

Original Article

## Renal Distribution of Vasohibin-1 in Patients with Chronic Kidney Disease

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Experimental studies have demonstrated the involvement of angiogenesis-related factors in the progression of chronic kidney disease (CKD). There have so far been no reports investigating the distribution and clinical roles of Vasohibin-1 (VASH-1), a negative feedback regulator of angiogenesis, in CKD. We recruited 54 Japanese CKD patients and 6 patients who had normal renal tissues excised due to localized renal cell carcinoma. We evaluated the correlations between the renal expression level of VASH-1 and the clinical/histological parameters. VASH-1 was observed in renal endothelial/mesangial cells, crescentic lesions and interstitial inflammatory cells. Significant positive correlations were observed between 1) crescent formation and the number of VASH-1<sup>+</sup> cells in the glomerulus ( $r=0.48$ ,  $p=0.001$ ) or cortex ( $r=0.64$ ,  $p<0.0001$ ), 2) interstitial cell infiltration and the number of VASH-1<sup>+</sup> cells in the cortex ( $r=0.34$ ,  $p=0.02$ ), 3) the glomerular VEGFR-2<sup>+</sup> area and the number of VASH-1<sup>+</sup> cells in the glomerulus ( $r=0.44$ ,  $p=0.01$ ) or medulla ( $r=0.63$ ,  $p=0.01$ ). These results suggest that the renal levels of VASH-1 may be affected by local inflammation, crescentic lesions and VEGFR-2.

**Key words:** chronic kidney disease, inflammation, Vasohibin-1, VEGF-A, VEGFR-2

Chronic kidney disease (CKD) is associated with an increased risk of end-stage renal disease (ESRD) and cardiovascular morbidity and mortality. Since the number of patients with ESRD is increasing worldwide, clarification of the pathological factors promoting renal function deterioration and prevention of the progression of CKD is required.

Glomerulosclerosis and tubulointerstitial altera-

tions are the most common characteristic histological alterations observed in patients with renal disorders. In general, deterioration of the renal function correlates better with the degree of tubulointerstitial injury than do glomerular alterations [1]. Experimental and biopsy studies have shown that the loss of podocytes [2-5] and renal capillaries [6] is closely linked with the progression of CKD. Angiogenesis is regulated by the balance between proangiogenic and anti-angiogenic

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factors. In the normal healthy kidney, vascular endothelial growth factor (VEGF)-A, a major pro-angiogenic factor [7], is primarily expressed by podocytes and tubular epithelial cells [8]. VEGF-A plays an important role in maintaining the glomerular capillary structure and repairing glomerular endothelial cells and peritubular capillaries following injuries [6, 9, 10]. Physiological levels of VEGF-A are also required for maintenance of the glomerular filtration barrier. In the early stages of diabetic nephropathy, the number of glomerular capillaries and the glomerular levels of VEGF-A and its receptor VEGFR-2 are increased [11, 12]. Previous reports have also demonstrated altered distributions of VEGF-A and its receptors VEGFR-1 and VEGFR-2 in patients with glomerular disorders [13, 14].

Vasohibin-1 (VASH-1), an endogenous angiogenesis inhibitor, was originally identified in a microarray analysis assessing genes up-regulated by VEGF-A in endothelial cells [15]. The human VASH-1 protein is composed of 365 amino acid residues and regulates the proliferation and migration of endothelial cells in an autocrine manner; thus it is considered to be a negative feedback regulator of angiogenesis [15]. A more recent report has described the critical role of VASH-1 in the maintenance of endothelial cells against cellular stressors, and the regulatory mechanisms of its synthesis via posttranscriptional regulation mediated by the binding of HuR proteins to AU-rich elements in the 3' untranslated region of VASH-1 mRNAs [16]. VASH-1 does not contain a classic signal sequence; rather, a small vasohibin-binding protein (SVBP) serves as its secretory chaperone and contributes to its antiangiogenic effects [17]. To date, no cell surface receptors for VASH-1 have been identified. The therapeutic efficacy of VASH-1 in experimental models of tumors, atherosclerosis, proliferative retinopathy [15, 18, 19] and diabetic nephropathy [20, 21] has been reported. However, the renal distribution of VASH-1 in patients with CKD has not yet been thoroughly examined.

In the present study, we aimed to elucidate the renal distribution of VASH-1 in CKD patients. We determined the renal level of VASH-1 by immunohistochemistry and evaluated the correlations between the renal VASH-1 level and both clinical and histological variables, including angiogenesis-associated factors. This is the first study to examine the renal

distribution of VASH-1 in CKD patients, and the first to suggest a potential role for VASH-1 in protecting against inflammatory and crescentic lesions in patients with CKD.

## Materials and Methods

**Study population.** We studied 54 Japanese CKD patients and their renal biopsy samples. Normal renal tissue samples were also obtained from six patients who had undergone surgical excision of distant portions of the kidneys due to localized renal cell carcinoma.

All patients attended the Department of Nephrology at Okayama University Hospital (Okayama, Japan) between the years 2007 and 2009. The histological evaluations of the biopsies were performed by 2 renal pathologists. To measure the estimated glomerular filtration rate (eGFR), the modified MDRD equation for Japanese individuals [22] was used. The medical records from the day of the renal biopsy were evaluated. The protocol of the current study was approved by the Institutional Ethical Review Board of Okayama University Hospital (No. 471, Oct. 23, 2007), and written informed consent was obtained from all subjects.

The patients were followed up annually for three years. We defined a composite renal event as an eGFR decline of greater than 30% from baseline, initiation of renal replacement therapy or kidney disease-related death.

**Histological analysis.** Formalin-fixed, paraffin-embedded sections were stained with periodic acid-Schiff (PAS), periodic acid-methenamine-silver (PAM) or Masson's trichrome for light microscopic observation. Renal histological alterations were semiquantitatively evaluated by scoring as follows [23]: for mesangial hypercellularity and mesangial sclerosis, 0 = none, +1 = 1-25%, +2 = 26-50%, and +3 = 51-100% of the glomerulus; for interstitial cell infiltration, interstitial fibrosis and tubular atrophy, 0 = none, +1 = mild, +2 = moderate, and +3 = severe; and for vascular sclerosis, 0 = none, +1 = mild, and +2 = severe. The mean percentage of glomeruli with crescent formation and global sclerosis was also determined. The histological evaluation was performed in a blinded fashion by 2 investigators, and the results were averaged.

**Immunohistochemistry.** Immunohistochemistry was performed as previously described [1, 21]. Following deparaffinization and hydration, formalin-fixed, paraffin-embedded sections (4- $\mu$ m-thick) were placed in a hot bath in the target retrieval solution, pH9.0 (Dako, Glostrup, Denmark) at 98°C for 40 min. The following primary antibodies were used: (1) monoclonal mouse anti-human VASH-1 (2 $\mu$ g/mL) [15]; (2) monoclonal mouse anti-human CD31 (JC70A; Dako); (3) monoclonal mouse anti-human VEGF-A (JH121; NeoMarkers, Fremont, CA, USA) [24]; and (4) monoclonal mouse anti-human VEGFR-2 (sc-6251; Santa Cruz Biotechnology, Santa Cruz, CA, USA) [24]. The samples were then washed and the color was developed using the EnVision+ system (Dako) according to the manufacturer's instructions. The chromogen used was diaminobenzidine. Sections were counterstained with hematoxylin.

The mean number of glomerular VASH-1<sup>+</sup> cells was evaluated as follows: the total number of VASH-1<sup>+</sup> cells was divided by the number of glomeruli. The number of VASH-1<sup>+</sup> cells in the tubulointerstitium in the renal cortex and medulla were evaluated as follows: the number of VASH-1<sup>+</sup> cells was divided by the number of observed high power fields (original magnification  $\times$  400). The glomerular CD31<sup>+</sup> endothelial areas were semiquantitatively evaluated. All glomeruli were observed, and images were analyzed to determine the mean percentages of the glomerular VEGF-A<sup>+</sup> and VEGFR-2<sup>+</sup> areas.

Double immunofluorescent staining was performed as previously described [25, 26]. Frozen sections were incubated with the following primary antibodies: monoclonal mouse anti-human VASH-1 [15], rat anti-mouse CD31 (PharMingen) or polyclonal rabbit anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Epitomics, Burlingame, CA, USA). For negative control, we performed immunostaining without the usage of primary antibody. Subsequently, the sections were incubated with the following secondary antibodies: Alexa Fluor 546-labeled goat anti-mouse IgG (VASH-1; Invitrogen), Alexa Fluor 488-labeled goat anti-rat IgG (CD31; Invitrogen) or Alexa Fluor 488-labeled donkey anti-rabbit IgG ( $\alpha$ -SMA; Invitrogen). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Chemicon). The sections were observed under a BIOZERO fluorescent microscope (BZ-800; Keyence, Osaka, Japan) and images were obtained.

**Statistical analysis.** All values are expressed as the mean  $\pm$  standard deviation (SD). Pearson's correlation coefficients were used to evaluate the association between 2 continuous variables. The Mann-Whitney U-test was used to compare the expression levels of VASH-1, CD31, VEGF-A and VEGFR-2 in the control subjects and patients with renal disorders. Statistical significance was defined as a *p*-value of  $< 0.05$ . The composite renal event rates were estimated using the Kaplan-Meier method and compared using the log-rank test. All statistical tests were performed using the JMP version 9 software package (SAS Institute Inc., Cary, NC, USA).

## Results

**Characteristics of the CKD patients.** The mean age of the patients was  $45 \pm 18$  years, the mean eGFR was  $75.8 \pm 28.1$  mL/min/1.73m<sup>2</sup> and the mean daily level of proteinuria was  $2.2 \pm 4.8$  g/day. The prevalence of hypertension, diabetes mellitus and dyslipidemia was 34%, 10% and 55%, respectively (Table 1). The baseline clinical data classified accord-

**Table 1** Clinical parameters of the CKD patients

		(n = 54)
Age	45 $\pm$ 18	(17-82)
Gender (male/female)	22 / 32	
BMI (kg/m <sup>2</sup> )	22.9 $\pm$ 3.8	(16.6-31.8)
SBP (mmHg)	126.2 $\pm$ 20.4	(82-161)
DBP (mmHg)	76.7 $\pm$ 13.6	(49-107)
Hemoglobin (g/dL)	13.2 $\pm$ 1.7	(8.5-16.6)
HbA1c (NGSP) (%)	5.8 $\pm$ 0.8	(4.8-8.2)
FPG (mg/dL)	107 $\pm$ 30	(74-224)
T-cho (mg/dL)	233 $\pm$ 86	(106-590)
LDL-C (mg/dL)	139 $\pm$ 64	(49-417)
HDL-C (mg/dL)	63 $\pm$ 22	(29-132)
TG (mg/dL)	138 $\pm$ 74	(42-361)
eGFR (mL/min/1.73m <sup>2</sup> )	75.8 $\pm$ 28.1	(19-132)
Daily proteinuria (g/day)	2.2 $\pm$ 4.8	(0.03-30)
Hypertension (%)	34.1	
Diabetes mellitus (%)	10.0	
Dyslipidemia (%)	54.8	

BMI, body mass index; CKD, chronic kidney disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NGSP, national glycohemoglobin standardization program; SBP, systolic blood pressure; T-cho, total cholesterol; TG, triglycerides. The values are presented as the means  $\pm$  SD. The values in the parenthesis are minimum to maximum.

ing to the diagnoses of the renal disorders are shown in Table 2. Only the age, serum creatinine and eGFR were available for the control subjects, and are shown in Table 2. No significant differences were observed among any of the groups.

**Distribution and number of renal VASH-1<sup>+</sup> cells in patients with CKD.** In the normal control subjects (Fig. 1, panels a, b and c) and the patients with mild glomerulonephritis (Fig. 1, panels e and f: IgA nephropathy), VASH-1<sup>+</sup> cells were faintly observed in endothelial cells. However, VASH-1<sup>+</sup> cells were not observed in extraglomerular arterioles in the normal control subjects (Fig. 1, panel d). In the patients with purpura nephritis, VASH-1<sup>+</sup> cells were observed in cellular crescents and were also distributed in vasa recta in the renal medulla (Fig. 1, panels g and h). VASH-1<sup>+</sup> cells were also observed in endothelial and inflammatory cells in the patients with granulomatosis with polyangiitis (Fig. 1, panels i and j). In the patients with diabetic nephropathy, no VASH-1<sup>+</sup> cells were observed in the nodular lesions of the glomeruli, although they were observed in interstitial inflammatory cells in the cortex (Fig. 1, panels k and l).

The number of glomerular VASH-1<sup>+</sup> cells was significantly elevated in the patients with membranous nephropathy compared with that observed in the normal controls and patients with IgA nephropathy and diabetic nephropathy (Fig. 2, panel A). The numbers of VASH-1<sup>+</sup> cells in the cortex of patients with membranous nephropathy, IgA nephropathy, crescentic glomerulonephritis, lupus nephritis and other conditions were increased compared with those observed in

the normal controls (Fig. 2, panel B). The numbers of VASH-1<sup>+</sup> cells in the medulla in the patients with membranous nephropathy, IgA nephropathy and crescentic glomerulonephritis were also increased compared with those observed in the normal controls (Fig. 2, panel C).

**Correlations between the number of renal VASH-1<sup>+</sup> cells and various clinical/histological parameters.**

The numbers of VASH-1<sup>+</sup> cells in the glomeruli, cortex and medulla were positively correlated (Fig. 3). The number of VASH-1<sup>+</sup> cells in the cortex was inversely correlated with the diastolic blood pressure (Table 3). The number of VASH-1<sup>+</sup> cells in the medulla was inversely correlated with the age and systolic/diastolic blood pressure and positively correlated with the eGFR. The renal level of VASH-1 did not show a significant correlation with proteinuria. The numbers of VASH-1<sup>+</sup> cells in the glomeruli and cortex positively correlated with the presence of crescent formation. The number of VASH-1<sup>+</sup> cells in the cortex were positively correlated with the presence of interstitial cell infiltration. There were no significant correlations between the numbers of VASH-1<sup>+</sup> cells and other clinical parameters (gender, body mass index, hemoglobin, HbA1c, fasting plasma glucose, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and triglycerides) (data not shown). No other histological parameters were significantly correlated with the levels of VASH-1 (Table 3).

**Correlations between VASH-1 and the renal levels of CD31, VEGF-A and VEGFR-2.** Next, we investigated the correlations between the renal

**Table 2** Characteristics of the patients and controls

	n	Age (years)	sCr (mg/dL)	eGFR (mL/min/1.73m <sup>2</sup> )	Proteinuria (g/day)	Number of glomeruli
Control	6	65 ± 11	0.6 ± 0.3	87.3 ± 18.1	—	20.0 ± 0.0
MN	3	66 ± 9	1.3 ± 0.2	53.5 ± 14.8	4.9 ± 2.5	7.7 ± 3.8
MC	5	37 ± 7	0.7 ± 0.2	88.0 ± 11.4	9.6 ± 1.9	5.4 ± 3.6
Crescentic GN	5	64 ± 7	1.2 ± 0.2	52.1 ± 11.4	0.5 ± 1.9	5.8 ± 3.5
IgA N	18	39 ± 4	0.8 ± 0.1	79.6 ± 6.0	0.6 ± 0.5	7.2 ± 6.9
Lupus N	3	43 ± 9	1.1 ± 0.2	54.7 ± 14.8	1.1 ± 2.5	5.7 ± 4.0
Diabetic N	4	48 ± 8	1.0 ± 0.2	56.7 ± 12.8	3.1 ± 3.0	6.3 ± 3.5
Nephrosclerosis	3	65 ± 9	0.6 ± 0.2	88.2 ± 14.8	0.1 ± 2.5	6.7 ± 7.0
Others	13	39 ± 4	0.8 ± 0.1	86.8 ± 7.1	2.4 ± 1.2	11.9 ± 9.9

eGFR, estimated glomerular filtration rate; GN, glomerulonephritis; MC, minimal change; MN, membranous nephropathy; N, nephropathy; sCr, serum creatinine. The values are expressed as the means ± SD.

levels of VASH-1, CD31 (a marker for endothelial cells), VEGF-A and VEGFR-2. The glomerular CD31 immunoreactivity was specifically localized to endothelial cells in the glomerulus and peritubular capillaries and arterioles in the normal controls (Fig. 4, panel a) and the diseased glomeruli (Fig. 4, panel b–f). The glomerular CD31 (glomerular endothelial area) score tended to be lower in the patients with renal disorders than in the normal controls (Fig. 5, panel A). The glomerular CD31 score was not significantly correlated with the renal levels of VASH-1 (Table 4). Glomerular VEGF-A immunoreactivity was primarily localized to podocytes in the normal controls (Fig. 4, panel g) and was markedly increased in the podocytes and additionally observed in glomerular endothelial cells (Fig. 4, panels h–j and l), crescents (Fig. 4, panel i) and interstitial inflammatory cells (Fig. 4, panel k) in the diseased glomeruli. In tubular

epithelial cells, VEGF-A immunoreactivity was slightly observed in the normal controls (Fig. 4, panel g) and was markedly increased in the sections of kidney diseases (Fig. 4, panel h–l). Recent studies reported that VEGF-A was expressed not only in podocytes, but also in tubular epithelial cells [13, 14]. The glomerular VEGF-A<sup>+</sup> areas were significantly increased in the patients with minimal change disease, IgA nephropathy and other renal disorders compared with that observed in the normal controls (Fig. 5, panel B). The presence of glomerular VEGF-A<sup>+</sup> areas was not correlated with the number of VASH-1<sup>+</sup> renal cells (Table 4). Glomerular VEGFR-2<sup>+</sup> cells were found in endothelial cells and podocytes in the normal controls (Fig. 4, panel m) and were enhanced in the patients with renal disorders (Fig. 4, panels n and p). Additionally, immunoreactivity for VEGFR-2 was observed in inflammatory cells and crescents (Fig. 4, panel o

**Table 3** Correlations between the renal levels of Vasohibin-1 and the clinical/histological parameters

	No. of VASH-1 <sup>+</sup> cells in glomeruli		No. of VASH-1 <sup>+</sup> cells in cortex		No. of VASH-1 <sup>+</sup> cells in medulla	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Age (years)	0.00	0.98	0.08	0.55	−0.40	0.03 <sup>a</sup>
SBP (mmHg)	−0.14	0.37	−0.27	0.09	−0.55	0.02 <sup>a</sup>
DBP (mmHg)	−0.15	0.35	−0.34	0.03 <sup>a</sup>	−0.52	0.02 <sup>a</sup>
eGFR (mL/min/1.73 m <sup>2</sup> )	0.12	0.40	−0.15	0.27	0.39	0.04 <sup>a</sup>
Proteinuria (g/day)	0.07	0.64	0.07	0.64	0.34	0.64
Mesangial hypercellularity	0.09	0.56	0.07	0.65	−0.14	0.54
Mesangial sclerosis	−0.04	0.79	0.20	0.20	−0.01	0.96
Crescent formation	0.48	0.001 <sup>b</sup>	0.64	<0.0001 <sup>b</sup>	0.22	0.33
Global sclerosis	−0.08	0.60	0.03	0.83	−0.19	0.41
Interstitial cell infiltration	0.20	0.18	0.35	0.02 <sup>a</sup>	−0.06	0.79
Interstitial fibrosis	0.07	0.67	0.25	0.10	−0.14	0.92
Tubular atrophy	−0.01	0.96	0.11	0.46	−0.15	0.49
Arteriosclerosis	0.14	0.39	0.21	0.17	0.14	0.55

DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; No., number; SBP, systolic blood pressure; VASH-1, Vasohibin-1. <sup>a</sup>*p* < 0.05. <sup>b</sup>*p* < 0.01.

**Table 4** Correlations between the glomerular CD31<sup>+</sup> areas, VEGF<sup>+</sup> areas, VEGFR-2<sup>+</sup> areas and renal expression of Vasohibin-1

	The score of CD31 <sup>+</sup> area		VEGF <sup>+</sup> area		VEGFR-2 <sup>+</sup> area	
	<i>r</i> value	<i>p</i> value	<i>r</i> value	<i>p</i> value	<i>r</i> value	<i>p</i> value
No. of VASH-1 <sup>+</sup> cells in glomeruli	0.04	0.80	0.01	0.96	0.44	0.01 <sup>a</sup>
No. of VASH-1 <sup>+</sup> cells in cortex	−0.14	0.33	−0.08	0.63	0.25	0.20
No. of VASH-1 <sup>+</sup> cells in medulla	0.02	0.93	0.35	0.14	0.63	0.01 <sup>a</sup>

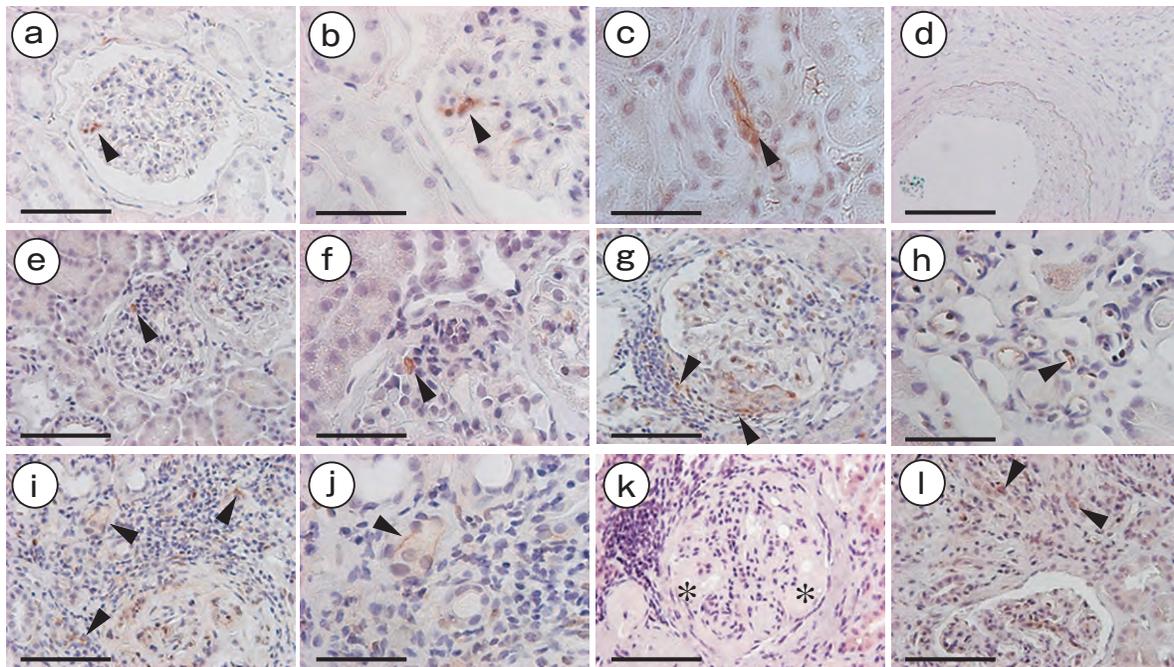
No., number; VASH-1, Vasohibin-1. <sup>a</sup>*p* < 0.05. <sup>b</sup>*p* < 0.01.

and q) but not in sclerotic areas (Fig. 4, panel o and r). VEGFR-2 immunoreactivity was moderately observed in the tubular epithelial cells in sections of the control and the renal disorders (Fig. 4, panels m-r). Recent study showed similar VEGFR-2 staining pattern in tubular epithelial cells [14]. The number of VEGFR-2<sup>+</sup> areas in the glomeruli was significantly increased in the patients with minimal change disease compared with that observed in the normal controls (Fig. 5, panel C). The number of glomerular VEGFR-2<sup>+</sup> areas was positively correlated with the numbers of VASH-1<sup>+</sup> cells in the glomeruli and medulla (Table 4).

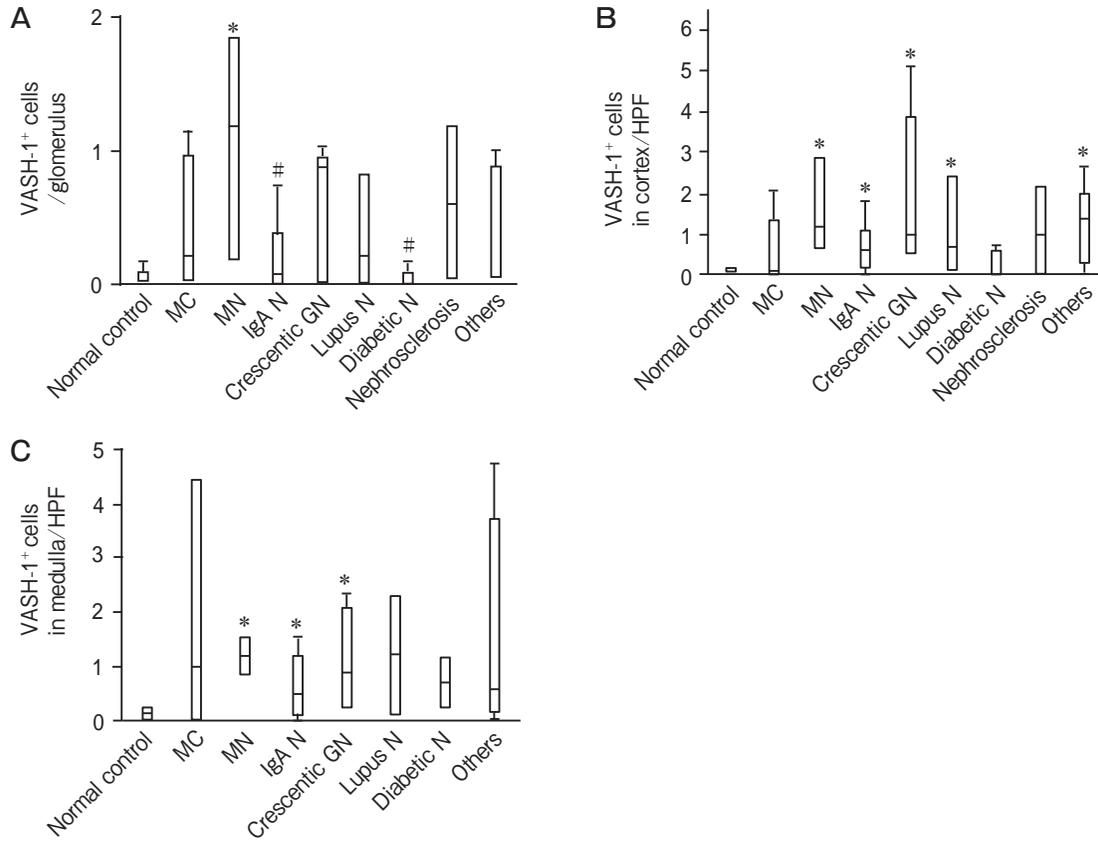
**Double immunostaining for VASH-1,  $\alpha$ -SMA and CD31.** We next performed double immunostaining to confirm the localization of VASH-1. In the normal kidney, endogenous VASH-1 was slightly observed in the glomeruli and the peritubular capillaries (Fig. 6, panels a and g). Immunoreactivity for

VASH-1 was increased in the mesangial ( $\alpha$ -SMA<sup>+</sup>) and endothelial (CD31<sup>+</sup>) areas in the patients with lupus nephritis (Fig. 6, panels d-f and j-l) and was also increased in the crescents in the patients with IgA nephropathy (Fig. 7, panel a-d).

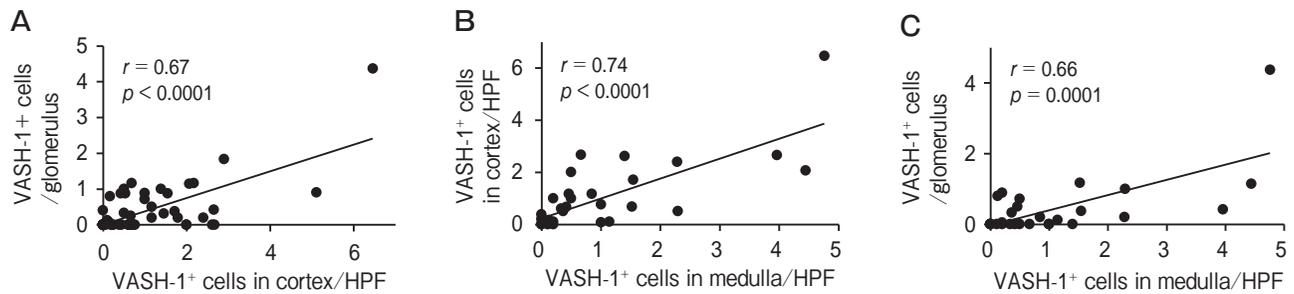
**Correlation between the renal levels of VASH-1 and renal outcomes.** We next performed a Kaplan-Meier analysis to evaluate the potential clinical usefulness of the renal levels of VASH-1 in predicting renal outcomes. The patients were stratified into 2 groups for the Kaplan-Meier analysis, with the cutoff values for the renal levels of VASH-1 being as shown in Fig. 8. The patients with elevated renal levels of VASH-1 tended to experience a higher incidence of composite renal events, but the difference was not statistically significant (Fig. 8).



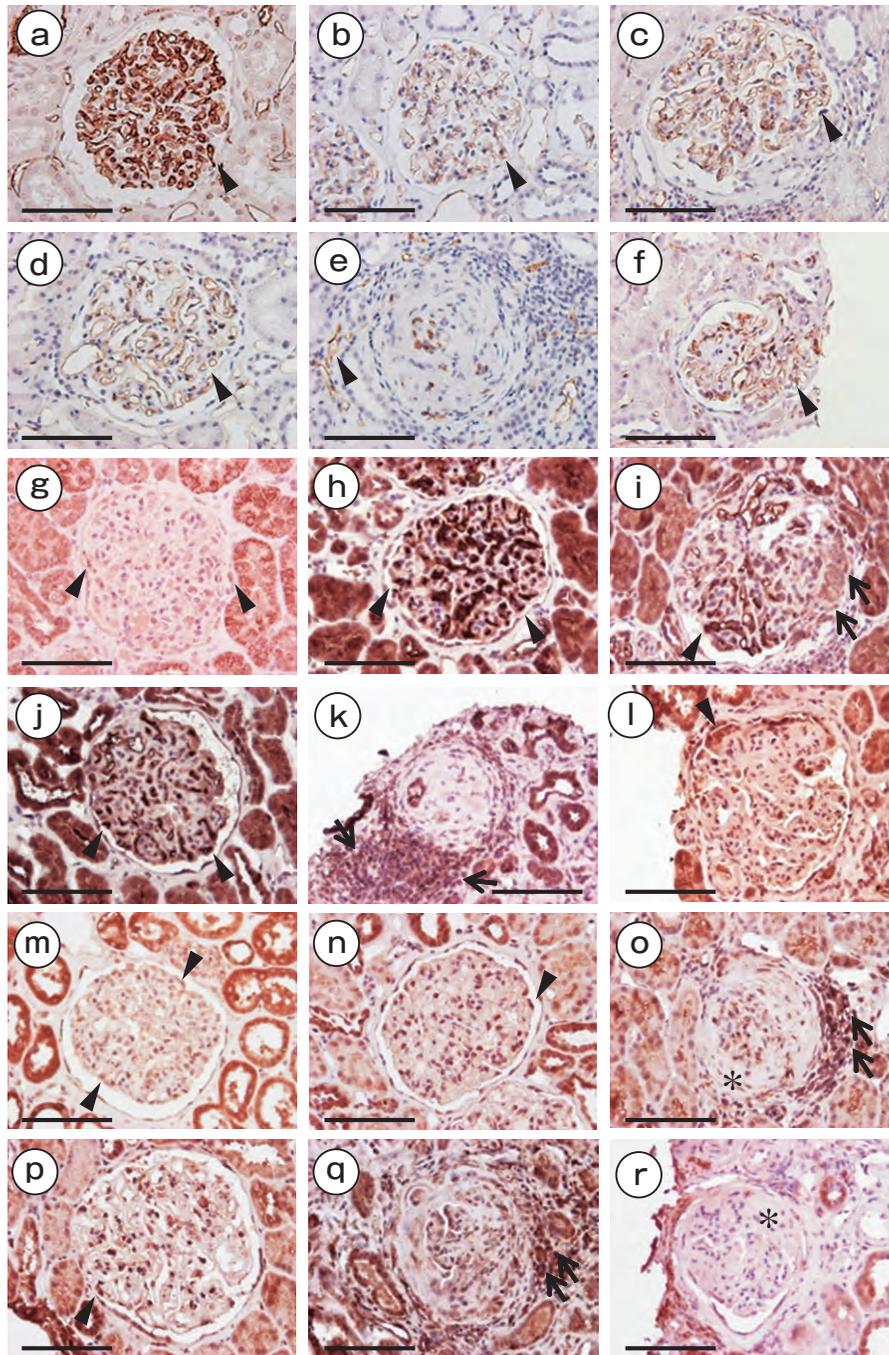
**Fig. 1** Distribution of Vasohibin-1<sup>+</sup> cells in the kidneys. In the normal control subjects, VASH-1<sup>+</sup> cells were faintly observed in glomerular endothelial cells (a, b: arrowheads) and peritubular capillaries in the renal cortex (c: arrowhead). However, VASH-1<sup>+</sup> cells were not observed in extraglomerular arterioles (d). In the patients with IgA nephropathy, VASH-1<sup>+</sup> cells were faintly observed in glomerular endothelial cells (e, f: arrowheads). In the patients with purpura nephritis, VASH-1<sup>+</sup> cells were observed in cellular crescents in the glomerulus (g: arrowheads) and in the endothelial cells of the vasa recta (h: arrowhead). In the patients with granulomatosis with polyangiitis, VASH-1<sup>+</sup> cells were observed in endothelial and interstitial inflammatory cells (i, j: arrowheads). In the patients with diabetic nephropathy, no VASH-1<sup>+</sup> cells were observed in the nodular lesions of the glomerulus (k: asterisks), although they were observed in inflammatory cells in the cortex (l: arrowheads). VASH-1, Vasohibin-1. Scale bars: 200  $\mu$ m (d); 100  $\mu$ m (a, e, g, i, k and l); 50  $\mu$ m (b, c, f, h and j).



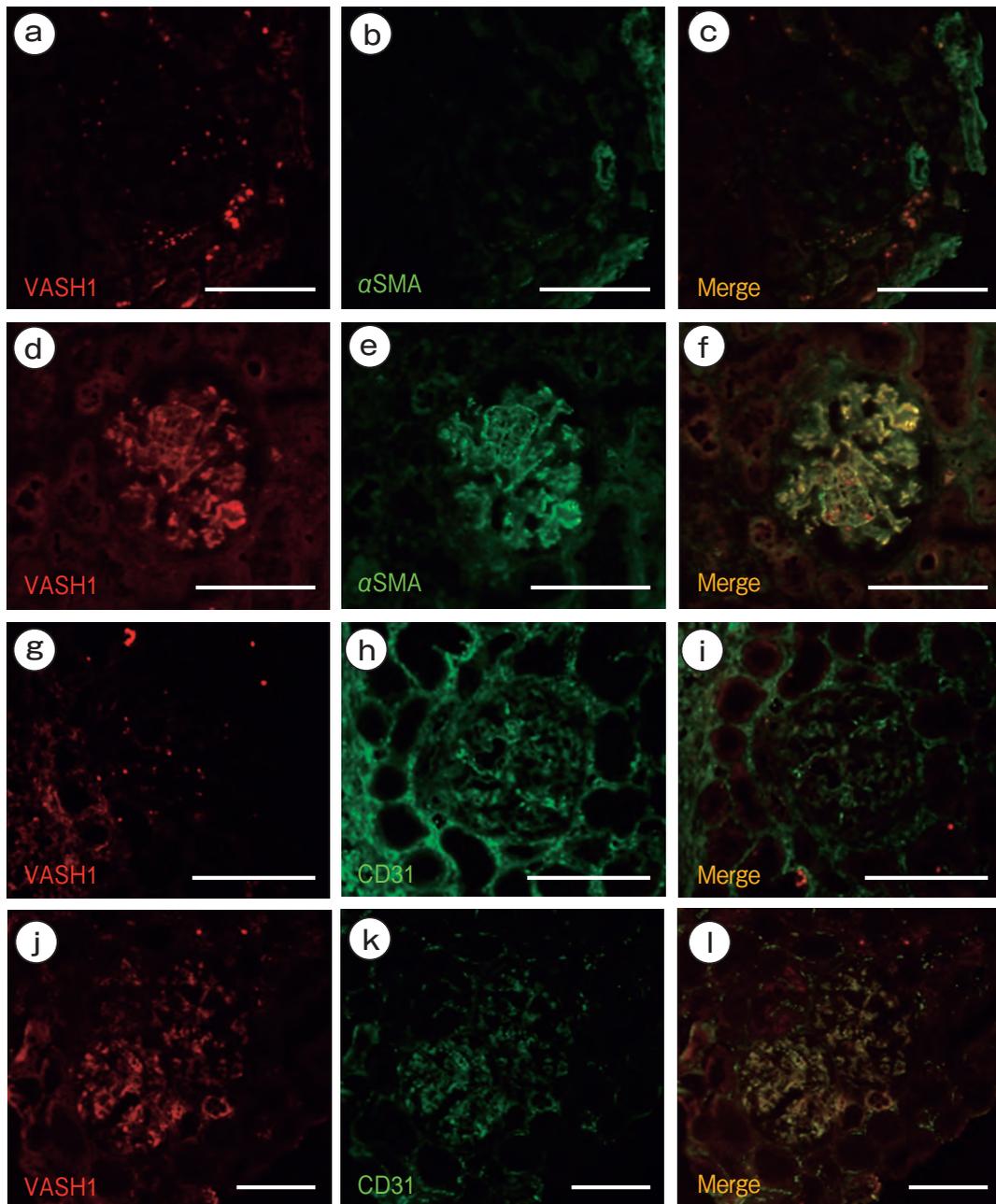
**Fig. 2** Renal expression of Vasohibin-1 in patients with various renal disorders. **(A)** The glomerular expression of VASH-1 in patients with various renal disorders. **(B)** The number of VASH-1<sup>+</sup> cells in the cortex in patients with various renal disorders. **(C)** The number of VASH-1<sup>+</sup> cells in the medulla in patients with various renal disorders. \**p* < 0.05 versus normal controls. #*p* < 0.05 versus membranous GN. GN, glomerulonephritis; HPF, high power field; MC, minimal change disease; MN, membranous nephropathy; N, nephropathy; VASH-1, Vasohibin-1.



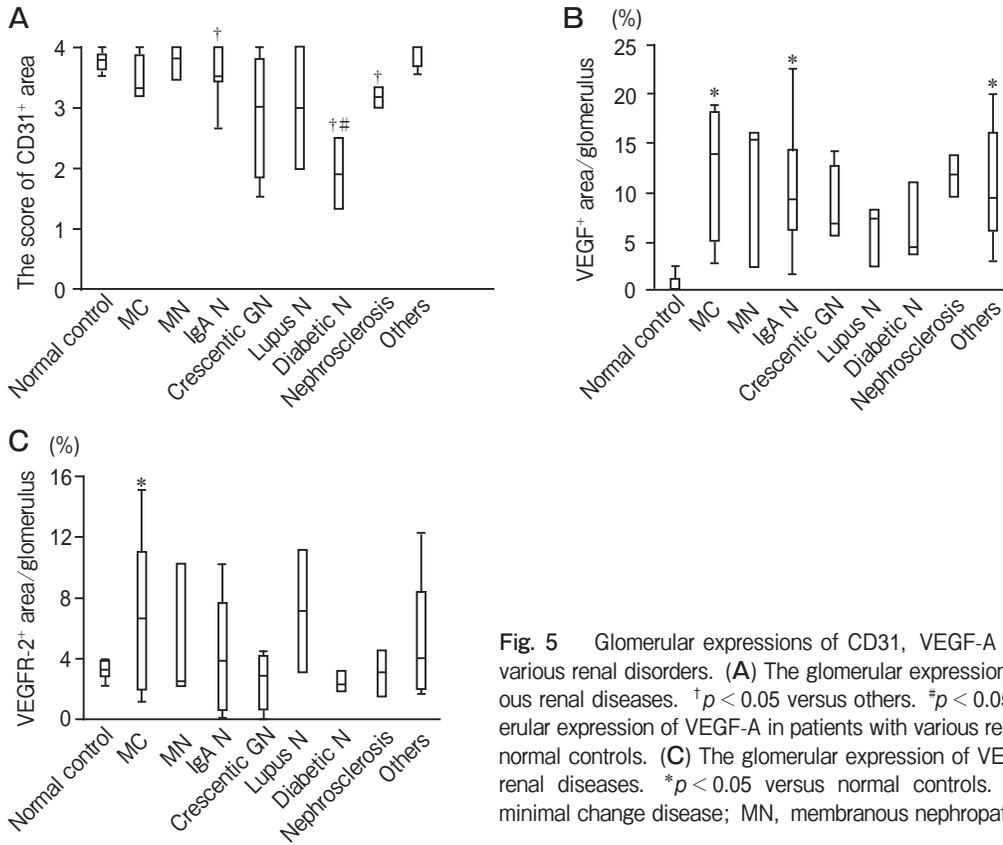
**Fig. 3** Correlations between the numbers of renal Vasohibin-1<sup>+</sup> cells. **(A, B, C)** Correlation between the glomerular, cortical and medullary expressions of VASH-1. HPF, high power field; VASH-1, Vasohibin-1.



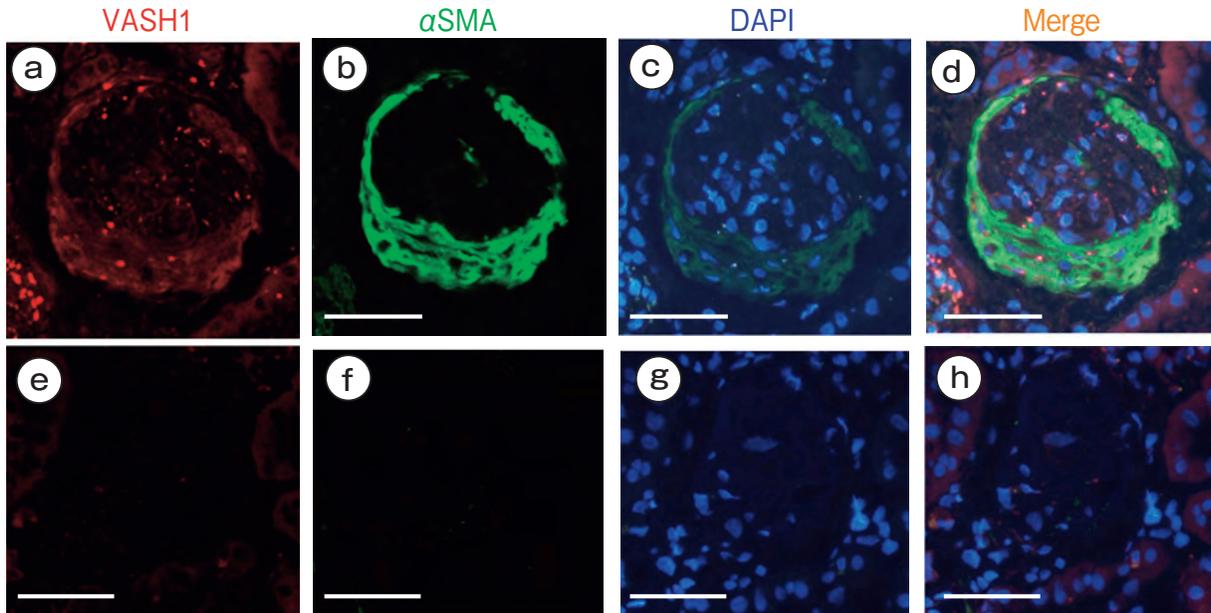
**Fig. 4** Immunohistochemistry for CD31, VEGF-A and VEGFR-2. Representative photomicrographs of CD31 staining (a-f), VEGF-A staining (g-l) and VEGFR-2 staining (m-r) in the following: the glomeruli of a control subject (a, g, m) and patients with IgA nephropathy (b, h, n), purpura nephritis (c, i, o), membranous glomerulonephritis (d, j, p), granulomatosis with polyangiitis (e, k, q) and diabetic nephropathy (f, l, r). The CD31<sup>+</sup> glomerular endothelial areas indicate the capillary density of the glomerulus (a-f; arrowheads). The glomerular VEGF-A<sup>+</sup> areas were primarily localized to podocytes in the normal controls (g; arrowheads) and were markedly increased in podocytes and additionally observed in glomerular endothelial cells (h-j and l; arrowheads), crescents (i; arrows) and interstitial inflammatory cells (k; arrows) in the diseased glomeruli. Glomerular VEGFR-2<sup>+</sup> cells were found in endothelial cells and podocytes in the normal controls (m; arrowheads) and were enhanced in the patients with renal disorders (n and p; arrowheads). Additionally, immunoreactivity for VEGFR-2 was observed in inflammatory cells and crescents (o and q; arrows), but not in sclerotic areas (o and r; asterisks). Scale bars: 100 $\mu$ m.



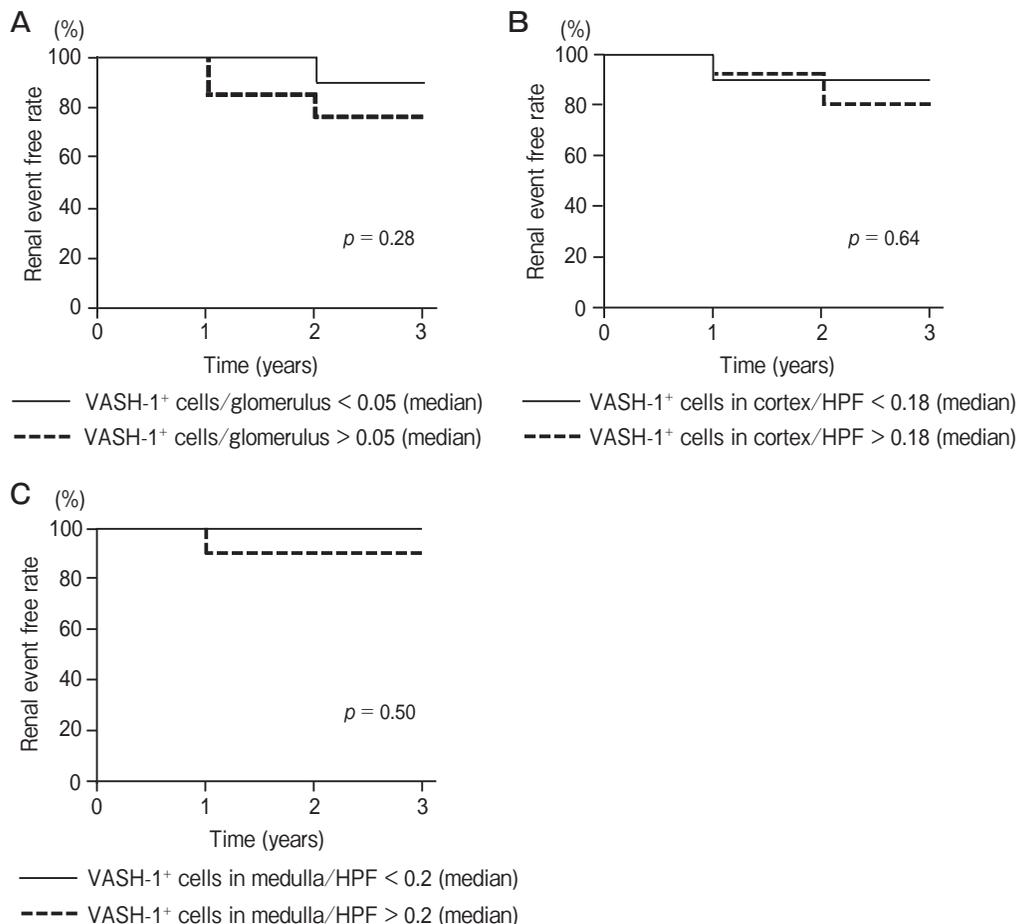
**Fig. 6** Double immunofluorescent staining for endogenous Vasohibin-1, CD31 and  $\alpha$ -SMA. Double immunofluorescent staining of VASH-1 (red) and  $\alpha$ -SMA (green) and merged images in the kidneys in a normal control (a-c) and a patient with lupus nephritis (d-f). Although VASH-1 was faintly observed in the normal control glomeruli (a), increased immunoreactivity for VASH-1 was observed (d) and was merged with that of  $\alpha$ -SMA in patients with lupus nephritis (f). Double immunofluorescent staining of VASH-1 (red) and CD31 (green) and merged images in the kidneys in a normal control (g-i) and a patient with lupus nephritis (j-l). VASH-1 was merged with CD31 in the patients with lupus nephritis (j-l).  $\alpha$ -SMA, anti- $\alpha$ -smooth muscle actin; VASH-1, Vasohibin-1. Scale bars: 100  $\mu$ m.



**Fig. 5** Glomerular expressions of CD31, VEGF-A and VEGFR-2 in patients with various renal disorders. **(A)** The glomerular expression of CD31 in patients with various renal diseases. †*p* < 0.05 versus others. \**p* < 0.05 versus IgA N. **(B)** The glomerular expression of VEGF-A in patients with various renal disorders. \**p* < 0.05 versus normal controls. **(C)** The glomerular expression of VEGFR-2 in patients with various renal diseases. \**p* < 0.05 versus normal controls. GN, glomerulonephritis; MC, minimal change disease; MN, membranous nephropathy; N, nephropathy.



**Fig. 7** Double immunofluorescent staining for endogenous Vasohibin-1,  $\alpha$ -SMA and DAPI in the crescentic lesion. Double immunofluorescent staining of VASH-1 (red),  $\alpha$ -SMA (green), DAPI (blue) and merged images in the kidney in a patient with IgA nephropathy (a-h). In the crescentic lesions, immunoreactivity for VASH-1 was observed and co-localized with  $\alpha$ -SMA (a-d). Panels e-h show the negative control staining results for the same glomerulus used in panel a-d.  $\alpha$ -SMA, anti- $\alpha$ -smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; VASH-1, Vasohibin-1. Scale bars: 50  $\mu$ m.



**Fig. 8** Kaplan-Meier analysis of the composite renal endpoint based on the renal levels of Vasohibin-1. A composite renal event was defined as a decline in the eGFR of more than 30% of the baseline value, initiation of renal replacement therapy or death associated with a renal disorder. In order to perform a Kaplan-Meier analysis of the renal levels of VASH-1, the patients were stratified into 2 groups using the median as the cutoff point. The log-rank test was used to compare differences between the 2 groups. Increased renal levels of VASH-1 tended to be correlated with worse renal outcomes. HPF, high power field; VASH-1, Vasohibin-1.

### Discussion

Angiogenesis is involved in pathological disorders as well as physiological processes [27]. Experimental studies have demonstrated the involvement of an imbalance in angiogenesis-related factors in the progression of CKD [11, 28–33] and the potential therapeutic effects on CKD achieved by modulating these factors [6, 9, 10, 34–39]. The clinical usefulness of angiogenesis-associated factors, such as VEGF-A, soluble VEGFR-1/2 and Angiotensin-1/2, in CKD patients has recently been demonstrated [8, 14, 40–42]. There are as yet no reports investigating the roles of VASH-1 in CKD patients.

In the normal kidney, VASH-1<sup>+</sup> cells were faintly observed in the glomerular as well as peritubular capillary endothelial cells, and their expression was less remarkable than that observed in the CKD patients. In the CKD patients, VASH-1 was observed in the glomeruli and interstitial inflammatory cells. No tubular localization of VASH-1 was evident, excluding the possibility of the tubular epithelium as the primary source of renal VASH-1. The numbers of VASH-1<sup>+</sup> cells in the glomeruli and cortex were positively correlated with the presence of crescent formation, and the number of VASH-1<sup>+</sup> cells in the cortex was positively correlated with the presence of interstitial cell infiltration. In general, VASH-1 is involved in the

maturation of neovessels, and the anti-inflammatory actions of VASH-1 have been observed in experimental animal models [19–21, 43]. Therefore, VASH-1 is thought to be upregulated in interstitial inflammatory lesions in a compensatory manner in order to stabilize peritubular capillaries and prevent further infiltration of inflammatory cells. Relevant to the present results, previous reports have demonstrated the expression of VASH-1 in monocytes/macrophages in association with inflammatory lesions [44–47]. Double immunofluorescent staining has further revealed that VASH-1 is present in glomerular endothelial and mesangial cells in patients with renal disorders.

Inverse correlations between the number of VASH-1<sup>+</sup> cells in the medulla and the age/blood pressure and a positive correlation between the number of VASH-1<sup>+</sup> cells in the medulla and the eGFR were observed in CKD patients. Furthermore, an inverse correlation between the number of VASH-1<sup>+</sup> cells in the cortex and the diastolic blood pressure was observed in CKD patients. In general, VASH-1 is primarily synthesized and secreted by vascular endothelial cells. These results suggest that the production of VASH-1 by vascular endothelial cells is reduced in elderly and hypertensive patients due to systemic arteriosclerotic alterations accompanied by endothelial cell dysfunction, consistent with recent findings suggesting that VASH-1 is an important factor for the maintenance of endothelial cells [16].

Immunoreactivity for VASH-1 was not observed in the sclerotic area of glomeruli. Because VASH-1 has been shown to ameliorate the accumulation of matrix proteins in the mesangial area in animal models of diabetic nephropathy, we expected to observe increased immunostaining for VASH-1 in the nodular and diffuse lesions of our patients with diabetic nephropathy. Our findings indicate that VASH-1 may be expressed in endothelial and mesangial cells at a relatively earlier stage of disease progression, rather than at the later stage accompanied by sclerotic lesions.

A recent report demonstrated that VASH-1 is primarily expressed in the cytoplasm and membrane of renal tubular epithelial cells and partly in vascular endothelial cells and glomerular mesangial cells [48]. This discrepancy may be attributable to the use of distinct anti-VASH-1 antibodies. In most reports examining the VASH-1 expression in human tissues [19, 24, 46, 49–51], including the present study, a

murine monoclonal antibody against a synthetic peptide corresponding to the 286–299 amino acid sequence of VASH-1 [15] was used. However, distinct anti-VASH-1 antibodies were used in the above-described report [48], partly explaining the discrepant findings.

VASH-1 was originally identified as a gene that was up-regulated by VEGF-A in endothelial cells [11]. Therefore, we next aimed to investigate the correlation between VEGF-A/VEGFR-2 and the VASH-1 levels in the kidney. In the present study, no significant correlations between the glomerular VEGF-A level and the VASH-1 levels were observed. A recent report indicated that in cancer-associated endothelial cells, the increase in endothelial EZH2, a member of the polycomb-group (PcG) proteins, is a direct result of VEGF-A stimulation by a paracrine circuit that promotes angiogenesis by methylating and silencing VASH-1 [52]. The levels of VASH-1 observed in CKD patients may be affected by several pathways downstream of VEGF-A stimulation, and may therefore be partly attributable to the posttranscriptional regulation mediated via the HuR induced by oxidative stress, as recently demonstrated by Miyashita *et al.* [16].

The expression of VASH-1 was not significantly correlated with the presence of CD31<sup>+</sup> glomerular endothelial areas or chronic lesions (mesangial sclerosis, global sclerosis, interstitial fibrosis, tubular atrophy and arteriosclerosis), although it was positively correlated with the presence of crescent formation, interstitial cell infiltration and VEGFR-2<sup>+</sup> areas in the glomeruli and medulla. These results suggest that the renal levels of VASH-1 may be affected by the status, but not the number of endothelial cells. Similarly, previous reports have demonstrated a positive correlation between the levels of VASH-1 and VEGFR-2 in tumors and ocular lesions [24, 46, 49, 53, 54]. On the other hand, crosstalk between the glomerular endothelial cells and podocytes is crucial for the maintenance of the glomerular architecture, as well as the glomerular filtration barrier. The involvement of the VEGF-A/VEGFR-2 system in this regulatory mechanism has been extensively studied to date [55]. VASH-1 may be regulated downstream of VEGFR-2 signaling in endothelial cells, and then may indirectly affect podocytes, inflammatory cells and mesangial cells in association with its potential renoprotective role. We previously demonstrated a renoprotective role of VASH-1 via its direct effects on

glomerular endothelial and mesangial cells and podocytes, and showed that this renoprotection was due to various mechanisms in both experimental animal models and cell cultures. Therefore, direct protective effects of endogenous VASH-1 on renal component cells may also play a role in suppressing the progression of CKD.

The Kaplan-Meier analysis further indicated that elevated renal levels of VASH-1 at baseline tended to predict a poor renal outcome, although the differences in the data did not reach statistical significance. Studies employing a larger number of patients both in total and in each disease category may lead to the identification of a significant difference in the future. Elevated expressions of VASH-1 have been reported to predict worse clinical outcomes in patients with various cancers, which are similar to the findings in the present study [24, 46, 49–51, 56]. In various animal disease models, including those of tumors and diabetic nephropathy, the injection of adenoviral vector encoding VASH-1 results in therapeutic effects [15, 19–21, 43, 57–59]. More recent findings have demonstrated the role of VASH-1 in enhancing the stress resistance of endothelial cells [16]. Therefore, we conjecture that endogenous VASH-1 is upregulated in response to increased disease activity and endothelial cell stress in a compensatory manner in patients with renal disorders.

Our study had several limitations. The sample size was not adequate for making comparisons between the patients with various renal disorders. In addition, the mechanisms underlying the relationships among the renal expression of VASH-1, inflammatory stimuli and VEGFR-2 were not fully clarified. To further elucidate these precise mechanisms, basic research on VASH-1 *in vitro* and *in vivo*, including studies using animal models of renal disorders, is required.

In conclusion, we investigated the renal distribution of VASH-1 in patients with CKD for the first time, and demonstrated an association between the renal VASH-1 levels and renal histological alterations, such as inflammation and crescent formation, as well as the VEGFR-2 levels, thus suggesting a potential renoprotective role for VASH-1 in response to various stresses in patients with CKD.

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