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Serum and Urine ANGPTL8 Expression Levels are Associated with Hyperlipidemia and Proteinuria in Primary Nephrotic Syndrome

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Abstract

Background: This study aimed to investigate the expression characteristics of ANGPTL8 in patients with primary nephrotic syndrome and its possible correlation

with hyperlipidemia and proteinuria.

Methods: ANGPTL8 levels were determined using Enzyme-linked immunosorbent assay in 133 subjects with PNS, and 60 subjects with healthy controls.

Results: Subjects with primary nephrotic syndrome had higher levels of serum and urine ANGPTL8 than healthy controls subjects ($P < 0.001$). In primary nephrotic syndrome patients, serum ANGPTL8 was positively correlated with cholesterol ($r = 0.209$, $P < 0.05$) and triglycerides ($r = 0.412$, $P < 0.001$), while no correlation with 24hUTP. Urine ANGPTL8 was positively correlated with high density lipoprotein-cholesterol ($r = 0.181$, $P < 0.05$), while urine ANGPTL8 was significantly negatively correlated with creatinine ($r = -0.323$, $P < 0.001$) and 24hUTP ($r = -0.268$, $P = 0.002$). Interestingly, urine ANGPTL8 concentrations were different between membranous nephropathy and mesangial proliferative glomerulonephritis pathological types.

Conclusions: Serum and urine ANGPTL8 levels in primary nephrotic syndrome patients were correlated with blood lipid levels and proteinuria, respectively, suggesting that ANGPTL8 may play a role in the development of primary nephrotic syndrome hyperlipidemia and proteinuria.

Background

The typical clinical manifestations of PNS include massive proteinuria, hypoproteinemia, edema and (or) hyperlipidemia [1], among which, marked proteinuria is the core clinical manifestation, and its severity is often parallel to the degree of hyperlipidemia [2-4]. The mechanism of PNS complicated with hyperlipidemia has not been well understood.

Angiopoietin-like proteins are a family of proteins that identified by Kim et al in 1999 according to the homology of Ang1's and Ang2's amino acid sequence [5]. ANGPTLs consist of 8 members (angiopoietin like protein 1-8) [6]. ANGPTL8, as a newly recognised ANGPTLs family member, has been found to be highly expressed in mouse liver as well as the brown adipose tissue, and moderately elevated in subcutaneous adipose tissue, kidney, small intestine and heart [7-9].

Previous studies report ANGPTL8 detected both in human serum and mouse serum [9], as well as in human urine samples [10]. It's also recently observed that the serum levels of ANGPTL8 is significantly increased in patients with metabolic diseases such as type 2 diabetes [10,11], hyperlipidemia [12-14], non-alcoholic fatty liver [15] and so on. Moreover, in type 2 diabetes patients, the elevated serum ANGPTL8 level are associated with their increased risk of diabetic nephropathy, suggesting ANGPTL8's possible role in kidney damage [10].

This study aims to investigate the characteristics of serum and urine ANGPTL8 levels in PNS patients, and further to look at its relationship with patients' lipid metabolism parameters as well as severity of urinary protein. We found that there was a significant correlation between serum ANGPTL8 and lipid metabolism indexes, such as TG. And urine ANGPTL8 was significantly correlated with the degree of urinary protein and pathological types.

Methods

Subjects. This cross-sectional study was carried out between November 2017 to November 2019 in the Gansu Provincial People's Hospital. The study was approved by

the Medical Ethics Committee of Gansu University of Traditional Chinese Medicine (No. syll20160037), and all methods were carried out in accordance with relevant guidelines and regulations. All participants gave their oral and written informed consent prior to participation. A total of 193 subjects were recruited including 133 patients with PNS and 60 HC. Subjects underwent a detailed medical history record, medical examination, comprehensive urine analysis, 24-hour urine protein assay, routine blood test, endocrine profile, biochemical analysis, and ultrasound examination of urinary system. 78 PNS patients performed renal biopsy.

The inclusion criteria of PNS patients were as follows: heavy proteinuria [24-hour urine total protein (24hUTP) > 3.5g or urine protein/creatinine ratio > 3.0mg/mg or 24hUTP > 50mg/kg] and hypoalbuminemia [serum albumin (ALB) < 25g/L], or various degrees of edema and hyperlipidemia [16,17].

The HC had no concomitant health problems, and fasting blood lipid levels [18] [cholesterol (CHOL) < 200mg/dl, triglycerides (TG) < 150 mg/dl, and low density lipoprotein-cholesterol (LDL-C) < 130mg/dl] and urinary proteins (urinary microalbumin $\leq$ 150mg/dl or urine qualitative test was negative) were within normal range.

The exclusion criteria were as follows: secondary nephrotic syndrome, previous history of other acute and chronic kidney disease, patients with abnormal ultrasound examination of the urinary system (deformities, cysts, hydrops, stones, etc.), identified acute or chronic illness (diabetes mellitus, thyroid dysfunction, polycystic ovary syndrome, obesity, fatty liver, familial hypercholesterolemia), and other system

diseases, such as hematological diseases, cardiovascular diseases, connective tissue diseases, tumors, and obvious infections.

Anthropometric and laboratory measurements. The medical records of following parameters were collected for the study: age, gender, body mass index (BMI) calculated as weight divided by height squared (kg/m^2), blood pressure, disease state including initial treatment (the first onset of PNS, without immunosuppressive drugs and other medical treatment, the course of disease is generally less than 2 months) and relapse[(for 3 consecutive days, urine protein changed from negative to (+++) or (++++), or $24\text{hUTP} \geq 50\text{mg/kg}$, or urine protein/creatinine ≥ 2.0 (mg/mg)](19), pathology result of renal biopsy provided by Guangzhou KingMed Diagnostics (a independent clinical laboratories certified by ISO15189), including minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN), and mesangial proliferative glomerulonephritis (MsPGN). In addition, the data of levels of serum CHOL, TG, high density lipoprotein-cholesterol (HDL-C), LDL-C, ALB, creatinine (CREA), urea(Ur) [measured by automated biochemical analyzer (ARCHITECT c1600, Abbott, USA)], 24hUTP , urinecreatinine (UCr) [measured by automated chemiluminescence immunoanalyzer (UniCel DxI 800, Beckman-Coulter, USA)], and urine protein [measured by automated urine analyzer (FUS-2000, DIRUI, China)] were also collected. All assays were performed according to the routine procedures specified by the clinical laboratory center (certified by ISO15189), the Gansu Provincial People's Hospital. Severity of proteinuria were divided into three categories according to levels of 24hUTP : mild ($\leq 1\text{g/d}$), moderate($1\sim 3.5\text{g/d}$), and

severe($\geq 3.5\text{g/d}$) proteinuria. The estimated glomerular filtration rate (eGFR) was calculated from serum creatinine using the Cockcroft-Gault formula equation [20].

Enzyme-linked immunosorbent assay (ELISA). Blood samples were collected from the antecubital vein of subjects between 07:30 and 09:00a.m. After an overnight (at least 10h) fast. Collected blood were separated by centrifugation for 20 min at 3,000 rpm at 4°C after coagulating for 30 min at 4°C. Furthermore, a clean midstream first-morning urine sample was obtained from all subjects. Assay samples were immediately stored at -20°C until ELISA was performed, and we also extensively avoided repeated freeze-thaw. Serum and urine ANGPTL8 concentrations were measured using ELISA kits (Jiangsu Meimian Industrial Co., Ltd, Jiangsu, China, Catalogue No. E1766H1), procedures were conducted in compliance with manufacturer's protocol.

Statistical analysis. The distribution pattern of continuous variables were tested for normality using Kolmogorov-Smirnov ($n \geq 100$) / Shapiro-Wilk's test ($n < 100$). For normally distributed variables, the data were presented as mean $\pm$ standard deviations (S.D.); differences were assessed by independent samples t test / Two-tailed Student's t-test / One-way ANOVA; Pearson correlation coefficients were estimated to determine the correlation. For nonnormally distributed variables, the data were presented as median [interquartile range]; differences were assessed by Mann-Whitney U test / Kruskal-Wallis test / Kruskal-Wallis one-way ANOVA; correlations were analyzed by Spearman correlation method. Categorical variables were reported as frequency or percentage; differences were assessed by Pearson χ^2 / continuity modified χ^2 test. For grade variables, differences were assessed by Wilcoxon symbol rank and test. A two-

sided P value < 0.05 was considered as statistically significant. All statistical analyses were carried out using SPSS software (version 26.0).

Result

Baseline characteristics of the total samples

Table 1 summarizes the clinical characteristics of the total samples. 133 cases were enrolled in PNS group, including 94 males (70.68%) and 39 females (29.32%) that male to female ratio was 2.41. 60 cases were enrolled in control group, including 40 males (66.67%) and 20 females (33.33%) that male to female ratio was 2.41. There were no difference in gender, age and blood pressure between the two groups ($P > 0.05$).

Serum and urine ANGPTL8 levels significantly increased in PNS patients

Figure 1 and 2 shows that, when investigating serum and urine ANGPTL8 in relation to PNS, patients with PNS had significantly higher serum and urine ANGPTL8/UCr concentrations compared with control group. However, no statistical difference was found in the expression of serum ANGPTL8 and urine ANGPTL8/UCr between recurrent and newly diagnosed PNS patients. (Table. 2)

Serum ANGPTL8 level correlated with blood lipid level in PNS.

As shown in table 3, in PNS patients, serum ANGPTL8 level was significantly positively associated with CHOL ($r = 0.209$, $P < 0.05$) and TG ($r = 0.412$, $P < 0.001$), while no significant association was found between serum ANGPTL8 level and 24h UTP.

Urine ANGPTL8 level associated with PNS pathological types.

We further investigated the serum and urine ANGPTL8 level in 78 PNS patients with

renal biopsy pathology results which includes 21 MCD, 8 MsPGN, 38 MN and 11 FSGS. No overall significant difference of serum ANGPTL8 level was found across different PNS types, however, for urine ANGPTL8/UCr level, significant difference was observed between MN and MsPGN group ($P < 0.05$, table 4).

Urine ANGPTL8/UCr level associated with degree of proteinuria in PNS patients.

PNS patients were further grouped into three proteinuria groups according to levels of 24hUTP. As shown in table 5, serum ANGPTL8 level were not significantly different among three proteinuria groups ($P > 0.05$), while urine ANGPTL8/UCr level were positively associated with degree of proteinuria.

Correlation analysis on urine ANGPTL8 and lipid metabolism indicators showed: urine ANGPTL8/UCr level was positively correlated with HDL-C ($r = 0.201$, $P < 0.05$), and was negatively correlated with CREA ($r = -0.323$, $P < 0.001$) and 24hUTP ($r = -0.268$, $P < 0.05$) (Table 3).

Discussion

The molecular mechanism of massive proteinuria complicated with hyperlipidemia in PNS has yet not been elucidated. Recent studies have shown that the degree of hyperlipidemia in PNS is often parallel to the severity of proteinuria [2-4]. Hyperlipidemia symptom occurs in PNS patients involving two main mechanisms: first, increased glomerular permeability results in massive urinary protein loss, as a result, this severe hypoproteinemia stimulates the synthesis of protein in the liver, leading to increased production of lipoproteins; Second, reduced concentration and decreased activity of lipid lipase (LPL) in the blood, a key rate-limiting enzyme in lipid

catabolism, impaired clearance of low density lipoproteins (LDL) and very low density lipoprotein (VLDL) [21]. In this study, we analyzed the level of ANGPTL8 in patients with PNS to control group, and its possible association with levels of parameters related to hyperlipidemia, so as to explore a new potential molecular target for the study of the mechanism of lipid metabolism disorder in PNS.

It is known that ANGPTL8, a potent inhibitor of LPL activity, reduces the clearance of TG and increases the level of serum TG [7]. Study on gene editing mice showed higher level of serum TG in ANGPTL8 overexpression mice, while lower level in ANGPTL8 knockout mice [7,8]. In the current study, we detected ANGPTL8 in blood and urine of patients with PNS using ELISA method, and found that PNS patients had significantly higher level of serum and urine ANGPTL8 as compared with healthy controls, which implicated the potent role of ANGPTL8 in the pathophysiology of PNS.

Previous studies showed serum ANGPTL8 was positively correlated with TG, and negatively correlated with CHOL and HDL in patients with type 2 diabetes [10]; ANGPTL8 was also related to TG and CHOL in patients with hyperlipidemia after adjusting age, sex, BMI, alanine aminotransferase, hypersensitive C-reactive protein and CREA. In agreement to our findings on PNS patients, serum ANGPTL8 level was found to be positively associated with CHOL, but not with HDL-C and LDL-C [14]. In the type 2 diabetes study [10], ANGPTL8 level was found to be related to urinary albumin excretion, and there is a positive correlation between serum ANGPTL8 and urinary albumin /creatinine ($r = 0.427$, $P < 0.001$). While in our study on PNS patients, there was no statistically significant difference between serum ANGPTL8 and 24hUTP,

however, serum ANGPTL8 is positively correlated with both CHOL and TG, either using ANGPTL8 as continuous variable or categorical variable. Thus, in contrast to diabetic nephropathy, in PNS patients, serum ANGPTL8 showed stronger correlation with metabolism disorders. The possible mechanism of the effect of ANGPTL8 on the blood lipid level of PNS needs to be further studied.

Further analysis on different pathology types of PNS patients showed urine ANGPTL8/UCr level were significant higher in MsPGN group compared to MN groups, however for serum ANGPTL8 the difference was not found, suggesting urine ANGPTL8 is more likely to be associated with renal damage. Moreover, the correlation analysis between urine ANGPTL8/UCr and main PNS parameters showed in PNS patients ANGPTL8/UCr was positively correlated with HDL-C and negatively correlated with CREA and 24hUTP ($r = -0.268$, $P < 0.05$).

Taking these findings into consideration, we would like to suggest further urine ANGPTL8 studies should be addressed on mechanisms related to renal damage. Notably, as a small molecule of secretory glycoprotein, the source of ANGPTL8 detected in urine still needs to be identified. Whether ANGPTL8 could be synthesized and secreted in the kidney is the primary hypothesis to be tested when studying the pathophysiological significance of ANGPTL8 in renal damage, and we are carrying out related experiments. Taken together, we preliminarily revealed the characteristic of serum and urine ANGPTL8 in patients with PNS and examined the association with main disease indicators. This study might be helpful in understanding the pathogenesis of PNS and explore a new potential target for treatment.

Conclusions

There were significant differences in levels of serum and urine ANGPTL8 both in PNS and health control subjects. In PNS patients, serum ANGPTL8 level was significantly positively associated with serum lipid indicator CHOL, moreover urine ANGPTL8/UCr level was negatively correlated with proteinuria degree 24h UTP.

ANGPTL8 might be involved in the development of PNS.

Fig.1 Comparison of serum ANGPTL8 levels box-plot between PNS group and control group, patients with PNS had significantly higher serum ANGPTL8 levels than healthy controls ($P<0.001$).

Fig.2 Comparison of urine ANGPTL8 levels box-plot between PNS group and control group, patients with PNS had significantly higher urine ANGPTL8 levels than healthy controls ($P<0.001$).

Table 1 Anthropometric and laboratory characteristics of healthy individuals and patients with PNS.

Variables	PNS n = 133	Controls n = 60	P value
Sex (F/M)	94 / 39	40 / 20	0.576
Age (years)	33.23 ± 20.16	36.05 ± 16.40	0.276
BMI (kg/m ²)	22.94 ± 2.78	22.24 ± 1.75	0.051
SBP (mmHg)	120.00 (107.50, 129.00)	117.00 (108.25, 126.75)	0.466
DBP (mmHg)	73.39 ± 10.03	73.50 ± 8.32	0.941
CHOL (mmol/L)	8.25 (6.42, 10.33)	4.39 (3.74, 4.95)	<0.001*

TG (mmol/L)	2.77 (1.91, 3.29)	1.11 (0.96, 1.43)	<0.001*
HDL-C (mmol/L)	1.66 (1.25, 1.98)	1.31 (1.19, 1.48)	<0.001*
LDL-C (mmol/L)	5.11 (3.61, 6.64)	2.51 (2.13, 2.91)	<0.001*
ALB (g/L)	23.40 (17.90, 28.95)	46.25 (44.13, 47.96)	<0.001*
CREA (μmol/L)	62.50 (46.60, 78.10)	58.65 (50.13, 76.15)	0.891
Ur (mmol/L)	6.10 (4.50, 8.30)	4.26 (3.67, 5.39)	<0.001*
Urine protein	1~4	0	<0.001*
Serum ANGPTL8 (ng/ml) ^a	28.29 (22.31, 31.30)	23.21 (19.55, 25.11)	<0.001*
Urine ANGPTL8 (ng/ml) ^a	42.36 (37.45, 76.44)	36.38 (33.59, 39.41)	<0.001*

F: female, M: male. In the qualitative test of urine protein, '0' represents '-', '1' represents '+', '2' represents '++', '3' represents '+++', '4' represents '++++'.

* $P < 0.05$ was considered significant.

Table 2 Comparison of ANGPTL8 levels of PNS patients by disease status.

initial treatment	Serum ANGPTL8 (ng/ml)	Urine ANGPTL8/UCr (ug/g)
initial treatment (n=99)	28.72(22.71,32.27)	57.36(39.34,106.62)
Relapse (n=34)	25.29(19.75,29.96)	56.48(39. 31,87.47)
<i>P</i> value	0.055	0.585

Table 3 Correlation between ANGPTL8 levels and main clinical indicators in patients with PNS.

Variables	Serum ANGPTL8		Urine ANGPTL8/UCr	
	r	<i>p</i> value	r	<i>p</i> value
CHOL	0.209	0.016*	0.073	0.406
TG	0.412	0.000*	0.036	0.682
HDL-C	0.065	0.445	0.201	0.020*
LDL-C	0.129	0.140	0.113	0.193
ALB	-0.027	0.757	-0.068	0.434
CREA	-0.017	0.845	-0.323	0.000*
Ur	0.115	0.188	-0.089	0.309
eGFR	-0.137	0.116	0.016	0.853
24hUTP	0.087	0.321	-0.268	0.002*

Spearman's correlation analysis was used.

r: Spearman's correlation coefficient.

* P value < 0.05 was significant correlation.

Table 4 Comparison of ANGPTL8 levels of PNS patients by different pathological types.

Pathological types	Serum ANGPTL8 (ng/ml)	Urine ANGPTL8/UCr (ug/g)
MCD (n=21)	25.76 ± 9.09	52.78 (44.21, 86.77)
MsPGN (n=8)	26.42 ± 5.72	91.68 (53.16, 130.32) *
MN (n=38)	26.00 ± 9.47	45.08 (31.06, 69.86)
FSGS (n=11)	27.13 ± 6.79	76.01 (35.64, 134.98)
P value	0.977	0.010

* The significance values were adjusted by Bonferroni correction, which was statistically significant compared with the MN group ($P < 0.05$).

Table 5 Comparison of ANGPTL8 levels of PNS patients by different pathological types.

Pathological types	Serum ANGPTL8 (ng/ml)	Urine ANGPTL8/UCr (ug/g)
MCD (n=21)	25.76 ± 9.09	52.78 (44.21, 86.77)
MsPGN (n=8)	26.42 ± 5.72	91.68 (53.16, 130.32) *
MN (n=38)	26.00 ± 9.47	45.08 (31.06, 69.86)
FSGS (n=11)	27.13 ± 6.79	76.01 (35.64, 134.98)
P value	0.977	0.010

* The significance values were adjusted by Bonferroni correction, which was statistically significant compared with the MN group ($P < 0.05$).

Abbreviation

PNS: primary nephrotic syndrome

HC: healthy controls

24hUTP: 24hour urine total protein

ALB: serumalbumin

CHOL: cholesterol

TG: triglycerides

LDL-C: low density lipoprotein-cholesterol

BMI: mass index

MCD: minimal change disease

FSGS: focal segmental glomerulosclerosis

MN: membranous nephropathy
MsPGN: mesangial proliferative glomerulonephritis
HDL-C: high density lipoprotein-cholesterol
CREA: creatinine
Ur: urea
UCr: urinecreatinine
ELISA: Enzyme-linked immunosorbent assay
S.D.: standard deviations
LDL: low density lipoproteins
VLDL: very low density lipoprotein
LPL: lipid lipase

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Authors' contributions

Dr.Xia Gao designed the study and wrote the article. Dr. Qingju Liu performed the statistical analysis and data entry. Dr. Chengdong Kang took part in preparation of the article. Dr. Weijing Cui collected the patients' information. Dr. Zichuan Xu and Fu Zhong finished the ELISA tests

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Availability of data and materials

The datasets generated during and/or analysed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Gansu University of Traditional Chinese Medicine (No. syll20160037), and all methods were carried out in accordance with relevant guidelines and regulations. All participants gave their oral and written informed consent prior to participation.

Consent for publication

The patients signed an informed consent from for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Comparison of serum ANGPTL8 levels box-plot between PNS group and control group, patients with PNS had significantly higher serum ANGPTL8 levels than healthy controls ($P < 0.001$).

Figure 2

Comparison of urine ANGPTL8 levels box-plot between PNS group and control group, patients with PNS had significantly higher urine ANGPTL8 levels than healthy controls ($P < 0.001$).