Proteomic analysis of Down's syndrome patients with gout

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Abstract

Background: In this study, the expression of hypoxanthine-guanine phosphoribosyl transferase (HPRT) in Down's syndrome patients with gout (DS/G) was determined, and possible underlying mechanisms of gout were characterized using proteomic tools.
Methods: Serum was obtained from DS/G, healthy controls and gout patients (without DS), recruited from the rheumatology clinic. Baseline enzyme assays were recorded and RT-PCR used to identify HPRT gene expression. 2-D electrophoresis and mass spectrometry were utilized to determine a plausible explanation concerning the mechanisms leading to increased uric acid levels in DS patients.
Results: Two DS patients were diagnosed with gouty arthritis. Their HPRT enzyme activity was slightly lower than that of normal controls. HPRT expression was also slightly decreased in DS/G patients compared with controls. Serum protein profiles of these two DS/G patients revealed that haptoglobin α chain and apolipoprotein A1 (ApoA1) were both significantly down-regulated. Protein expression was validated by immunoblot.
Conclusion: Our results revealed that low levels of haptoglobin in the two DS/G patients were related to renal dysfunction may have affected uric acid excretion and caused gout. However, decreased ApoA1 revealed a positive correlation between defective lipid metabolism and gouty arthritis in DS/G patients.
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1. Introduction

Down's syndrome (DS) patients are considered susceptible to oxidative injury and the development of multiple pathologies derived from an extra copy of chromosome 21 [1]. It has previously been demonstrated that DS patients suffer from increased oxidative stress such as lipid peroxidation, which is followed by cell apoptosis. In the present study, we found that two DS subjects showed elevations in serum urate levels and had symptoms of gouty arthritis [2]. The peak age of onset of gouty arthritis is between the fourth and sixth decades [3]. Onset before the age of 30 years is rare [4,5]. The etiology of gout in DS patients remains unclear, as does the potential etiological role, if any, of genes such as hypoxanthine-guanine phosphoribosyl transferase (HPRT).

The purine salvage enzyme, HPRT, is associated with two distinct inherited sex-linked recessive diseases [6]. Complete deficiency of HPRT leads to the Lesch-Nyhan syndrome, whereas partial deficiency causes severe gout and mental retardation. However, to the best of our knowledge, there has been no previous study concerning HPRT in patients with DS and gout (DS/G). We measured the activity and expression of this enzyme to discern whether gouty arthritis in DS patients may result from a HPRT deficiency [7,8]. Uric acid is regarded to be an important antioxidant in humans. An imbalance in the antioxidant enzyme defense system or renal dysfunction

may cause accumulation of uric acid in DS/G patients [9,10]. We also tried to investigate speculations about the possible pathogenetic mechanisms leading to the increase in uric acid levels in DS/G patients with proteomic tools, which allow the global study of protein expression and regulation in a biological system [11–13].

Serum protein profiles provide important information about the biological behavior of the cell, and changes detected in protein expression may offer an opportunity to understand the potential pathogenesis of gout in DS patients.

2. Materials and methods

2.1. Patients

Case 1 and Case 2 were both males with DS and were 27 and 30 years old at time of consultation respectively. The first attack of gout in both patients involved the left ankle joint, and they were respectively aged 24 and 27 years at that time. No tophus was found in either patient. Case 1 exhibited neurological manifestations of DS at the age of 15 months, whereas Case 2 first exhibited them at 18 months.

Blood samples from three age-matched healthy controls, three patients suffering from gout (without DS) and two DS/ G patients were obtained from patients recruited at the hospital rheumatology outpatient clinic [3]. All subjects gave their informed consent to the study. The Committee on Research Involving Human Subjects of the Chang Gung Memorial Hospital in Kaohsiung, Taiwan, approved the study.

2.2. Enzyme activity assay

A non-radiochemical HPLC-linked method was used to determine the activity of HPRT [14]. Blood spots of approximately 20 μl were adsorbed onto filter paper and allowed to dry for at least 3–4 h at room temperature. Spots were immediately used after different periods (24, 48 and 72 h, and 15 days). Discs, 7 mm in diameter, were punched from each blood spot, transferred to Eppendorf tubes and soaked with 500 μl 0.1 mol/l Tris–HCl buffers, pH 7.4, until complete elution. The paper was spun down in a centrifuge at $12,000 \times g$ for 5 min, and the supernatant used to assay for HPRT activity by HPLC as previously described [14].

2.3. Evaluation of HGPRT expression by RT-PCR

Total RNA was prepared from peripheral blood by an RNA extraction kit. The first strand cDNA was synthesized with Molonney murine leukemia virus reverse transcriptase (Promega, USA) using 2 μg of total RNA and oligo(dT) primers, then the target region was amplified by PCR. Two oligonucleotide primers (sense: 5′-GAATTCCTCCTTCCT-GAGCAGTCAG-3′, antisense: 5′-AAAGCTTTACTAAG-CAGATGGCCAC-3′) were used to flank the entire HPRT coding region of 654 base pairs [15]. Amplification was carried out after a 5 min 95 °C hot start, and Taq enzyme addition for 30 cycles, using the following conditions: denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extending at 72 °C for 30 s. PCR was performed at 30 cycles for HPRT, and 25 cycles for GAPDH. PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. The target bands were analyzed densitometrically by using a GS-700 Imaging Densitometer (Bio-Rad, CA, USA). All experiments were repeated at least three times.

2.4. Albumin depletion from serum and 2D electrophoresis

Using a Millipore Montage Albumin Deplete Kit, 30 μL of serum from DS/G patients, controls and gout patients was diluted with 170 μL of equilibration buffer (kit reagent) before application to the column. The protocol used has previously been described [16]. The protein amount was measured using a BCA kit (Pierce, USA). Ten micrograms of albumin-depleted fractionations were applied onto an 8–16% gradient SDS-PAGE at 150 V at 4 °C, and visualized by silver stain.

Albumin-depleted fraction (150 μg) was precipitated using a 3:1 v/v acetone-to-sample solution. Protein pellets were collected, dried, and solubilized in 2-DE lysis buffer that contained 7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue, 2% IPG buffer (pH 3–10 linear) and 65 mM DTE. The samples were then separated by 13 cm Immobiline DryStrip (pH 3–10 linear) on the IPGphor Isoelectric Focusing System (Amersham Bioscience) in the first dimension. The running condition of the IEF was as follows: 30 V, 12 h; 100 V, 1 h; 250 V, 1 h; 500 V, 0.5 h; 1000 V, 0.5 h; 4000 V, 0.5 h; 8000 V, up to 60 kV h. Before 2-D SDS-PAGE, the IPG strips were equilibrated in a solution containing 50 mM Tris–HCl pH 8.8, 6 M urea, 2% SDS, 30% glycerol and a trace of bromophenol blue, which included 15 min exposure to 2% DTT for the first equilibration, and replacement with 2.5% iodoacetamide for the second equilibration for a further 15 min. Two-dimensional SDS-PAGE was carried out on 12% acrylamide gradient gels at 24 mA/gel until the bromophenol blue dye front reached the bottom of the gel. After approximately 5 h, all gels were visualized by silver staining and scanned using an ImageScanner (Amersham Bioscience). Protein spots were quantified using the Nonlinear Progenesis software (technical support by J&H Technology Company, Taiwan). All experiments were repeated at least three times.

2.5. Tryptic in-gel digestion of 2-D PAGE-resolved proteins and MALDI-TOF mass spectrometry

Protein spots were excised from the polyacrylamide gel, washed twice with 100 μl of 50% acetonitrile/25 mM

Table 1
The clinical characteristics of uric acid and HGPRT activities between DS/G patients and the controls

	Case 1	Case 2	Controls[a]
Uric acid (mg/dL)	10.6	12.2	<7
Uric acid excretion (mg/dL)	704	368	594±143
Enzyme activities (mmol/h/mg Hb)	86	96	107±15

[a] The biochemical definitions were quoted from [27].

ammonium bicarbonate buffer pH 8.0 for 15 min, washed once with 100 μl of 100% acetonitrile and dried. The dried gel pieces were swollen in 10 μl of 25 mM ammonium bicarbonate containing 0.1 μg trypsin (Promega, Madison, WI, USA). The gel pieces were then crushed with a siliconized blue stick and incubated at 37 °C for at least 16 h. Peptides were subsequently extracted with 50 μl of 50% acetonitrile/5% trifluoroacetic acid, and dried once again. The peptides were then resuspended in 10 μl of 0.1% trifluoroacetic acid. Application of the sample to the sample target (Anchorchip, Bruker Daltonics, Bremen, Germany) was performed manually. The peptide mixture (0.2 μl) was simultaneously applied with 0.2 μl of matrix solution, consisting of 20 mg/ml α-cyano-4-hydroxycinnamic acid and the standard peptide BSA fragment (m/z=927.49, 1479.79) and adrenocorticotropic hormone fragment 18–39 (2465.20) as internal calibrate. These samples were analyzed using a Reflex/Biflex™ (Bruker Daltonics, Bremen, Germany). The measured monoisotopic masses of peptide were analyzed using World Wide Web search programs such as Mascot, provided by Expasy (http://www.matrixscience.com), with MSDB database. Search parameters were set as follows: maximum allowed peptide mass error of 50 ppm, consideration of one incomplete cleavage per peptide, and a pI range between three and ten [17].

2.6. Western blot analysis

Fifteen micrograms of albumin-depleted serum was collected from each DS/G patient, each gout patient (without DS), and controls, and was diluted in two-fold sample buffer and incubated at 60 °C for 10 min [18]. Samples were separated on 10% SDS-PAGE and then transferred to nitrocellulose membrane (PALL, Carlsbad, CA). The following primary antibodies were utilized: rabbit anti-human haptoglobin polyclonal antibody (DakoCytomation, Carpinteria, CA) and goat anti-human apolipoprotein A1 polyclonal antibody (Santa Cruz Biotechnology, CA). Nitrocellulose membranes were incubated with individual primary antibodies diluted 1:2000 for 2 h at room temperature. Membranes were then incubated for 45 min with goat anti-rabbit (Sigma) or donkey anti-goat secondary alkaline phosphatase-conjugated antibody (Santa Cruz) diluted 1:3000. Protein bands were visualized by using enhanced chemiluminescence (CDP-star; PerkinElmer Life Science, Boston, MA, USA). Densitometric analyses of scanned immunoblotting images were performed with NIH Image software.

3. Results

3.1. Clinical characteristics and HPRT expression in DS patients with gout and the control

Both DS patients were suspected on clinical grounds to have gout; their serum uric acid levels were 625.4 μmol/l (Case 1) and 719.8 μmol/l (Case 2), respectively. The 24-h renal excretions of uric acid were 704 mg (Case 1) and 368 mg (Case 2) while on a purine-free diet. Other serum biochemical parameters for Case 1 were: total cholesterol (194 mg/dl), triglyceride (512 mg/dl), and HDL-cholesterol (34 mg/dl); and for Case 2: total cholesterol (162 mg/dl), triglyceride (236 mg/dl), and HDL-cholesterol (48 mg/dl). Their respective body mass indices were 29.3 kg/m^2 and 25.1 kg/m^2. In addition, these two DS/G patients had normal renal and liver function, and were diagnosed with hyperuricemia. As listed in Table 1, Case 1 showed normal uric acid excretion and Case 2 showed reduced uric acid excretion. The enzyme activity was within the normal range for both DS/G patients. However, the expression of HPRT was lower in both DS/G patients compared with normal controls (Fig. 1), which implied decreasing RNA amount in these two patients. These expression profiles were consistent with normal enzymatic activity.

3.2. Differential display of plasma proteins among normal control, gout patients and DS/G patients

To reveal whether other proteins may also be associated with the development of gout in DS patients, we compared the protein profiles of DS/G patients, the controls and gout patients (without DS). The albumin-depleted serum samples were separated by 2-D electrophoresis (pH from 3 to 10) and visualized by silver staining (Fig. 2). With Nonlinear Progenesis software, three protein spots with significant differences between normal controls and DS/G patients were counted. Of note, two spots appearing in control samples were markedly increased in volume. These protein spots were subsequently identified by peptide mass fingerprinting (PMF) and aligned with the protein sequence in databases (www.expasy.ch/Mascort) as listed in Table 2. Among the identified proteins in DS/G samples, the two downregulated spots (Fig. 2, spots 1 and 2), ApoA1 and haptoglobin α chain, were confirmed.
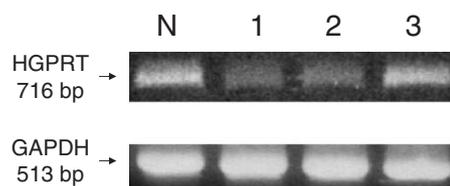

Fig. 1. Messenger RNA expression of HPRT in DS/G patients. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression (GAPDH) was used as the control for quantification. N indicates the normal control; lanes 1 and 2 indicate DS/G Cases 1 and 2, respectively, and lane 3 indicates the gout patient.
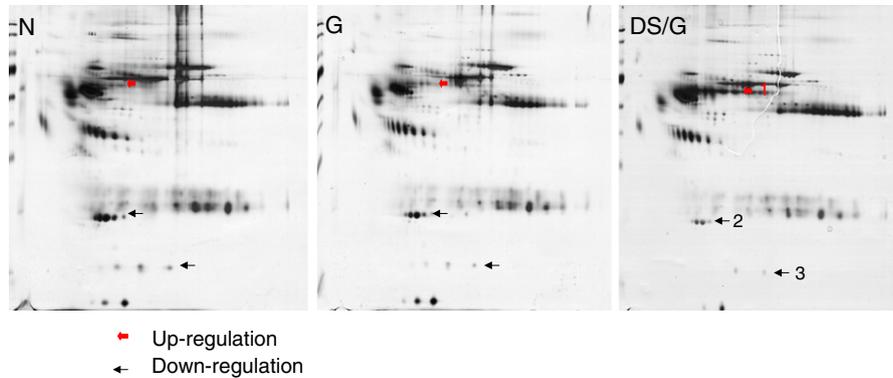
Fig. 2. Two-dimensional electrophoresis patterns of plasma proteins obtained from healthy control (N), gout (G) and DS/G patients. Patients' sera (150 μg) were focused on a linear 3–10 IPG, and then migrated at a right angle in SDS-PAGE on a 12% polyacrylamide gradient. Each spot volume was determined and quantified from the intensity of spots derived from the silver-stained 2DE.

3.3. Western blot analysis for relative protein quantity

According to results from the proteome alternation profiles, we postulated that changes in ApoA1 and haptoglobin α chain may be an important index in the serum of DS/G patients. To accurately quantify relative expression levels of these two proteins we analyzed serum samples from normal controls, gout patients, and DS/G patients using Western blot analysis. Regression analysis of total amount of protein loaded versus chemiluminescence was performed, demonstrating a strong, linear relationship for all antibodies tested. As shown in Fig. 3, the amount of ApoA1 and haptoglobin α chain was significantly decreased in the two DS/G subjects, especially in Case 2. There were no obvious changes in expression of haptoglobin β chain for the controls and DS/G patients. This finding was consistent with 2-DE protein expression profiles presented by DS/G subjects and controls.

4. Discussion

Although hyperuricemia is frequently found in DS patients, only a small proportion will develop gouty arthritis. The etiology remains unclear. It has been reported that impairment of renal excretion of uric acid is found in DS/G patients [19]. However, Case 1 showed a mild decrease in uric acid excretion, while Case 2 revealed normal uric acid excretion.

HPRT catalyzes an early step in the salvage pathway for guanine and hypoxanthine metabolism. HPRT deficiency usually causes hyperuricemia, precocious gout and uric acid nephrolithiasis [20]. According to our results, although these two DS/G patients showed a moderate reduction in amount of HPRT, its activity remained within normal ranges, which were 86 and 96, respectively. Some defects may be located on the promoter region rather than on the gene itself leading to low levels of RNA and cDNA. The function of HPRT would be maintained in this circumstance. Alternatively some polymorphisms may exist at the intron region, resulting in preserved enzyme activity but reduced RNA expression.

Our findings indicate that HPRT gene did not contribute to pathology in the DS/G patients. We suggest that oxidative stress caused by overexpressed genes on chromosome 21 may be associated with hyperuricemia in DS subjects [21]. Serum protein profiles of the two DS/G patients revealed a significant decrease in haptoglobin (HP) expression compared to those obtained from age-matched controls with and without gout. HP, a conserved plasma glycoprotein, forms complexes with free hemoglobin. It is considered to reduce hemoglobin-induced renal damage. Previous reports demonstrated that once the HP gene was knocked out in mice, some parameters would lead to severe tissue damage, and kidney was the most affected tissue [22]. There were higher levels of renal lipid peroxidation in HP-knockout mice and various oxidative products may activate the antioxidant enzyme system leading to acute or chronic renal damage

Table 2
List of identified protein spots

| Spot no.	Protein name	Swissport no.	Function	Process
Up-regulation				
1	IgA	P01876	Infection and inflammation	Immune response
Down-regulation				
2	Apo A1	P02647	Lipid binding and transporter activity	Cholesterol metabolism and lipid transport
3	Haptoglobin α chain	P00738	Plasma glycoprotein and hemoglobin binding	Defense response, proteolysis and peptidolysis
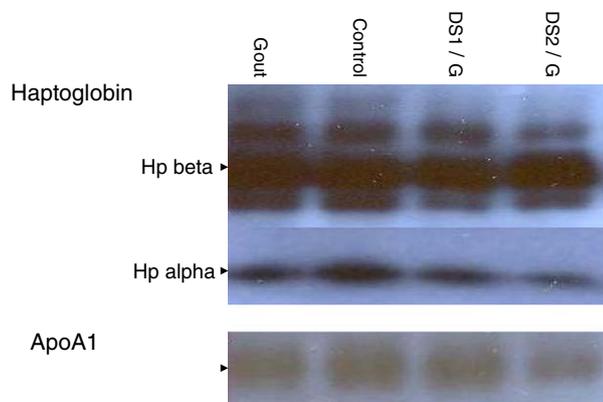
Fig. 3. The validation of protein levels on haptoglobin alpha chain and ApoA1 through Western blotting. Serum samples were extracted from DS/G patients (DS/G1 and DS/G2 designating Cases 1 and 2 respectively), gout (G) and normal controls (N). Normal controls and gout samples expressed more abundant haptoglobin alpha chain than DS/G patients. The amount of haptoglobin beta chain showed no significant differences while ApoA1 haptoglobin alpha chain was down-regulated in DS/G2.

[22]. This may explain the correlation between the reduction of plasma HP and pathogenesis of gout in DS patients. We also addressed the finding that ApoA1 showed threefold downregulation in DS/G samples and it inhibited the activity of enzyme catalyzing lecithin-cholesterol acyltransferase associated with cholesterol excess from cells [23,24]. Young DS/G patients are often obese with lipid metabolic problems. These results suggest that the plasma protein expression actually elicits the physiological uric acid turnover in DS/G patients [25]. On the other hand, the amount of IgA was slightly higher in DS/G patients than in controls. Our findings implicate that the determination of IgA serves to distinguish inflammatory gout and noninflammatory arthritis such as degenerative rheumatism [26]. These results clearly show that serum protein profiles may help to build the basis for the future identification of new drug targets and therapeutic strategies for gout in DS patients.

Despite these results, we can not exclude the possibility of other etiologies causing hyperuricemia in DS and subsequent gouty arthritis. It is also possible that other factors are required in order for the clinical features of gout to develop in DS patients. The other oxidative stress associated proteins in DS/G patients should be further investigated under redox proteomics analysis.

In conclusion, this is the first report to discuss the HPRT and serum proteins in DS associated with gout. Thus, 2-DE based serum proteome analysis may be useful in the screening of DS patients for the potential development of gouty arthritis.

Acknowledgements

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