대한소아신장학회지 제13권 제2호 2009년
DOI 10.3339/jkspn.2009.13.2.170

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The Comparison between FSGS and MCNS Using Proteomic Method in Childhood Nephrotic Syndrome; Preliminary Study

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= **Abstract** =

Purpose : FSGS do not respond well to any kind of therapy and gradually progress to end-stage renal disease. This study was conducted to investigate the difference of protein expression between MCNS and FSGS as a preliminary study for understanding the pathophysiology of FSGS.
Methods : Renal biopsy samples of MCNS and FSGS were obtained, which was diagnosed by one pathologist. They were solubilized with a conventional extraction buffer for protein extraction. The solution was applied on immobilized linear gradient strip gel (pH 4-7) using IPGphor system. Silver staining was carried out according to standard method. Protein identification was done by searching NCBI database using MASCOT Peptide Mass Fingerprint software.
Results : The differences in protein expressions between MCNS and FSGS were shown by increased or decreased protein spots. Most prominently expressed spot among several spots in FSGS was isolated and analyzed, one of which was glutathione S-transferase (GST) P1-1, whereas it was not found in MCNS. So GSTP1-1 was considered as the one of the key biomarkers in pathogenesis of FSGS.
Conclusion : This result would be helpful in diagnosing FSGS and researching FSGS. Further studies for glutathione S-transferase P1-1 might be necessary to elucidate the mechanisms regarding FSGS. **(J Korean Soc Pediatr Nephrol 2009;13:170-175)**

Key Words : Nephrotic syndrome, Glomerulosclerosis, Proteomics, Glutathione S-transferase

Introduction

Idiopathic nephrotic syndrome is one of the most common renal diseases that occurs in children. It has a reported incidence of two to seven cases per 100,000 children and is most commonly caused by minimal change nephrotic syndrome (MCNS) or sometimes by focal segmental glomerulosclerosis (FSGS) [1]. MCNS generally has a favorable long-term prognosis. Prompt administration of steroids has decreased morbidity and mortality of disease. However several patients especially who have a pathology of FSGS do not respond well to corticosteroid therapy and gradually progress to end-stage renal disease despite the use of other immunosuppressive agents [2, 3]. Therefore FSGS is a disease with substantial

접수 : 2009년 7월 31일, 수정 : 2009년 9월 12일
승인 : 2009년 9월 14일
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morbidity and it is necessary to study the difference between both diseases. Recently proteomic techniques are emerging as a promising and useful tool for understanding cell physiology and pathogenesis of diseases [4]. Attention has turned to assess changes of protein expression in a given genome since the completion of the human genome in 2002. As a consequence, techniques using proteomics have been used at research area to examine not only the presence or absence of particular proteins, but also post-synthetic changes [3, 4]. This study was conducted to investigate the difference of protein expression using proteomic analysis tool between MCNS and FSGS as a preliminary study to understand pathogenesis of FSGS.

Methods

1. Subjects

We had underdone renal biopsy for steroid resistant INS before cyclosporine or cyclophosphamide treatment.

We obtained 2 or 3 cores of kidney tissues by ultrasound guided renal biopsy from patients.

Most of them were sent to the department of pathology for diagnosis and small amounts of cortex portion (about 0.2-0.5 cm) were kept in a deep-freezer of -70℃ for reconfirmation of the diagnosis if needed.

Among them, we used samples from 4 patients with MCNS and 4 patients with FSGS to elucidate the different characteristics between MCNS and FSGS. Four patients with MCNS were steroid resistant or frequent relapser, but responsive to cyclophosphamide. The four patients with FSGS didn't respond to any kind of immunosuppressive agents and finally progressed to chronic kidney disease.

2. Methods

1) Protein extraction

Their samples were solubilized with a conventional extraction buffer for protein extraction. The solution was applied on immobilized linear gradient strip gel (pH 4-7) using IPGphor system. Silver staining was carried out according to the method of Heukeshoven and Dernick using a silver staining kit. The proteins were identified by searching NCBI database using MASCOT Peptide Mass Fingerprint software.

2) MALDI-TOF-MS and MALDI-TOF/TOF Tandem MS (MS/MS)

The resulting tryptic peptides were dissolved in 0.5% trifluoroacetic acid (TFA) solution and then desalted using ZipTipC18 (Millipore, Bedford, MA) tip. Peptides were eluted directly onto MALDI target by α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix solution (10 mg/mL CHCA in 0.5% TFA/50% acetonitrile (1:1 v/v). All mass spectra were acquired at a reflection mode by a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). External calibration was performed using a standard peptide mixture of des-Arg Bradykinin, Angiotensin I, Glu-Fibrino-peptid B, Adrenocortico-tropic hormone (ACTH) clip 1-17, ACTH clip 18-39, and ACTH clip 7-38. Internal calibration was also performed using two autolysis peaks of trypsin ($[M+H]^+$=842.5099 and 2,211.1046). When the protein spots were not identified by peptide mass fingerprinting (PMF),

fragmentation patterns of tryptic peptide molecular ions ($[M+H]^+$) were analyzed by MS/MS methods for obtaining their partial sequences using MALDI-TOF/TOF technique. All samples were irradiated with UV light (355 nm) of an Nd :YAG laser shots were averaged to normal mass spectra and MS/MS spectra, respectively. The samples were analyzed at 25 kV of source acceleration voltage with two-stage reflection in MS mode. In the MS/MS experiment, collision energy, which was defined by the potential difference between the source acceleration voltage (8kV) and the floating collision cell (7kV), was set to 1kV.

3) Database Searching and Identification of Proteins

The proteins were identified by searching NCBI nonredundant database using MASCOT Peptide Mass Fingerprint software (Matrixscience, London) and MSFit (Protein Prospector; UCSF, San Francisco, CA). All mass spectra were searched in the database of rodent species or all entries. The search parameters were considered to allow the modifications of N-terminal Gln to pyroGlu, oxidation of methionine, acetylation of protein N-terminus, carbamidomethylation of cysteine, and acrylamide-modified cysteine. The criteria for positive identification of proteins were set as follows: (i) minimum for matching peptide masses, (ii) 50-100 ppm mass accuracy, and (iii) molecular weight and pI obtained from image analysis. For MS/MS search, fragmentation of selected peptide molecular ion peak was used to identify the protein in the same manner by searching NCBI nonredundant database using MASCOT MS/MS ion search program.

Results

The mean age of the patients was 11.3±0.8 years in MCNS and 12.5±0.5 years in FSGS. Serum biochemistry data did not show any statistical difference between MCNS and FSGS at the time of renal biopsy. However, FSGS patients gradually progressed to CRF after 3 years later (Table 1).

Protein spots of MCNS were isolated by immobilized linear gradient strip gel (pH 4-7) using IPGphor system and silver staining (Fig 1). The differences in protein expressions between MCNS

Table 1. Biochemistry Findings at the Time of Initial Renal Biopsy and 3 Years Later

	Renal biopsy*		3 years later		*P*
	MCNS (Mean±SD)	FSGS (Mean±SD)	MCNS (Mean±SD)	FSGS (Mean±SD)	
Protein (g/dL)	4.2±0.5	4.3±0.5	6.2±1.9	4.3±1.3	0.04
Albumin (g/dL)	2.1±0.2	2.0±0.6	3.6±1.2	2.0±0.8	0.03
BUN (mg/dL)	15.8±6.8	8.8±3.7	12.8±5.3	25.0±9.5	<0.001
Creatinine (mg/dL)	0.7±0.1	0.5±0.1	0.7±0.2	1.5±0.5	0.001
Calcium (mg/dL)	8.2±0.5	8.6±0.6	9.4±2.6	8.7±2.5	NS
Phosphorus (mg/dL)	4.8±0.7	5.4±0.2	4.5±1.3	5.2±1.6	NS
Uric acid (mg/dL)	5.5±1.2	4.4±0.2	5.4±1.7	7.1±2.5	NS

Abbreviation: NS, Not significant. *P=NS

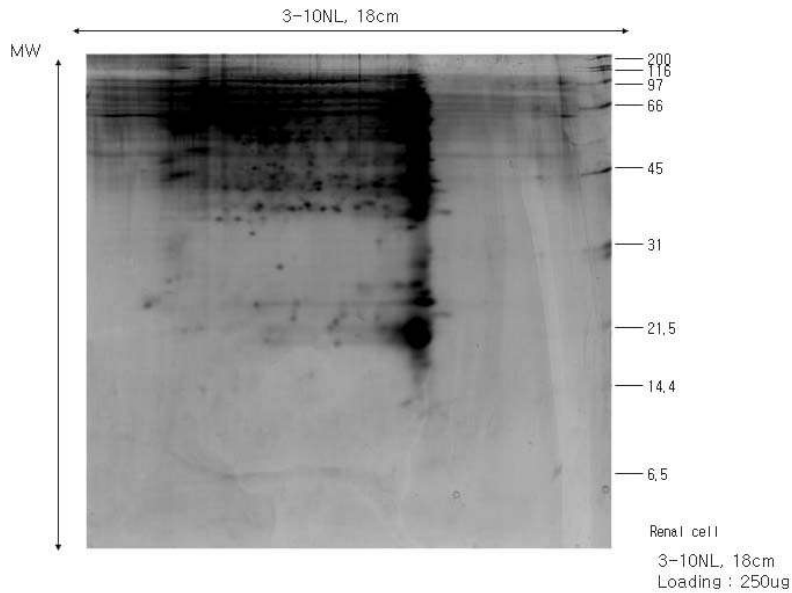

Fig. 1. Protein spots of MCNS were isolated by immobilized linear gradient strip gel (pH 4-7) using IPGphor system and silver staining.

and FSGS were shown by significantly increased or decreased protein spots. Most prominently expressed spot among several spots in FSGS was glutathione S-transferase (GST) P1-1, whereas it was not found in MCNS. So GSTP1-1 was considered as the one of the key biomarkers in pathogenesis of FSGS.

Discussion

MCNS is the most common cause of idiopathic nephrotic syndrome in children. However, FSGS is somewhat less but do not respond well to steroid therapy and gradually progress to renal failure. In children the diagnosis of FSGS may be suspected when the condition is steroid-resistant. The initial clinical presentation of FSGS may be identical to that of MCNS, but FSGS is characterized by steroid resistance, progressive worsening of renal function, and a typical glomerular pathology. Recent reports suggest that there has been an increase in the frequency of FSGS as a pathological picture of idiopathic NS in the pediatric population. There is evidence that FSGS is becoming more common : In one series, between 1976 and 1979, 15% of cases were due to FSGS, and in a later cohort between 1995 and 1997, the proportion with FSGS had risen to 35% [4]. Despite the increase of incidence, the mechanisms or pathogenesis for FSGS in children with INS still are obscure.

In an attempt to elucidate the pathogenesis of FSGS, we conducted a proteomic analysis for renal biopsy samples of FSGS. We tried to find out the differences in protein expressions between MCNS and FSGS by significantly increased or decreased protein spots. However, it was not sufficient to analyze protein expression because the biopsy samples were not so much to analyze it. So we had to examine the most prominently ex-

pressed pot among them, which was glutathione S-transferase P1-1, whereas it was not found in MCNS. So glutathione S transferase P1-1 was considered as the one of key biomarkers in pathogenesis of FSGS.

Human GST enzymes can be subdivided into five main classes, alpha (A), mu (M), pi (P), theta (T), and zeta (Z). Each class includes one or more isoenzymes with different, but sometimes overlapping, substrate specificity. GSTP1-1 is a heterodimeric enzyme with subunits varying between 23 and 28 kDa. GSTP1-1 is a phase II drug metabolism enzyme playing an important role in cell detoxification by conjugating electrophilic compounds to glutathione, allowing their export through the GS-X pump [5, 6]. GSTP1-1 over-expression was shown in various cancers such as cholangiocarcinoma, ovary carcinoma and leukemia. Over-expression of GSTP1-1 in tumors suggests that it may be a significant factor that leads to drug resistance. Indeed, GSTP1-1 over-expression has been reported with cisplatin resistance in head and neck squamous cell carcinoma. Study in kidney disease showed that the detection of GSTP1 protein released from damaged tubular cells has been useful in the study of both acute and chronic renal injury in a variety of clinical and experimental situations [7-12]. Other studies reported that patients with GSTP1 polymorphism in NS had a significantly lower rate of sustained remission compared to homozygous wildtype after cyclophosphamide treatment [13].

Therefore urinary GST-P1 level or genetic polymorphisms might be useful to plan treatment for patients with steroid resistant NS. In the present study, there are some limitations that need to be acknowledged and addressed. First, the sample size for each group is too small. Second, we identified only one prominent spot among several spots. So it is difficult to certain significant differences between two groups from the data. Therefore further studies of large-scale will be needed to reveal the difference between MCNS and FSGS.

Although our study is preliminary trial and some limitations, this result would be helpful in researching the pathogenesis of FSGS and GSTP associated enzymes are thought to be important factor for drug response in FSGS. Further studies for glutathione S-transferase P1-1 are warranted to confirm and elucidate the mechanisms of FSGS.

Acknowledgements

This research was supported by the Korean Society of Pediatric Nephrology research fund (2003 year Ferring pharmaceuticals).

요 약

단백질체학을 이용하여 국소성 분절성 사구체 경화증과 미세 변화형 신증후군의 비교

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목 적 : 국소성 분절성 사구체 경화증 환아들은 어떤 종류의 약물치료에도 잘 반응하지 않고 점차 말기 신부전으로 진행되는 경우가 많다. 본 연구는 미세 변화형 신증후군과 국소성 분절성 사구체 경화증 사이의 단백질 발현의 차이를 알아보고자 시행하였다.

방 법 : 미세 변화형 신증후군과 국소성 분절성 사구체 경화증의 신장 조직 샘플로부터 단백질을 추출

하였다. 추출한 단백질들에 대해 2차원 전기영동 시스템을 이용하여 개개의 단백체로 분리한 후 실버 염색을 하였다. 분리한 단백질은 MASCOT Peptide Mass Fingerprint 프로그램을 이용하여 동정하였다.

결 과 : 미세변화형 신증후군과 국소성 분절성 사구체 경화증의 신장 조직에서 서로 상반된 발현 양상을 보여주었다. 이중 가장 크고 두드러지게 발현되는 부위를 잘라내어 단백질 분석을 시행한 결과 국소성 분절성 사구체 경화증에서만 glutathione S-transferase P1-1 단백질이 발현 되었다.

결 론 : 상기 결과는 비록 국소성 분절성 사구체 경화증의 병태생리를 알기 위한 기초연구였으나 본 연구에서 밝혀진 glutathione S-transferase P1-1은 향후 질병의 기전을 밝히는데 있어서 중요한 소견으로서 향후 지속적인 연구가 필요할 것으로 사료된다.

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