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Activated YAP causes renal damage of type 2 diabetic nephropathy

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Abstract. – OBJECTIVE: Yes-associated protein (YAP) is a critical factor of Hippo pathway. It can control organ size, regulate proliferation, differentiation, and apoptosis of cells, and mediate epithelial-mesenchymal transition and cell contact inhibition. It has gradually become a hot spot in the field of diabetic nephropathy (DN). Tea domain (TEAD) is a factor with a deoxyribose nucleic acid (DNA) binding domain, which combines with activated YAP to control the expression of their important target factor - connective tissue growth factor (CTGF).

PATIENTS AND METHODS: We have investigated the role of YAP in type 2 diabetic nephropathy and evaluated the correlation between YAP and the progress of type 2 diabetic nephropathy. We have detected the expression of YAP, TEAD and CTGF in normal people (n=10) and patients with DN (n=51) by immunohistochemical and immunofluorescence staining and evaluated the relationship among clinical, pathologic data and YAP expression in type 2 diabetic nephropathy.

RESULTS: In kidneys of type 2 diabetic nephropathy, YAP, TEAD and CTGF were highly expressed in the nucleus of glomerular podocytes. In those healthy kidneys, however, all three of the above factors were mainly expressed in cytoplasm. Furthermore, the high expression of YAP in DN had relevance to increasing systolic blood pressure (SBP) (r=0.484, p=0.019), blood urea nitrogen (BUN) (r=0.522, p=0.032), creatinine (Cr) (r=0.496, p=0.031), progression of DN stage (r=0.647, p=0.001) and progression of DN pathologic classification (r=0.298, p=0.033). In addition, decreasing serum albumin (SAlb) (r=-0.656, p=0.001) and estimated glomerular filtration rate (eGFR) (r=-0.607, p=0.006) were also correlated with the high expression of YAP in DN.

CONCLUSIONS: High expression of YAP, TEAD and CTGF in kidney tissues suggested that YAP played a significant role in the renal damage of type 2 diabetic nephropathy. YAP that is correlated with SBP, BUN, Cr, DN stage, DN pathologic classification, SAlb and eGFR, suggested that inhibition of the activity of YAP might have the effect in delaying DN progression.

Key Words
Yes-associated protein, Tea domain, Connective tissue growth factor, Type 2 diabetic nephropathy.

Introduction

Diabetic nephropathy (DN) is one of the most critical kidney diseases underlying End-stage renal disease (ESRD) and has risen to the first cause in incidence globally[1]. Therefore, it has become an urgent task to study the mechanism of DN and control its progression. Yes-associated protein (YAP) is known as a transcriptional co-activator, which has only a transcription activation domain and lacks of a DNA binding domain. As a critical member of Hippo signaling pathway, YAP drives embryonic growth[2], stem cell proliferation and differentiation[3,4], vascular remodeling[5], progression of nervous system diseases and tumors[6-12]. First discovered by Sudol et al[13,14] in 1994, YAP, a protein with the molecular weight of 65 kDa encoded on the human 11q22 chromosome, had been under-recognized to be associated with a variety of human tumors. Under normal physiologic conditions, phosphorylated YAP combines with 14-3-3 regulatory protein which controls the subcellular localization, retains in cytoplasm together, and regulates the expression[15] of target factors such as Cyclin E, Death-associated inhibitor of apoptosis 1 (DIAP1) and CTGF to

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maintain the balance of cell survival. If Hippo pathway had been blocked or inhibited, dephosphorylated YAP would migrate from cytoplasm to nucleus. Functionally activated YAP then interacts with TEAD, which includes a DNA binding domain. Then, the combination of YAP and TEAD activates the overexpression of these target genes, and mediates cellular proliferation and extracellular matrix synthesis[16]. While a mutation occurs at a significant site of YAP-TEAD binding domain, YAP mediated expression of target gene would be inhibited[17]. In recent years, YAP has been identified to take part in the renal damage repairment of acute kidney injury (AKI) and chronic kidney disease (CKD). YAP nuclear accumulation can not only promote the complete repairment of damaged cells in response to slight injury with increased transient nuclear expression, but also the continuous incomplete repairment in response to serious injury with increased continuous nuclear expression[18]. Like in focal segmental glomerulosclerosis (FSGS), autosomal dominant polycystic kidney disease (ADPKD) and DN, YAP is highly expressed in the renal nucleus to activate the continuous cell proliferation and repairment of damaged kidney. It plays an important role in promoting the occurrence and development of renal fibrosis[19]. Although YAP has attracted more and more attention in prior renal studies, researches about YAP in DN are still sparse. As far as we know, there is no report on the complete study of YAP pathway in human DN specimens. In order to clarify the role of YAP in the occurrence and progression of DN for later research, this paper aims at discussing YAP-TEAD-CTGF pathway through immunohistochemical and immunofluorescence staining of renal biopsy specimens of patients with DN and exploring the relationship between clinical, pathologic data and YAP.

Patients and Methods

Patients

In Qilu Hospital of Shandong University and Yuhuangding Hospital Affiliated to Qingdao University, 51 patients with DN had been collected as a case group (DN group) from January 2012 to January 2018, who were in the DN III-V stages according to the standard of Mogensen[20], and diagnosed by renal puncture pathology. Their pathologic classification was based on the DN pathological classification system established by Research Committee of the Renal Pathology Society[21]. These patients were divided into 3 groups defined as DN 1 group (DN III stage, n=21), DN 2 group (DN IV stage, n=25) and DN 3 group (DN V stage, n=5). Exclusion criterion was: pathology showed diabetes mellitus complicated with other glomerular diseases. Another 10 cases were collected from patients with renal tumors, who were undergone radical nephrectomy from May 2017 to August 2017 in Department of Urology of Qilu Hospital of Shandong University, and tissues of negative surgical margin, which were proven by pathology, were taken as the healthy control group (HC group). Our studies were approved by the Qilu Hospital of Shandong University and Yuhuangding Hospital Affiliated to Qingdao University Ethics Committee. Informed consent was signed by each subject.

Clinical Indicators

A uniform form was used to collect basic information of patients, including age, sex, course of disease and systolic blood pressure (SBP). Venous blood was collected from all patients for centralized inspection after 8 hours of fasting. Laboratory indicators including urinary microalbuminuria (U-MA), urinary albumin/creatinine (UAcr), SAlb, fasting blood glucose (FBG), glycated hemoglobin (GHbA1c), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), BUN, Cr, uric acid (UA), and cystatin c (Cys-C) were checked. eGFR was calculated using MDRD formula[22,23].

Research Methods

Immunohistochemical Staining on Renal Tissues

Renal tissues were paraffin-embedded and cut into sections with a thickness of 2-3 mm. Immunohistochemical staining was performed to observe the expression of YAP, TEAD and CTGF in HC group and DN group. Firstly, tissues were covered with the first antibody and placed at 4°C overnight. Then, rabbit secondary antibody was added to the slices and incubated. At last, renal tissue samples were photographed using a confocal microscope (Olympus, Tokyo, Japan). Semi-quantitative analysis was scored using the product of staining intensity and stained cells percentage: 0 point was negative, 1-4 points were weakly positive, and > 4 point was positive. Dyeing degree: none staining, 0 point; light staining, 1 point; dark staining, 2 points. The percentage of stained cells in counting cells: ≤ 5 %, 0 point;

6-25%, 1 point; 26-50 %, 2 points; ≥ 51 %, 3 points. The scores were independently evaluated using blind method by three pathologists, and the final score was averaged.

Immunofluorescence Staining on Renal Tissue Samples

Immunofluorescence staining was performed to observe the co-localization of YAP, WT1 and 4',6-diamidino-2-phenylindole (DAPI) (Solarbio, Beijing, China) in HC group and DN group. Firstly, frozen renal sections with a thickness of 9 mm were immunostained with antibodies directed against YAP and podocyte specific marker WT1. Then, after secondary antibodies, nuclear marker DAPI was counterstaining. At last, renal tissue samples were photographed under an immunofluorescence microscope (Olympus, Tokyo, Japan).

Antibodies

Antibodies used in this study were Rabbit anti-YAP (Novus Nb11058358, Littleton, CO, USA), Rabbit anti-TEAD (Abcam Ab97460, Cambridge, MA, USA), Rabbit anti-CTGF (Abcam Ab6992, Cambridge, MA, USA) Mouse anti-WT1 (Biorbyt Orb317387, Cambridge, MA, USA), Alexa Fluor 594 donkey anti-rabbit IgG (Proteintech, Rosemont, IL, USA), and Alexa Fluor 488 donkey anti-mouse IgG (Proteintech, Rosemont, IL, USA).

Statistical Analysis

Statistical comparison and analysis were performed using Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism for Mac 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were presented as means±SD (standard deviation). Clinical data of DN group were carried out using normal distribution test and calculated using nonparametric test and one-way analysis of variance (ANOVA), followed by post-hoc test LSD (Least Significant Difference). Spearman correlation analysis was used to compare scores of YAP, TEAD and CTGF expression, as well as the correlation between clinical, pathologic data and YAP. $p<0.05$ was considered statistically significant.

Results

Comparison of Clinical Data in Different Stages of DN

All clinical data were listed in Table I. No statistically significant differences of age, sex among all groups. Comparing the clinical data of DN group, we found that U-MA, UAcr, TG, TC, Cr, UA, Cys-c increased with the progress of disease staging ($p<0.05$). On the other side,

Table I. Comparison of clinical indexes in DN III-V stage.

Indexes	DN 1 Group	DN 2 Group	DN 3 Group	p
Age (years)	52±12.51	47.12±9.02	53±1.58	0.263
Sex (male/female)	11/10	14/11	4/1	0.237
Duration (years)	5.29±2.25	8.05±3.36	16±0.01	0.133
SBP (mmHg)	160.13±22.77	173.75±24.17	154.19±21.41	0.259
U-MA (mg/L)	1284.96±608.98	2938.07±1370.12*	472.08±0.01	0.001
UAcr (g/mmol)	2.18±1.28	6.31±3.15*	2.64±1.21	0.000
SAlb (g/L)	34.49±7.86	27.35±6.94*	23.58±4.82$^{\#}$	0.002
FBG (mmol/L)	8.11±4.15	8.19±4.71	6.61±2.61	0.896
HbA1C (%)	8.47±1.93	8.58±2.58	5.6±0.03	0.241
TG (mmol/L)	2.36±0.86	2.04±0.9	2.44±0.59$^{\#\&}$	0.011
TC (mmol/L)	6.36±2.57	7.76±2.8	5.54±2.16$^{\#\&}$	0.005
LDL-c (mmol/L)	3.17±0.98	4.77±2.08	4.57±0.01	0.507
HDL-c (mmol/L)	1.32±0.38	1.63±0.42	0.66±0.01	0.119
BUN (mmol/L)	10.82±5.25	10.33±4.76	35.2±15.55	0.065
Cr (umol/L)	128.95±60.97	145.82±97.39	633.25±241.84$^{\#}$	0.025
UA (umol/L)	421.21±101.47	342.5±66.8*	526.33±107.36$^{\&}$	0.002
eGFR (mL/min/1.73m^2)	62.23±30.21	65.39±32.75	8.25±2.53$^{\#\&}$	0.005
Cys-C (mg/L)	1.95±0.77	1.69±0.74	4.78±0.01	0.000

*DN 2 Group VS DN 1 Group; $^{\#}$DN 3 Group VS DN 1 Group; $^{\&}$DN 3 Group VS DN 2 Group. Data was expressed as means ± SDs.

SAlb and eGFR gradually declined ($p<0.05$). Meanwhile, there were no statistically significant differences during the course of disease, SBP, FBG, GHbA1c, LDL-c, HDL-c, BUN in all DN patients ($p>0.05$). Although no significant differences of FBG and GHbA1c were observed, the indexes in DN 2 group were higher than those in DN 1 group. Interestingly, these indexes almost returned to normal in DN 3 group.

The YAP Expression in DN Immunohistochemical

As shown in Figure 1, in HC group, YAP, TEAD, and CTGF were mainly expressed in cytoplasm of renal tissues. On the contrary, in DN group, YAP, TEAD, and CTGF were mainly expressed in the nucleus. YAP, TEAD, and CTGF staining scores of DN were compared respectively. The staining score of DN 3 group was higher than DN 1-2 groups ($p<0.05$).

Immunofluorescence

As shown in Figure 2, in DN group, YAP staining was co-localized with nuclear marker DAPI. While in HC group, YAP staining was co-localized with podocyte marker WT1 in the cytoplasm.

Correlation Analysis

Correlation Analysis Between YAP and Clinical Data

Spearman correlation analysis showed that YAP scores of immunohistochemical staining were positively correlated with SBP, BUN, Cr, DN stage and negatively correlated with SAlb, eGFR ($p<0.05$) (Figure 3) in DN group. However, YAP scores had no statistical correlation with age, sex, course of disease, U-MA, UAcr, FBG, GHbA1c, TG, TC, LDL-c, HDL-c, UA, Cys-c ($p>0.05$).

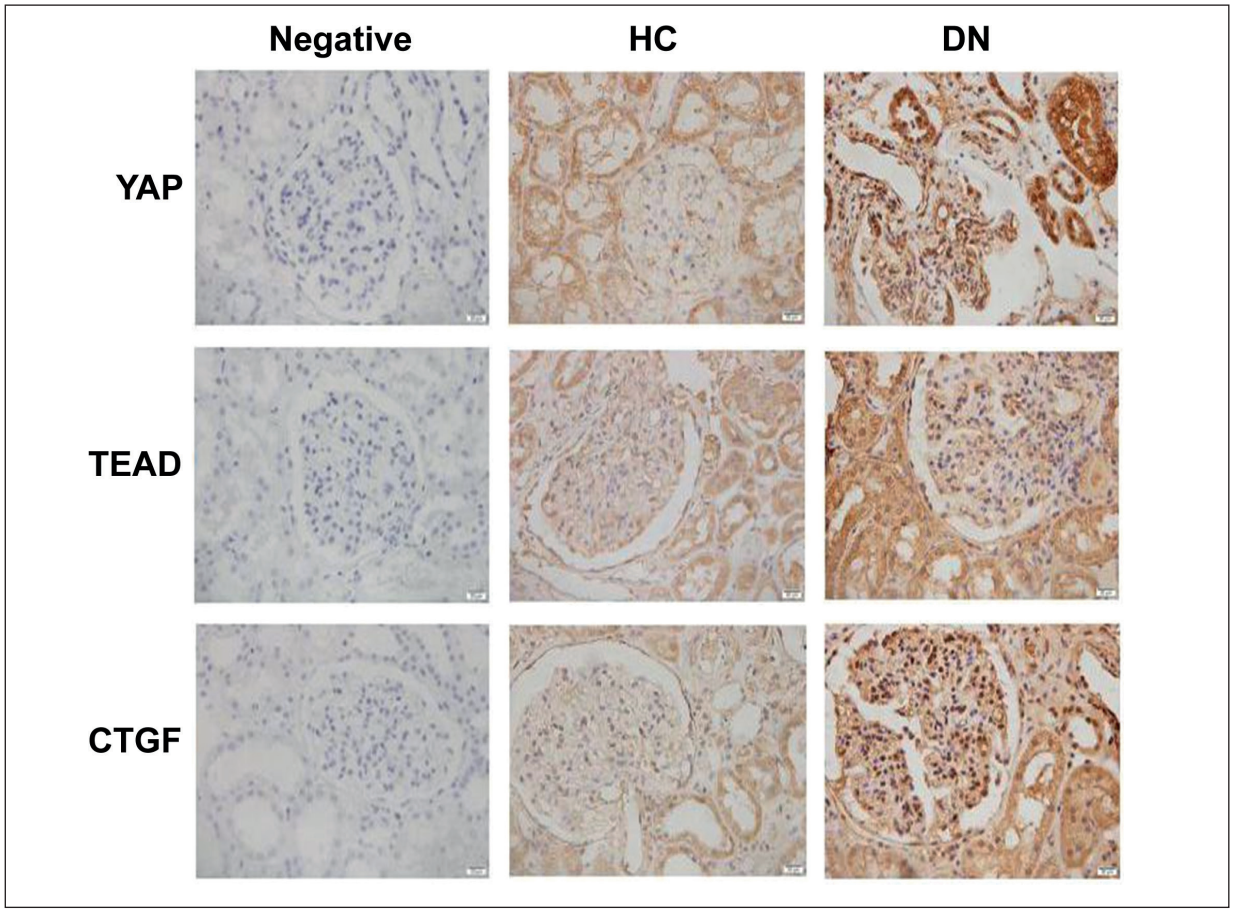

Figure 1. Immunohistochemical. HC, health control group; Negative, PBS replaces the primary antibody group; DN, diabetic nephropathy group (Magnification × 400).

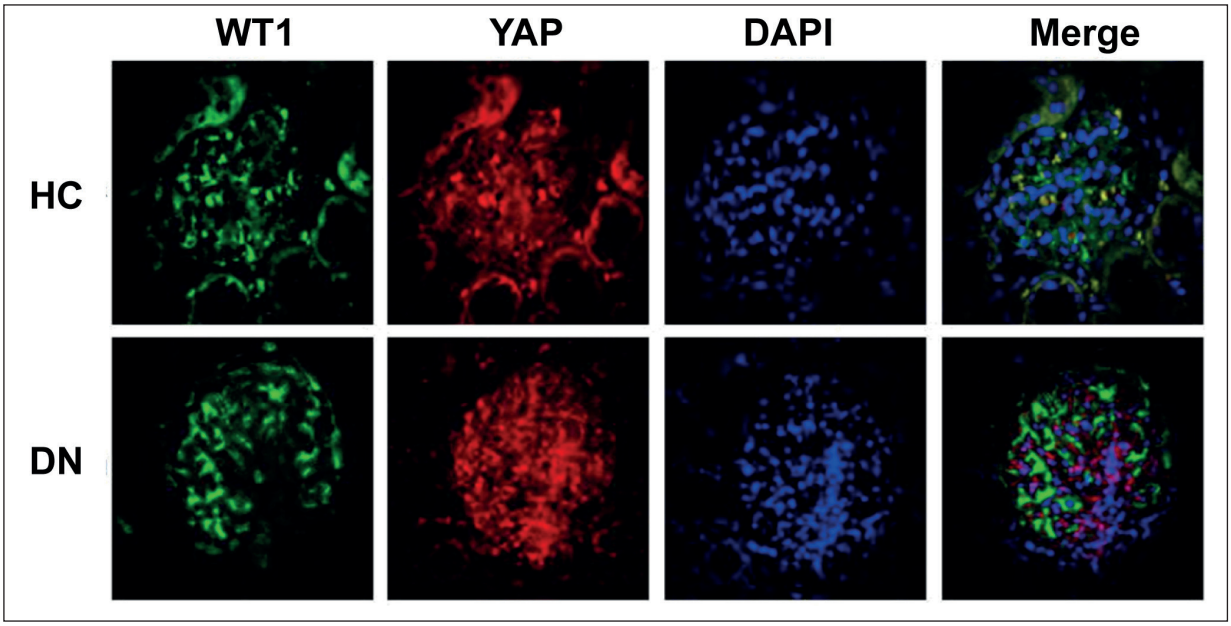

Figure 2. Immunofluorescence labeling of YAP in glomeruli. Lower panels: In DN, YAP (red) was co-localized with nuclear marker DAPI (blue). Red and blue overlap to show pink. Upper panels: In normal renal tissue, YAP was co-localized with podocyte marker WT1 (green) in the cytoplasm. Red and green overlap to show yellow (Magnification × 400).

Correlation Analysis of YAP, TEAD and CTGF

Spearman correlation analysis showed that, in nuclei of DN group, YAP expression was positively correlated with TEAD (r=0.704, $p<0.001$) and CTGF (r=0.504, $p=0.012$) expression. TEAD expression was positively correlated with CTGF (r=0.845, $p<0.001$) expression (Figure 4).

Correlation Analysis of YAP and Pathologic Classification

Spearman correlation analysis showed that YAP score had positively correlation with pathologic classification (r=0.298, $p=0.033$) (Figure 5).

Discussion

In this study, we have testified the expression of YAP, TEAD and CTGF in nuclear of type 2 diabetic nephropathy, especially in glomerular podocytes. YAP expression was positively correlated with TEAD and CTGF, suggested that there was a regulatory relationship in YAP-TEAD-CTGF pathway. The pathway might participate in renal damage of DN. Interestingly, according to the scores of YAP, TEAD and CTGF in different stages of DN, we noted that there was a higher expression of the above three proteins in DN V stage than DN III-IV stage. It was, therefore, possibly verified that the trend of cell proliferation and fibrosis in DN V stage was more obvious than that in DN III-IV stages. Histologically, featuring characteristics of early DN include microalbuminuria, thickening and stiffness of basement membrane, extracellular matrix accumulation in glomerular mesangium and renal interstitial tissue, and eventually lead to glomerulosclerosis, albuminuria and podocyte loss[24, 25]. In this view, DN is treated as one of podocyte diseases. Under normal physiologic conditions, YAP is considered as a physiological antagonist of podocyte apoptosis, which plays an important role in maintaining the integrity of glomerular filtration barrier and reducing urinary protein production. When YAP is activated, it translocates in the nucleus and combines with TEAD, and then mediates many transcription factors to cause fibrosis, especially CTGF. This pro-fibrosis reaction will lead to thickening and stiffness of the basement membrane and further enhancement of YAP activation, thus aggravate renal pathological changes[26]. Studies[22] of high glucose-treated proximal renal tubule cells and DN mice models have verified the above theory. Glomerular transcriptome data analysis of three DN mouse models showed a significant increase in expression of six classical target genes (CTGF,

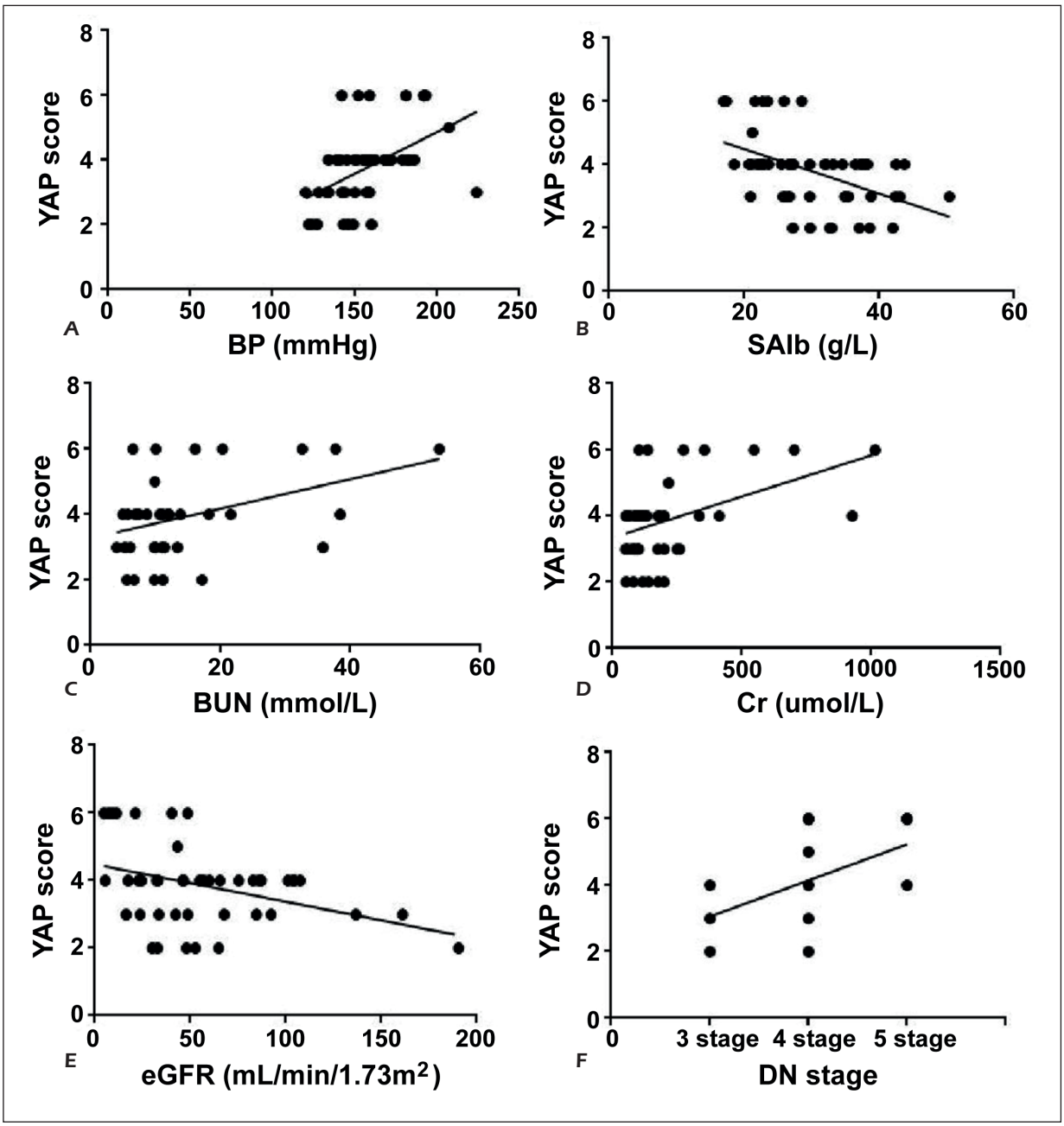

Figure 3. **A**, Correlation analysis between YAP and SBP, Spearman r=0.484, p=0.019. **B**, Correlation analysis between YAP and SAlb, Spearman r=-0.656, p=0.001. **C**, Correlation analysis between YAP and BUN, Spearman r=0.522, p=0.032. **D**, Correlation analysis between YAP and Cr, Spearman r=0.496, p=0.031. **E**, Correlation analysis between YAP and eGFR, Spearman r=-0.607, p=0.006. **F**, Correlation analysis between YAP and DN stage, Spearman r=0.647, p=0.001.

Cyr61, Ankrd1, Itgb2, Col8a1 and Axl) of YAP[22]. Immunohistochemical staining of renal biopsy specimens from 8 DN patients showed that CTGF expression was significantly higher than that in normal podocytes[27]. Taken together, these reports are in line with our results. Previous studies had shown that when podocyte injury led to cytoskeleton reorganization and cell morphological change, the filter barrier could be damaged and protein might leak into urine[27]. Thus, microalbuminuria is considered to be the first clinical indicator in the diagnosis of DN. UA is the end product of purine metabolism and about 70% of UA is removed from kidney. UA can cause endothelial

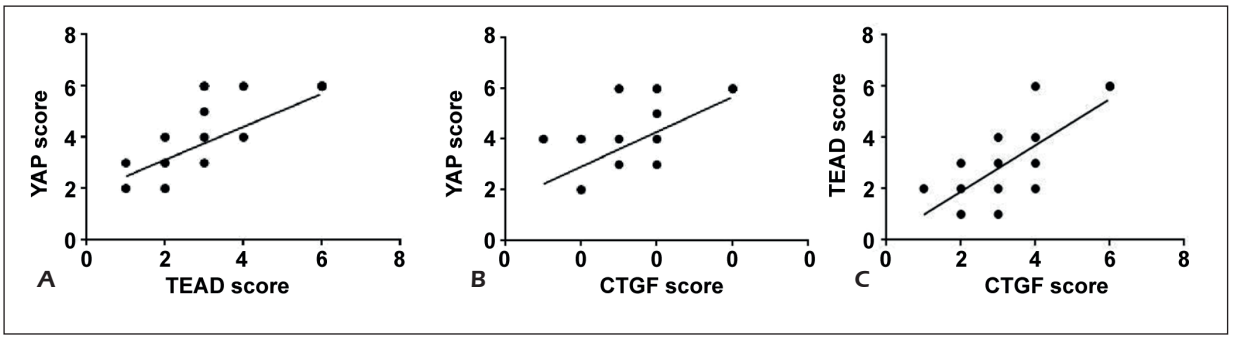

Figure 4. A, Correlation analysis between YAP and TEAD, Spearman r=0.704, p<0.001. **B**, Correlation analysis between YAP and CTGF, Spearman r=0.504, p=0.012. **C**, Correlation analysis between TEAD and CTGF, Spearman r=0.845, p<0.001.

dysfunction, activate RAAS system and pro-fibrosis cytokines, induce inflammatory cascade reaction and kidney injury in DN[28]. Based on this hypothesis, we analyzed the correlation between YAP expression and clinical data of DN patients, which was expected to provide theoretical basis for subsequent YAP clinical research. In comparison of clinical data in our study, DN V stage had higher U-MA, UAcr, TG, TC, Cr, UA, Cys-c than DN III-IV stage. Meanwhile, lower eGFR and SAlb were observed in DN V stage than in DN III-IV stage. Moreover, SBP, BUN, and Cr were positively correlated with YAP expression, while eGFR and Alb were negatively correlated with YAP expression. In this regard, active YAP expression may be related to the severity of renal dysfunction. According to the clinical standard stage of DN, the renal tissues collected in our study ranged from DN III stage to DN V stage. In spearman correlation analysis, the expression of YAP was positively correlated with DN stage, indicating that high YAP expression is involved in the progress of DN stage. No patients with DN I - II stages were collected in this study due to the limitation of clinical renal puncture standards. Based on the DN pathological classification system established by Research Committee of the Renal Pathology Society, our DN subjects' pathologic classification ranged from Class II - IV. As is known to all, glomerular lesions best reflect the natural course of progressive DN[29]. In our finding, YAP expression was positively correlated with DN pathological classification, which indicated that high YAP expression was accompanied by the aggravation of DN pathological damage. It is worth mentioning that high glucose stimulates high nuclear accumulation of YAP. Then YAP, to be positively interacted with TEAD, causes over-expression of target gene CTGF in the kidney[30,31]. However, YAP expression was not significantly correlated with FBG and GHbA1c in the statistical results of our study. Small sample size might be the explantation.

Our finding suggests that activated YAP may induce DN renal fibrosis representing a thrilling advance. Some studies have found that miRNAs are involved in various cell injuries in DN. In the study of mesangial cells in type 1 and type 2 diabetic rats, it was found that inducing up-regulation of miR-192 expression could lead to the deposition of type 1 collagen α2 and promote the accumulation of extracellular matrix. Inducing up-regulation of miR-216a and miR-217 expression could promote cell apoptosis. Increased miR-29c expression in kidney of type 2 diabetes mice model and high glucose-induced podocytes could result in podocyte apoptosis and extracellular matrix deposition[32]. Therefore, subsequent studies can verify the role of YAP in the progression of DN by detecting the expression of YAP and miRNAs. Moreover, researchers[18,34,35] have suggested that the

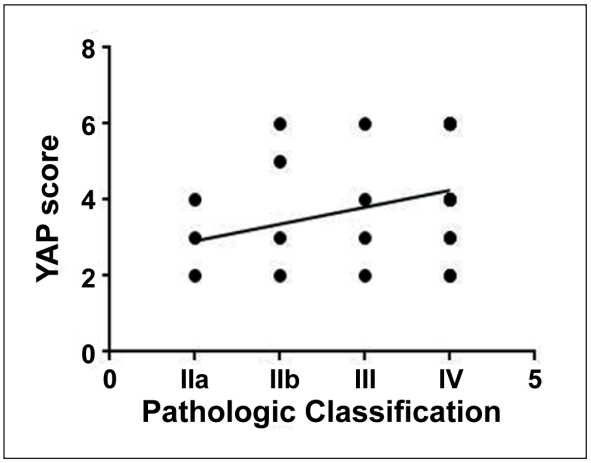

Figure 5. Correlation analysis between YAP and Pathologic Classification, Spearman r=0.298, p=0.033.

occurrence and development of DN can be treated by inhibiting YAP level. Recently, verteporfin (VP) is widely identified as a potent YAP inhibitor in animal experiments by inhibiting the combination of YAP-TEAD, which can reduce urinary protein, and inhibit the progress of renal fibrosis[34-36]. Since there is no specific, safe and effective treatment for renal fibrosis at present, the use of YAP inhibitors to reduce YAP activity and rebalance YAP expression has become an important thought for antifibrotic therapies of DN. Further studies should evaluate the role of YAP in type 2 diabetic nephropathy pathogenesis and the potential utility of its inhibitors as a therapeutic target.

Conclusions

We showed that high expression of YAP, TEAD and CTGF in kidney tissues suggested that YAP played a significant role in the renal damage of type 2 diabetic nephropathy. YAP that is correlated with SBP, BUN, Cr, DN stage, DN pathologic classification, SAlb and eGFR, suggested that inhibition of the activity of YAP might have the effect in delaying DN progression.

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Conflict of Interests

The authors declared no conflict of interest.

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