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A(-20)C polymorphism of the angiotensinogen gene and progression of IgA nephropathy

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A(-20)C polymorphism of the angiotensinogen gene and progression of IgA nephropathy.

Background. The M235T polymorphism of the angiotensinogen gene (*AGT*) is associated with an increased risk of primary hypertension, which may then lead to progressive renal disease. Recent studies showed that nucleotide substitution in the 5′ upstream core promoter region of *AGT* affects the basal transcription rate of the gene.

Methods. To evaluate the role of *AGT* polymorphisms in the progression of IgA nephropathy (IgAN), we analyzed the association of A(-20)C and M235T polymorphisms with renal prognosis in histologically-proven IgAN patients using the Kaplan-Meier method and Cox proportional hazards regression model.

Results. The incidence of hypertension during the course was associated with T235, but not with C(-20). The renal survival rate for 137 patients with creatinine clearance (C_{Cr}) of 70 mL/min or greater at the time of renal biopsy, and follow-up time of two years or more was significantly lower in the patients with C(-20) ($P = 0.008$). The Cox proportional hazards regression model showed an increased hazard ratio (HR) for urinary protein (more than 2 g/day) of 28.3 (95% CI, 7.3 to 109.8; $P < 0.001$), hypertension at the time of renal biopsy of 4.6 (95% CI, 1.8 to 11.9; $P = 0.002$), and C(-20) of 3.6 (95% CI, 1.5 to 8.7; $P = 0.004$).

Conclusion. This work provides evidence that the C(-20) polymorphism of *AGT*, a subset of T235 alleles, is associated with progression of renal dysfunction in IgAN.

Immunoglobulin A nephropathy (IgAN) is the most common glomerulonephritis among patients undergoing renal biopsy throughout the world. It is characterized by mesangial proliferative glomerulonephritis with predominant IgA deposits. The actuarial renal survival at 10 years is assumed to range between 80% and 85% from apparent onset [1]. Familial clustering of IgAN and inter-individual differences in the clinical course suggests that genetic factors may contribute to the development and progression of this disease.

Previous studies provided definitive evidence that angiotensinogen gene (*AGT*) variants are important in the pathogenesis of cardiovascular diseases such as hypertension. Changes in the 5′ upstream core promoter region of *AGT*, which is essential for the transcription of angiotensinogen mRNA, may cause functional differences that may contribute to pathogenesis [2]. One mutation in particular, an adenine-to-cytosine transition at nucleotide -20 of the 5′ upstream core promoter region [A(-20)C] has been shown to increase the basal promoter activity of *AGT* by increasing the affinity of adenoviral major late transcription factor (MLTF) to this region of the promoter [3].

The existence of an association between *AGT* polymorphisms and the progression of IgA nephropathy is a controversial issue. Pei et al showed that patients with the *AGT* 235MT and TT genotypes have a faster rate of deterioration in creatinine clearance (C_{Cr}) than those with the MM genotypes [4]. However, whether variations in the core promoter region of *AGT* are associated with an actuarial long-term renal prognosis in patients with IgAN is yet to be fully investigated.

METHODS

Patients

Patients were recruited from Niigata University Hospital (Niigata, Japan) as well as other hospitals in the Niigata prefecture. The ethics committee of each institute approved the study. Informed consent was obtained from all participants in the genetic studies.

IgAN was diagnosed by renal biopsy as a mesangial proliferative glomerulonephritis with predominant IgA

Key words: mesangial proliferative glomerulosclerosis, progressive renal disease, glomerulonephritis, hypertension, M235T.

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and C3 depositions in the mesangium. Henoch-Schönlein purpura and secondary IgAN as hepatic glomerulosclerosis were excluded from the analysis.

To analyze the renal survival rate, 137 IgAN patients with a C_{Cr} level of 70 mL/min or greater and a follow-up time of two years or greater were studied. Patients whose C_{Cr} value was less than 70 mL/min at the time of the renal biopsy were excluded because there may have been considerable differences in the onset of IgAN in these patients. Patients whose follow-up time was less than two years also were excluded in order to eliminate the influence of factors other than glomerulonephritis itself. Clinical characteristics including age, sex, duration of observation (in months), body mass index (BMI; kg/m^2), level of urinary protein excretion (g/day), serum creatinine (S_{Cr}; mg/dL), and C_{Cr} (mL/min) were investigated in these patients. Hypertension was defined by the use of one or more antihypertensive medications and/or a blood pressure greater than or equal to 140 mm Hg systolic or 90 mm Hg diastolic blood pressure. The primary end point was defined as the date at which S_{Cr} levels doubled after the time of diagnosis, or when patients underwent their first hemodialysis. For statistical analysis, patients with C(-20) ($N = 55$) were compared with those without C(-20) ($N = 82$) for age, sex, BMI, blood pressure, proteinuria, S_{Cr}, C_{Cr} at the time of renal biopsy, and medical therapy.

A similar analysis was performed in subgroups of the patients with C_{Cr} levels of 70 mL/min or greater who were followed for more than three ($N = 120$) or five years ($N = 92$).

DNA analysis

Genomic DNA from each patient was prepared from peripheral leukocytes in blood samples using an automatic DNA isolation system (NA-100; Kurabo, Osaka, Japan).

To determine the A-C transition at nucleotide -20 of the 5′ upstream region of the core promoter of the *AGT* gene, the following primers were constructed: 5′-primer, 5′-AGAGGTCCCAGCGTGAGTGTC-3′ (nucleotides −166 to −144); 3′-primer, 5′-AGCCCACAGCTCAGT TACATC-3′ (nucleotides 81 to 101) [5]. Polymerase chain reaction (PCR) was performed in a final volume of 50 μL containing 100 ng DNA, 10 pmol of each primer, 250 mmol/L of each of the four dNTPs, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol Tris-HCl at pH 8.4, and 2 U of *Taq* polymerase (Takara, Shiga, Japan). The PCR conditions were as follows: 30 cycles of 94°C for 30 seconds, 64°C for one minute, and 72°C for one minute. After PCR, 265-bp products including the 5′ upstream core promoter region were obtained. Then, 8.5 μL of the unpurified product was digested with 2 U of *Eco*O109I (Takara) for at least three hours at 37°C. These samples were separated by 3% agarose gel electrophoresis, and visualized by ethidium bromide staining.

The M235T variant of *AGT* at exon two was determined as described previously [6].

Statistical analysis

Pair-wise linkage disequilibrium (LD) coefficients were estimated by the maximum-likelihood method and the extent of disequilibrium was expressed as $D' = D/D_{max}$ or D/D_{min}, according to Thompson et al [7]. Haplotype frequencies for pairs of alleles were estimated using the Estimating Haplotype-Frequencies software program (ftp://linkage.rockefeller.edu/software/eh).

Statview 5.0J software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. Continuous variables were expressed as mean ± SD or percentage according to clinical features. When the baseline characteristic was continuous (age, disease duration, BMI, urinary protein, S_{Cr}, C_{Cr}), the unpaired *t* test and Mann-Whitney *U* test were used. The χ^2 test was used when indicated. The Kaplan-Meier method and the Cox proportional hazards regression model analyzed the time course from renal biopsy to the end point (initiation of dialysis or when the S_{Cr} level doubled after the time of diagnosis). In the Cox regression model, we tested covariates [age, sex, BMI, urinary protein, the category of hypertension, steroid therapy, and administration of angiotensin-converting enzyme inhibitor (ACEI), and the gene polymorphism] by a stepwise backward method and several covariates were selected. The effects of these covariates were expressed by a hazard ratio. A *P* value less than 0.05 was considered statistically significant.

RESULTS

The genotype distribution in this study was not different from that in Hardy-Weinberg equilibrium. The genotype and allele frequencies of *AGT* M235T and A(-20)C did not differ from previously reported in Japanese studies [5, 8–10]. Haplotype analysis showed a LD between these two alleles (LD coefficient: D′, 1.00). Because the *AGT* variant at -20 was observed only in a subset of the 235T alleles, the following haplotypes were determined: T235 & C(-20); T235 & A(-20); and M235 & A(-20) (Table 1).

Clinical characteristics of the patients investigated are listed in Table 2. A comparison between patients either homozygous or heterozygous for C(-20) and those without C(-20) showed no significant differences in age, sex, BMI, S_{Cr}, C_{Cr}, urinary protein excretion, blood pressure at the time of renal biopsy, or in the percentage of cases treated by ACEI. The percentages of patients administered an antihypertensive agent were not different between the patients with C(-20) and those without C(-20).

Table 1. Genotype, allele, and haplotype frequencies

Genotype distribution	M235T		A(-20)C	
	MM	2	AA	82
	MT	41	AC	49
	TT	94	CC	6
Allele frequency	M235T		A(-20)C	
	M	0.16	A	0.22
	T	0.84	C	0.78
Haplotype frequency				
	T235 & C(-20)		0.22	
	T235 & A(-20)		0.62	
	M235 & A(-20)		0.16	

Glucocorticoids were administered significantly more frequently in patients with C(-20) than those without C(-20).

Because polymorphisms of *AGT* were reported to be associated with essential hypertension in a previous study, the frequencies of genotype M235T were compared between hypertensive and normotensive subjects at the time of renal biopsy and during the observation period (Table 3). The frequency of TT235 was significantly higher in the patients with hypertension during the clinical course and significantly associated with the number of antihypertensive drugs used during the study period. To determine whether C(-20) and the haplotype including C(-20) are associated with hypertension, the frequencies of genotype A(-20)C and the haplotype T235 & C(-20), T235 & A(-20) and M235 & A(-20) were compared between hypertensive and normotensive subjects. C(-20) was not associated with any category of hypertension. The significant increase of M235 & A(-20) in normotensive subjects was observed, which reflected the symmetrical decrease of T235 allele; however, no significant difference in the frequencies of the haplotype T235 & C(-20) between hypertensive and normotensive subjects was observed in both categories.

To examine the effect of A(-20)C polymorphism on disease progression, we compared the survival rate from renal biopsy to the end point in those patients with C_{Cr} level of 70 mL/min or greater at renal biopsy and a follow-up time of two years or more. The renal survival rate in patients with C(-20) was significantly less ($\chi^2 = 7.0$, $P = 0.008$) than in patients without C(-20) (Fig. 1). Moreover, in patients with a C_{Cr} level of 70 mL/min or greater at renal biopsy and follow-up time of more than three years ($N = 120$; mean observed periods, 116.4 months) or five years ($N = 92$; mean observed periods, 137.5 months), the renal survival rate was significantly lower in patients with C(-20) ($P = 0.02$ at 3 years and $P = 0.04$ at 5 years). The renal survival rate in TT235 patients ($N = 43$) also was significantly lower than in MM/MT235 patients ($N = 94$; $P = 0.02$; Fig. 2).

The Cox proportional hazards regression model showed an increased hazard ratio (HR) for C(-20), 3.6 (95% CI, 1.5 to 8.7; $P = 0.004$) from multivariate analysis, including several covariates selected by stepwise backward analysis (hypertension at the time of renal biopsy, proteinuria more than one or two grams per day; Table 4). The HR for urinary protein more than two grams per day (vs. <1 g/day) was extensively increased, which was 28.3 (95% CI, 7.3 to 109.8; $P < 0.001$) from multivariate analysis. Hypertension at the time of renal biopsy was demonstrated to be a statistically significant risk factor in multivariate analysis including urinary protein and C(-20) (HR 4.6; 95% CI, 1.8 to 11.9; $P = 0.002$).

DISCUSSION

This study demonstrated that the renal survival rate was significantly lower in patients with C(-20) in Japanese patients with IgAN. The Cox proportional hazards regression model showed an increased hazard ratio, 3.6 in multivariate analysis, indicating that this polymorphism is an independent risk factor for progression to end-stage renal failure.

Previously, association studies of *AGT* A(-20)C and essential hypertension in the Japanese population were reported [5, 8–10]. However, to our knowledge an association of the *AGT* A(-20)C polymorphism with the progression of IgAN was not investigated. Our study clearly demonstrates that C(-20) is an independent risk factor for the progression of IgAN in patients whose renal function was preserved at the time of renal biopsy. Many confounding factors are known to affect the progression of IgAN, including immune-mediated events [11–13], hemodynamic factors [14, 15], cell proliferation and an increase in extracellular matrix [12, 16, 17]. In this study, systemic hypertension may play a role in the decline of renal function in patients with *AGT* TT235, because the TT235 was significantly associated not only with hypertension during the clinical course, but also with the renal prognosis. These observations were assumed to reflect the influence of A(-6), which has been known to increase the level of transcription of angiotensinogen, because A(-6)G and M235T polymorphism of *AGT* are in complete LD [18, 19]. In contrast and unexpectedly, C(-20) was significantly associated with renal prognosis independently of hypertension. Although the exact mechanism that explains these dissociated results on A(-20)C and M235T polymorphisms remained unclear, these results suggest that the transcriptional regulation of *AGT* in the renal tissue is distinct from the systemic circulation. It has been reported that angiotensin II in renal interstitial fluids are much higher than plasma levels, suggesting the compartmentalization and independent regulation of renal angiotensin II [20]. Furthermore, recent haplotype studies demonstrated that LD between M235T and A(-20)C was not more complete than M235T and A(-6)G [18, 21],

Table 2. Clinical characteristics at the time of renal biopsy

	All patients (N = 137)	*AGT* AA(-20) (N = 82)	*AGT* AC/CC(-20) (N = 55)
Observed periods *months*	105.7 ± 68.3	104.6 ± 69.4	107.2 ± 67.1
Background 1st renal biopsy			
Age *years*	34.9 ± 12.4	35.8 ± 12.8	33.5 ± 11.7
Male %	45.3	43.9	47.3
BMI kg/m^2	22.5 ± 2.8	22.9 ± 3.0	22.0 ± 2.4
U_{prot} *g/day*	1.2 ± 1.1	1.1 ± 0.7	1.4 ± 1.4
S_{Cr} *mg/dL*	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
C_{Cr} *mL/min*	104.6 ± 23.5	106.6 ± 24.8	101.6 ± 21.2
Blood pressure *mm Hg*			
Systolic	125.8 ± 16.9	126.3 ± 18.0	125.1 ± 15.1
Diastolic	75.6 ± 12.6	76.4 ± 13.0	74.4 ± 12.0
Treatment during the course			
Glucocorticoid %	24.3	16.0	36.4[a]
Antihypertensive drugs %	53.3	47.6	61.8
ACEI %	40.1	39.0	41.8

Abbreviations are: BMI, body mass index; U_{prot}, urinary protein; S_{Cr}, serum creatinine; C_{Cr}, creatinine clearance; ACEI, angiotensin-converting enzyme inhibitor.
[a] $P < 0.05$ vs. *AGT* AA(-20) patients

Table 3. Genotype and haplotype frequencies in hypertensive and normotensive subjects

	At the renal biopsy			During the course			Number of anti-HT drugs	
Genotype	HT %	NT %	*P*	HT %	NT %	*P*	mean ± SD	*P*
MM/MT235	6.6	24.8		12.4	19.0		0.7 ± 1.1	
TT235	25.5	43.1	0.08	47.4	21.2	0.001	1.2 ± 1.3	0.003
AA(-20)	19.7	40.1		32.8	27.0		1.0 ± 1.3	
AC/CC(-20)	12.4	27.7	0.85	27.0	13.1	0.16	1.1 ± 1.2	0.36

	At the renal biopsy			During the course		
Haplotype	HT %	NT %	*P*	HT %	NT %	*P*
T235 & C(-20)	21.6	22.6	0.49	25.0	18.0	0.12
T235 & A(-20)	69.3	58.1	0.048	64.6	57.3	0.14
M235 & A(-20)	9.1	19.5	0.02	10.4	24.5	0.002

Abbreviations are: HT, hypertensives; NT, normotensives; *P*, *P* value.

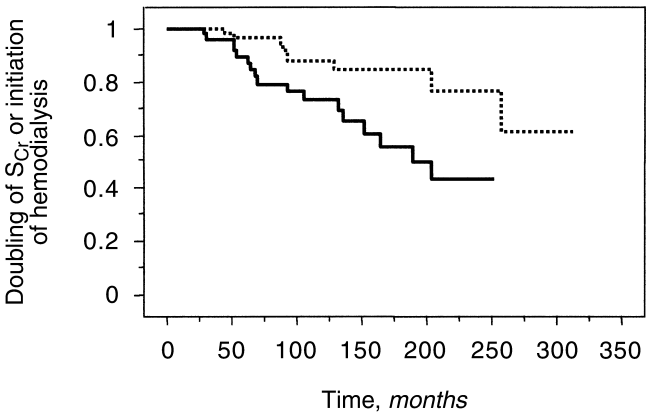

Fig. 1. *AGT* A(-20)C and the renal survival rate in patients with IgAN. The renal survival rate in patients with C(-20) (N = 55) was less than that in patients without C(-20) (N = 82). Symbols are: (dotted line) AA(-20); (solid line) AC/CC(-20). Log-rank test, P = 0.008.

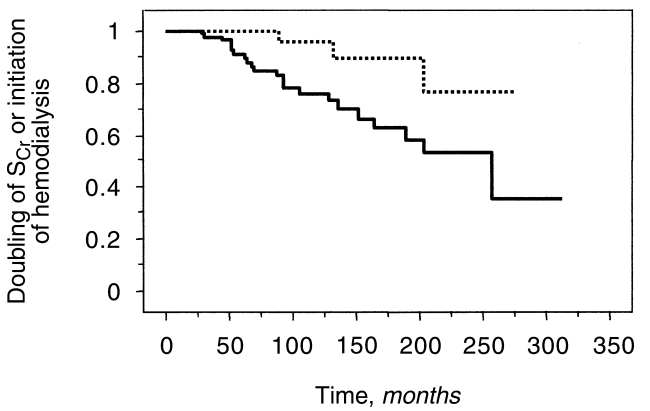

Fig. 2. *AGT* M235T and the renal survival rate in patients with IgAN. The renal survival rate in patients with MM/MT235 (dotted line; N = 94) was less than that in patients with TT235 (solid line; N = 43). Log-rank test P = 0.02.

Table 4. Cox proportional hazards regression model

Variable	*P* value	HR	95% CI
U_{prot}			
>1 g/day, <2g/day	0.01	5.4	1.5 to 20.4
>2 g/day	<0.001	28.3	7.3 to 109.8
HT at renal biopsy	0.002	4.6	1.8 to 11.9
AGT C(-20)	0.004	3.6	1.5 to 8.7

Abbreviations are: U_{prot}, urinary protein; HT, hypertension; HR, hazard ratio; CI, confidence interval.

although C(-20) is a subset of T235. There is a possibility that the A(-20)C polymorphism may be chiefly related to the local activation of renin-angiotensin system through a different transcriptional regulation, leading to renal dysfunction, whereas the M235T polymorphism may be implicated with renal dysfunction through systemic hypertension. Further study is necessary to explore the molecular mechanism of transcriptional regulation of *AGT* in the kidney under both physiological and pathological conditions.

Several earlier studies have analyzed an association of T235 variants of *AGT* with the progression of IgAN. Pei et al showed that patients with MT and TT genotypes had a faster rate of deterioration of renal function than those with MM genotypes [4]. Because a large proportion of patients in their study were treated with antihypertensive drugs (56 to 82%) and renal function in patients with MT or TT was moderately impaired, the effects of this polymorphism might be directly on blood pressure rather than having an independent effect on deterioration of renal function. In contrast, Hunley et al failed to find an association between the *AGT* T235 variant and any clinical categories of deterioration in IgAN [22]. However, in their study the length of clinical observation (6 to 7 years) appeared to be short for classifying the patients into categories. "Observation bias" would tend to misclassify the patients who may be destined to be in the disease progression group, to the stable renal function group [23]. Recently, a large and well-designed study (IGARAS) investigated the role of renin-angiotensin system gene polymorphisms in the progression of IgAN [24]. In their study, the distribution of *AGT* M235T genotypes was not different among the patients grouped by S_{Cr} and proteinuria at the time of renal biopsy. The Cox proportional hazards regression model did not find predictive values of *AGT* polymorphisms for renal survival; however, information about the long-term effect of each polymorphism on the progression of IgAN with preserved renal function at the time of renal biopsy was not available.

The limitation of this study may be that the patients who had different degrees of renal injury and had different rates of progression of renal dysfunction were recruited, although we selected the patients whose C_{Cr} at the time of renal biopsy was more than 70 mL/min. Histopathological analysis of the patients who reached end points within several years revealed severe expansion of mesangial matrix, tuft adhesion, and crescent formation. However, the mean time to the end points in our study was nearly 10 years, and a substantial proportion of the patients had stable or slowly declining renal function during the follow-up period. D'Amico et al's study found that the speed of progression of end-stage renal failure in IgAN patients was quite variable [25]. In fact, there was a large inter-individual difference in the time course to reach the end point, which was the very point we investigated here as the genetic background. We employed a time-to-event analysis because it is suggested that, in a study including patients with stable renal function, an analysis of the time-to-event approach is favored over an analysis of mean slope of renal function [26].

Our study could not provide evidence that the *AGT* C(-20) influenced the therapeutic effect of angiotensin II blockade by ACEI. In this respect, further investigation with a long-term prospective observation of a large number of cases is necessary. However, our study suggests that genotyping of *AGT* at -20 and precise clinicopathological assessments can lead to more accurate estimations of the prognosis, and to more proper and active usage of ACEI or ARB in patients with IgAN.

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