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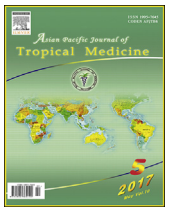

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Identification of novel biomarkers for adult-onset-immunodeficiency (AOID) syndrome using serum proteomics

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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