Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:63-74
DOI: 10.1159/000491663
Published online: July 12, 2018
Accepted: May 04, 2018

Original Paper

Expression of Endothelial Cell Injury Marker Cd146 Correlates with Disease Severity and Predicts the Renal Outcomes in Patients with Diabetic Nephropathy

Ying Fan[a] Yang Fei[a] Li Zheng[b] Jiemin Wang[b] Wenzhen Xiao[c] Jiejun Wen[a] Yanping Xu[b] Yiyun Wang[a] Li He[a] Jian Guan[d] Jia Wei[b] John Cijiang He[c,e] Niansong Wang[a]

[a]Department of Nephrology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, [b]Portfolio & Project management, Asia & Emerging Market iMED, AstraZeneca R&D, Shanghai, China, [c]Department of Medicine, Division of Nephrology, Icahn School of Medicine at Mount Sinai, New York, USA, [d]Department of Otolaryngology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China, [e]Renal Section, James J Peter Veterans Administration Medical Center, New York, USA

Key Words
Cd146 • Diabetic nephropathy • Endothelial dysfunction • Chronic kidney disease • Albuminuria

Abstract
Background/Aims: Glomerular endothelial cell injury plays a crucial role in the development of diabetic nephropathy (DN). CD146, an endothelial marker, was shown to increase in chronic kidney disease (CKD), but its role in DN remains unknown. We aim to assess whether CD146 could be used to evaluate disease severity and predict renal outcomes in DN at early stages. ***Methods:*** 159 non-dialysis type 2-DN patients from 2008 to 2015 were enrolled to measure the plasma concentration of soluble CD146 (sCD146). 94 type 2 diabetes mellitus patients without DN and 100 healthy subjects were used as controls. The patients with CKD stage 1-3 were referred as early stage patients. Another independent cohort of 48 patients with biopsy-proved DN was used for the immunohistochemistry study of CD146. Renal outcomes were defined as doubling of serum creatinine, initiation of renal replacement therapy or death. ***Results:*** We found that plasma level of sCD146 was upregulated and associated with renal function in DN patients. sCD146 was proved to be a more optimal marker than urine albumin creatinine ratio to evaluate disease severity in these DN patients. The kidney expression of CD146 was co-localized with endothelial marker CD31 and increased in DN. CD146 staining in kidney was correlated with the severity of pathological changes in DN patients. Survival analysis suggested that both plasma and biopsy expression of CD146 were correlated with renal outcomes. ***Conclusions:*** CD146 is associated with kidney injury and could be a good marker to predict renal outcomes in patients with early stages of DN.

Y. Fan and Y. Fei contributed equally to this work.

Niansong Wang, M.D.,
John Cijiang He, M.D.
and Li Zheng, M.D.

Department of Nephrology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital
600 Yishan Road, Shanghai, 200233 (China) Tel. +86-021-64369181, Fax +86-021-64701361
E-Mail wangniansong2012@163.com, cijiang.he@mssm.edu, Li.Zheng@astrazeneca.com

Introduction

Diabetic nephropathy (DN) occurs in approximately 10-30% of patients with diabetes mellitus (DM) and has become the leading cause of end-stage renal disease (ESRD) in most countries [1, 2]. DN is clinically characterized by persistent albuminuria and a progressive decline of renal function. Histologically, glomerular basement membrane (GBM) thinckening, mesangial expansion and later glomerulosclerosis with classic Kimmelstein-Wilson nodules, tubulointerstitial changes and vascular lesions may be observed [3]. Albuminuria has been widely viewed as a conventional biomarker for the progression and prognosis in patients with type 2 DM (T2D). However, it has been challenged in recent years advocating that albuminuria does not uniformly predict the progression of DN and a significant proportion of patients with DM who progress to ESRD do not have overt proteinuria [4, 5]. Furthermore, renal function may be lost years before proteinuria develops, suggesting an alternative pathway of progression to severe DN [6, 7]. Therefore, it is urgent to identify better biomarkers that can more effectively evaluate the renal injuries and predict the renal outcomes at early stages of DN.

Endothelial dysfunction is well documented as an initiating event in the development of DN [8]. Impairment of endothelial glycocalyx and loss of endothelial fenestrations [9, 10]; alteration of endothelial adhesion molecule and activation of inflammation [11, 12] are all major events in endothelial injury. However, the exact role of endothelial dysfunction in DN and how endothelial injuries contribute to the progression of DN remains largely unknown.

CD146 is a transmembrane glycoprotein that is constitutively expressed in human endothelium [13]. Studies have demonstrated the function of CD146 is not only limited to cell adhesion but also involved in cell signaling, migration, angiogenesis, proliferation and differentiation [14-16]. In addition to the membrane-anchored form of CD146, a soluble form of CD146 (sCD146) has been identified in the supernatant of cultured human endothelial cells and in the blood of healthy or diseased subjects [17-19]. The plasma concentration of sCD146 was regulated in inflammatory diseases, angiogenesis and tumor metastasis [20-22]. Recent studies have shown that sCD146 increases in chronic kidney diseases (CKD) reflecting endothelial dysfunction in these patients [18, 23]. However, the role of sCD146/CD146 in the pathogenesis and progression of DN has not been well studied.

In the current study, we examined the circulating levels of sCD146 in plasma and renal expression of CD146 in kidney biopsies of DN patients at different CKD stages. We evaluated whether CD146 could be used to assess the severity of disease and predict renal outcomes in DN at early stages.

Materials and Methods

Patients and Cohorts

This is a retrospective cohort study comprised of two cohorts of type 2 diabetic patients who were recruited from 2008 to 2015 for the study of circulating sCD146 and kidney expression of CD146 respectively. The diagnosis of T2D and DN is based on the criteria proposed by WHO in 1999 [24] and Mogensen et al. [25]. In the study of plasma sCD146 cohort, 159 of 238 type 2 DN cases were finally eligible for the enrollment. 94 T2D patients without DN and 100 healthy subjects were enrolled as controls. While in the study of biopsy CD146 cohort, 48 of 60 adult patients with kidney biopsy were recruited with the confirmed diagnosis of DN [26]. Kidney biopsies from 8 patients with minimal change disease (MCD) and 10 nephrectomy samples were used as controls. The research protocols and exclusion criteria were presented in (Fig. 1A, B). The study was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and was carried out in full accordance with the principles of the declaration of Helsinki. Written informed consents were obtained from all participants prior to blood sampling and kidney biopsy.

Laboratory Measurements

Blood and urine samples were obtained for the laboratory measurements. The urinary albumin creatinine ratio (UACR) was determined during the period of hospitalization. The estimated glomerular

filtration rate (eGFR) was calculated through Modification of Diet in Renal Disease (MDRD) formula: $eGFR=170\times sCr^{-0.999}\times age^{-0.176}\times BUN^{-0.170}\times alb^{0.318}\times (0.762$ female)

Definition of CKD

The total of 159 DN patients were categorized into 5 stages, including 32 cases of CKD1, 28 cases of CKD2, 35 cases of CKD3, 31 cases of CKD4 and 33 cases of CKD5, based on eGFR level (MDRD calculation) according to the KDOQI classification [26]. 95 cases of the DN patients at CKD stages (eGFR≧60ml/min) were defined as early eGFR loss [7].

Measurement of plasma sCD146

Blood samples were collected in tubes containing EDTA and centrifuged at 3000 rpm at 4°C for 15 min. The supernatants were aliquot and stored at −80°C until assayed. The plasma concentration of sCD146 was determined with a commercial enzyme-linked immunosorbent assay (CY-QUANT ELISA, BioCytex, Marseille, France) according to the manufacturer's instructions.

Renal Biopsy and Pathological Classification

Kidney samples were cut into 3 μ m sections after biopsy and stained with hematoxylin and Eosin, Periodic Acid Schiff and Masson trichrome staining. All the kidney specimens were processed for EM diagnosis. The classification of DN and histological scoring were performed by two independent renal pathologists according to the criteria of Tervaert et al. [3].

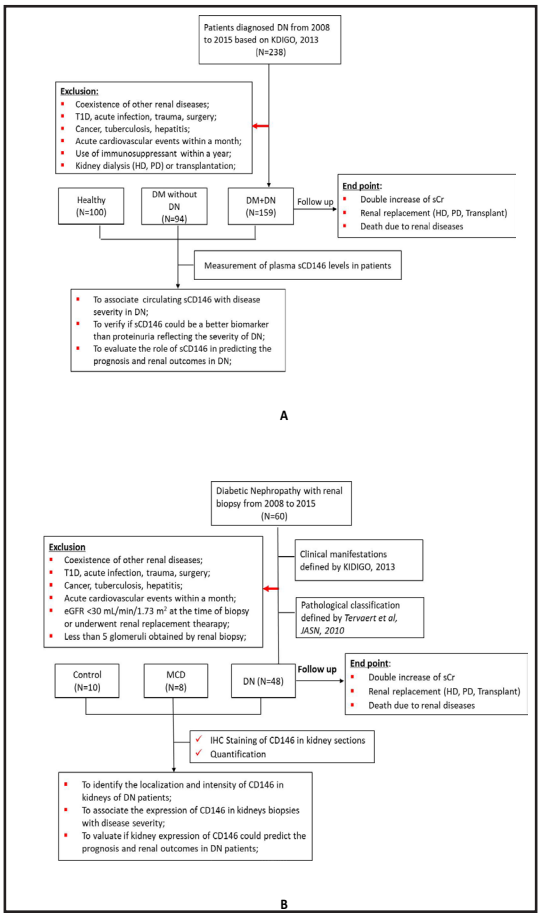

Fig. 1. Workflow of the CD146 study cohorts. (A) Work flow of circulating sCD146 study in diabetic patients cohort. (B) Work flow of CD146 study in Kidney biopsy cohort of DN patients.

Immunohistochemistry and immunofluorescence staining

Immunohistochemistry staining of CD146 (ABCAM, ab75769) was performed on paraffin embedded human kidney sections. A double-immunofluorescence technique was used for CD146 and endothelial marker CD31 (ABCAM, ab76533) on human frozen sections. The semi-quantitative analysis based on the positive area of CD146 was performed in both glomerulus and tubular compartment in a scale of 0-4 according to previously described [27].

Follow-up and End-point

Follow-up was from the date of initial hospital admission to March 2016. The endpoints were defined as either one of the following: doubling of baseline serum creatine (sCr) with the level of at least 2.3mg/dl, initiation of renal replacement therapy including dialysis or renal transplantation, and death due to renal disease.

Statistical Analysis

Analysis was computed using SAS 9.4 for Windows and $P<0.05$ was considered significant. Data were presented as mean ± SD, median (IQR) or percentages when appropriate. T-test, Wilcoxon test and ANOVA test were employed when comparing parameters between two groups or among multiple groups. Categorical variables were compared by Chi-square tests. Receiver operating characteristic curves (ROC) for CKD stage classification by sCD146 and UACR were generated and the area under the curve (AUC) was

calculated to compare the CKD stage discrimination ability of the test. Survival analysis by sCD146 or tissue CD146 level was performed to determine its effect on time to renal outcome. Hazard Ratio estimates were also assessed in the Cox proportional Hazard's model.

Results

Patients Baseline Characteristics

Baseline clinical characteristics among healthy controls and T2D patients with or without DN were summarized in (Table 1). All subjects in healthy controls had negative results in the qualitative test of albuminuria. There was no statistical difference of gender, post-prandial blood glucose and low-density lipoprotein-C (LDL-C). However, DN patients were much older and had longer duration of DM course, also with higher blood pressure, higher body mass index (BMI), higher level of total cholesterol (TC) and triglyceride (TG) than healthy controls and DM patients without DN. The levels of sCr and UACR increased while levels of eGFR, hemoglobin (Hgb) and serum albumin decreased in DN patients as compared with those without DN and healthy controls.

Table 1. Baseline characteristics of diabetic patients in the patient cohort for the study of plasma sCD146. Data were presented as mean ±SD or as median (IQR) when they were not normally distributed. ANOVA test was used in comparison of these variables among three groups. *P<0.05 was considered significant compared with DM and # P<0.05 was considered significant compared with health controls. Wilcoxon test was used in comparison of these variables between two groups

Characteristics	Health Control	DM	DN	P value
Patients, n	100	94	159	
Sex, male, n(%)	65(65%)	60(63.8%)	101(63.5%)	0.961
Age, years	45±13	54±14#	62±13#*	0.000
BMI, kg/m²	23.2±3.1	24.8±3.7#	26.7±4.4#*	0.000
SBP, mmHg	120±16	126±16#	140±21#*	0.000
DBP, mmHg	74±10	78±9#	78±11#	0.013
Course of DM, years	/	8±8	13±8*	
s-Alb, g/L	47.7±2.3	41.6±3.6#	37.9±6.4#*	0.000
UACR, mg/g	NA	7.70 (5.37)	1625.51 (2894.48)*	
24h-UP, mg/24hr	NA	7.80 (6.63)	1272 (2890)*	
sCr, mg/dL	0.79±0.18	0.72±0.16	2.65±0.21#*	0.025
eGFR, ml /min/1.73m²	101.0±39.4	113.4±26.3#	51.1±39.3#*	0.027
FBG, mmol/L	5.04(0.54)	7.06 (3.57)#	6.78 (3.89)#	0.000
2hr-PBG, mmol/L	NA	12.11±4.23	11.67 (5.47)	
HbA1c, %	5.4(0.5)	8.2 (3.4)#	7.4 (3.0)#	0.000
Hgb, g/L	149±20	140±14	117±27#*	0.001
CRP, mg/dL	NA	0.72 (1.84)	1.59 (3.10)*	
TG, mmol/L	1.27(0.94)	0.97(0.76)	1.54 (1.03)#*	0.375
T-Chol, mmol/L	4.82(1.46)	4.49 (1.24)#	4.92 (2.20)*	0.018
HDL-C, mmol/L	1.45(0.53)	1.16 (0.35)#	1.08 (0.44)#	0.000
LDL-C, mmol/L	2.80(1.0)	2.77 (1.20)	2.92 (1.50)	0.358

Plasma concentration of sCD146 was upregulated in DN patients

We found increased plasma level of sCD146 in T2D when compared with healthy controls (434.7 vs 358.3 ng/ml, *P*<0.01), and the level of sCD146 was even higher in DN group than DM patients without DN (644.2 vs 434.7 ng/ml, *P*<0.01) (Fig. 2A). In DN patients, sCD146 levels increased at the stage 2 of CKD and peaked at the stage 3. Interestingly, a declining trend of sCD146 was observed in CKD4 and CKD5 patients probably due to the diffuse glomerular endothelial atherosclerosis in these ESRD patients (Fig. 2B). These data suggest that sCD146 increased mostly in early stages of CKD and might be a good biomarker for early stages of DN (CKD stage 1-3). Therefore, we focused on the role of CD146 in early DN (CKD stage 1-3) for the following studies.

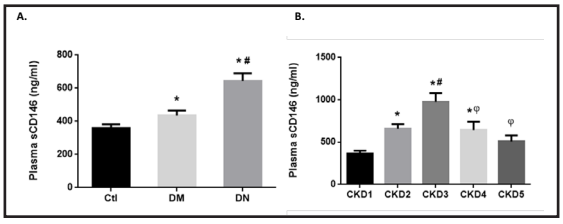

Fig. 2. Plasma concentration of sCD146 was upregulated in patients with DN. A) The comparison of plasma sCD146 among DM without DN (n=94), DN (n=159) and healthy controls (n=100). *P<0.05 compared with healthy controls; #P<0.05 compared with DM patients without DN (Turkey adjusted ANOVA test). B) The concentration of plasma sCD146 in DN patients among CKD stage 1-5. *P<0.05 compared with CKD stage 1; #P<0.05 compared with CKD stage 2; φP<0.05 compared with stage 3. (Turkey adjusted ANOVA test).

The association of plasma sCD146 with clinical variables in DN patients with CKD stages 1-3

Spearman analysis was conducted to study the association between plasma sCD146 and clinical variables in DN patients across all CKD stages and found sCD146 was significantly correlated with s-Alb, UACR, LDL-c, HDL-c and TC

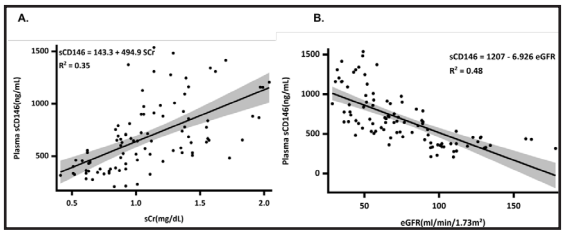

Fig. 3. Elevated plasma sCD146 correlates with renal function in DN patients with CKD stages 1-3. A) The concentration of plasma sCD146 was positively correlated with sCr in DN patients with early eGFR loss. R^2=0.35, P<0.01. B) The plasma sCD146 was inversely correlated with eGFR in DN patients with early stage CKD. R^2=0.48, P<0.01.

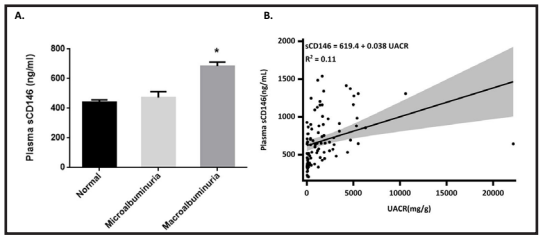

Fig. 4. The association of plasma sCD146 with UACR in diabetic patients. A) Comparison of the plasma sCD146 concentration among the three UACR levels in DM patients without DN (n=94) and DN patients with early stages of CKD (n=48). *P<0.01 compared with microalbuminuria group (Turkey adjusted ANOVA test). B) Plasma sCD146 was significantly but weakly correlated with UACR in DN patients at early stages. (R^2= 0.11, P<0.01).

(P<0.05). In simple linear regression, plasma sCD146 showed a good positive correlation with sCr (R^2=0.35, P<0.01) and a negative correlation with eGFR (R^2=0.48, *P*<0.01) (Fig. 3A, B) in DN patients at CKD stages 1-3. After adjusting for the factors in the multivariable linear regression model, plasma sCD146 was still significantly associated with renal function in these patients (R^2=0.580, *P*<0.001) (Table 2).

We then categorized diabetic patients with CKD stages 1-3 into 3 groups by UACR level, defined as normal (UACR<30mg/g), microalbuminuria (30≤UACR<300mg/g) and macroalbuminuria (UACR≥300 mg/g). The mean plasma levels of sCD146 was slightly increased with no statistical significance in microalbuminuria group as compared to DM patients without DN. Whereas sCD146 level was much higher in DN patients with macroalbuminuria compared to those with microalbuminuria group (684.9 vs 472.3 mg/g, *P*<0.001) (Fig. 4A). In addition, a significant but weak correlation between plasma concentration of sCD146 and UACR was found in DN patients. (R^2=0.11, P<0.01) (Fig. 4B).

These data suggest that plasma sCD146 correlates well with renal function but poorly with albuminuria at CKD1-3.

Table 2. Multivariate linear regression model of sCD146 in DN patients with CKD stages 1-3. The adjusted variables for the stepwise model selection of sCD146 included eGFR, age, sex, s-ALB, UACR, FBG, PBG, GA, HbA1C, Hb, CRP, TG, LDL_C, HDL-C, BMI, Course, SBP, DBP; The entry alpha=0.10, n=95. Model test: P<0.001, adjusted R-square=0.580, collinearity has been checked by VIF. Abbreviations: eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; Hgb, hemoglobin; LDL-C, low density lipid-cholesterol; CRP, C reaction protein

Variables	β	SE	P
eGFR, ml/min	-5.17	0.71	<0.01
FBG, mmol/L	19.95	6.32	0.002
Hgb, g/L	-2.81	1.04	0.008
LDL-C, mmol/L	65.92	15.59	<0.001
CRP, mg/dL	-6.27	3.12	0.048

Plasma sCD146 level is a better marker than UACR to evaluate disease severity in DN Patients with CKD stages 1-3

ROC analysis of plasma sCD146 levels and UACR were therefore performed to determine which biomarker is better for evaluation disease severity in DN patients within CKD1-3. Data showed that in DN patients with CKD1 and CKD2, the area under the ROC curve (AUC) of sCD146 was greater than those of UACR (0.964 vs 0.818, *P*=0.020). The AUC of sCD146 was consistently and significantly higher in DN with CKD2 than that of UACR in CKD3 patients (0.804 vs 0.575, *P*=0.011) (Fig. 5A, B). This suggests that sCD146 could be a more effective marker than UACR to evaluate disease severity in early CKD stages of DN patients.

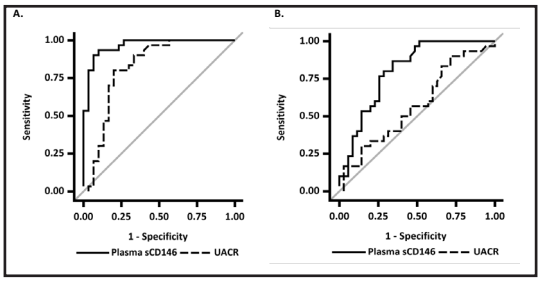

Fig. 5. ROC curve of plasma sCD146 and UACR in DN patients at CKD stages 1-3. A) Comparison of plasma sCD146 and UACR by ROC analysis in DN patients stages 1 (n=30) vs. stage 2 (n=30). B) Comparison of plasma sCD146 and UACR by ROC analysis in DN patients stages 2 (n=30) vs. stage 3 (n=35).

Expression of CD146 in kidney biopsies of DN patients

Next, we studied the local expression of CD146 in human kidneys. The baseline of clinical characteristics was shown in (Table 3). Immunohistochemistry staining of CD146 was performed on paraffin embedded kidney sections of patients with DN, MCD as well as normal kidney sections of nephrectomy samples. The expression of CD146 was significantly increased in DN compared to MCD and normal kidney sections, mainly localized in glomerular tufts, kidney arterioles and tubular compartments (Fig. 6A). Quantification of the staining revealed that more advanced stages of DN had more abundant positive areas of CD146 expression in the kidney. (Fig. 6B). Given that CD146 is constitutively expressed in endothelial cells, we performed co-immunofluorescence staining for CD146 (green) and CD31 (red), an endothelial marker, on frozen kidney sections from DN patients and normal control. Data showed that CD146 co-localized with CD31 in glomerular endothelial cells and substantially increased in DN compared to normal controls, confirming that endothelial cell localization of CD146 and increased expression of CD146 may contribute to vascular endothelial dysfunction in DN (Fig. 6C).

Table 3. Baseline Characteristics of DN Patients in the study cohort of biopsy-proven DN. Data were presented as mean ± SD or as median (IQR) when they were not normally distributed. ANOVA test was conducted in the comparison among CKD stages. T-test was performed in the comparison between two groups. P<0.05 was considered significant. *P<0.05, compared with DN-CKD1 group, # P<0.05, compared with DN-CKD2 group

Characteristics	CKD-1	CKD-2	CKD-3	P value
Patients, n	15	18	15	
Age, years	52.56±9.19	55.39±10.46	60.64±10.51	0.095
Sex, male, n (%)	11 (73.33)	8 (44.44)	10 (66.66)	0.211
Course of DM, years	6.00±3.92	11.78±6.92*	9.25±5.38*	0.021
BMI, kg/m^2	23.76±3.13	25.02±3.90	22.42±1.91	0.082
SBP, mmHg	145.3±22.05	143.8±17.52	149.6±20.06	0.706
DBP, mmHg	85.81±9.99	84.22±5.71	83.93±9.24	0.819
FBG, mmol/L	8.53±3.20	7.77±2.72	8.08±3.12	0.759
2hBG, mmol/L	9.99 (4.86)	11.15±3.51	13.27±5.22	0.380
HbA1C, %	7.82±1.68	7.63±1.37	8.57±1.65	0.225
Hgb , g/dl	143.90±17.57	119.20±23.76*	120.10±24.47*	0.004
s-ALB , g/L	38.69±7.62	36.56±5.95	34.43±5.53	0.207
T-Chol, mmol/L	3.86±2.56	4.68±1.46	5.73±2.73	0.089
TG, mmol/L	1.50 (0.7)	1.18 (0.98)	1.48 (0.80)	0.758
HDL, mmol/L	1.30 (2.12)	1.22 (0.59)	1.17 (0.79)	0.259
LDL, mmol/L	3.50 (1.24)	2.90±0.65	3.24±1.11	0.106
sCr, mg/dl	0.78±0.15	1.04±0.25*	1.50±0.41*#	<0.0001
eGFR, ml/min/1.73m^2	116.60±21.70	72.49±9.28*	48.60±9.71*#	<0.0001
Proteinuria, g/24hr	2.37±3.32	2.71±1.65	4.40±3.11	0.101
UACR, mg/g	1116±1438	2201±1422	4211±3013*#	0.0038
ACEI-ARB, %	73.3	55.6	60	0.576
Insulin therapy, %	67.6	61.1	53.3	0.876

Kidney expression of CD146 is associated with disease severity in DN patients at CKD stage 1-3

To determine whether kidney expression of CD146 is associated with disease severity in DN patients at stage 1-3, we firstly examined the association of CD146 in kidney sections with clinical parameters. The intensity of CD146 expression in kidney was found to be positively correlated with sCr (R^2= 0.42 in glomeruli, R^2= 0.58 in tubular, P<0.01) and inversely with eGFR (R^2= 0.55 in glomeruli, R^2=0.65 in tubular, P<0.01) in DN patients by fitting linear regression model (Fig. 7A-D), suggesting a pivotal role of this biomarker in kidney to evaluate renal function and disease severity at CKD stage 1-3. However, only a weak relationship was

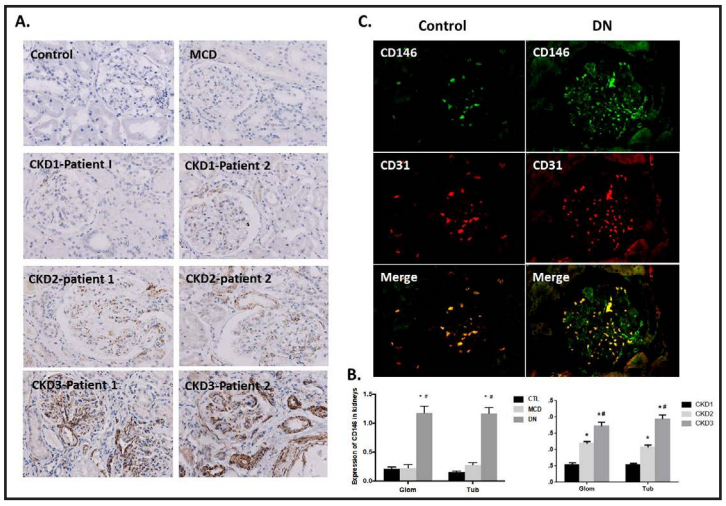

Fig. 6. The expression of CD146 in kidney biopsies of DN patients. A) Representative immunostaining of CD146 in kidney sections of patients with DN (n=48), MCD (n=8) and normal kidneys (n=10). Original magnification × 400. B) Semi-quantitative scoring of CD146 staining for both glomerular and tubular interstitial compartments summarized in a bar graph in DN and control kidney sections. *P<0.05 compared with control; #P<0.05 compared with MCD; Quantification of CD146 expression in diabetic kidneys of different CKD stages. The ANOVA with Bonferroni correction was used. *P<0.05, compared with CKD1; #P<0.05, compared with CKD2. C) The co-immunofluorescence staining for CD146 (green) and CD31 (red) on frozen kidney sections from DN patients and nephrectomy sections.

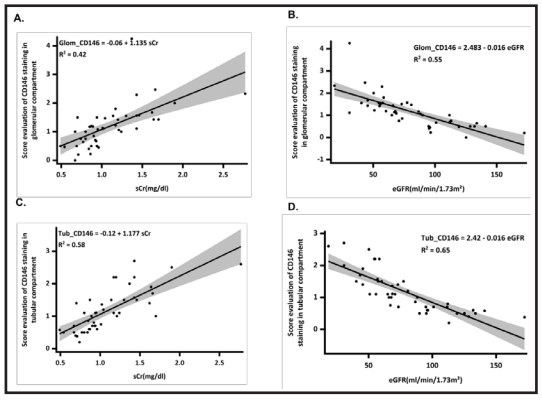

Fig. 7. Association of CD146 expression in biopsy kidneys with renal function in DN patients. A,B) Correlation between the intensity of CD146 staining in glomerular compartment and renal function. n=48. C,D) Correlation between the intensity of CD146 staining in tubular compartment and renal function. n=48.

found between kidney expression of CD146 and UACR in these biopsy patients (R^2=0.16 in glomeruli, R^2=0.18 in tubular, *P*<0.01).

The correlations between histopathological findings and CD146 scores were then examined in the kidneys of DN patients using the Spearman correlation coefficient. The CD146 staining score in glomeruli showed a strong correlation with glomerular class classification (r=0.49, *P*=0.001) and ateriosclerosis (r=0.52, *P*<0.001), and a weak correlation with the IFTA score (r=0.32, *P*=0.038), interstitial inflammation score (r=0.39, *P*=0.011) and arteriolar hyalinosis score (r=0.38, *P*=0.015). Also, CD146 score in tubular compartment was well correlated with interstitial inflammation (r= 0.53, *P*<0.001) and IFTA scores (r=0.42, *P*=0.007) (Table 4). These findings suggest that the expression of CD146 in kidneys correlates with renal histopathological injury in DN patients.

CD146 was associated with the renal outcomes in DN patients at CKD stage 1-3

To evaluate the role of sCD146/CD146 in the progression and prognosis of DN, we retrospectively followed up 80 of the 95 DN patients who were at CKD stages 1-3. The medium follow-up duration was 24(IQR 12) months. During the follow-up, 19 patients (23.75%) reached the end point, including 10 patients (52.63%) had doubling of baseline sCr, 5 (26.32%) progressed to ESRD with renal replacement therapy and 4 (21.05%) were died due to renal diseases. We then categorized patients into three groups based on tertiles of sCD146 level and found that patients with higher sCD146 level tend to have a poorer renal

outcome comparing survival between patients with upper and lower tertiles of CD146 by Kaplan-Meier analysis (*P*=0.049) (Fig. 8). Data suggests that plasma sCD146 could be a predictive marker for the progression of DN

A longitudinal study was also performed in the biopsy study cohort to examine whether increased expression of CD146 in kidney was associated with renal outcomes. A total of 36 DN patients who underwent kidney biopsy were followed up for a median duration of 28 (IQR14) months and the overall renal survival rate was 80.56% in these patients. Using univariate Cox analysis, we found the expression of CD146 in kidneys of DN was strongly associated with the renal outcomes, both in glomeruli (HR=4.11 [1.66, 10.18], *P*=0.002) and in tubular-interstitium (HR=7.89 [2.08, 29.98], *P*=0.002), suggesting that renal expression of CD146 might be a predictive marker for the progression of DN.

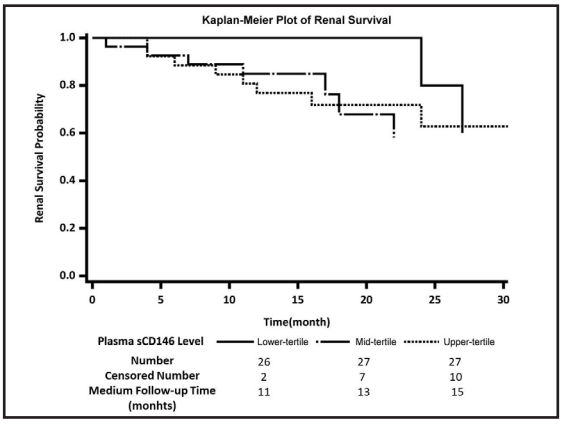

Fig. 8. Kaplan-Meier Curve of renal survival by plasma sCD146 in DN with early stages. Patients with upper tertile of sCD146 level had poorer survival rate than those with lower tertiles by Wilcoxon test (P=0.049).

Table 4. Correlations between histopathological changes and CD146 scores in the kidneys of DN patients. Abbreviation: Glom, glomerular; Tub, Tubular. P<0.05 was considered significant

DN pathologic Classification	CD146 Scores in Glom		CD 146 Scores in Tub	
	R	P value	r	P value
Glomerular Class (I-IV)	0.49	0.001	0.42	0.007
IFTA Score (0-3)	0.32	0.038	0.42	0.007
Interstitial inflammation (0-2)	0.39	0.011	0.53	<0.001
Arteriolar hyalinosis (0-2)	0.38	0.015	0.36	0.022
Arteriosclerosis (0-2)	0.52	<0.001	0.38	0.018

Discussion

Endothelial dysfunction has been considered as a critical mechanism underlying the microvascular injury in DN [28-30]. Thus measuring biological markers of vascular endothelial function *in vivo* may provide insights into the progression and prognosis of DN [10, 31]. The endothelial intercellular junctions exert a key function in chronic inflammation through their ability to modulate leukocyte trafficking and play a critical role during angiogenesis. Studies have confirmed an increased expression of cell adhesion molecules, such as ICAM-1 [32, 33], VCAM-1 [34, 35] and E-selectin [31, 36] occurs in response to the disruption of glomerular endothelial cell homeostasis in DN. CD146 has been identified as an adhesion molecule expressed at the intercellular junction of endothelial cells [15]. More recently, CD146 was found to be involved in endothelial cell activity and angiogenesis [37-39]. The membrane glycoprotein CD146 is rarely found in the blood of healthy subjects, while a soluble form of CD146, which is generated probably by extracellular shedding, proteolysis or DNA splicing from damaged endothelium, was confirmed to be increased in the peripheral circulation of patients with tumor [22], inflammatory diseases [20] or CKD [18], suggesting a pivotal role of this soluble biomarker in the pathogenesis of angiogenesis and vascular endothelial alteration.

In the current study, we demonstrated that T2D patients with DN showed higher plasma level of sCD146 compared with diabetic patients without DN or healthy controls. The concentration of sCD146 increased from CKD1 to CKD3 in DN patients. In addition, sCD146 was shown to correlate better with kidney injury than UACR to evaluate kidney injury in DN patients at early CKD stages. All these data suggest a critical role of sCD146 in evaluating disease severity at early stages of DN. A clinical study with a small cohort in Japan found persistently elevated circulating levels of sCD146 correlated with disease severity in DN patients [23]. However, our study demonstrated that the concentration of plasma sCD146 increased progressively in DN patients from CKD stage 1-3 and then declined slightly in

CKD stages 4-5 patients. It is well recognized that abnormal angiogenesis occurs in early DN and is associated with neovasculation, elevated length and surface area of capillaries, and increased endothelial numbers in kidney glomeruli [40, 41]**.** Nevertheless, studies have proved that glomerular endothelial cell numbers were decreased in severe DN [42], reduced angiogenesis and capillary loss also occurred in the later stages of DN [10, 42, 43]. Based on these strong findings, it is reasonable to speculate that the down-regulation of plasma sCD146 level in severe DN patients is most likely due to the extensive glomerular sclerosis and a decline of endothelial cell number at the end stage of DN.

The measurement of circulating sCD146 is a convenient and noninvasive way to assess the severity of DN. However, plasma sCD146 concentration may be changed due to various disorders including infection, trauma, tumor and some other chronic diseases [20, 22, 44, 45]. Studies showed tissue expression of CD146 was increased in active inflammatory bowel diseases [20], rheumatoid arthritis [46], as well as in kidney biopsies from CKD patients [18]. However, the alteration of CD146 expression in kidney tissues and its association with kidney diseases hasn't been fully understood. In this study, we were able to obtain 48 samples from early to moderate stage of DN patients, which is a reasonable sample size for the human study in the setting of DN due to the difficulty in obtaining biopsy samples of DN patients. To our knowledge, this is the first study to demonstrate a clear association of CD146 expression in kidney with clinical findings in DN. CD146 was confirmed to be co-localized with CD31 in kidney arterioles and glomeruli tufts, suggesting a pivotal role of CD146 in kidney endothelium. Interestingly, CD146 was found not only restrict to glomerular endothelial cells, but was also abundant in tubular compartment of DN patients, indicating the multifunctional role of CD146 in kidney cells. Immunochemistry assessment of kidney biopsy specimens showed increased expression of CD146 was associated with the severity of renal function in DN patients, which was consistent with the findings on circulating sCD146 levels in DN patients at early CKD stages. Since histological classification is a golden standard for both diagnosis and evaluation of DN, we performed histological scoring on each kidney section in DN patients. We found CD146 scoring in either glomeruli or tubular compartment could be a valuable marker evaluating glomerular endothelial injury, vascular angiogenesis, inflammatory infiltration, and tubular interstitial fibrosis in DN. The increased expression of CD146 in kidney was synchronized with the elevated plasma level of sCD146 in DN patients with early CKD stages, corroborating a reliable and synergetic role of both CD146 and sCD146 in evaluating disease severity and progression of DN.

Currently it is still challenged to identify early DN patients who are at high risk of developing progressive nephropathy [47]. Studies have indicated that albuminuria failed to be a specific prognostic biomarker for the progressive DN, especially when urinary albumin excretion is <300 mg /24 h [6, 48]. In patients with DN, circulating sCD146 has been shown to be elevated when compared with the non-diabetic population or T2D without DN [49, 50]. However, these studies had small sample sizes and were unable to ascertain whether sCD146 could be used as biomarkers to predict patients at risk of progressive DN. A very recent study by Ilie et al. [22] demonstrated that plasma sCD146 levels together with circulating endothelial cells (CECs) counts were associated with clinical outcome in non-small cell lung cancer (NSCLC) patients undergoing surgery, suggesting a predictive role of sCD146 in tumor metastasis and prognosis. Hereby we first conduct a survival study of sCD146/CD146 in DN patients. We report that both plasma concentration of sCD146 and the intensity of CD146 expression in kidney sections predict renal outcomes and progression at early stages of DN.

The study has several potential concerns and limitations. It's a single center and retrospective cohort study with relatively limited sample size, including two independent data sets from plasma and biopsy cohorts respectively. It would be more persuasive if we could recruit DN patients who have both blood and kidney biopsy samples, with long-term follow-up from early disease stage, to evaluate the role of sCD146/CD146 in the progression of DN. The result of survival analysis for biopsy-proven DN patients was not strong enough due to the limited sample size and small event number. Moreover, since the pathogeneses of DN is multifactorial, a single biomarker might not be strong enough to predict the disease

progression for DN. Finally, the underlying mechanisms explaining how CD146 contributes to the progression of DN require further investigation.

In conclusion, our findings demonstrate that sCD146/CD146 could be a useful biomarker to evaluate disease severity and predict renal outcomes in patients with early stages of DN. The aberrant expression of CD146 may reflect underlined endothelial dysfunction and vascular angiogenesis in DN.

Acknowledgements

J.C.H. is supported by NIH 1R01DK109683, NIH 1R01DK078897, NIH 1R01DK088541, and NIH P01-DK-56492. N.W. is supported by National Natural Science Foundation of China (81270824, 81670657), National "Twelfth Five-Year" Plan for Science & Technology Support (2013BAI02B01-01) and Translational Medicine Funding of Shanghai Jiao Tong University (152H2011). Y.F. is supported by National Natural Science Foundation of China (81400735) and Chinese Medical Association Funding (15020140602).

Disclosure Statement

The authors declare to have no competing interests.

References

1 Molitch ME, Adler AI, Flyvbjerg A, Nelson RG, So WY, Wanner C, Kasiske BL, Wheeler DC, de Zeeuw D, Mogensen CE: Diabetic kidney disease: a clinical update from Kidney Disease: Improving Global Outcomes. Kidney Int 2015;87:20-30.

2 Thomas MC, Cooper ME, Zimmet P: Changing epidemiology of type 2 diabetes mellituand associated chronic kidney disease. Nat Rev Nephrol 2003;12:73-81.

3 Tervaert TW, Mooyaart AL, Amann K, Cohen AH, Cook HT, Drachenberg CB, Ferrario F, Fogo AB, Haas M, de Heer E, Joh K, Noël LH, Radhakrishnan J,Seshan SV, Bajema IM, Bruijn JA, Renal Pathology Society: Pathologic classification of diabetic nephropathy. J Am Soc Nephrol 2010;21:556–563.

4 Kramer HJ, Nguyen QD, Curhan G, Hsu CY: Renal insufficiency in the absence of albuminuria and retinopathy among adults with type 2 diabetes mellitus. JAMA 2003;289:3273–3277.

5 MacIsaac RJ, Ekinci EI, Jerums G: Progressive diabetic nephropathy. How useful is microalbuminuria?: contra. Kidney Int 2014;86:50–57.

6 Perkins BA, Ficociello LH, Roshan B, Warram JH, Krolewski AS: In patients with type 1 diabetes and new-onset microalbuminuria the development of advanced chronic kidney disease may not require progression to proteinuria. Kidney Int 2010;77:57–64.

7 Gohda T, Niewczas MA, Ficociello LH, Walker WH, Skupien J, Ficociello LH, Rosetti F, Cullere X, Johnson AC, Crabtree G, Smiles AM, Mayadas TN, Warram JH, Krolewski AS: Circulating TNF receptors 1 and 2 predict stage 3 CKD in type 1 diabetes. J Am Soc Nephrol 2012;23:516–524.

8 Nakagawa T, Tanabe K, Croker BP, Johnson RJ, Grant MB, Kosugi T, Li Q: Endothelial dysfunction as a potential contributor in diabetic nephropathy. Nat Rev Nephrol 2011;7:36–44.

9 Weil EJ, Lemley KV, Mason CC, Yee B, Jones LI, Blouch K, Lovato T, Richardson M, Myers BD, Nelson RG: Podocyte detachment and reduced glomerular capillary endothelial fenestration promote kidney disease in type 2 diabetic nephropathy. Kidney Int 2012;82:1010–1017.

10 Oltean S, Qiu Y, Ferguson JK, Stevens M, Neal C1, Russell A, Kaura A, Arkill KP, Harris K, Symonds C, Lacey K, Wijeyaratne L, Gammons M,Wylie E, Hulse RP, Alsop C, Cope G, Damodaran G, Betteridge KB, Ramnath R, Satchell SC, Foster RR, Ballmer-Hofer K, Donaldson LF,Barratt J, Baelde HJ, Harper SJ, Bates DO, Salmon AH: Vascular Endothelial Growth Factor-A165b Is Protective and Restores Endothelial Glycocalyx in Diabetic Nephropathy. J Am Soc Nephrol 2015;26:1889–1904.

11 Roy MS, Janal MN, Crosby J, Donnelly R: Markers of endothelial dysfunction and inflammation predict progression of diabetic nephropathy in African Americans with type 1 diabetes. Kidney Int 2015;87:427–433.

12 Navarro-González JF, Mora-Fernández C, Muros de Fuentes M, García-Pérez J: Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. Nat Rev Nephrol 2011;7:327–340.
13 Lehmann JM, Riethmüller G, Johnson JP. MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. Proc Natl Acad Sci U.S.A. 1989;86: 9891–9895.
14 Anfosso F, Bardin N, Vivier E, Sabatier F, Sampol J, Dignat-George F: Outside-in signaling pathway linked to CD146 engagement in human endothelial cells. J Biol Chem 2001;276:1564-1569.
15 Bardin N, Anfosso F, Massé JM, Cramer E, Sabatier F, Le Bivic A, Sampol J, Dignat-George F: Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. Blood 2001;98:3677-3684.
16 Ye Z, Zhang C, Tu T, Sun M, Liu D, Lu D, Feng J, Yang D, Liu F, Yan X: Wnt5a uses CD146 as a receptor to regulate cell motility and convergent extension. Nat Commun 2013;4:2803.
17 Bardin N, Francès V, Combes V, Sampol J, Dignat-George F: CD146: biosynthesis and production of a soluble form in human cultured endothelial cells. FEBS Lett 1988;421:12-14.
18 Bardin N, Moal V, Anfosso F, Daniel L, Brunet P, Sampol J, Dignat George F: Soluble CD146, a novel endothelial marker, is increased in physiopathological settings linked to endothelial junctional alteration. Thromb Haemost 2003;90:915–20.
19 Fürstenberger G, von Moos R, Senn HJ, Boneberg EM: Real-time PCR of CD146 mRNA in peripheral blood enables the relative quantification of circulating endothelial cells and is an indicator of angiogenesis. Br J Cancer 2005;93:793–798.
20 Bardin N, Reumaux D, Geboes K, Colombel JF, Blot-Chabaud M, Sampol J, Duthilleul P, Dignat-George F: Increased expression of CD146, a new marker of the endothelial junction in active inflammatory bowel disease. Inflamm Bowel Dis 2006;12:16–21.
21 Stalin J, Harhouri K, Hubert L, Subrini C, Lafitte D, Lissitzky JC, Elganfoud N, Robert S, Foucault-Bertaud A, Kaspi E, Sabatier F, Aurrand-Lions M, Bardin N,Holmgren L, Dignat-George F, Blot-Chabaud M: Soluble melanoma cell adhesion molecule (sMCAM/sCD146) promotes angiogenic effects on endothelial progenitor cells through angiomotin. J Biol Chem 2013;288:8991-9000.
22 Ilie M, Long E, Hofman V, Selva E, Bonnetaud C, Boyer J, Vénissac N, Sanfiorenzo C, Ferrua B, Marquette CH, Mouroux J, Hofman P: Clinical value of circulating endothelial cells and of soluble CD146 levels in patients undergoing surgery for non-small cell lung cancer. Br J Cancer 2014;110:1236-1243.
23 Saito T, Saito O, Kawano T, Tamemoto H, Kusano E, Kawakami M, Ishikawa SE: Elevation of serum adiponectin and CD146 levels in diabetic nephropathy. Diabetes Res Clin Pract 2007;78:85–92.
24 Alberti KG, Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998;15:539-553.
25 Mogensen CE: Progression of nephropathy in long-term diabetics with proteinuria and effect of initial anti-hypertensive treatment. Scand J Clin Lab Invest 1976;36:383–388.
26 KDOQI: KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Diabetes and Chronic Kidney Disease. Am J Kidney Dis 2007;49(2 Suppl 2):S12–154.
27 Fan Y, Xiao W, Li Z, Li X, Chuang PY, Jim B, Zhang W, Wei C, Wang N, Jia W, Xiong H, Lee K, He JC: RTN1 mediates progression of kidney disease by inducing ER stress. Nat Commun 2015;6:7841.
28 Fu J, Lee K, Chuang PY, Liu Z, He JC: Glomerular endothelial cell injury and cross talk in diabetic kidney disease. Am J Physiol Renal Physiol 2015;308:F287–F297.
29 Reidy K, Kang HM, Hostetter T, Susztak K: Molecular mechanisms of diabetic kidney disease. J Clin Invest 2014;124:2333–2340.
30 Rask-Madsen C, King GL: Kidney complications: factors that protect the diabetic vasculature. Nat Med 2010;16:40-41.
31 Lopes-Virella MF, Carter RE, Gilbert GE, Klein RL, Jaffa M, Jenkins AJ, Lyons TJ, Garvey WT, Virella G: Risk factors related to inflammation and endothelial dysfunction in the DCCT/EDIC cohort and their relationship with nephropathy and macrovascular complications. Diabetes Care 2008;31:2006–2012.
32 Okada S, Shikata K, Matsuda M, Ogawa D, Usui H, Kido Y, Nagase R, Wada J, Shikata Y, Makino H: Intercellular adhesion molecule-1-deficient mice are resistant against renal injury after induction of diabetes. Diabetes 2003;52:2586–2593.

33 Lin J, Glynn RJ, Rifai N, Manson JE, Ridker PM, Nathan DM, Schaumberg DA: Inflammation and progressive nephropathy in type 1 diabetes in the diabetes control and complications trial. Diabetes Care 2008;31:2338–2343.
34 Tan SM, Sharma A, Stefanovic N, Yuen DY, Karagiannis TC, Meyer C, Ward KW, Cooper ME, de Haan JB: Derivative of bardoxolone methyl, dh404, in an inverse dose-dependent manner lessens diabetes-associated atherosclerosis and improves diabetic kidney disease. Diabetes 2014;63:3091-3103.
35 Liu JJ, Yeoh LY, Sum CF, Tavintharan S, Ng XW, Liu S, Lee SB, Tang WE, Lim SC, SMART2D study: Vascular cell adhesion molecule-1, but not intercellular adhesion molecule-1, is associated with diabetic kidney disease in Asians with type 2 diabetes. J Diabetes Complications 2015;29:707-712.
36 Lee HJ, Jeong KH, Kim YG, Moon JY, Lee SH, Ihm CG, Sung JY, Lee TW: Febuxostat ameliorates diabetic renal injury in a streptozotocin-induced diabetic rat model. Am J Nephrol 2014;40:56-63.
37 Yan X, Lin Y, Yang D, Shen Y, Yuan M, Zhang Z, Li P, Xia H, Li L, Luo D, Liu Q, Mann K, Bader BL: A novel anti-CD146 monoclonal antibody, AA98, inhibits angiogenesis and tumor growth. Blood 2003;102:184-191.
38 Zeng Q, Wu Z, Duan H, Jiang X, Tu T, Lu D, Luo Y, Wang P, Song L, Feng J, Yang D, Yan X: Impaired tumor angiogenesis and VEGF-induced pathway in endothelial CD146 knockout mice. Protein Cell 2014;5:445–456.
39 Wang P, Luo Y, Duan H, Xing S, Zhang J, Lu D, Feng J, Yang D, Song L, Yan X: MicroRNA 329 suppresses angiogenesis by targeting CD146 Mol Cell Biol. 2013;33:3689-3699.
40 Guo M1, Ricardo SD, Deane JA, Shi M, Cullen-McEwen L, Bertram JF: A stereological study of the renal glomerular vasculature in the db/db mouse model of diabetic nephropathy. J Anat 2005;207:813–821.
41 Nakagawa T, Kosugi T, Haneda M, Rivard CJ, Long DA: Abnormal angiogenesis in diabetic nephropathy. Diabetes 2009;58:1471-1478.
42 Hohenstein B, Hausknecht B, Boehmer K, Riess R, Brekken RA, Hugo CP: Local VEGF activity but not VEGF expression is tightly regulated during diabetic nephropathy in man. Kidney Int 2006;69:1654–1661.
43 Baelde HJ, Eikmans M, Lappin DW, Doran PP, Hohenadel D, Brinkkoetter PT, van der Woude FJ, Waldherr R, Rabelink TJ, de Heer E, Bruijn JA: Reduction of VEGF-A and CTGF expression in diabetic nephropathy is associated with podocyte loss. Kidney Int 2007;71:637–645.
44 Harhouri K, Kebir A, Guillet B, Foucault-Bertaud A, Voytenko S, Piercecchi-Marti MD, Berenguer C, Lamy E, Vely F, Pisano P, Ouafik L, Sabatier F, Sampol J,Bardin N, Dignat-George F, Blot-Chabaud M: Soluble CD146 displays angiogenic properties and promotes neovascularization in experimental hind-limb ischemia. Blood 2010;115:3843-3851.
45 Ciftci Dogansen S, Helvaci A, Adas M, Deniz Onal S: The relationship between early atherosclerosis and endothelial dysfunction in type 1 diabetic patients as evidenced by measurement of carotid intima-media thickness and soluble CD146 levels: a cross sectional study. Cardiovasc Diabetol 2013; 12:153.
46 Yeremenko N, Noordenbos T, Cantaert T, van Tok M, van de Sande M, Cañete JD, Tak PP, Baeten D: Disease-specific and inflammation-independent stromal alterations in spondylarthritis synovitis. Arthritis Rheum 2013;65:174-185.
47 Brosius FC, Saran R: Do we now have a prognostic biomarker for progressive diabetic nephropathy? J Am Soc Nephrol 2012;23:376-377.
48 Levey AS, Cattran D, Friedman A, Miller WG, Sedor J, Tuttle K, Kasiske B, Hostetter T: Proteinuria as a surrogate outcome in CKD: report of a scientific workshop sponsored by the National Kidney Foundation and the US Food and Drug Administration. Am J Kidney Dis 2009;54:205–226.
49 Malyszko J, Malyszko JS, Brzosko S, Wolczynski S, Mysliwiec M: Adiponectin is related to CD146, a novel marker of endothelial cell activation/injury in chronic renal failure and peritoneally dialyzed patients. J Clin Endocrinol Metab 2004;89:4620-4627.
50 Wang F, Xing T, Wang N, Liu L: Clinical significance of plasma CD146 and P-selectin in patients with type 2 diabetic nephropathy. Cytokine 2012;57:127–129.