble solids. The clear supernatants were stored at −80 °C until use. Cold ACN (−20 °C) was added to the PDE samples to a final concentration of 75%. The mixture was stored overnight at −20 °C and then centrifuged at 10,000*g* at 4 °C for 15 min to obtain a pellet. The pellets were dissolved with a solubilizing buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 2% (v/v) ampholytes (pH 4–7). Albumin and IgG were removed from the sample/lysis buffer solution using ProteoPrep Immunoaffinity Albumin and IgG Depletion kit according to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO, USA). Protein concentration was determined with the 2-D Quant kit (GE Healthcare).

2.5. Protein labeling

Protein extracts were labeled using the fluorescent cyanine dyes (CyDye) developed for two-dimensional differential gel electrophoresis (2-D DIGE) technology following the manufacturer's recommended protocols (GE Healthcare), as described previously [12]. In brief, the internal standard was prepared by combining equal portions of each of the four test samples. Sample proteins (50 μg) were labeled with Cy3 or Cy5, and the internal standard mixture with the same protein amount was labeled with Cy2. Mixed solutions were incubated on ice for 30 min in the dark. The reactions were then quenched with the addition of 1 mL of 10 mM lysine for 10 min on ice in the dark. The quenched Cy3- and Cy5-labeled samples and the Cy2-labeled internal standard were pooled prior to 2-DE analysis. Meanwhile, two preparative gels, each containing 500 mg of unlabeled internal standard mixture proteins, were analyzed.

2.6. 2-D DIGE technique

Immobilized drystrips (linear pH gradient of 4–7, 24 cm long; GE Healthcare) were rehydrated for 12 h in 450 mL rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% v/v IPG buffer pH 4–7) using an Ettan IPGphor IEF system (GE Healthcare). Once rehydration was complete, samples were focused at 500 V for 1 h, 1000 V for 1 h, and 8000 V for a total of 65,000 V $*$ h at 20 °C. Strips were incubated in equilibration buffer I (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris–HCl (pH 8.8), 1% DTT) for 15 min at room temperature. The strips were then soaked in equilibration buffer II (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris–HCl (pH 8.8), 2.5% iodoacetamide) for an additional 15 min. The equilibrated strips were embedded in 0.5 w/v agarose on top of 12.5% acrylamide slab gels. Second dimension separations were performed on an Ettan DALTsix electrophoresis system (GE Healthcare). The proteins were separated initially at 15 °C, 2 W/gel for 50 min, followed by 17 W/gel until the dye front reached the bottom of the gel. Two preparative gels were stained with deep purple total protein stain (RPN6306, GE Healthcare). Sample gels, internal standard gels, and preparative gels were individually imaged using a Typhoon Variable Mode Imager 9400 (GE Healthcare). The following emission filters with a resolution of 100 μm were used: 488 nm (Cy2), 532 nm (Cy3), 633 nm (Cy5), and 560 LP (deep purple).

2.7. Protein identification

The DeCyder differential in-gel analysis (DIA) module allowed for a direct comparison of the intensities of specific protein spots between test samples to the internal standard within the same gel. The DeCyder biological variation analysis (BVA) module was then used simultaneously to match all 12 protein-spot maps from the four DIGE gels. The standardized spot intensity was then averaged across the triplicate gel. The final values for the expression ratio of specific protein spots between four groups was then determined as described [13]. The protein spots of interest were excised manually from the gels and protein identification was performed with an Ultraflex III mass spectrometer (Bruker Daltonics, Bremen, Germany). A standard peptide mixture with a mass range 800–4000 Da (Bruker Daltonics) was used for external calibration. The subsequent MS/MS analysis was performed in a data-dependent manner, and the five most abundant ions fulfilling certain pre-set criteria were subjected to LIFT for a post-source decay analysis. Peptide mass fingerprints (PMFs) and MS/MS analyses were searched by the BioTools software (version 3.0, Bruker Daltonics) against the SwissProt protein database. Protein identifications were accepted when the peptide score was higher than the threshold value ($P < 0.05$), and manual interpretation was used to confirm agreement between the spectra and peptide sequence.

2.8. Enzyme-linked immunosorbent assay (ELISA) analysis

The levels of vitamin D-binding protein, complement C3 and apolipoprotein A-I in PDE were measured using ELISA (Assay Pro, St. Charles) according to the manufacturer's instructions. 100 μl of anti-vitamin D-binding protein polyclonal antibody (1:1000 dilution) and 100 μl of anti-complement C3 polyclonal antibodies (1:1000 dilution) or anti-apolipoprotein A-I polyclonal antibodies (1:1000 dilution) were added to 100 μl of each sample (1:400

dilution for all the three proteins in serum; 1:3 dilution for complement C3 in PDE; 1:20 dilution for vitamin D-binding protein in PDE; 1:100 dilution for apolipoprotein A-I in PDE). After development with a chromogen-substrate solution, the reaction was terminated by adding 100 μl of stop solution. Optical density values were read at 450 nm and concentrations were automatically calculated according to the standard curve.

2.9. Statistical analysis

Data were presented as the case numbers or means ± SD. Enumeration data were compared with χ^2 test. Homogeneity of variance tests was conducted for the measurement data. If the data accord with normal distribution and homogeneity of variance one-way ANOVA or independent *t* test was performed, otherwise a rank sum test was used. Statistical analyses were performed using the Statistical Package for Social Science program, version 16.0 (SPSS Inc. Chicago, IL, USA). A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Demographic and clinicopathological characteristics of patients with different peritoneal transport properties

A total of 76 patients who received PD therapy were enrolled in this study, and 27 subjects who met the inclusion criteria were recruited for proteomic analysis. According to their D/Pcr value, these patients were classified into H (6 cases), HA (8 cases), LA (8 cases) and L (5 cases) transporters. The demographic and clinicopathological properties of these patients were summarized in Table 1. No significant difference was found in the gender, age, BMI value, dialysis duration, serum albumin, serum triglyceride, serum cholesterol levels, RRF, Kt/V, creatinine clearance, or normalized protein catabolic rate among patients with different peritoneal transport properties. However, the H and HA transporters had significantly greater albumin levels in PDE than LA and L transporters. Moreover, the 4-h ultrafiltration volumes in H and HA transporters were significantly lower than those in LA and L transporters.

3.2. Proteomic analysis

The representative 2-D gels of proteins derived from the PDE of patients with different peritoneal transport properties are shown in Fig. 1. Quantitative intensity analysis using DIA and BVA of the data obtained from four groups indicated that 10 protein spots presented significantly different intensity levels among different groups. PMFs and MS/MS analyses were applied to identify these proteins. As shown in Table 2, the majority of these proteins were identified as acute phase reactants, including complement C3, complement factor C4A, haptoglobin and alpha-1 antitrypsin. Besides, vitamin D-binding protein, apolipoprotein A-I, immunoglobulin kappa light chain, alpha-2-microglobulin, retinol-binding protein 4 and transthyretin were also identified.

3.3. ELISA analysis

Next, we expanded our sample size, and evaluated the expressions of vitamin D-binding protein; complement C3 and apolipoprotein-A1 in PDE and blood serum derived from patients with different peritoneal transport properties. For this purpose, H (11 cases), HA (17 cases), LA (13 cases) and L (8 cases) transporters were included for ELISA analysis. As shown in Fig. 2, the levels of all three proteins in PDE derived from H transporters were significantly increased as compared with those derived from HA, LA and L transporters. In addition, HA transporters showed a significant increase in PDE complement C3 and apolipoprotein A-I concentrations when compared to LA and L transporters. However, no significant difference was found in the serum levels of these proteins among different groups. The expressions of other identified proteins in patients with different peritoneal transport properties have also been determined, whereas the altered levels of these proteins were not as significant as vitamin D-binding protein, complement C3 and apolipoprotein A-I (data not shown).

4. Discussion

Emerging lines of evidences suggest that increased peritoneal membrane transport is associated with decreased survival and poor outcomes for patients undergoing continuous PD therapy [14,15]. A meta-analysis indicates a summary mortality relative risk of 1.15 for every 0.1 increase in the D/Pc, and compared with patients with low transport status; the mortality risk for high transporters in increased by 77.3% [16]. Patients with a greater rate of membrane solute transport are hypothesized to have enhanced small solute clearance and protein loss, leading to malnutrition and diminished fluid removal [16]. The proteome profile of PDE obtained from ESRD patients who have different types of peritoneal membranes was first determined in 2007 [7]. Here, the following five differentially-expressed proteins were identified in patients

Table 1
Demographic and clinicopathological characteristics of patients with different peritoneal transport properties.

	H	HA	LA	*L*	*P* value
Cases	6	8	8	5	
Male	3	5	4	1	0.449
Female	3	3	4	4	
Age (yr)	44.00 ± 12.57	39.67 ± 9.99	34.33 ± 10.03	42.40 ± 16.50	0.543
BMI (kg/m^2)	24.27 ± 6.52	20.85 ± 3.31	19.91 ± 2.07	22.62 ± 2.65	0.243
Dialysis duration (d)	104.17 ± 57.27	58.67 ± 34.53	37.67 ± 21.50	58.00 ± 38.18	0.055
sALB (g/L)	40.99 ± 5.21	38.85 ± 3.57	37.42 ± 2.17	38.72 ± 3.50	0.927
sTG (mM)	1.79 ± 0.95	1.80 ± 0.70	1.52 ± 0.76	2.56 ± 0.28	0.526
sCHO (mM)	4.03 ± 1.04	4.40 ± 1.63	5.13 ± 1.52	5.45 ± 1.77	0.542
PDE albumin (mg/L)	1204.50 ± 171.54	832.34 ± 203.07	760.61 ± 478.11	679.14 ± 164.41	**0.042**
4 h Ultrafiltration vol. (mL)	86.67 ± 117.58	317.78 ± 162.08	378.33 ± 135.41	490.00 ± 44.16	**<0.001**
RRF (mL/min/1.73 m^2)	4.24 ± 1.87	2.71 ± 0.68	3.02 ± 0.76	2.81 ± 0.73	0.353
Kt/V	3.14 ± 1.10	2.02 ± 0.71	2.36 ± 0.22	1.66 ± 0.36	0.056
CCL (L/wk/1.73 m^2)	124.01 ± 37.55	85.35 ± 17.75	98.09 ± 12.04	60.45 ± 13.51	0.062
nPCR (g/kg/d)	0.81 ± 0.10	0.93 ± 0.22	0.91 ± 0.09	0.73 ± 0.15	0.488

BMI, body mass index; sALB, serum albumin; sTG, serum triglyceride; sCHO, serum cholesterol; PDE, peritoneal dialysate effluent; RRF, residual renal function; CCL, creatinine clearance; nPCR, normalized protein catabolic rate. *P* values lower than 0.05 were considered significant, and indicated in bold.

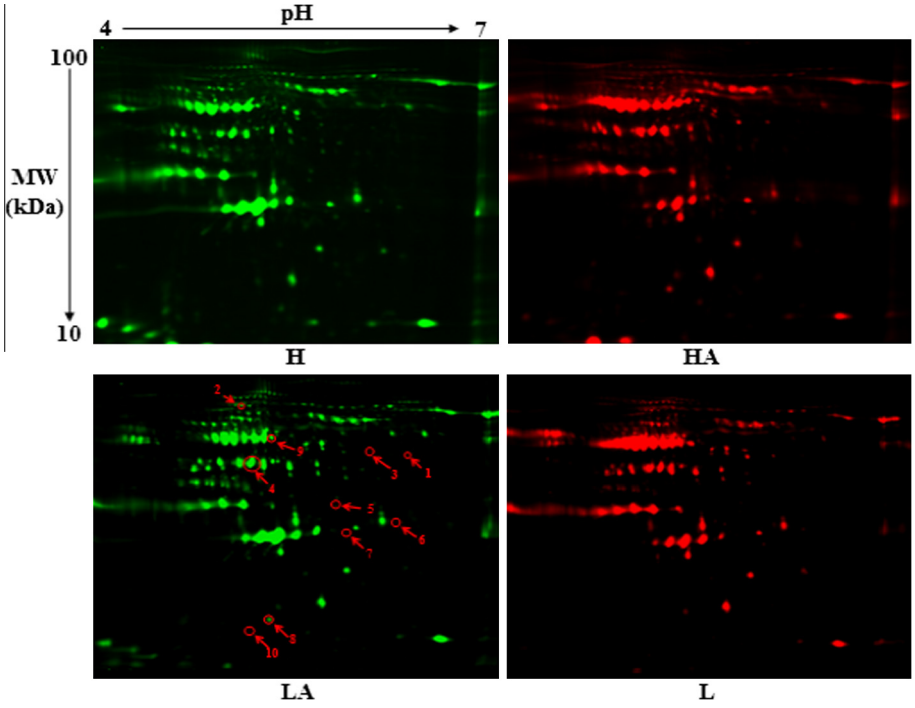

Fig. 1. Representative 2-D gel images of PDE proteins derived from different types of peritoneal membranes. Protein samples derived from H and LA groups were labeled with Cy3 (green); samples from HA and L groups were labeled with Cy5 (red). Quantitative intensity analysis revealed 10 protein spots whose intensity levels significantly differed among groups. These protein spots (circled and indicated with numbers) were subsequently identified by PMFs and MS/MS analyses (Table 2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Proteins identification using MALDI-TOF MS/MS.

Protein ID	Accession no.	Mr (kDa)	pI	Protein score	Protein name
1	P01026	31.36	6.02	54	Complement C3
2	P00751	86.85	6.67	32	Complement factor C4A
3	P02774	54.523	5.40	31	Vitamin D-binding protein
4	P00738	45.86	6.13	46	Haptoglobin
5	P02874	30.85	5.26	62	Apolipoprotein A-I
6	P09610	24.92	5.53	57	Immunoglobulin kappa light chain
7	O43866	38.09	5.50	64	Alpha-2-microglobulin
8	P02753	23.34	5.76	83	Retinol-binding protein 4
9	P01372	46.29	5.37	78	Alpha-1 antitrypsin
10	P00843	15.88	5.52	166	Transthyretin

pI, isoelectric point.

with different types of peritoneal membranes: serum albumin in complex with myristic acid and triiodobenzoic acid, alpha1-antitrypsin, complement component C4A, immunoglobulin kappa light chain, and apolipoprotein A-I [7]. Peritoneal transport characteristics change significantly within the first month of PD therapy, while the PET measurements conducted more than one year following PD commencement generally agreed closely with one-month measurements [17]. Thus, we conducted a 2-D DIGE analysis using PDE samples derived from patients with different peritoneal membrane transporters and identified 10 proteins with altered expressions. We found the numbers and category of the identified expression proteins differed from that reported by Sritippayawan *et al.* These differences might be due to the different proteomic technology applied; precipitation with 75% ethanol, no removal of high abundance proteins, and 2-D gel analysis were used in their study. However, we precipitated the samples with 75% acetonitrile, removed the high abundance proteins, and used the more sensitive 2-D DIGE technique. The samples precipitated with ACN had the greatest number of protein spots visualized in 2-DE gels and removal of abundant PDE proteins facilitated the detection of less abundant proteins [10].

In this study, the majority of the proteins with differed expressions were confirmed as acute phase reactants, such as complement C3, complement factor C4A, haptoglobin and alpha-1 antitrypsin. Acute phase reactants are closely associated with infections and inflammatory diseases [18]. A cross-sectional study of a cohort of 84 patients with ESRD who were receiving continuous ambulatory peritoneal dialysis (CAPD) for a minimum of 36 months shows that H and HA transporters had higher C-reactive protein (CRP) levels when compared with the L and LA types [19]. Additionally, the high transporter peritoneal membrane characteristic is confirmed as a risk factor for inflammatory state in patients with ESRD [19]. Consistent with these findings, we also found increased acute phase reactants, such as complement C3, in patients with high transporter peritoneal membranes. ELISA analysis further revealed that the level of complement C3 was significantly enhanced in PDE of H transporters as compared with HA, LA and L transporters. These data, combined with previous studies linking acute phase reactants to H and HA transporters, suggest that the high transporter peritoneal membrane characteristic could be linked to the inflammatory status. High-density lipolipoprotein (HDL)-associated apolipoprotein A-I is recognized as a negative acute phase reactant, whose level is lowered by more than 25% during the acute phase [20]. The decrease of plasma levels of HDL-associated apolipoprotein A-I upon acute inflammation is a sign of the possible development of chronic inflammation [21]. In contrast, we found that the PDE level of apolipoprotein A-I is also increased in high transporters. Future studies are necessary to explore the potential correlation between elevated apolipoprotein A-I levels and high transporter peritoneal membrane characteristics.

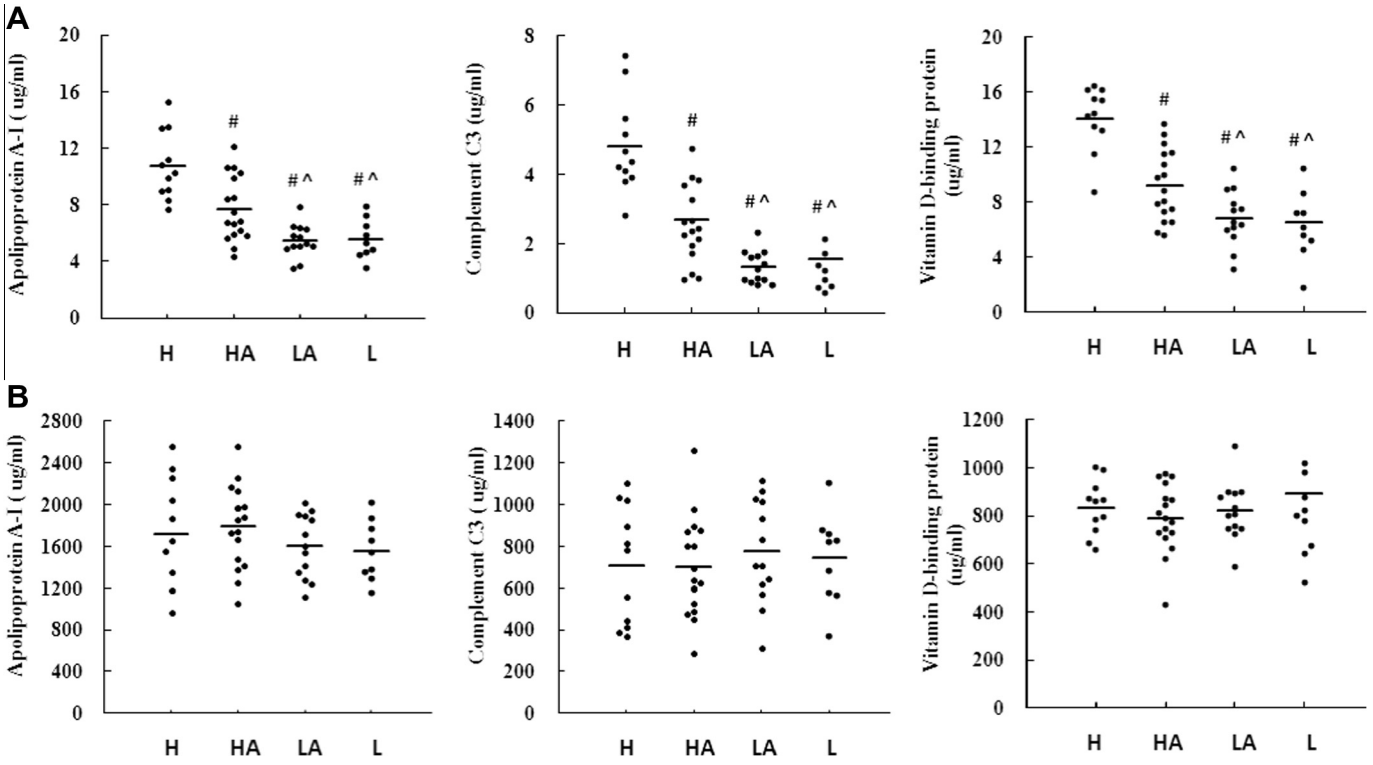

Fig. 2. Vitamin D-binding protein, complement C3 and apolipoprotein-A1 levels in PDE and serum in a validation set of 49 patients. The number of cases in each group was as follows: H, 11 cases; HA, 17 cases; LA, 13 cases; L, 8 cases. The level of vitamin D-binding protein, complement C3 and apolipoprotein-A1 in PDE (A) and serum (B) was determined by ELISA. $^{*}P < 0.05$ vs. H; $^{\wedge}P < 0.05$ vs. HA. No significant difference was found in the serum protein levels among different groups ($P > 0.05$).

Vitamin D-binding protein is a polymorphic serum protein exerting many physiological, biochemical and molecular functions [22]. CAPD may lead to the loss of DBP, whereas the peritoneal loss of DBP is well compensated by biosynthesis in the liver and does not result in serum deficiency [23,24]. In accordance with these findings, we detected a significant increased level of vitamin D-binding protein in PDE of patients with high transporter peritoneal membrane characteristics as compared with other groups. Nevertheless, the serum level of this protein is well maintained under homeostasis and no significant difference was found among different groups of patients.

In summary, this study identified ten differentially-expressed proteins associated with the characteristics of peritoneal membrane transport. These results provide a framework for future studies, which will be conducted to investigate the association between these proteins and specific peritoneal membrane transport properties. Further, the correlation between the levels of these proteins and the outcomes of PD therapy will also be examined.

Conflict of interest

The authors have declared no conflict of interest.

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