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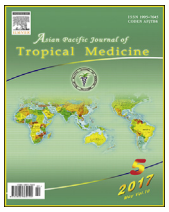

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Identification of novel biomarkers for adult-onset-immunodeficiency (AOID) syndrome using serum proteomics

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ABSTRACT

Objective: To identify the candidate protein biomarkers of adult-onset-immunodeficiency (AOID) syndrome using serum proteomics.

Methods: Screening and verification phases were performed in the study. A total of 97 serum samples were classified into three groups: AOID patients with opportunistic infections (active AOID), AOID patients without opportunistic infections (inactive AOID), and healthy control. In the screening phase, pooled sera collected from patients and healthy control in each group were separated by 2D-gel electrophoresis, analyzed for differentially expressed proteins and identified for biomarkers using LC/MS. In the verification phase, the protein candidates were selected for confirmation by western blotting.

Results: The analysis revealed 35 differentially expressed proteins. Three proteins including haptoglobin, gelsolin, and transthyretin, were selected for verification. The results showed that the levels of haptoglobin in both active and inactive AOID groups were significantly higher than that in the control group, while the levels of gelsolin in the active AOID group were significantly lower than that in the inactive AOID group. The level of transthyretin in the active AOID group was also significantly lower than that in the control group.

Conclusions: The comparison of serum proteins between the three groups revealed three candidates which are related to chronic inflammatory diseases. Haptoglobin and transthyretin biomarkers could be applied in clinical assessment for monitor of disease outcome, including for the study of AOID pathogenesis.

1. Introduction

Adult-onset-immunodeficiency (AOID) syndrome is an emerging syndrome which is associated with disseminated infections caused by common pathogenic organisms, for example, non-tuberculous mycobacteria, Salmonella species, *Burkholderia pseudomallei*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Penicillium marneffei*, and Varicella-zoster virus [1–3]. The syndrome has been reported in Thailand, Taiwan and China [1]. Autoantibodies against interferon-gamma (IFN-ϒ) may be the cause of immunodeficiency in the patients. The autoantibody was detected in approximately 88% of the patients with multiple opportunistic infections. A further study reported the prevalence of the autoantibody in northern Thailand, with prevalence of 100% in non-HIV patients who were repeatedly infected with unusual intracellular pathogens. In addition, patients with active opportunistic infections had significantly greater mean

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concentrations of the antibody to IFN-ϒ, approximately 3.5-fold compared to cases without active opportunistic infections [4]. The mechanism of disease remains obscure and several studies have attempted to elucidate the pathogenesis of this syndrome. Genetic factor is one of the possible explanations that associated with this condition. HLA-DRB1*16:02, DQB1*05:01 [5,6], HLADRB1*15:01, and DQB1*05:01 [5] are significantly associated with disseminated opportunistic infections with acquired anti-IFN-ϒ autoantibody. Another factor that plays a crucial role in the immunodeficiency condition of these patients is cell-mediated immunity (CMI). It has been reported that AOID cases had reduced production of both IL-2 by CD4 T cells and TNF-α by CD4 and CD8 T cells [7]. Recently, the AOID group was reported to have increased white blood cells, monocytes, and natural killer cells [8]. However, the IFN-ϒ and TNF-α cytokine production was found to have been up-regulated and the levels of IL-4 and IL-17 were observed to remain unchanged upon TCR activation. For these reasons, CMI cascades in AOID patients still require further studies.

Since the anti-IFN-ϒ autoantibody is the only biomarker of AOID, this project aimed to compare differentially expressed proteins in sera between active AOID cases, inactive AOID cases, and healthy control. The proteins were subsequently identified using LC/MS and verified between new enrolled active AOID cases, inactive AOID cases, and healthy control by western blotting. The differentially expressed proteins obtained from this study could be used as potential biomarkers in long-term follow-up for AOID patients.

2. Materials and methods

2.1. Study design and participants

The patients included in the study consisted of 27 active AOID and 40 inactive AOID patients who were followed up at Maharaj Nakorn Chiang Mai Hospital. Additional 30 healthy individuals were recruited as control. This study is a part of a project entitled, 'Targeted research proposal on the integrated studies for diagnosis and treatment of anti-interferon-gamma antibody-related adult-onset immunodeficiency in northern Thailand.' The study was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University (105/2557), and the Human Experimentation Committee, Research Institute for Health Sciences (RIHES), Chiang Mai University (13/56). Written informed consent was obtained from all participants from before any of the procedures was initiated. Briefly, serum samples were obtained on the day of the scheduled patient follow-up. Samples were prepared and screened for HIV virus antibody (anti-HIV), CD4, and anti-IFN-ϒ autoantibody levels. All the serum samples were stored at −70 °C until analysis. The inclusion criteria for AOID cases are male or female; negative for anti-HIV antibody; presented with at least one episode of culture or histopathology proven infections caused by unusual intracellular pathogens; for example, non-tuberculous mycobacteria, disseminated fungal infections, non-typhoidal Salmonella bacteremia; and positive for the anti-IFN-ϒ antibody. Active AOID patients are AOID cases that have symptoms and signs of infections as in the criteria, whereas inactive AOID patients are AOID cases that have no symptoms or signs of any infections. The inactive AOID cases were still on treatment for the prevention and treatment of opportunistic infections. Healthy controls are male or female, negative for the anti-HIV antibody and anti-IFN-ϒ autoantibody, no history of mycobacterial infection, no active infection during the past 1 month, and no underlying medical conditions that may compromise the immune status.

2.2. Experimental design

The serum samples for screening and verification were analyzed separately in the present study. In the screening phase, the serum protein of the active AOID group (n = 12), the inactive AOID group (n = 12), and the healthy control group (n = 12) were prepared as three pools per group for biological replicates, and then each group of pooled samples was divided into three aliquots and processed as technical replicates. The data for each pool were obtained by averaging the results from the three technical replicates. In the verification phase, the protein biomarkers were selected for confirmation by western blotting in duplicate using serum that consisted of the active AOID group (n = 15), the inactive AOID group (n = 28), and the control group (n = 18).

2.3. Two-dimensional gel electrophoresis (2-DE) and liquid chromatography–mass spectrometry analysis

Albumin was removed from the sera in each of the pools using the ProteoExtract Albumin removal kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's recommendations. Then, the protein concentrations were measured by the Bio-Rad Bradford total protein assay kit (Bio-Rad Laboratories, United States) using bovine serum albumin as the standard curve. Then 300 μg of protein was mixed with 340 μL of rehydration buffer (8 M urea, 4% CHAPS, 0.001% bromophenol blue, and 3 mM dithiothreitol) containing 1% (3–10) NL IPG buffer. The sample was loaded on to 18-cm IPG strips with a pH range of (3–10) NL of an isoelectric focusing system (Ettan IPGphore III). The samples were run through the steps of strip rehydration (20 °C, 16 h) and isoelectric focusing (500 V for 500 V-h, 1 000 V for 800 V-h, and 10 000 V to reach 36 000 V-h). The maximum current was maintained at 75 μA per strip. After the complete process was accomplished, the strip was equilibrated twice (15 min each) in equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.03% bromophenol blue) supplemented with 65 mM DTT and 135 mM iodoacetamide to allow the cysteine residues to be reduced, and then made to undergo carbamidomethylation. The strip was subjected to the second dimensional separation (Ettan DALTsix) using an SDS-polyacrylamide gel (12.5%). The separation of protein was executed under an applied voltage of 10 W per gel at 20 °C until the bromophenol blue dye front reached 0.5 cm from the bottom of the gel. The gels were stained with colloidal Coomassie blue staining according to the standard recommendation. The differentially expressed protein spots were removed from the blot and subjected to in-gel tryptic digestion according to the method modified from [9]. The peptide mixtures were analyzed by Dionex Ultimate 3000 (Thermo Scientific) in combination with an electrospray ionization (ESI)/quadrupole ion trap mass spectrometer (amaZon SL, Bruker Daltonik, Germany). The LC separation was performed on a reversed phase column (Hypersil GoLD 50 mm × 0.5 mm, 5 μm C18), protected by a guard column, eluted at a flow rate of 100 μL/min under gradient conditions of 5%–80% B over 50 min. Mobile phase A consists of water/formic acid (99.9: 0.1, v/v), and B consists of acetonitrile (100, v). The mass spectral data from 150 m/z to 1 500 m/z were collected in the positive

ionization mode. The MS/MS spectrometry data were searched against the NCBI database using the MASCOT search engine, as described elsewhere [10].

2.4. Verification of proteomic data by western blotting

For protein preparation and western blotting, sera of active/inactive AOID and control was individually albumin depleted, as described above, and made into a concentrate using a Vivaspin 2 ultrafiltration column with a 10 kDa molecular weight cut-off (GE Healthcare, Buckinghamshire, UK) in accordance with the manufacturer's recommendations. The protein concentration was determined as before and the samples were stored at −70 °C. SDS-PAGE and western blot analysis were employed to determine the enrichment of the sera. A quantity in the range of (10–20) μg of concentrated sera was separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and analyzed by Western blotting with antibodies against transthyretin (pre-albumin), transferrin, gelsolin, and haptoglobin (all from Abcam plc, Cambridge, UK). The secondary antibodies used were a goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a goat anti-mouse IgG peroxidase (Sigma–Aldrich, Milwaukee, WI). ECL detection was performed according to the manufacturer's protocol (Merck KGaA, Darmstadt, Germany). The antibody against transferrin was applied as the internal control. Image J software was employed to determine the optical density values of bands for relative comparisons. The experiment was performed in duplicate.

2.5. Statistical analysis

The data were expressed as mean ± SEM. To compare the clinical parameters and the expression level of the biomarker proteins between active/inactive AOID and control, the statistical significance of the intensity of the bands was determined using one-way ANOVA with Bonferroni's multiple comparison tests using the Prism 5 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characteristics of study population

The participants were classified based on the two phases: screening phase and verification phase (Table 1). As far as the screening phase is concerned, the age and sex ratios between the patients and the healthy controls were found to be comparable in all the three groups. In addition, the CD4 count of active AOID group was not statistically different from the inactive AOID group, whereas the anti-IFN-ϒ autoantibody of the active AOID group was significantly higher than those of the inactive AOID and the control groups. In the case of the verification phase, 15 active AOID, 28 inactive AOID, and 18 healthy controls were recruited. The age and sex ratios demonstrated similar results as found in the screening phase. There was significant difference in the CD4 count between the active and the inactive cases. The anti-IFN-ϒ autoantibody of the active AOID group was also significantly higher than those of the inactive AOID and the control groups.

3.2. Serum proteomic analysis

Prior to 2D gel electrophoresis, serum samples were divided into three pools for biological replicates. Each pooled sample was subsequently divided into three equal aliquots and processed as technical replicates. The data for each pool were obtained by averaging results from the three technical replicates. Equal quantities of the pooled sera were albumin depleted, measured for protein concentration, and cleaned up for being subjected to 2D gel electrophoresis. The proteomic profiles of the control, active AOID, and inactive AOID were analyzed. The criteria to define significant changes in protein abundance consist of (i) P values must be less than 0.05, (ii) the ratio of the percentage volume of active or inactive AOID compared to the control must be either more than 1.5-fold (significant increase) or less than 0.67 (significant decrease). Thirty-five protein spots were found to be differentially expressed between the three groups. The fact that in some spots we have found the proteins only once or twice in three biological replicates depends on the inter-individual differences on the protein pattern. Therefore only 11 spots that have been found in three biological replicates and have similar trends in the comparison between the three groups were selected and identified by mass spectrometry: haptoglobin, transthyretin, retinol-binding protein 4, anti-thrombin 3, gelsolin, prothrombin, and serum albumin (Table 2). The predicted and experimental values of isoelectric point (pI), molecular weight (MW), and relative expression levels for all the identified proteins are summarized in Table 2. The comparison between the active and the inactive AOID groups revealed that haptoglobin is the significantly up-regulated protein which showed increased expression levels (more than 1.5-fold compared to the inactive AOID group).

Table 1
Demographic and clinical data of healthy controls, active AOID patients, and inactive AOID patients in biomarker-screening phase and verification phase.

Groups	Age (years)		Sex (male/female)		Absolute CD4+ count (cells/mm^3)		Anti-IFN-ϒ autoantibody (Arbitrary unit/mL)	
	A	B	A	B	A	B	A	B
Control	53.50 ± 1.43	50.94 ± 1.15	6:6	4:14	ND	ND	1.933 ± 0.270	1.434 ± 0.163
Active AOID	56.92 ± 2.74	51.93 ± 1.89	6:6	9:6	787.58 ± 128.50	1042.36 ± 180.31	469.29 ± 155.02*	423.82 ± 107.28*
Inactive AOID	56.00 ± 2.07	52.50 ± 1.61	7:5	12:16	483.58 ± 74.54	620.28 ± 62.00$^{\#}$	46.44 ± 21.48$^{\#}$	107.35 ± 41.22$^{\#}$
P value	0.514	0.782	0.894	0.090	0.053	0.009	0.001 3	<0.000 1

A: Screening phase, $n = 12$ in each group. B: Verification phase, $n = 18$ in control group; $n = 15$ in active AOID group and $n = 28$ in inactive AOID group. Data are expressed as mean ± SEM. ND: not done. *$P < 0.05$ compared with control group. $^{\#}P < 0.05$ compared with active AOID group.

Table 2
Differentially expressed proteins in sera of healthy controls, active AOID patients, and inactive AOID patients, identified by LC/MS.

	Spot ID	Protein	Swiss-protein accession number	MS score	pI	MW	Relative intensity (mean ± SEM)			Ratio			*P* value
							Control	Active AOID	Inactive AOID	AP/CP	IP/CP	AP/IP	
Acute-phase response	No. 1	Haptoglobin	P00738	96	5.57	15.94	0.073 0 ± 0.000 2	0.527 ± 0.068	0.213 ± 0.107	7.22	2.92	2.47	0.033
	No. 25	Haptoglobin	Q9UC67	356	6.13	45.86	0.566 ± 0.027	1.097 ± 0.118	0.714 ± 0.143	1.94	1.26	1.54	0.024
	No. 26	Haptoglobin	Q9UC67	247	6.13	45.86	0.257 ± 0.004	0.564 ± 0.062	0.356 ± 0.098	2.20	0.63	1.58	0.024
	No. 36	Prothrombin	Q9UCA1	133	5.64	71.48	0.048 ± 0.003	ND	ND	–	–	–	<0.000 1
Transport protein	No. 2	Transthyretin	P02766	154	5.52	15.99	0.526 ± 0.081	0.217 ± 0.038	0.565 ± 0.100	0.41	1.07	0.38	0.036
	No. 7	Retinol-binding protein 4	P02753	127	5.76	23.34	0.154 ± 0.021	0.088 ± 0.003	0.157 ± 0.021	0.57	1.02	0.56	0.036
	No. 8	Retinol-binding protein 4	P02753	123	5.76	23.34	0.090 ± 0.015	0.049 ± 0.009	0.155 ± 0.014	0.54	1.72	0.32	0.003
	No. 31	Antithrombin-III	Q9UC78	189	6.32	53.02	0.227 ± 0.019	0.122 ± 0.005	0.226 ± 0.010	0.54	1.00	0.54	0.002
	No. 32	Serum albumin	Q86YG0	1187	5.92	71.32	3.066 ± 0.461	0.985 ± 0.170	1.803 ± 0.225	0.32	0.59	0.55	0.009
	No. 33	Serum albumin	Q86YG0	935	5.92	71.32	1.167 ± 0.019	0.566 ± 0.023	1.168 ± 0.017	0.48	1.00	0.48	0.000 3
Apoptosis, actin binding	No. 34	Gelsolin	Q8WVV7	218	5.90	86.04	0.105 ± 0.013	0.051 ± 0.003	0.113 ± 0.003	0.48	1.08	0.45	0.034

AP: active pools; IP: inactive pools; CP: control pools. ND: not detected.

Transthyretin, retinol binding protein-4, anti-thrombin III, albumin, and gelsolin are the significantly down-regulated proteins which showed decreased expression levels (less than 0.67-fold compared to the inactive AOID group). Haptoglobin has well-known functional roles as acute phase proteins. The remaining down-regulated proteins played important roles in transport and apoptosis.

3.3. Verification of biomarkers

Three proteins (haptoglobin, gelsolin, and transthyretin, or, as previously called, prealbumin) were then chosen for further protein verification. Haptoglobin and transthyretin have been reported as protein markers that are found in acute phase response to inflammation [11]. Gelsolin has been reported to have an association with autoimmune diseases and may be employed as a prognostic marker for inflammatory condition [12].

Densitometry analysis from western blot detection of haptoglobin, gelsolin, transthyretin and transferrin revealed that haptoglobin levels of the active AOID and the inactive AOID groups were significantly elevated when compared to the control group (Table 3). However, gelsolin and transferrin densitometric values showed no significant difference between the three groups, the result was not statistically significant. Transthyretin in the active AOID was significantly different from control and the inactive group. To standardize data analysis, the haptoglobin/transferrin, gelsolin/transferrin and transthyretin/transferrin ratios were calculated for each group, to allow more accurate comparison between the three groups (Figures 1 and 2). The relative haptoglobin levels (Figure 1A) demonstrated similar trend as in the pre-standardized results. In addition, the haptoglobin level in the active AOID group was comparable to that in the inactive AOID group. The level of serum gelsolin in the active group was significantly lower than that in the inactive group (Figure 1B). The analysis of transthyretin expression by western blot revealed that the transthyretin expression in the active group was significantly lower when compared to the healthy control group (Figure 2) and slightly lower than the inactive AOID group.

4. Discussion

Proteomic pattern is the most important breakthrough for the identification of proteins associated with diseases [13–16]. Therefore, this study was based on the screening, identification, and verification of proteins found in the sera of AOID patients for the identification of the proteins associated with the severity of the disease. These proteins might be employed as biomarker for evaluating the severity of the disease. The 2D-gel electrophoresis approach was chosen to investigate the differentially expressed proteins in the sera of active AOID, inactive AOID, and healthy control specimens. In this study, 11 differentially expressed proteins were identified in the AOID groups in comparison to the control, using LC-MS/MS, which suggests that many proteins are associated with the AOID syndrome. Although the number of sera of AOID patients and control are small, we employed sera specimens from different enrollment for screening phase and verification phase to increase the reliability of the assay results.

Haptoglobin is an acute phase protein produced mainly by the liver. This protein engages in non-covalent interaction with hemoglobin. Because of this, the hemoglobin-haptoglobin complex is rapidly cleared by the reticuloendothelial system in the liver [17]. The functional properties of haptoglobin consist of anti-oxidative capacity, defense against pathogenic bacteria, and inhibition of prostaglandin synthesis, including control of immunity and inflammation [18]. A significant increase in the haptoglobin level in autoimmune diseases has been previously reported in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [19,20]. Haptoglobin has been previously reported as one of the biochemical markers for evaluating the disease activity of patients with RA [20]. In addition, a decreased level of haptoglobin has been reported as a biomarker in predicting the outcome of methotrexate treatment in RA patients [21]. In our study, we observed a significant increase in the level of haptoglobin in active AOID and inactive AOID patients compared to healthy controls (Figure 1A), suggesting that chronic inflammation did occur in AOID patients, thereby increasing the haptoglobin level. As there is no specific treatment for AOID diseases, the treatment at present is based on personal symptoms and patient follow-up. The level of haptoglobin in the active AOID patients was not significantly different from that of the inactive group even though the level was observed to have slightly decreased in the latter group after receiving symptomatic treatment, suggesting no disease activity. Therefore, haptoglobin can be a predictive biomarker after treatment and could be further developed as an appropriate drug.

Table 3

Densitometry Analysis of haptoglobin, gelsolin, transthyretin and transferrin in serum samples of the healthy controls and the AOID patients.

Group	N	Haptoglobin	Gelsolin	Transthyretin	Transferrin
Control	18	68.006 ± 2.954	47.399 ± 4.488	86.946 ± 4.556	62.142 ± 3.482
Active AOID	15	86.448 ± 5.355*	32.038 ± 4.411	68.068 ± 4.006*	60.473 ± 4.246
Inactive AOID	28	85.566 ± 2.877*	45.870 ± 5.125	83.300 ± 3.853#	63.344 ± 1.753

The mean density values are expressed as mean ± SEM. *$P < 0.05$ compared with control group. #$P < 0.05$ compared with active AOID group.

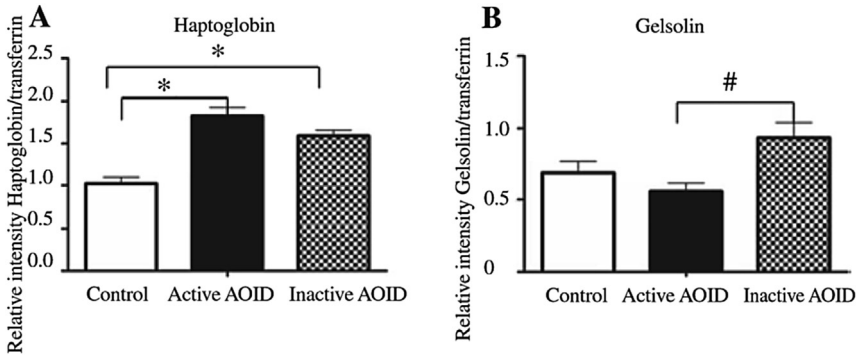

Figure 1. Western blot analysis of haptoglobin (A) and gelsolin (B) expressions in the serum of the healthy controls and the AOID patients. The data are plotted as mean ± SEM. *$P < 0.05$ compared with control group.#$P < 0.05$ compared with active AOID group.

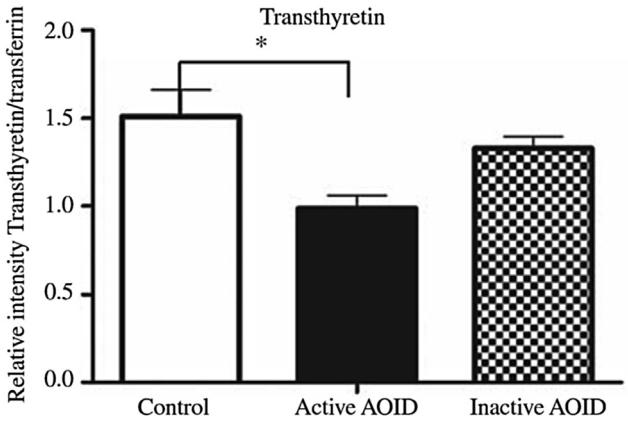

Figure 2. Western blot analysis of transthyretin expression in the serum of the healthy controls and the AOID patients.
The data are expressed as mean ± SEM. *P < 0.05 compared with control group.

The mechanism to explain the increased haptoglobin level in active AOID patients is based on several reasons as haptoglobin is a multifunctional protein. When there is an inflammation due to autoimmune reactions, haptoglobin participates in all the processes including polymorphonuclear leukocyte recruitment, and tissue repair and regeneration [18]. Haptoglobin also modulates the balance between Th1 and Th2. Expression of haptoglobin has been associated with diverse inflammatory autoimmune diseases, for example, SLE, celiac disease, diabetes type I, and inflammatory bowel diseases [22]. Basically, there are two alleles of haptoglobin proteins: HP^1 and HP^2. The HP^1 allele is predominant in West Africa, East Africa, and South America, while HP^2 is predominant in North America, Europe, Australia, and Asia [23]. There are evidences of Hp2-2 being over-represented in autoimmune diseases such as RA and SLE [24,25]. Haptoglobin 2-1 and haptoglobin 2-2 phenotypes have been reported to be present in 37.1% and 54.8% in Thai population. Both the phenotypes have weaker hemoglobin binding, antioxidative capacity, and inhibition of prostaglandin synthesis than the Hp1-1 phenotype [26]. Therefore, the severity of symptoms in AOID patients can be explained using the model of the role of haptoglobin in inflammatory response [18]. In general, this is after danger signals from stressed cells induce the expression of IL-6 which, in turn, induces the expression of Hp. Hp of Hp1-1 significantly decreases the ROS generation, Hb-binding, and anti-inflammatory role, thereby triggering Th2-dominant response initiating healing and repair. In contrast, our subjects are likely to have possessed the Hp2-2 phenotype; therefore, the persistence of inflammation caused by weak ROS led to Th1 response and increased oxidative stress. The weak hemoglobin binding, in addition to the antioxidant and pro-inflammatory activity of Hp2, led to the Th1-dominant cytokine expression. There was an evidence of up-regulated IFN-ϒ and TNF-α cytokines by Th1 of patients with IFN-ϒ autoantibody which strongly support the model [8]. Haptoglobin genotype modulated AOID pathogenesis requires further studies.

Another candidate identified in this study is gelsolin. Gelsolin is a calcium-regulated actin-binding protein involved in severing and capping proteins [27]. There are two forms of gelsolin: intracellular and secreted [28]. The secreted form differs from the cytoplasmic form in that it has 24 amino acid signaling peptides. Reduced plasma gelsolin levels have been reported in autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic carditis [12,29–31]. In addition, gelsolin was employed as the diagnostic marker of acute rheumatic carditis and might be further developed as the prognostic marker since there is a correlation between gelsolin level and inflammatory condition [12]. The mechanism of blood plasma gelsolin depletion is not known. The possible reasons include cellular damage in the inflammatory condition causing the release of actin into the blood stream. Then, the gelsolin function involves fast severing and removal of the actin filaments. In addition, gelsolin accumulation has been reported at the injury sites of its interaction with the released actin, for example, in the inflamed brain of multiple sclerosis mouse

models [32]. Gelsolin-actin complexes in the synovial fluid of patients with RA have also been reported [29]. We found lower serum gelsolin levels in the active AOID group than in the inactive AOID group but not with the control group (Figure 1B). Although gelsolin could not totally correlate with disease activities but remain of interest for possible future validation in larger cohorts of patients. The possible reason of gelsolin depletion in the active group is that it could bind abundant actin in cells exposed by tissue breakdown including bioactive inflammatory mediators as reported in other inflammatory disease [33] and the increase of gelsolin in the inactive group suggested that supportive treatment based on the symptoms at the present can ameliorate systemic injury and inflammation thereby no cell destruction and no release of actin from damaged cells. Clinical application of serum gelsolin in therapy has reported in multiple sclerosis mouse models. Gelsolin administration in mouse decreased the extracellular actin and disease activity, and increased the survival rates [32]. At present gelsolin administration is a standard treatment in many diseases [33–35]. In Rheumatoid arthritis treatment, the principle is usually based on the monitoring of plasma gelsoin level as predetermined level. A level of gelsolin in patients at or below the predetermined level is indicative that the subject is at an elevated risk of developing the inflammatory disease. In addition, other cytokine markers of inflammation should be also evaluated. Only the subjects having or at risk of developing an inflammatory diseases will get effective amount of gelsolin. Therefore, gelsolin administration approach might establish itself as a further therapeutic strategy in AOID disease treatment.

Transthyretin, or prealbumin, is a tetrameric protein secreted by the liver. This protein is involved in the dual transportation of the thyroid hormone and vitamin A, and also has peptidase activity [36,37]. Serum transthyretin is a nutritional biomarker. Low transthyretin levels have been associated with inadequate protein calorie consumption or malnutrition. It is also employed as a prognostic biomarker as well as an index for tracking the adequacy of nutritional supplementation [11,38]. In addition, the level of transthyretin in the serum is affected by a nutrition-independent mechanism, the systemic inflammatory response syndrome (SIRS) [39]. The syndrome represents an acute physiologic stress reaction to various conditions, including major trauma, burns, and infection. The mechanism of SIRS is mediated by proinflammatory cytokines such as TNF, IL-1, IL-12, IFN-gamma, and IL-6, thereby increasing the hepatic synthesis of acute phase proteins like haptoglobin and CRP, and decreasing the synthesis of transthyretin. Low serum transthyretin levels in critically ill trauma patients have been associated with poor clinical outcomes [40]. Active AOID patients are infected by disseminated infections caused by common pathogenic organisms, thereby lowering the transthyretin level, in comparison with inactive and control groups. For autoimmune diseases, transthyretin has been employed as the specific biomarker for RA [41]. High levels of transthyretin in the serum were reported in severe RA compared to non-severe and healthy control. In addition, transthyretin is specific and can be applied for RA diagnosis since the level of transthyretin in the plasma of RA patients was 6.6-fold and 5-fold more than the levels of OA and SLE, respectively. Unlike the observation for RA, the active AOID group was found to have increased haptoglobin levels but decreases transthyretin levels; therefore, transthyretin in the autoimmune condition still requires further study. Our 2D-gel electrophoresis and LC/MS demonstrated new finding by identifying proteins involved in acute phase response; haptoglobin and transthyretin. This finding may lead to the application of C-reactive protein [42] which is a marker protein for inflammation as an additional biomarker for a determination of disease progress in AOID patients. Normally, C-reactive protein assay is highly cost-effective which is also appropriate in resource-limited settings. Therefore C-reactive protein, erythrocyte sedimentation rate (ESR) and simple biomarkers *e.g.* complete blood cell count including blood chemistry test will be further studied and applied for routinely used in clinical practice. In our study, we employed transferrin as the internal loading control for the comparison of protein loading of control, active and inactive AOID groups. Transferrin is loading control commonly used for serum western blot. Normally, inflammation can lower plasma transferrin concentration approximately 30–60% [43]. We determined the expression of transferrin in serum by comparing mean ± SEM of densitometric values of the three groups by image J analysis from western blots and found no statistically significant difference between the three groups (Table 3). Therefore, transferrin is likely to be the appropriate loading control in this study.

In summary, the comparison of proteins in sera between active AOID patients, inactive AOID patients, and healthy controls revealed three biomarkers, namely haptoglobin, transthyretin, and gelsolin, which are related to chronic inflammatory diseases. Haptoglobin and transthyretin are potential biomarkers that could be applied in clinical assessment for the monitoring of disease severity, including the study of AOID pathogenesis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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