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Endothelin-1 *K198N* Polymorphism Modifies Clinical and Histopathological Manifestations of IgA Nephropathy

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Summary. Endothelin 1 (ET-1) is a potent vasoconstrictor that plays an important role in the progression of renal injury. IgA nephropathy (IgAN), the most prevalent form of primary glomerulonephritis, is one of the major causes of end stage renal disease (ESRD) and the susceptibility to progressive renal dysfunction in this disease may be determined by genetic variations. In the present study, we investigated 308 patients with biopsy-proven IgAN for possible associations between *ET-1 K198N* polymorphism and clinical and histopathological manifestations. At the time of biopsy, the *ET-1 198KK* genotype was significantly associated with a higher incidence of hypertension and lower renal function, and the renal survival of patients with the *ET-1 198KK* genotype was significantly worse than those with other genotypes. Moreover, with respect to the histopathological characteristics of renal biopsy findings, the *198KK* genotype was significantly associated with higher scores of global sclerosis, interstitial fibrosis, and arteriosclerosis of large arteries than other genotypes, whereas no difference was observed in other histological parameters including mesangial cell proliferation, mesangial matrix increase, the adhesion of glomerular tufts to Bowman's capsule, and crescent formation. The results of the present study indicate that the *K198N* polymorphism can be a marker for severe renal vascular injury and poor renal survival in patients with IgAN.

Key words— IgA nephropathy; endothelin; renal failure.

INTRODUCTION

IgA nephropathy (IgAN), the most common form of primary glomerulonephritis worldwide, is one of the major causes of end stage renal disease (ESRD).[1, 2] The proliferation of mesangial cells and expansion of the extracellular matrix are found in IgAN patients, both subsequently causing progressive glomerular and interstitial sclerosis, and eventually leading to ESRD. The clinical course of IgAN varies widely, with patients showing very different rates of progression to ESRD; some of these with renal function deterioration at the time of diagnosis experiencing no progression at all, even after decades. It has been proposed that genetic factors, at least partially, contribute to the onset and the progression of this disease. Previous studies have proved several genetic correlations between hemodynamic factors and the progression of IgAN, such as associations between gene polymorphisms of the renin-angiontensin system (RAS) and progressive renal dysfunction.[3,4,5]

Endothelin (ET)-1, another potent vasoactive factor, is a 21 amino-acid peptide converted by ET-converting enzymes (ECEs) from a 38 amino-acid big ET-1, which is cleaved from ET-1 mRNA encoding a 212-amino acid preproendothelin-1.[6] ET-1 is the major isoform synthesized in the kidney among three isoforms, ET-1,

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Abbreviations – Ccr, creatinine clearance; ESRD, end stage renal disease; ET, endothelin; GFR, glomerular filtration rate; IgAN, IgA nephropathy; PRD, progressive renal disease; RAS, renin-angiontensin system; SBP, systolic blood pressure; sCr, serum creatinine.

ET-2, and ET-3. Preproendothelin-1 mRNA and ET-1 are localized in golmerular capillary walls, mesangial and glomerular epithelial cells as well as in the brush border of some proximal tubules, and in small vessels.[7] ET-1 reduces the renal blood flow by increasing renal vascular resistance through the vasoconstriction of renal afferent and efferent arterioles as well as arcuate and interlobular arteries.[8] A systemic administration of ET-1 reduces the glomerular filtration rate (GFR) and is likely to cause antinatriuresis, which in part is related to the stimulation of aldosterone.[9]

Moreover, ET-1 has been demonstrated to participate pathophysiologically in renal failure and diabetes.[10] Primary disease effects in genetic studies showed that transgenic animals overexpressing the human ET-1 gene develop glomerulosclersis and display reduced GFR and proteinuria.[11] ET-1 also has been proved to be involved in the development of albuminuria.[12]

Although accumulating evidence shows that ET-1 plays a crucial role in the progression of renal diseases, little is known whether or not ET-1 is involved in the inter-individual variations of clinical and histopathological manifestations of IgAN. One of potential interesting genetic variant is *K198N*, which is located in the exon 5 of the ET-1 gene and results in Lys/Asn amino acid substitution at coden 198 of preproendothelin-1. This genetic polymorphism has been reported to be associated with hypertension in obese subjects and with high systolic blood pressure (SBP) in gravida.[13]

In the present study, we investigated the possible association of the *K198N* polymorphism of ET-1 with clinical and histopathological characteristics of IgAN in Japanese patients.

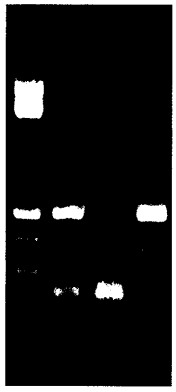

Fig. 1. Representative gel electrophoresis of PCR-RFLP. Genomic DNA was amplified by PCR and applied in an agarose electrophoresis after restriction digestion with CAC8 I as described in the Methods. Each genotype is indicated below the gel.

METHODS

Study subjects

The ethics committee of the institution approved the protocol for the genetic study. Patients were eligible for inclusion in the analysis when: 1) they had been diagnosed as having IgAN by kidney biopsy at our institute between 1976 and 2003; 2) they had no evidence of systemic diseases such as hepatic glomerulosclerosis, Schönlein-Henoch purpura, or rheumatoid arthritis; 3) they had been followed-up for at least 12 months in our institute; and 4) informed consent for genetic study was obtained. A total of 310 patients were analyzed. In all cases, the diagnosis of IgAN was based on immunofluorescent microscopy of a kidney biopsy specimen, which showed dominant or co-dominant deposition of IgA in the glomerular mesangium.

The clinical characteristics of the patients at the time of diagnosis including gender, age, office blood pressure, urinary protein excretion (g/day), (serum creatinine (sCr) level; (mg/dL)), and (24-hour creatinine clearance (Ccr); ml/min) were investigated. Because blood pressure without any antihypertensive treatment was not always available, actual values of blood pressures were recorded and hypertension was defined by the use of one or more antihypertensive medications and/or a blood pressure greater than or equal to 140 mmHg systolic or 90 mmHg diastolic. The primary endpoint (progressive renal disease (PRD)) was defined as the date when the sCr level was double that at the time of diagnosis, or when patients underwent their first renal replacement therapy.

DNA preparation and genotype determination

Genomic DNA was extracted from the peripheral blood cells (PBC) of patients by an automatic DNA isolation system (NA-1000, Kurabo, Osaka). The genotype of *ET-1 K198N* was determined by the PCR-RFLP Method using restriction endonuclease Cac8 I (NewEngland BioLabs, MA, USA). Primers used for the PCR reaction were 5'-TAAGCATAGGGGCAGGCTTT-3' (sense) and 5'-CCACTGATGGAAGCCAGTGA-3' (antisense). The reaction mixture contained 10 × Ex Taq buffer, 200 mmol/l deoxynucleotide triphosphates (dNTPs), 1 unit Taq DNA polymerase (Takara, Kyoto), 10 pmol of each primer, and 50-100 ng genomic DNA. The PCR amplification reaction consisted of a cycle at 95°C for five min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, and extension at 72°C for 30 sec. A final extension was performed at 72°C

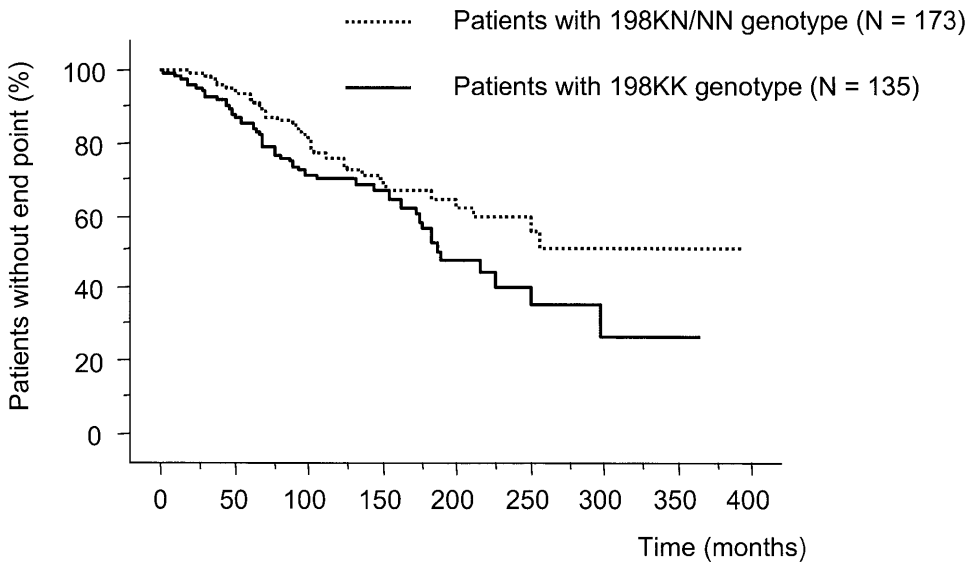

Fig. 2. *ET-1 K198N* and the renal survival rate in patients with IgA nephropathy (IgAN). The renal survival rate in patients with *198KK* (N = 135) was less than that in patients without *198KK* (N = 173). Log-rank test $P = 0.0344$.

Table 1. Clinical data of the subjects both at the time of diagnosis and during observation

	All patients	Genotype of *ET-1 K198N*			*P* value	
		KK	*KN*	*NN*		
	N = 308	N = 135	N = 138	N = 35	Among three genotypes	*KK vs* others
Gender, MF (%)	45.1	50.4	41.3	40	0.2612	0.1025
Time to diagnosis (months)	61.8 ± 59.8	62.8 ± 58.3	61.2 ± 52.4	60.5 ± 57.1	0.8856	0.7559
At the time of diagnosis						
Age (year)	36.8 ± 13.8	38.1 ± 14.3	35.8 ± 13.2	35.7 ± 13.7	0.3982	0.1764
SBP (mmHg)	127.3 ± 18	129.7 ± 18.8	125.6 ± 17.0	125.7 ± 18.5	0.2512	0.0963
DBP (mmHg)	76.9 ± 13.7	78.9 ± 14.1	75.6 ± 13.0	74.6 ± 14	0.1494	0.0667
UP (g/day)	1.3 ± 1.3	1.5 ± 1.5	1.3 ± 1.4	1.1 ± 0.9	0.5062	0.2522
Cr (mg/dl)	0.97 ± 0.59	1.06 ± 0.80	0.91 ± 0.35	0.84 ± 0.32	0.1497	0.0904
Ccr (ml/min/1.73m^2)	89.7 ± 33.1	85.8 ± 36.7	90.2 ± 29.0	102.5 ± 31.6	0.0533	0.0738
Hypertension (%)	36.0	44.4	30.4	25.7	0.0296	0.0097
During observation						
Observation (months)	111.4 ± 77.2	108.7 ± 75.6	113.4 ± 79.1	113.4 ± 76.9	0.8965	0.6401
Endopoint (%)	28.9	35.5	26.1	14.3	0.0263	0.0206
Mean SBP (mmHg)	127.7 ± 16.1	129.5 ± 17.3	126.7 ± 15.4	124.7 ± 13.6	0.3129	0.1540
Mean DBP (mmHg)	76.5 ± 11	77.6 ± 12.4	75.8 ± 9.8	75.4 ± 9.9	0.6445	0.3683

Time to diagnosis, time from the first urine abnormality to renal biopsy; SBP, systolic blood pressure; DBP, diastolic blood pressure; UP, urinary protein excretion; Cr, creatinine; Ccr, 24-hour creatinine clearance. The endpoint was defined as described in the Methods.

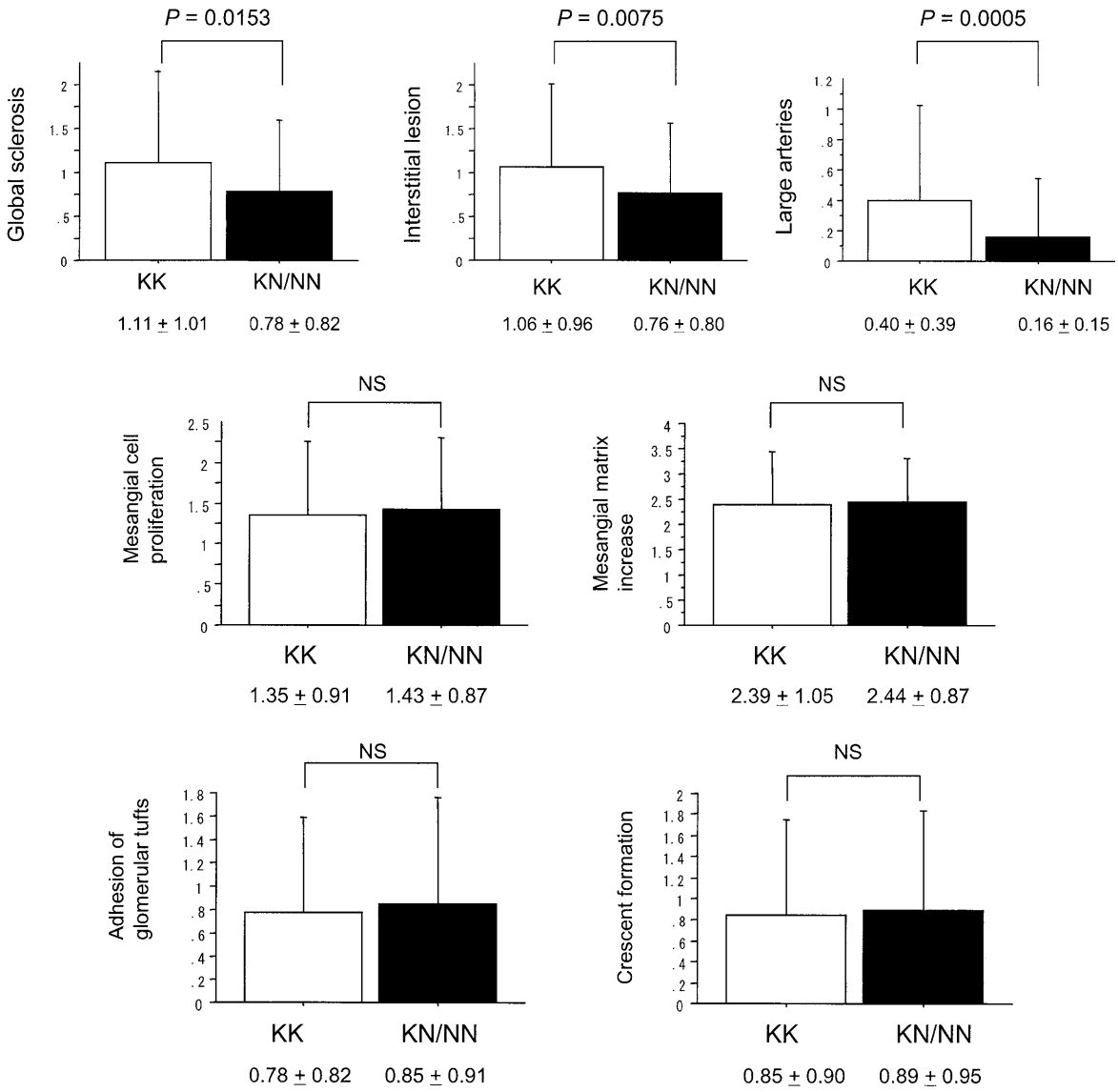

Fig. 3. Mean values of each histopathological grading score in IgAN patients with the KK (□) and KN/NN (■) genotypes of the *ET-1 K198N* polymorphsim. Glomerular changes were scored for each glomerulus, and the mean score of each was calculated as described in the Methods. Data are given as mean ± SD.

for seven min. The 506bp PCR products were digested with restriction endonuclease Cac8 I (Biolabs) and electrophoresed on a 1.5% agarose gel (Fig.1).

Histopathological examination

Histopathological findings were classified according to the classification described previously.[14, 15] A single pathologist evaluated all specimens by light microscopy in a double blind fashion. Glomerular changes were scored for each glomerulus, and the average score of each patient was calculated. The scores of cellular proliferation and the matrix increase in the mesangium were ranked into five grades ranging from zero (minimal change) to four (diffuse global marked). Other glomerular changes including endocapillary proliferation, duplication of glomerular basement membrane (GBM), crescent formation, and adhesion of tufts to Bowman's capsule as well as tubulointerstitial lesions were graded zero to four according to their incidence. Grades zero to four represent an incidence of the lesion in 0-4%, 5-24%, 25-49%, 50-74%, and 75-100% of all cases, respectively.

Statistical analysis

Statview 5.0 statistical software (Abacus Concepts Inc, Berkeley, CA, USA) was used for statistical analyses. The Hardy-Weinberg equilibrium was tested by a Chi-square test with 1 *df*. A Chi-square test for independence analysis was used when comparing allele frequencies between groups. Continuous variables were compared using the Kruskal-Wallis test or Mann-Whitney U-test. The Kaplan-Meier method was used to analyze the time course from renal biopsy to end point. A value of p less than 0.05 was considered to indicate statistical significance.

RESULTS

The allele frequencies of *K* and *N* of the *ET-1 K198N* polymorphism were 0.662 and 0.338, respectively. The potential frequencies of the genotypes, which are assumed to be under the Hardy-Weinberg equilibrium, were no different from the observed genotype distribution. Clinical characteristics of patients with IgAN at the time of renal biopsy and during observation are listed in Table 1. There was no differences in gender, time from the first urine abnormality to renal biopsy, or age at the diagnosis. On the other hand, though not statistically significant, systolic and diastolic blood pressures tended to be, higher in patients with the *KK* genotype of *ET-1 K198N* polymorphism than those with other genotypes. Moreover, the incidence of hypertension was significantly higher in the *KK* genotypes. The odds ratio (OR) for the patients with the *198KK* genotype was 1.914 (95% confidence interval (CI), 1.195 to 3.066; $P = 0.0097$, *KK vs* other genotypes). In addition, *KK* genotype carriers tended to have higher levels of sCr, lower 24-hour Ccr, and urinary protein excretion in comparison to the other genotypes though these differences did not attain statistical significance.

The mean duration of the observation period was 111.4 ± 77.2 months. No difference was noted for the duration of observation and mean systolic and diastolic blood pressures. However, during the observation, the incidence of progressive renal disease was significantly higher in the patients with the *KK* genotype (OR = 1.776; 95% CI, 1.081 to 2.920; $P = 0.0206$, *KK vs* other genotypes).

To examine the effect of *K198N ET-1* polymorphism on disease progression, we compared survival rates from the renal biopsy to the endpoint. The renal survival rate in patients with the *198KK* genotype was significantly less ($\chi^2 = 4.5$, $P = 0.0344$) than in patients without this genotype (Fig.2).

Next, possible associations between the *ET-1 K198N* genotype and quantitatively estimated scores of histopathological findings in kidney biopsies were invested. Patients carrying the *198KK* genotype had severer histopathological damage including global sclerosis, interstitial lesions, and arteriosclerosis of large arteries than those with other genotypes (Fig.3A). In contrast, no difference was observed between patients with the *198KK* and *198KN/NN* genotype in mean values of other histological scores including mesangial cell proliferation, matrix increase, the adhesion of glomerular tufts to Bowman's capsule, and crescent formation (Fig.3B).

DISCUSSION

In the present study, we investigated patients with IgAN for a possible association of *ET-1 K198N* polymorphism with clinical and histopathological manifestations and found that the *198KK* genotype was associated with a higher incidence of hypertension and lower renal function at the time of renal biopsy. The renal survival of patients with the *198KK* genotype was significantly worse than those with other genotypes. Moreover, with respect to the histopathological characteristics of renal biopsy findings, the *198KK* genotype was significantly associated with higher scores of global sclerosis, interstitial fibrosis, and arteriosclerosis of large arteries than other genotypes, whereas no difference was observed in other histological parameters including mesangial cell proliferation, mesangial matrix increase, the adhesion of glomerular tufts to Bowman's capsule, and crescent formation.

ET-1 has been well documented to play an important role in hypertension. It has been shown that the plasma level of ET-1 is significantly higher in patients with essential hypertension.[16] In the presence of an enhanced production of ET-1 in some hypertension models, ET-1 contributes to the remodeling of large and small arteries.[17] Furthermore, ET-1 infusion can cause profound renal vasoconstriction in healthy volunteers, whose systemic blood pressure was negligibly affected.[18] ET-1 also plays numerous other functions in many different organs. In the kidney, ET-1 gene expression and the renal synthesis of ET-1 are upregulated in animal models with an obvious loss of nephrons due to immune or nonimmune damage.[19] Also, increased urinary ET-1 has been reported in patients with PRD, including membranoproliferative glomerulonephritis, lupus nephritis, and IgAN.[20] In addition to its direct vasoconstrictive action, ET-1 can stimulate the conversion of angiotensin I to angiotensin II and potentiate the angiotension II-induced production of aldosterone,which can increase sodium water retention.[21]

Therefore, if the *ET-1 K198N* polymorphism directly influences ET-1 synthesis in renal injury, it is quite reasonable that the patients with the *198KK* genotype

had more severe arteriosclerosis, glomeruloscresosis, and interstitial fibrosis, as observed in the present study, and that these patients accordingly had higher incidences of high blood pressure and lower renal function, and lower renal survival than those with other genotypes. However, we could not examine the relationship between the *ET-1 K198N* polymorphism and systemic or local production of endothelin in renal tissue because plasma and urine samples of the patients before any anti-hypertensive and immunosuppressive treatments were not available.

In this study, we examined *K198N* polymorphism, the transversion of G-to-T in exon 5 of ET-1, which causes the amino acid substitution of Lys-to-Asn at coden 198 of proendothelin-1, more above either big ET-1 (coden 53-93) or mature ET-1 (coden 53-74), but just in signal peptide.[22,23] Therefore, it is unlikely that the *K198N* polymorphism directly affects ET-1 production, while there is a likelihood that another genetic variant, which is in linkage disequilibrium with the *K198N*, influences the production of ET-1 in local tissue in renal inflammatory injury. An alternative explanation for the observed association is that the *K198N* affects the activation of preproendothelin. Recently, it has been reported that *K198N* polymorphism could affect the conversion of preproendothelin and thereby influence the biosynthesis of active ET-1.[24] In this regard, further studies will be required regarding the functional significance of this polymorphism. The results of the present study, if established by a replication study and by functional analysis, indicate that the *K198N* polymorphism can be a marker for severe renal vascular injury and poor renal survival not only in patients with IgAN, but also in those with other types of chronic kidney diseases.

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