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Original Article

Immunohistochemistry of Vasohibin-2 in Human Kidney Disease: Implications in Impaired Glucose Tolerance and Reduced Renal Function

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Several angiogenesis-related factors are known to play important roles in the pathogenesis of kidney disease. Vasohibin-2 (VASH-2) was recently reported as a novel proangiogenic factor. Although VASH-2 was demonstrated to accelerate tumor angiogenesis, its roles in non-tumor processes including renal disease have not been well elucidated yet. Here, we performed a retrospective study including an immunohistochemical analysis of human kidney biopsy specimens from 82 Japanese patients with a variety of kidney diseases, and we evaluated the correlations between the immunoreactivity of VASH-2 and the patients' clinicopathological parameters. VASH-2 immunoreactivity was detected in varying degrees in renal tubules as well as in peritubular capillaries and vasa recta. The cortical and medullary tubule VASH-2$^+$ scores were correlated with the presence of hypertension, and the medullary tubule VASH-2$^+$ score was significantly correlated with the blood glucose ($p=0.029$, $r=0.35$) and hemoglobin A1c levels ($p=0.0066$, $r=0.39$). Moreover, decreased VASH-2$^+$ scores in the vasa recta were associated with reduced renal function ($p=0.0003$). These results suggest that VASH-2 could play an important role in the pathogenesis of renal diseases, and that VASH-2 is closely associated with hypertension and impaired glucose tolerance.

Key words: vasohibin-2, kidney disease, vasa recta, medullary tubules

Chronic kidney disease (CKD) affects more than 10% of the world's population; it is particularly common in the elderly and in the presence of comorbidities such as hypertension, diabetes, and hyperlipidemia [1]. When these underlying comorbidities and risk factors are not adequately controlled, CKD often results in end-stage renal disease (ESRD) [2]. The role of metabolic abnormalities in the progression of kidney disease is thus receiving increasing attention. Progressive kidney disease is often accompanied by histopathologic evidence of glomerulosclerosis and tubulointerstitial alterations including tubular atrophy and interstitial fibrosis [3,4]. Such changes are thought to be the results of a loss of renal capillaries and are known to be highly predictive of the subsequent deterioration of renal function and CKD progression [5]. Indeed, several angiogenesis-related factors such as vascular endothelial growth factor (VEGF)-A, soluble Flt-1, angiopoietins, and vasohibin-1 (VASH-1) have been shown

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to be related to the severity of interstitial fibrosis in CKD [6-9].

VEGF-A is a proangiogenic factor that is expressed mainly by podocytes and tubular epithelial cells in the normal kidney [6], and it is upregulated in renal tubules in patients with early stage IgA nephropathy with peritubular capillary loss [10]. VASH-1 is an endogenous angiogenesis inhibitor that is up-regulated by VEGF-A in endothelial cells in an autocrine manner; it acts as a negative feedback regulator of angiogenesis [11]. The therapeutic efficacy of VASH-1 has been demonstrated in animal models of malignancy [12, 13], atherosclerosis [14], and diabetic nephropathy [15, 16], and we recently demonstrated an association between elevated urinary and plasma levels of VASH-1 and the progressive decline of the renal function in patients with CKD [17].

Plasma VASH-1 levels were also shown to be negatively correlated with blood pressure and positively correlated with the mean percentage of glomeruli with crescent formation in renal biopsies [18]. We have also observed that VASH-1 is expressed in renal endothelial/mesangial cells, crescentic lesions and interstitial inflammatory cells, and that there is a significant association between the number of VASH-1-positive cells with crescent formation and interstitial infiltration in crescentic glomerulonephritis [17].

Vasohibin-2 (VASH-2) is a homologue of VASH-1 that is generally considered to be an endogenous proangiogenic factor [11, 19, 20]. VASH-2 was first identified in a mouse model of subcutaneous angiogenesis as in infiltrating bone marrow-derived mononuclear cells at the sprouting point of neo-vessels [21], and it has been shown to be expressed in various tumor tissues including serous ovarian adenocarcinoma [22], hepatocellular carcinoma [23], and gastrointestinal carcinoma [24]. Most of the published studies on VASH-2 have focused on VASH-2 expression in tumor angiogenesis [25-27], but the possible roles of VASH-2 in non-tumor pathological conditions have not been determined.

Considering its homology to VASH-1 (an important factor in renal diseases), we have looked for any possible association of VASH-2 in human kidney diseases. In the present study, we examined kidney biopsy specimens from patients with kidney disease to determine the specimens' VASH-2 expression, and we examined the correlations between renal VASH-2 immunoreactivity and various clinicopathological parameters.

Methods

Study design. We conducted a retrospective review of the cases of patients who had undergone a renal biopsy at Okayama University Hospital during the years 2007-2009 and 2014-2015. Written informed consent for the use of their specimens was obtained from all patients, and the study was approved by the Institutional Ethical Review Board of Okayama University Hospital (No. 471, Oct. 23, 2007 and No. 2063, May 22, 2014). We reviewed the clinical charts of these patients to examine the relevant clinical characteristics that are generally known to affect the prognosis of renal or cardiovascular diseases. Thirteen clinical parameters were considered: age, gender, body mass index (BMI), blood pressure, creatinine (Cr), Cr-based estimated glomerular filtration rate (eGFR) [28], blood urea nitrogen (BUN), proteinuria, uric acid (UA), phosphorus (P), total cholesterol (T-Cho), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), triglyceride (TG), post-prandial plasma glucose (PPG), and hemoglobin A1c (HbA1c; based on the National Glycohemoglobin Standardization Program value). The diagnostic criteria for the clinical entities were as follows:

- Hypertension: systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure (DBP) ≥ 90 mmHg, or on antihypertensive medications
- Diabetes mellitus: HbA1c ≥ 6.5%, PPG ≥ 200 mg/dL, or on antidiabetic medications
- Dyslipidemia: T-Cho ≥ 220 mg/dl, LDL-C ≥ 140 mg/dl, HDL-C ≤ 40 mmHg, or on antilipidemic medications

Biopsy specimens were stained and semi-quantitatively scored to assess renal histological alterations and immunostained to investigate the distributions of VASH-1 and VASH-2. In addition, paraffin-embedded normal kidney tissues were obtained from three patients who had undergone a surgical excision of localized renal cell carcinoma containing distant normal portions of the kidneys.

Histological scoring. Formalin-fixed, paraffin-embedded sections were stained with periodic acid-Schiff (PAS), periodic acid-methenamine-silver (PAM), and Masson's trichrome for light microscopic observation. The histological parameters are based on the Oxford classification of IgA nephropathy [29]. Renal histological alterations were semi-quantitatively evalu-

ated by scoring as follows [17, 30]. Mesangial hypercellularity and mesangial sclerosis scores: 0 = none, +1 = 1-25%, +2 = 26-50%, +3 = 51-75%, +4 = 76-100% of the glomerulus. Interstitial cell infiltration, interstitial fibrosis and tubular atrophy scores: 0 = none, +1 = mild, +2 = moderate, +3 = severe. Vascular sclerosis scores: 0 = none, +1 = mild, +2 = severe. The mean percentage of glomeruli with crescent formation and that of global sclerosis were also deter-mined. The histological evaluation was performed by 2 independent certified nephrologists.

Immunohistochemistry. Immunohistochemistry was performed as previously described [17, 31, 32]. Following the deparaffinization and hydration of formalin-fixed, paraffin-embedded 4-μm-thick sections, antigen retrieval was performed by boiling the sections in a hot bath in the target retrieval solution (Dako, Glostrup, Denmark), pH 9.0, at 98°C for 40 min. The following primary antibodies were used: (1) monoclonal mouse anti-human VASH-1 (clone 4E12; 2 μg/mL) [15, 33] and (2) monoclonal mouse anti-human VASH-2 (clone 1760; 1 μg/mL) [33, 34]. Both of these antibodies were provided by the Institute of Development, Aging, and Cancer, Tohoku University; their specificity on cancer cells was confirmed in human tissue specimens [33, 34].

The samples were washed with phosphate-buffered saline three times, and secondary detection was performed using a diaminobenzidine-based detection kit (EnVision+ system, Dako) according to the manufacturer's instructions. Sections were counterstained with hematoxylin. The immunohistochemistry sections were then descriptively reviewed to assess the localization and distribution of VASH-1 and VASH-2 staining. Sections were also semi-quantitatively scored for VASH-2-positive staining. Specifically, VASH-2$^+$ cells in the renal medulla (tubules and vasa recta [VR]) and cortex (tubules, glomeruli, and peritubular capillaries [PTCs]) were determined using the following criteria [17, 30]:

- Tubule score = the mean of % (none or score 1-3) × the intensity (score 1-3) in each 200× field
- VR score = the mean of the number of VASH-2$^+$ lumens of VR with VASH2$^+$ cells in each 200× field
- PTC score = the mean of the number of lumens of PTC with VASH2$^+$ cells in each 200× field
- Glomerular score = the mean of % (none or score 1-4) in each glomerulus

Statistical analysis. As the data were categorical (ranked) in nature, non-parametric tests were used for comparisons. Spearman's rank order test, the Steel test statistics, and the Mann-Whitney U-test were used as appropriate. All analyses were performed using JMP® 11.1.1 Microsoft Excel software for Mac 2011, ver. 14.6.4.

Results

Patient characteristics. A total of 82 patients with renal dysfunction were included in the study (Table 1). The patients were generally healthy with relatively well-controlled hypertension (n = 52), diabetes mellitus (n = 15), dyslipidemia (n = 59) and without extreme obesity (mean BMI, 22.2). Daily proteinuria varied among the patients, ranging from 0.29 to 2.89 g/day (mean, 0.81 g/day). On the basis of the World Health Organization (WHO) clinical classification of renal diseases, the majority of the cases were chronic glomerulonephritis (52.4%), followed by nephrotic syndrome (29.3%) and rapidly progressive glomerulonephritis (13.4%).

In terms of pathological diagnosis, the majority of the cases were mesangial proliferative nephritis (32.9%) including IgA nephropathy, followed by minimal change disease (MCD) (15.9%), and secondary focal segmental glomerulosclerosis (FSGS) and nephrosclerosis (8.43-12.0%).

Renal distribution of VASH-2. Of the 82 biopsy specimens, 81 specimens included the renal cortex and 51 included the renal medulla. Immunoreactivity for VASH-2 was not observed in the glomeruli in any of the 81 specimens from renal disease patients (Fig. 1A). VASH-2$^+$ cells were observed in PTCs (Fig. 1B; 50/81 specimens), VR (Fig. 1C; 38/51 specimens) and both medullary and cortical tubules (Fig. 1C, 38/51 specimens; Fig. 1D; 37/51 specimens).

In the control kidney specimens, VASH-2$^+$ cells were similarly observed in the VR and some PTCs, but the tubular VASH-2 staining was weak (Fig. 1E, F), suggesting that VASH-2 is present mainly in the VR in the basal condition and that tubular staining could be inducible in some renal diseases. Because the intensity of VASH-2 staining on the PTCs, the VR and the renal tubules showed wide variation among the specimens, we evaluated the intensity of VASH-2 staining using a

Table 1 The 82 patients' clinical, laboratory and histological characteristics

Clinical characteristics	
Age (y)	48.5 [32.0–64.3]
Gender (male/female; n)	37/45
Hypertension, n (%)	52 (63.4)
Diabetes mellitus, n (%)	15 (18.3)
Dyslipidemia, n (%)	59 (72.0)
BMI (kg/m^2)	22.2 [20.1–25.7]
SBP (mmHg)	130 [110–141]
DBP (mmHg)	78 [68–89]
Laboratory parameters	
Hemoglobin (g/dL)	13.4 [12.0–14.7]
Phosphorus (mg/dL)	3.5 [3.0–3.8]
Uric acid (mg/dL)	5.7 [4.6–7.1]
eGFR (mL/min/1.73 m^2)	69.0 [46.0–91.8]
eGFR <60, n (%)	21 (25.6)
Daily proteinuria (g/day)	0.784 [0.294–2.99]
PPG (mg/dL)	106.0 [91.5–136.5]
HbA1c (%)	5.7 [5.4–6.2]
T-Cho (mg/dL)	212 [172–251]
LDL-C (mg/dL)	129 [90.0–154.5]
HDL-C (mg/dL)	60 [43–76.5]
TG (mg/dL)	139 [93.5–213]
Clinical classification (WHO)	
CGN, n (%)	43 (52.4)
APHS, n (%)	4 (4.9)
NS, n (%)	24 (29.3)
RPGN, n (%)	11 (13.4)
Pathological classification	
MPN, n (%)	27 (32.9)
MCD, n (%)	13 (15.9)
Secondary FSGS and nephrosclerosis, n (%)	10 (12.2)
MN, n (%)	8 (9.8)
NCGN, n (%)	7 (8.5)
DMN, n (%)	4 (4.9)
Others, n (%)	14 (17.1)

Values for continuous variables are given as median [interquartile range]. Values for categorical variables are given as number (percentage). APHS, asymptomatic proteinuria and hematuria syndrome; BMI, body mass index; CGN, chronic glomerulonephritis; DBP, diastolic blood pressure; DMN, diabetic nephropathy; FSGS, focal segmental glomerulosclerosis; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MCD, minimal change disease; MN, membranous nephropathy; MPN, mesangial proliferative nephritis; NCGN, necrotizing and crescentic glomerulonephritis; NS, nephrotic syndrome; PPG, post-prandial plasma glucose; RPGN, rapidly progressive glomerulonephritis; SBP, systolic blood pressure; T-Cho, total lipoprotein cholesterol; TG, triglycerides.

semi-quantitative scoring system. The VASH2$^+$ scores on the PTCs were lower, with an exceptionally wide range (median 0.0; range 0-10.59). The tubular VASH2$^+$ scores were generally higher in the renal medulla than in the cortex (median 0.75; interquartile range [IQR] 0.0-1.33 vs. 0.17; IQR 0.0-1.0). The VASH2$^+$ scores on the VR were the highest among the positive regions (median 10.89; IQR 0.0-24).

Correlation between VASH-2$^+$ scores and histological parameters. The histological scoring (mean; range) showed variable degrees of renal pathology in the form of mesangial hypercellularity (0.167; 0-0.383), mesangial sclerosis (0.100; 0-0.357), crescent formation (0.0; 0-0.021), global sclerosis (0.150; 0-0.295), cell infiltration (1; 0-1), interstitial fibrosis (1; 1-2), tubular atrophy (1; 0-2), and vascular sclerosis (1; 0-1).

As shown in Table 2, none of these histological scores including tubular atrophy and interstitial fibrosis were significantly correlated with VASH-2$^+$ scores on the PTCs, VR, or cortical and medullary tubules. However, the presence of a histologically established diagnosis of diabetic nephropathy but not IgA nephropathy was significantly correlated with the cortical renal tubule VASH-2$^+$ scores, and this presence tended to be associated with the medullary tubule VASH-2$^+$ scores (Table 2 and Fig. 2).

Correlation between VASH-2$^+$ scores and clinical/laboratory parameters. We next examined whether the VASH-2$^+$ scores were associated with clinical or laboratory parameters (Table 3). VASH-2$^+$ scores on the medullary tubules were significantly correlated with age, PPG and the HbA1c level (Table 3), whereas the VASH-2$^+$ scores on the cortical tubules showed no association with theses parameters. With the use of Spearman's rank order test, it was revealed that the levels of PPG and HbA1c rose (that is, as the patients' glucose intolerance exacerbated), the medullary tubule VASH-2$^+$ scores increased (Fig. 3A, B). Elevated SBP was significantly correlated with the increase in the cortical tubule VASH-2$^+$ score but not the medullary tubule scores (Fig. 4A, B).

Decreased eGFR values tended to be associated with lower VASH-2 scores on the VR, but this association did not reach statistical significance (Fig. 4C). Considering the fact that the majority of the patients included in this study had well-controlled blood pressure and normal renal function, we divided the patients into subgroups with or without hypertension or renal

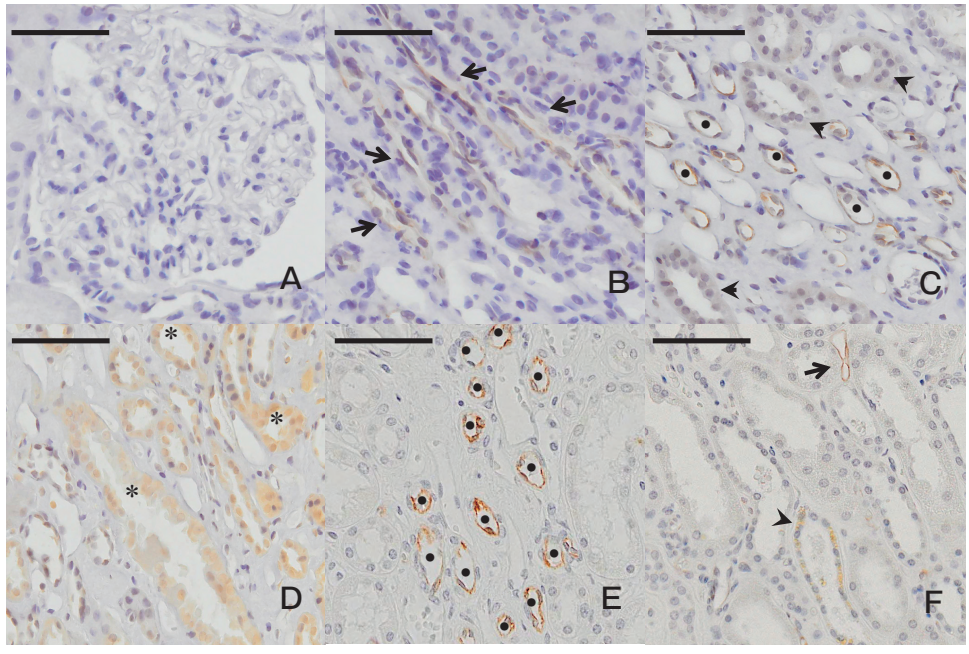

Fig. 1 Immunochemistry of VASH-2 in human kidney biopsy specimens. Representative VASH-2 immunohistochemical images from patients with kidney diseases. **A**, Glomerulus in obesity-related nephropathy; **B**, Peritubular capillaries in IgA nephropathy (IgAN); **C**, Vasa recta (VR) and medullary tubules in IgAN; **D**, Cortical tubules in membranousnephropathy. Immunoreactivity of VASH-2 is observed in endothelial cells of a PTC (*arrows*), the VR (*dots*), and cortical (*asterisks*) and medullary (*arrowheads*) tubules, at various intensities. In control kidney (**E**, **F**), VASH-2 positive cells were seen mainly in the VR (*dots*) and partially in PTCs (*arrow*). Tubular staining for VAHS-2 (*arrowhead*) was detected weakly in only a small fraction. Scale bars: 100 μm.

Table 2 Correlation between VASH-2$^+$ scores and histological parameters

	PTC score		Cortical tubule score		VR score		Medullary tubule score	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
%Mesangial hypercellularity	−0.0704	0.7065	0.1960	0.2906	−0.0347	0.8089	−0.1601	0.2617
%Mesangial sclerosis	−0.3416	0.0600	0.3344	0.0659	−0.0599	0.6761	0.0978	0.4947
%Crescent formation	0.1509	0.4178	0.0954	0.6098	0.0037	0.9794	−0.2579	0.0677
%Global sclerosis	−0.2451	0.1838	−0.0482	0.7969	0.2323	0.101	0.0727	0.6123
Cell infiltration	−0.3376	0.0633	0.1545	0.4065	0.0715	0.6179	0.2011	0.157
Interstitial fibrosis	−0.3052	0.0951	0.2112	0.2540	0.0674	0.6384	0.2289	0.1062
Tubular atrophy	−0.2340	0.2052	0.3088	0.0910	0.0857	0.55	0.2748	0.0510
Arteriosclerosis	−0.2224	0.2292	0.2086	0.2600	−0.1478	0.3008	0.0878	0.5403
DMN or not		0.2647		0.0035*		0.3326		0.0517
IgAN or not		0.8601		0.9398		0.7823		0.5789

DMN; diabetic nephropathy, IgAN; IgA nephronpathy. *$p < 0.05$.

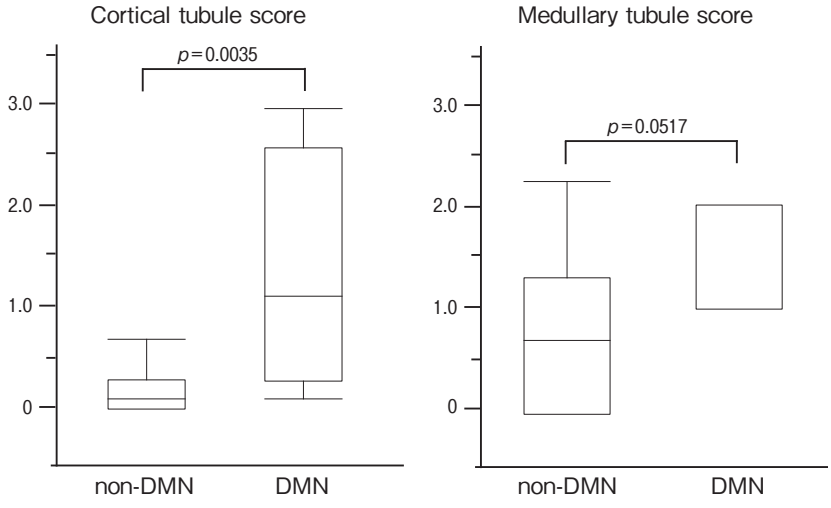

Fig. 2 Subgroup analysis of VASH-2$^+$ scores in the patients with and without diabetic nephropathy. Comparison between the patients with DMN and those without DMN by Mann-Whitney test. Among the VASH-2$^+$ scores, both the cortical and medullary tubule scores appear to be higher in DMN ($p=0.0035$ and $p=0.0517$, respectively).

Table 3 Correlation between VASH-2 scores and clinical/laboratory parameters

	PTC score		Cortical tubule score		VR score		Medullary tubule score	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age (yrs)	−0.2987	0.1026	0.0756	0.6862	−0.0456	0.7506	0.3466	0.0127*
BMI (kg/m^2)	−0.3533	0.0555	−0.0982	0.6056	−0.0618	0.6665	0.2299	0.1046
SBP (mmHg)	−0.4595	0.0106*	0.247	0.1883	0.1474	0.3018	0.2471	0.0804
Hb (g/dL)	−0.056	0.7689	−0.0331	0.862	0.0794	0.5798	0.1517	0.2881
P (mg/dL)	0.198	0.3126	−0.0482	0.8075	−0.2238	0.1305	−0.289	0.0488
UA (mg/dL)	−0.3685	0.0451*	0.235	0.2113	0.0318	0.8284	0.0106	0.9425
eGFR (mL/min/1.73 m^2)	0.2911	0.1121	−0.15	0.4205	−0.0189	0.8951	−0.2526	0.0738
UP (g/day)	0.009	0.9625	0.1263	0.5059	0.1306	0.3763	0.0249	0.8665
PPG (mg/dL)	−0.0101	0.9585	0.1741	0.3663	0.1553	0.2918	0.387	0.0066*
HbA1c (%)	−0.0262	0.9011	0.0008	0.997	−0.0939	0.5644	0.3465	0.0285*
T-Cho (mg/dL)	0.0508	0.7896	0.1379	0.4674	−0.1811	0.2082	−0.036	0.8042
LDL-C (mg/dL)	0.0611	0.7528	0.1639	0.3956	−0.1357	0.3577	−0.0718	0.6276
TG (mg/dL)	−0.3448	0.067	0.1944	0.3122	0.1776	0.2222	0.0853	0.5603

BMI, body mass index; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; LDL-C, low-density lipoprotein cholesterol; P, phosphorus; PPG, post-prandial plasma glucose; SBP, systolic blood pressure; T-Cho, total lipoprotein cholesterol; TG, triglycerides.; UA, uric acid; UP, urinary protein. *$p < 0.05$.

dysfunction (defined as eGFR ≤60 mL/min/1.73 m^2). The subgroup analysis revealed that the cortical and medullary tubule VASH-2$^+$ scores were significantly higher in the patients with hypertension (Fig. 5A, B). In addition, VASH-2$^+$ scores on the VR were significantly lower in the patients with renal dysfunction (Fig. 5C).

Correlation between VASH-2$^+$ score and VASH-1 staining in kidney. Consistent with our previous report about patients with CKD [17], immunoreactivity for VASH-1 was observed in glomeruli (some

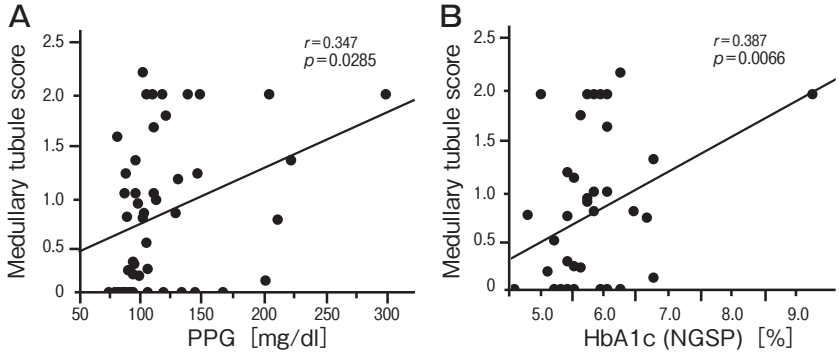

Fig. 3 Correlation analysis of VASH-2 scores and glucose intolerance. The correlations between the medullary tubule VASH-2$^+$ score and the post-prandial plasma glucose (PPG; **A**) or hemoglobin A1c (HbA1c; **B**) were evaluated by Spearman's rank order test. Both parameters were significantly correlated with the VASH-2$^+$ scores. The *r*-values are Spearman correlation coefficients.

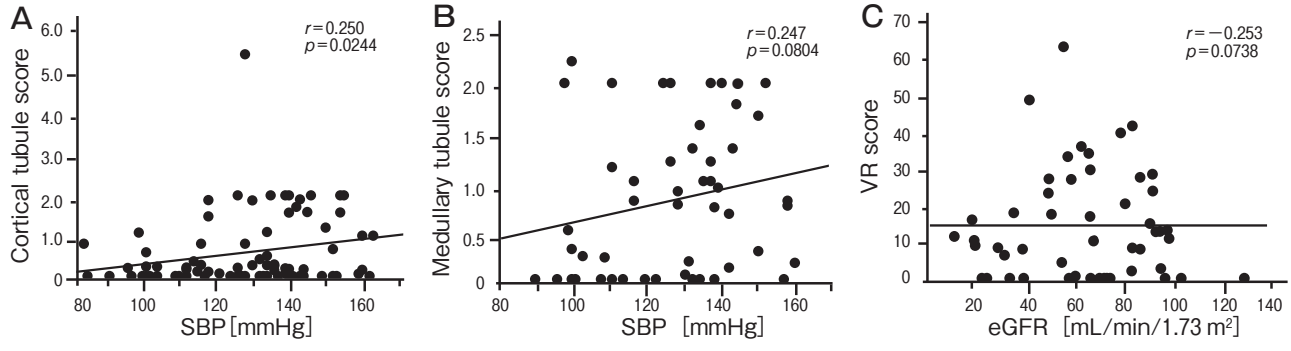

Fig. 4 Correlation analysis of VASH-2 scores and blood pressure or renal function. The correlations between the cortical tubule VASH-2$^+$ score and SBP (**A**), between the medullary tubule VASH-2$^+$ score and SBP (**B**), and between the VR VASH-2$^+$ score and eGFR (**C**) by Spearman's rank order test. SBP was significantly correlated with the cortical tubule VASH-2$^+$ score but not the medullary tubule VASH-2$^+$ score. eGFR tended to be inversely related with the VR VASH-2$^+$ score, but not significantly so. The *r*-values are Spearman correlation coefficients.

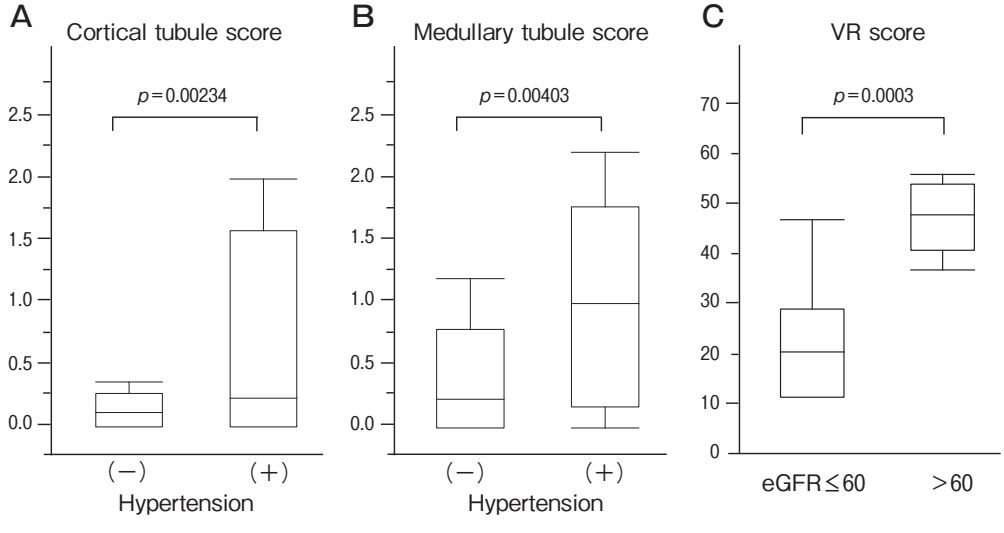

Fig. 5 Subgroup analysis of VASH-2$^+$ scores in the patients with and without hypertension or reduced eGFR. Comparison between the patients with and without hypertension (**A**, **B**) or declined eGFR (**C**: ≤60 ml/min/1.73 m^2) by the Mann-Whitney test. Among the VASH-2$^+$ scores, both the cortical and medullary tubule scores were significantly higher in the patients with hypertension ($p=0.00234$ and $p=0.0403$, respectively), whereas the VR scores were lower in the patients with reduced eGFR (**C**).

mesangial cells, Fig. 6A), renal cortex (endothelial cells and interstitial inflammatory cells, Fig. 6B), and medulla (VR endothelial cells, Fig. 6C). In the control kidneys, VASH-1-positive endothelial cells were confirmed in peritubular capillaries (Fig. 6D) as shown previously [17]. We found no association between any of the VASH-2^+ scores and the numbers of VASH-1^+ cells (Table 4).

Renal survival analysis. We performed a Kaplan-Meier analysis of the deterioration in renal function (defined as >30% reduction in the eGFR compared to baseline) according to VASH-2^+ scores, in order to evaluate the possible impact of VASH-2 on renal prognoses. This analysis used the mean and 75th quartile values of the respective scores as the cutoffs. In general, higher tubular VASH-2^+ scores (>75th quartile) especially in the medullary region ($p=0.0093$) were significantly associated with poorer outcomes over time (Fig. 7A-D). Although the subgroup with reduced eGFRs tended to have lower in VASH-2^+ VR scores (Fig. 5C), neither higher nor lower VR scores seemed to be associated with a sequential reduction in renal function (Fig. 7E, F). The PTC VASH-2^+ scores as well seemed to show no obvious association with renal survival (data not shown).

Discussion

The results of this immunohistochemical analysis suggest that VASH-2 plays a significant role in human kidney diseases. VASH-2-positive cells were not observed in glomeruli, but they were observed in the VR and peritubular capillaries as well as renal tubules in patients with kidney disease. Consistent with our

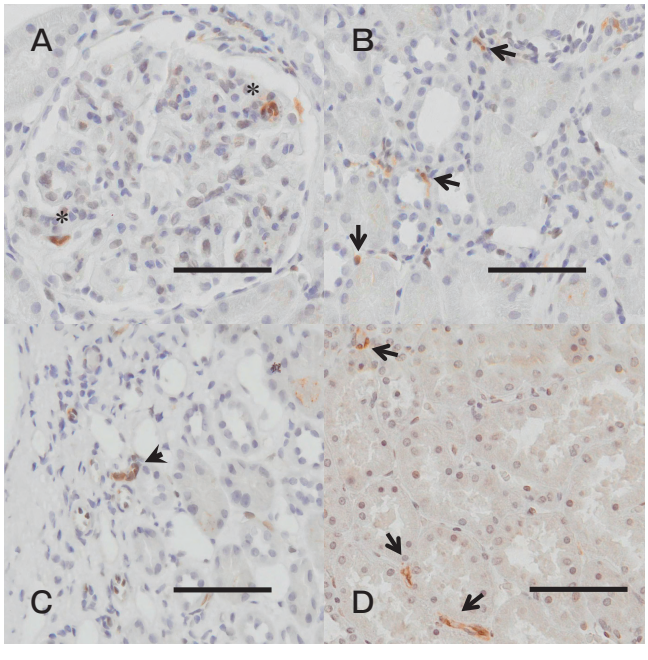

Fig. 6 Immunohistochemistry of the human kidney with VASH-1. Representative VASH-1 immunohistochemical images from patients with IgA vasculitis kidney diseases. **A**, Glomerulus; **B**, Cortical region; **C**, Medullary region. Immunoreactivity for VASH-1 was observed in some mesangial cells (*asterisk*, **A**), endothelial cells, and interstitial inflammatory cells in the cortex (*arrows*, **B**), and VR endothelial cells in the medulla (*arrowhead*, **C**). In the control kidney, VASH-1 staining was detected in endothelial cells (*arrows*, **D**). Scale bars: 100 μm.

Table 4 Correlation between VASH-2^+ scores and VASH-1 staining

	PTC score		Cortical tubule score		VR score		Medullary tubule score	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
VASH-1^+ score of glomeruli	−0.1536	0.4262	0.1678	0.3841	−0.2001	0.2980	−0.0982	0.6125
VASH-1^+ cells in cortex	0.2933	0.1225	0.1277	0.5091	−0.0971	0.6162	0.0125	0.9485
VASH-1^+ cells in medulla	0.1242	0.6018	−0.0720	0.7631	−0.1979	0.4030	−0.3395	0.1431

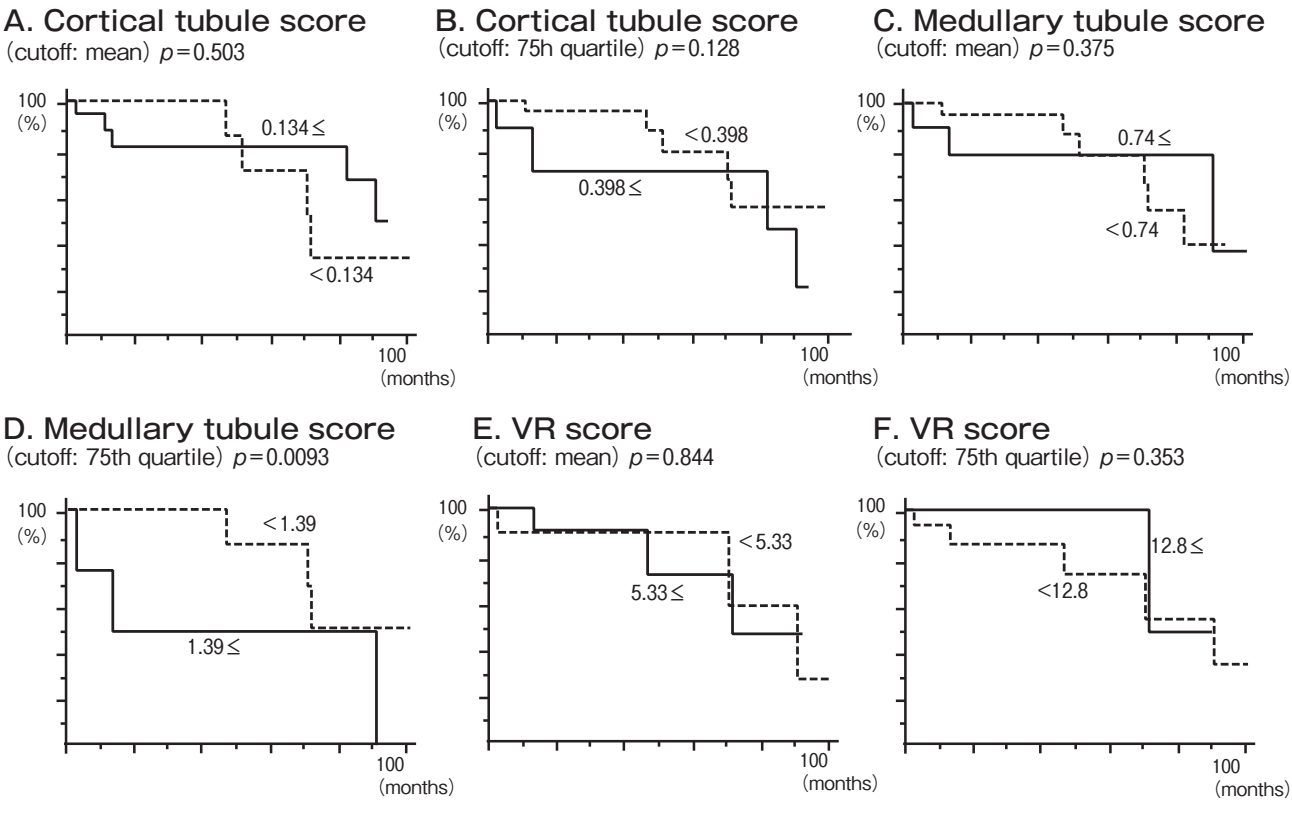

Fig. 7 Renal survival analysis according to VASH-2$^+$ scores. The outcome is defined as a $>30\%$ reduction in eGFR from the baseline within the follow-up duration of 100 months. The renal survival in the subgroup above (solid line) and below (dotted line) each cutoff is shown. **A**, **B**, Cortical tubule VASH-2$^+$ scores with the mean (0.134) and the 75th quartile (0.398) as the cutoff, respectively; **C**, **D**, Medullary tubule VASH-2$^+$ scores with the mean (0.74) and the 75th quartile (1.39) as the cutoff, respectively; **E**, **F**, VR VASH-2$^+$ scores with the mean (0.844) and the 75th quartile (0.353) as the cutoff, respectively. Overall, there were no significant associations between the subgroups regarding preserved renal function over time, except for our finding that higher tubular VASH-2$^+$ scores in medullary tubules are significantly associated with poor outcome ($p=0.0093$).

results, VASH-2 was previously shown to be expressed in blood vessels from human embryonic tissues, especially in endothelium [11].

Interestingly, we observed a correlation between renal tubular VASH-2 immunoreactivity and plasma glucose and HbA1c levels, suggesting a relationship between VASH-2 expression and impaired glucose tolerance (IGT). The VASH-2$^+$ tubule scores were also higher in the diabetic nephropathy group. Although these preliminary findings failed to demonstrate a cause and effect relationship between increased VASH-2 immunoreactivity and IGT, they suggest a potential role of hyperglycemia in inducing tubular VASH-2 expression. In diabetic nephropathy, hyperglycemia causes increased oxygen consumption in the kidneys, leading to tissue ischemia and hypoxia-related injury that first affect the interstitial regions in the renal medulla due to lower blood flow, before affecting glomeruli [35, 36]. It was reported that in diabetic nephropathy, hyperglycemia-induced hypoxia promotes the gene expression of factors such as glucose transporter 1 (GLUT-1) and VEGF, which control energy metabolism, cellular proliferation/differentiation, and angiogenesis [37, 38]. The preferential and higher expression of VASH-2 in the renal medulla observed in the present study suggests that VASH-2 could also play a similar role in diabetic nephropathy.

In this investigation, VASH-2 immunoreactivity was detected in the VR and peritubular capillaries but not in glomerular capillaries. This discrepancy cannot be explained on the basis of current knowledge about the regulatory mechanisms of VASH-2 gene expression. Considering the anatomy of the renal vasculature, the VR and peritubular capillaries are more likely to

become hypoxic compared to the glomeruli. In addition, whereas podocytes deliver a large amount of VEGF to endothelial cells in the glomeruli, the VR and peritubular capillaries receive VEGF from surrounding tubular cells to a lesser extent [39]. Thus, the VR and peritubular capillaries might require the angiogenic systems independent of VEGF in response to hypoxia.

Kimura *et al.* reported that VASH-2 was expressed in bone marrow-derived mononuclear cells in a mouse model of subcutaneous angiogenesis [21]. However, VASH-2 staining was found in cancer cells but not interstitial mononuclear cells in human ovarian carcinoma tissues [22]. These findings suggest that VASH-2 expression on mononuclear cells could be detected only in the active process of angiogenesis induced by hypoxia. Indeed, we did not detect VASH-2 immunoreactivity in the infiltrating mononuclear cells in the tubulointerstitial nephritis specimens.

We observed varying distributions and localizations of VASH-1 and VASH-2 in this study. Such differences in VASH-1 and VASH-2 distributions have also been demonstrated in human placental tissue, where VASH-2 was localized mainly in trophoblasts whereas VASH-1 was observed in capillaries [40]. Unlike VASH-1, the proangiogenic effect of VASH-2 has been shown to be VEGF-A-independent. For example, while VASH-1 was found to tightly correlate with VEGF-A expression in gastric cancer cell lines [24], there was not significant correlation between VASH-2 and VEGF-A expression. Another study also revealed that VASH-2 expression was scarcely stimulated by the factors that induced VASH-1, including VEGF in endothelial cells *in vitro* [11]. Taken together, these results suggest a novel mechanism of VASH-2 beyond angiogenesis. Indeed, non-angiogenic actions of VASH-2 was shown in human serous ovarian adenocarcinoma [22], where it influenced the migration of endothelial cells by affecting the epithelial-to-mesenchymal transition of cells. Other malignant cells such as those in ovarian, gastrointestinal and hepatocellular carcinoma cells have also been shown to express VASH-2 [23, 24]. In our present study, VASH-2 immunoreactivity varied within the renal tubules, being stronger in the renal medulla than the renal cortex. In addition, the tubular VASH-2$^+$ scores were significantly higher in the subgroup with hypertension, which was previously suggested to trigger interstitial injury including fibrosis via the renin-angiotensin pathway [41]. These findings suggest that the renal tubular expression of VASH-2 may reflect some alterations of cellular phenotype that accompany tubular atrophy and interstitial fibrosis during the disease progression in CKD.

Nevertheless, our study did not reveal a correlation between tubular VASH-2$^+$ scores and histopathological parameters including renal tubular atrophy and interstitial fibrosis. Considering the fact that our study subjects generally had normal renal function (with mean eGFR of 69.0 mL/min/1.73 m^2) and likely did not have advanced CKD, we speculate that such associations may only become apparent in advanced CKD. However, since a renal biopsy is generally not indicated in patients with advanced CKD, we were not able to investigate this relationship. In contrast to the tubular VASH-2$^+$ scores, higher VASH-2$^+$ scores in the VR were significantly associated with preserved renal function. Although the implication of vascular VASH-2 expression is not completely understood, the decreased expression of VASH-2 in the VR may reflect vascular injury related to renal dysfunction. Consistent with this hypothesis, the VR have been shown to be the main target of iodinated contrast media-induced renal injury [42].

In one of our previous studies, increased plasma and urinary VASH-1 levels predicted worse renal outcomes in CKD patients [16]. Unfortunately, since there are no established enzyme-linked immunosorbent assays (ELISAs) for VASH-2, we could not measure the plasma VASH-2 levels in the present patients with kidney diseases as well as malignancies. In contrast to VASH-1, which constitutively expresses in endothelial cells, the basal level of VASH-2 expression observed in various organs is quite low [21]. The plasma VASH-2 level is thus expected to be extremely low in humans, except for patients with highly VASH-2-expressing tumors.

There were several other limitations in this study. First, as a renal biopsy is not indicated in normal healthy humans or in advanced CKD patients, we were not able to obtain the ideal controls for comparative analyses. Second, the total sample size was not sufficient to allow comparisons among the patients with various renal disorders. Third, we did not fully elucidate the mechanisms underlying the relationships between VASH-2 expression and some clinical parameters including the plasma glucose level and the eGFR in this preliminary exploratory study. Systematic *in vitro*

and *in vivo* studies using appropriate controls are required to further clarify the precise mechanisms of VASH-2 action.

In conclusion, we investigated the renal distribution of VASH-2 in patients with kidney disease, and our results demonstrated a positive association between the renal tubular VASH-2 immunoreactivity and blood glucose levels, as well as a positive association between vasa recta VASH-2 and the eGFR. Further studies are required to fully understand the mechanisms and potential clinical roles of VASH-2 in kidney diseases.

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