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Proteomic analysis of CD4+ T-lymphocytes in patients with asthma between typical therapy (controlled) and no typical therapy (uncontrolled) level

Yang-Ching Ko[1], Wu-Huei Hsu[2], Jing-Gung Chung[3,4], Mong-Ping Dai[1], Chien-Chih Ou[1] and Wen-Pin Wu[1]

Abstract

T-lymphocyte (T-LC)-derived cytokines have been implicated in asthmatic pathogenesis. Proteomic technology is now widely accepted as a complementary technology to genetic profiling. We investigated the changes of proteins in T-LC of asthmatic patients from the no typical therapy (uncontrolled) to typical therapy (controlled) level by using standard proteome technology. Methods: The proteins of CD4+ T-LC were isolated from the whole blood of six asthmatic patients from uncontrolled to controlled levels over 3 months. Two-dimensional polyacrylamide gel electrophoresis was performed and coomassie blue stained protein spots were comparatively analyzed by using an image analyzer. Some differentially expressed spots were identified by liquid chromatography/mass spectrometry and database search. Our results showed that 13 proteins showed different expression. Six protein spots in the CD4+ T-LC of the uncontrolled asthmatic patients were increased and 7 spots were decreased compared to those of the controlled subjects. In conclusion, the proteomic examination of the CD4+ T-LC revealed some differentially expressed proteins in the uncontrolled and controlled asthmatic patients. The possibility of using the differentially expressed proteins as important biomarkers and therapeutic targets warrants further study.

Keywords

asthma, proteomics, T-lymphocytes

Introduction

The recognition of asthma as a chronic inflammatory disease in the 1980s led to new paradigms for asthma pathogenesis and treatment. Asthma is characterized by recurrent dyspnea, coughing, wheezing, and reversible airway obstruction mediated by airway inflammation[1-3] in which a variety of cell types, including eosinophils,[4] mast cells,[5] and T lymphocytes[6] contribute to a complex pathologic process that causes airway remodelling and ultimately leads to compromised lung function. It has been estimated that 5% to 10% of patients with asthma have severe disease that is not effectively controlled by typical therapies, and these patients are at high risk of asthma-related death.[7] However, there is still little information on the pathophysiological mechanisms responsible for uncontrolled asthma. It is well known that T-lymphocytes (T-LC) play a critical role in the

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initiation, progression and persistence of allergic disease, including asthma.[8] The critical role of T_H2 cells in asthma is also now widely accepted.[9] Natural killer T (NKT) cells are also believed to play a role in allergen sensitization and pathologic states in asthma.[10] Recently, advances have been made in defining mechanisms that control inflammation and induce immune tolerance to specific antigens. Subsets of CD4+ cells known as T regulatory cells still play an important role in directing these processes,[11] and recent experiments have begun to define crucial molecular and signalling pathways. However, the immunological basis of this disease is still controversial.

To understand the pathophysiologic mechanisms of disease, it is important to know which genes, gene transcripts, proteins, and metabolites are specifically expressed in the disease. Nair et al.[12] stressed the importance of the protein synthesis from the expression because the proteins produced by genes are ultimately responsible for most biological functions. Especially, because proteins undergo many post-translational modifications that affect their structures and functions, their ultimate phenotypes often differ from genetic information. Use of proteomic techniques to identify disease-specific protein biomarkers is a powerful tool for defining prognosis of disease and gaining deep insights into disease mechanisms in which proteins play a major role. Protein profiling has often been performed by the classical two-dimensional sodium dodecyl sulfate polyacryamide gel electrophoresis (2D-PAGE) based on the densitometric quantification of proteins visualized using dyes on gel. After in-gel enzymatic digestion of the subject protein spots, the resulting peptides are subjected to liquid chromatography/mass spectrometry (LC/MS). Several proteome profiles of bronchial asthmatic lungs have been reported; however, most of them were from animal studies.[13,14] The purpose of this study is to explore the differentially expressed proteins of the T-lymphocytes in patients with asthma between uncontrolled and controlled level with using 2D-PAGE and LC/MS.

Patients and methods

Study subjects

Six consecutive patients from 21 to 54 years of age (three females, three males; mean age, 34 years; Table 1) with asthma were enrolled on presentation to the pulmonary medicine clinic at St Martin De Porres Hospital, a regional hospital in central Taiwan, from June through December 2008, which satisfied the American Thoracic Society criteria,[15] and had asthmatic symptoms within 1 week before the first visit. Uncontrolled asthma was diagnosed according to the Global Initiative for Asthma guidelines.[16] Upon enrolment, all asthmatic patients underwent pulmonary function test, blood test for eosinophil count, chest radiography, and some necessary examinations for ruling out other pulmonary diseases. All subjects were non-smokers, and those who suffered from heart disease, autoimmune disease, endocrine disease, chronic liver and renal disease, and cancer were excluded. We also excluded both viral and bacterial infection-induced acute asthma according to the following signs: (1) no significant elevation of serum antibodies for influenza A and B virus; parainfluenza 1, 2, and 3 virus; and respiratory syncytial virus; (2) no fever elevation; (3) no purulent sputum; and (4) no increased peripheral neutrophils and serum C-reactive protein. Concomitant with the initial evaluation was the initiation of management with nebulized β-agonists with or without inhaled or systemic corticosteroids, oral theophylline, nedocromil, or cromolyn for at least 1 month. A blood specimen was obtained from each patient, and a standardized questionnaire of an action plan to maintain asthma control was administered by a trained investigator. The subjects who suffered from airway infection within 4 weeks immediately after the examinations were also excluded. The characteristics of the subjects are detailed in Table 1. Written informed consent was obtained from each subject before the study, and the study protocol was approved by the Institutional Review Board (IRB) of St. Martin De Porres Hospital.

For all participants, initial and subsequent therapies were ordered by the examining pulmonary medicine physicians. Three months later, these subjects had been free of symptoms of a respiratory infection and of any asthma exacerbation for at least the previous 4 weeks and had not used either inhaled or systemic corticosteroids, nedocromil, or cromolyn for at least 1 month before the second evaluation. Data from these six patients that satisfied these criteria for controlled asthma were included in the analysis and blood specimens were obtained again for proteomics.

Processing of samples

Heparinized peripheral venous from each subject was collected in a sterile vacuum tube as enrolled in uncontrolled asthma and subsequently controlled

Table 1. Demographics of patients from uncontrolled asthmatics to controlled level

Age (yr)	Gender	Lung function (FEV1)	Presentation
32	F	47	Exacerbation
30	F	79	4 asthma features[a]
21	M	63	Exacerbation
32	M	66	3 asthma features
54	F	58	4 asthma features
35	M	71	Exacerbation

F: female, M: male, FEV1: forced expiratory volume in the first second, expressed as the percentage of normal.
[a] Asthma features: daytime symptoms, limitations of activities, nocturnal symptoms/awakening, need for reliever/rescue treatment or abnormal lung function.

subjects 3 months later. Peripheral blood mononucleated cells were separated from 40 mL heparinized blood after washed and centrifuged by 40 mL of Histopaqu1-1077 solution (Sigma-Aldrich, Inc, St Louis, Missouri, USA) and 80 mL of normal saline. CD4+ TLC were independently purified from each blood sample by the methods of a T-cell-negative isolation procedure by using monoclonal antibody (MACS; Miltenyi Biotec; J&H Technology Co. Ltd, Taiwan ROC). Twelve samples of TLC from six persons were prepared using the processes described below.

Two-dimensional sodium dodecyl sulfate polyacryamide gel electrophoresisFor protein extraction, the CD4+ TLC pellet of each person was washed and centrifuged with 12,000 rpm, 10 min for three times to remove the serum. The cells were then treated with lysis buffer containing 7 M urea; 2 M thiourea; 4% CHAPS; and protease inhibitor. The mixture was then stored at −80°C until analysis after protein concentration was determined using a commercial reagent (2D Quant Kit, Cat. No. 80-6486-22, Amersham Biosciences, GE. Healthcare, NJ, USA).

Samples containing approximately 280 μg of total protein were precipitated with 2D Clean-Up Kit (Amersham Biosciences, Cat. No. 80-6484-51) and resuspended in 250 μL rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 1% immobilized pH gradient (IPG buffer), 0.002% Bromophenol Blue). The first-dimensional gel separation was carried out with 13 cm pH 3-10 IPG strips and isoelectric focusing (IEF) was performed using Ettan IPGPhor II (Amersham Biosciences) for 70 kVhr at 20°C. After IEF, strips were equilibrated for 15 min in 6 mmol/L urea, 2% sodium dodecyl sulfate, 50 mmol/L Tris-Cl (pH 8.8), and 20% glycerol containing 1% dithiothreitol and then equilibrated again for 15 min in the same buffer containing 2.5% iodoacetamide. Equilibrated IPG strips were placed on top of vertical slabs of 12% polyacrylamide gels and run in a PROTEAN II xi Cell tank (Bio-Rad Laboratories, Hercules, California, USA) at 35 mA per gel. The gels were visualized using Coomassie Brilliant Blue R250 staining. After staining, 2D gels were scanned using Powerlook 1120 (UMAX, Fremont, California, USA). The images were analyzed and compared using ImageMaster^{TM}2D Platinum version 5.0 (Amersham Biosciences). Analyses of spot-intensity calibration, spot detection, background abstraction, and matching of approximately 12 two-dimensional sodium dodecyl sulfate polyacryamide gel electrophoreses (2D-PAGEs) were performed using software (ProteomWeaver; Definiens; Munich, Germany). All selected spots were present in all gels. The differentially expressed spots were analyzed with using Mann-Whitney U test (SPSS, version 10.0.05; SPSS; Chicago, Illinois, USA). Correction for multiple comparisons was done according to the false discovery rate (FDR) method and p values larger than the FDR α value of 0.05 that was calculated using the software were considered statistically non-significant.

Identification of protein spots

Nano LC-MS/MS analysis was performed on an integrated nanoLC-MS/MS system (QSTAR XL) comprising an LC Packings NanoLC system with an autosampler, and a QSTAR XL Q-Tof mass spectrometer (Applied Biosystems, Foster City, California, USA) fitted with nano-LC sprayer. Online nanoESI-MS survey scan and data-dependent acquisition of CID MS/MS were fully automated and synchronized with the nanoLC runs under the full software control of AnalystQS. After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were combined and output as a single Mascot-searchable peak list file. The peak list

files were used to query the NCBI database using the Mascot program with the following parameters: peptide mass tolerance, 150 ppm; MS/MS ion mass tolerance, 0.15 Da; allowing up to one missed cleavage. Only significant hits as defined by Mascot probability analysis will be considered initially. In addition, a minimum total score of 20 comprising of at least a peptide match of ion score more than 20 was arbitrarily set as the threshold for acceptance.

Results

Two-dimensional sodium dodecyl sulfate polyacryamide gel electrophoresis

More than 100 spots were identified in the 2D-PAGE gels from the CD4+ TLC of the asthmatic patients from uncontrolled to controlled levels. The general distribution pattern of the spots in the coomassie blue-stained gels was similar in both groups (Fig. 1A and B, percent matches: 95.91%). The spots in the area of isoelectric point (pI) 3 to 10 and molecular weight of 10 to 100 kDa were analyzed by an image analysis program (ProteomWeaver). Protein spots of the uncontrolled and controlled asthmatic groups were compared, and 13 proteins showed different intensity, suggesting the differential expression. Among them, the intensities of six spots were significantly increased and the intensities of seven spots were decreased in the uncontrolled group compared to the controlled group. The 13 selected spots of the 2D-PAGE of an asthmatic, non-smoking, 32-year-old woman are shown in Figure 1C.

Identification of the differentially expressed proteins by LC/MS

After destaining, extraction, and lysis with trypsin, the individual spots were identified by LC/MS and this was followed by the MASCOT database search algorithm. On the list of 13 candidate proteins for each spot, the final protein was determined by comprehensively considering the corresponding experimental isoelectric point, the molecular masses, the number of matched peptides, and the sequence coverage. Six up-regulated and seven down-regulated proteins in the uncontrolled asthmatic group were identified (Table 2).

Discussion

In 2007, Jeong et al.[17] reported that proteomic examination of the peripheral T-lymphocytes revealed some differentially expressed proteins in the asthmatic patients. However, this is the first proteomic approach using the CD4+ TLC of blood in asthmatic patients and defining the difference in severity.

It is well-known that the asthmatic process that triggers the immune system can lead to excessive release of various cytokines and inflammatory mediators, which are produced by T-cells, infiltrated mononuclear cells, eosinophils, and local mast cells into the lung. Subsets of CD4+ cells known as T regulatory cells still play an important role in directing these processes,[11] and recent experiments have begun to define crucial molecular and signalling pathways. But the aetiology of airway hyperresponsiveness, a cardinal feature of asthma, has not been fully elucidated. Therefore, we performed proteomic analysis of the CD4+ TLC of controlled and uncontrolled asthmatic patients.

In the study, we identified several proteins that were previously unknown or known to be associated with the pathogenesis of asthmatic exacerbation. Our study showed the increase of the heat shock protein (HSP)-70 and HSP-90 in CD4+ TLC from the uncontrolled asthmatic group to controlled level. HSP is a ubiquitous, abundant, and conserved protein whose rate of synthesis is increased in response to cellular stress. It is often the target of humoral and T-cell-mediated immune responses to infection[18] and could protect tissue from the deleterious effects of numerous mediators, reactive oxygen species, or tumor necrosis factor-α. Several studies[19,20] have suggested that HSP is correlated with the severity of asthma exacerbation. It has also been proposed that the HSP-70 and HSP-90 chaperone associate with a range of cellular steroid receptor and may modulate the effectiveness of steroid in asthmatics.[21]

Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHA) is highly conserved molecule that functions as intracellular adaptors in a variety of biological processes, such as signal transduction, cell cycle control, and apoptosis. It is also an HSP that protects cells against physiological stress as its new cellular function.[22] Our study also showed the up-regulated expression of YWHA from the uncontrolled asthma group to controlled level.

Several cytoskeletal proteins were associated with the T-lymphocytes of asthma patients. Vimentin is a type III intermediate filament protein normally expressed in cells of mesenchymal origin and it attaches to the nucleus, endoplasmic reticulum, and

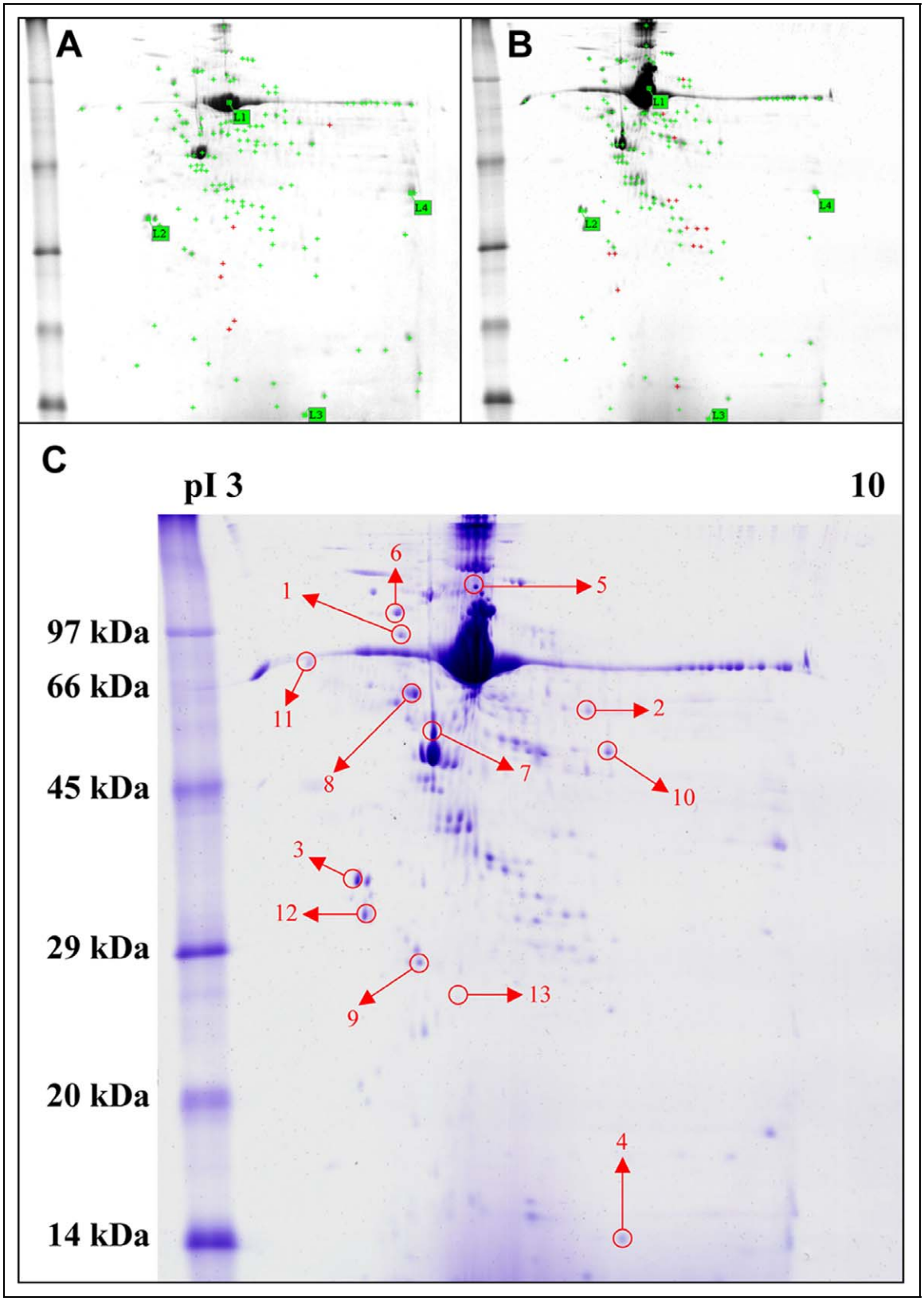

Figure 1. Representative two-dimensional sodium dodecyl sulfate polyacryamide gel electrophoresis (2D-PAGE) gels of the CD4+ T-lymphocytes from uncontrolled asthma patients (A) to control level (B). Each image shows a similar expression pattern of spots between A and B. Thirteen selected and identified spots are shown by the numbered arrows in a 2D-PAGE gel (C) of an uncontrolled, asthmatic, 32-year-old woman.

Table 2. Identification of the proteins with altered expression levels in the human CD4+ T-lymphocytes from the uncontrolled asthma patients to controlled level

No.	Protein name	Mascot program access N0.	Exp	Seq Cov, %	T Mass, kd/pI	O Mass, kd/pI	%Vol ratio	*p* Value
1	HSP-70	gi|16507237	↑	35	72/5.1	71/4.9	0.12/0.23	0.005
2	Fibrinogen β chain	gi|119625338	↓	55	52/8.3	51/8.1	0.34/0.21	0.014
3	Tropomyosin 3	gi|24119203	↑	33	29/4.8	33/4.8	0.54/0.60	0.009
4	Unnamed protein product	gi|29446	↓	88	16/7.1	15/6.8	3.43/0/46	0.024
5	ATP-dependent DNA helicase II	gi|10863945	↓	12	83/5.6	99/6.1	5.34/1.37	0.036
6	HSP 90	gi|61656603	↑	18	98/5.1	98/4.7	0.25/0.71	0.001
7	β-actin	gi|4501885	↓	53	42/5.3	58/5.1	0.72/0.20	0.019
8	Vimentin	gi|62414289	↓	42	54/5.1	66/5.2	0.49/0.48	0.044
9	Rho GDP dissociation inhibitor beta	gi|56676393	↑	47	23/5.1	28/5.1	0.04/0.65	0.012
10	Enolase 1	gi|4503571	↓	48	47/7.0	51/7.2	0.70/0.18	0.022
11	Calreticulin precursor	gi|4757900	↑	13	48/4.3	66/3.9	0.13/0.31	0.001
12	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein	gi|4507949	↑	24	28/4.8	32/4.9	0.04/0.32	0.031
13	Peroxiredoxin 2	gi|32189392	↑	18	22/5.7	26.5.1	0.02/0.12	0.018

Exp: expression, ↑: up expression levels, ↓: down expression levels, Cov: coverage, T: theoretic, O: observed, pI: isoelectric point, Vol: volume

mitochondria for the control of shape, motility, and migration of mesenchymal cells.[23] The increased expression of vimentin was observed in the uncontrolled asthma patients in our study. The cytoskeletal change may illustrate the functional change in the T-lymphocytes of asthma exacerbation. β-actin, also a cytoskeletal protein, functions in cellular shape and anchorage where transmembrane glycoproteins link fibronectin in the extracellular matrix with actin microfilaments on the cytoplasmic side of the membrane.[24] It is not surprising therefore that cellular proliferation, activation, and/or differentiation induces up-regulation of β-actin expression in CD4+ TLC of the uncontrolled asthmatic group and subsequent cytoskeletal remodelling.

Lung alveolar epithelial cells could produce fibrinogen when induced with proinflammatory mediators.[25] Fibrin is formed when thrombin, generated by the coagulation cascade factors and cofactors, cleaves fibrinopeptides A and B from fibrinogen. The most powerful protein inactivator of surfactant, to the best of our knowledge, is fibrin,[26] and immunohistochemically detected fibrin was seen along the luminal surface of the distal airways in a patient who died of status asthmatics.[27] Fibrinogen β-chain was also significantly increased in CD4+ TLC of the uncontrolled asthmatic group in this study.

The enolase 1 is a key glycolytic enzyme with highly conserved amino acid sequences from microbial organism to mammal.[28] This enzyme is ubiquitously expressed in nearly all cell types, including epithelial cells, endothelial cells, and hematopoietic cells.[28] Although enolase 1 is mainly a cytosolic protein, it is also expressed on cell surfaces and functions as a plasminogen receptor.[28] The enolase 1 was ever reported as an autoantigen associated with severe asthma[29] and upregulation of Enolase 1 has been detected in many types of human cancer. Its activity also increased in the uncontrolled asthmatic group in our study.

Peroxiredoxins are antioxidant enzymes involved in protein and lipid protection against oxidative injury and in cellular signalling pathways regulating apoptosis. Peroxiredoxin 2 is a member of the mammalian peroxiredoxin family of thiol proteins that is important in antioxidant defences and redox signalling. The decreased expression of peroxiredoxin 2 was reported to be associated with enhanced sensitivity of Down syndrome neurons to reactive oxygen species.[30] In our study, the up-regulation of peroxiredoxin 2 was also observed in CD4+ TLC from the uncontrolled asthmatic patients to controlled level.

Rho GTPases are molecular switches that regulate many essential cellular processes, including actin dynamics, gene transcription, cell-cycle progression, and cell adhesion. To date, most progress has been made in the cytoskeleton field, and several biochemical links have now been established between GTPases and the assembly of filamentous actin. Rho

GDP dissociation inhibitor beta (Rho-GDI beta), an inhibitor of Rho GTPase, is primarily expressed by hematopoietic cells. Rho-GDI beta was also observed to be down-regulated in interleukin-4 (IL-4)-treated T cells and IL-4 regulates the subsequent stages of T helper 2 cell–mediated diseases, such as allergies.[31] Our study also demonstrated that its activity increased in CD4+ TLC from the uncontrolled asthmatic patients to controlled level.

Nuclear DNA helicase II (NDH II) belongs to the DEXH helicase superfamily and is able to unwind both double-stranded DNA and RNA in the presence of one of the four ribo- or deoxyribonucleoside triphospates. In addition to transcription, NDH II has multiple physiological functions, such as in RNA processing and transport, in DNA repair, and also in tumorigenesis.[32] The helicase containing protein complexes may also facilitate the entry of transcriptional apparatus to the IL-4 responsive promoters.[33] However, change of NDH II might modify the exacerbation of asthma in a still unknown manner. Tropomyosins (Tm) are a group of proteins with multiple isoforms found in both muscle and nonmuscle cells and in association with the troponin complex play a central role in the actin-linked calcium regulatory system of muscle contraction.[34] The predominant colonic epithelial Tm isoform, hTm5, can induce both humoral (B–cells) and cellular (T–cells) response in patients with UC. Tm has also been described as relevant allergens.[35] Change of Tm 3 in CD4+ TLC was also observed in this study, but the relation of this protein with the asthmatic exacerbation is not yet clear. Calreticulin, a major Ca^{2+}-binding (storage) chaperone in the endoplasmic reticulum, is a key component of the calreticulin/calnexin cycle that is responsible for the folding of newly synthesized proteins and glycoproteins by binding monoglucosylated carbohydrate and for quality control pathways in the endoplasmic reticulum. Immunogenic death show that apoptotic cells also displaying calreticulin on their surface are processed by dendritic cells that induce a specific T cell–mediated immune response against these apoptotic cells. The expression of calreticulin, which is also formed in vascular smooth muscle cells and vascular endothelial cells exposed to hyperglycaemic conditions, underlies the process of down-regulation of glucose transport in vascular cells under high-glucose conditions.[36] The adaptive mechanism protects vascular cells against damaging effects of an uncontrolled influx of glucose in face of hyperglycemia. The expression of calreticulin precursor increased in CD4+ TLC from the uncontrolled asthma patients to controlled level in our study, the change could possibly interfere with glucose metabolism in response to asthmatic exacerbation.

From the above, it is apparent that proteomic analysis reveals differences in protein expression that come from the change in level of the asthma patients. Both strategies have unique strengths and limitations. 2D-PAGE resolves thousands of proteins in a single run and provides crucial molecular weight and isoelectric point information on intact protein; however, identification of some proteins may be hindered by their expression in various forms and the data may be skewed toward the more abundant proteins in the sample. Otherwise, although we selected nonsmokers and relatively young participants, it is known that depending on the different physiologic conditions, such as age, gender, physical activity, and the disease status, 2D-PAGE can show different panels.[12] Finally, whether the changes of such proteins are pathognomonic markers of asthma or of a reactive phenomenon is questionable.

In summary, we have used 2D-PAGE/LC/MS to examine the protein profiles and expression in CD4+ TLC from uncontrolled and controlled asthma patients. Identification of these proteins revealed significant increase in expression for eight proteins, whereas six showed a significant reduction. These differences provide novel information highlighting proteins that may be linked to the mechanism(s) that defines why the asthma patients develop acute deterioration. The possibility of using the differentially expressed proteins as important biomarkers and therapeutic targets in uncontrolled asthma patients warrants further study.

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