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ELECTRONIC LETTER

Renoprotective efficacy of renin–angiotensin inhibitors in IgA nephropathy is influenced by *ACE* A2350G polymorphism

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Immunoglobulin A nephropathy (IgAN) is the most prevalent form of primary glomerulonephritis and one of the principal causes of end stage renal disease (ESRD) throughout the world.[1 2] It is a complex disease, in which familial clustering suggests an inherited genetic predisposition. The disease has a variable clinical course, and one third of patients with IgAN progress to ESRD within 10–20 years of its onset.[3 4] The mechanisms of interindividual differences in the rate of disease progression are unclear.[5]

That increased production or activity of angiotensin II plays a detrimental role in the glomerular response to injury has been well documented. Recently, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blocker (ARB) treatments have been shown to decrease proteinuria by improving glomerular permselectivity in IgAN,[6 7] although the therapeutic effect was not recognised in about half of the patients.[6]

An insertion/deletion (I/D) polymorphism of the angiotensin I converting enzyme gene has been shown to influence the concentration of ACE in the circulation and local tissues.[8–10] Many studies have explored the association between the *ACE* I/D polymorphism and the development and progression of various cardiovascular diseases and renal diseases, including IgAN.[11–13] Moreover, several studies have investigated associations between the *ACE* I/D polymorphism and the therapeutic efficacy of ACE inhibitors. The DD homozygote of the *ACE* I/D polymorphism has been reported as a risk factor for progression to ESRD in patients with IgAN, as well as a predictive marker for responsiveness to the antiproteinuric effects of ACE inhibitors.[14] Other studies, however, reported that patients with the DD genotype were resistant to the renoprotective effects of ACE inhibitors, whether they had non-diabetic or diabetic nephropathy.[15–17]

Recently, an informative set of 13 single nucleotide polymorphisms (SNPs) across the entire *ACE* gene has been shown, and strong evidence from a large population based sample suggests that two SNPs—*ACE* A-240T and A2350G—are associated with plasma concentrations of ACE.[18] A polymorphism in exon 17, *ACE* A2350G, had the most significant effect on the concentration of ACE, whereas, after the effect of A2350G was adjusted for, the I/D polymorphism was not associated with the circulating concentration of ACE.

Taken together, it seems reasonable to hypothesise that these more functionally significant SNPs in the *ACE* gene may be involved in interindividual differences in the progression of glomerular injury. We investigated the possible role of these SNPs in the prognosis of renal function and on the therapeutic efficacy of ACE inhibitors and ARBs in patients with IgAN.

Key points

- Individual variations in responsiveness to the antiproteinuric and renoprotective effects of angiotensin converting enzyme (ACE) inhibitors in patients with immunoglobulin A nephropathy (IgAN) have been suggested to be influenced by genetic background. An insertion and deletion (I/D) polymorphism of the *ACE* gene is the candidate proposed to date, although the results are controversial. Recent evidence from a large population based sample suggested that two single nucleotide polymorphisms, *ACE* A-240T and A2350G, are strongly associated with plasma concentrations of ACE.
- 267 patients with histologically proven IgAN (114 of whom received ACE inhibitors or angiotensin receptor blocker (ARB), or both during their clinical course), were investigated retrospectively for clinical manifestations and renal prognosis, and associations with genotypes of *ACE* A-240T, I/D, and A2350G polymorphisms were evaluated.
- Renal prognosis was significantly better in patients who received ACE inhibitor or ARB, even though they had higher blood pressures and values of proteinuria at diagnosis. Proteinuria (>1.0 g/day), hypertension, no treatment with ACE inhibitor or ARB, and the AA genotype of A2350G were independent risk factors for progression of renal dysfunction; A-240T and I/D polymorphisms were not. The renoprotective effect of treatment with ACE inhibitor or ARB was remarkable in patients with the AA genotype (hazard ratio 7.473, $p<0.0001$), while the efficacy was not significant in those with other genotypes (1.767, $p=0.1119$).
- The *ACE* A2350G polymorphism may influence responsiveness to treatments that inhibit the renin–angiotensin system with respect to long term prognosis of renal function in Japanese patients with IgAN.

MATERIALS AND METHODS

Participants

The ethics committee of the University Graduate School of Medical and Dental Sciences approved the protocol for the genetic study. Japanese patients were eligible for inclusion in the analysis if they had been diagnosed as having IgAN by kidney biopsy at our institute between 1976 and 2001; had no evidence of systemic diseases such as hepatic glomerulo-

Abbreviations: IgAN, IgA nephropathy; ACE, angiotensin converting enzyme; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CI, confidence interval; I/D, insertion and deletion

Table 1 Clinical characteristics of patients with immunoglobulin A nephropathy. Values are mean (SD)

Characteristic	All patients (n=267)	ACEi or ARB: Received (n=114)	ACEi or ARB: Not received (n=153)	p Value	χ^2
Age (year)	37.0 (SD 13.5)	39.3 (SD 13.8)	35.3 (SD 13.0)	0.0230	
Sex (male %)	46.1	48.2	44.4	0.5377	0.380
Urinary protein excretion (g/day)	1.35 (SD 1.34)	1.51 (SD 1.29)	1.21 (SD 1.37)	0.0104	
Serum creatinine (mg/dl)	1.00 (SD 0.62)	0.93 (SD 0.32)	1.05 (SD 0.78)	0.7022	
Creatinine clearance (ml/min)	89.2 (SD 33.5)	90.4 (SD 30.6)	88.2 (SD 35.6)	0.4727	
Blood pressure (mm Hg)					
Systolic	127.9 (SD 18.4)	131.1 (SD 18.2)	125.4 (SD 18.3)	0.0099	
Diastolic	77.3 (SD 13.5)	79.3 (SD 13.9)	75.8 (SD 13.0)	0.0562	
Incidence of hypertension (%)	36.8	46.0	29.7	0.0069	7.311
Observed period (month)	91.7 (SD 67.6)	93.7 (SD 62.8)	92.0 (SD 62.2)	0.3384	
Incidence of PRD (%)	31.1	18.0	40.5	<0.0001	15.215
Glucocorticoid (%)	27.7	40.4	18.3	<0.0001	15.574
ACEi or ARB (%)	42.7	100	0		
Blood pressure (mm Hg)					
Systolic	128.6 (SD 16.6)	132.0 (SD 17.0)	127.4 (SD 17.4)	0.0774	
Diastolic	77.6 (SD 11.7)	80.7 (SD 11.8)	76.8 (SD 12.4)	0.1891	

ACEi, angiotensin I converting enzyme inhibitor; ARB, angiotensin receptor blocker; PRD, progressive renal disease.

sclerosis, Schönlein-Henoch purpura, and rheumatoid arthritis; had been followed up in our institute; and gave written informed consent for genetic study. Among 4493 patients who underwent renal biopsy at our institute between 1976 and 2001, 557 were diagnosed as having IgAN. In total, 267 patients fulfilled the criteria for inclusion and were recruited to the study. In all cases, the diagnosis of IgAN was based on a kidney biopsy that showed the presence of dominant or codominant glomerular mesangial deposits of immunoglobulin A, as assessed by immunofluorescence.

Clinical data and survival analysis

We retrospectively collected clinical characteristics of the patients, including age, sex, office blood pressure, level of urinary protein excretion (g/day), duration of observation (months), serum creatinine (mg/dl), and 24 hour creatinine clearance (ml/min), from medical records. Hypertension was defined by the use of one or more antihypertensive drugs or blood pressure ≥140 mm Hg (systolic) or 90 mm Hg (diastolic), or both. In the survival analysis, the primary endpoint (progressive renal disease) was defined as the date at which the concentration of serum creatinine was double that at the time of diagnosis or when the patient underwent their first haemodialysis. All patients were treated according to our study group's standard protocol for patients with primary glomerulonephritis. Corticosteroids generally were administered to patients with a urinary protein excretion >1.0 g/day at the time of renal biopsy, with the exception of cases with poor renal function (24 hour creatinine clearance <30 ml/min) and those aged ≥65 years. Antihypertensive agents were given in combination with or without ACE inhibitor or ARB to maintain the blood pressure <140 mm Hg (systolic) and <90 mm Hg (diastolic). Administration of glucocorticoids, antihypertensive agents, ACE inhibitors, and ARBs also was recorded for each patient. In total, 114 patients received ACE inhibitors or ARBs, or both, after the diagnosis and during their clinical course. About half of the ACE inhibitors prescribed were enalapril (2.5–10 mg/day; 56 patients); other ACE inhibitors prescribed included temocapril (1–4 mg/day; n = 19), quinapril (5–10 mg/day; n = 16), lisinopril (5–10 mg/day; n = 8), captopril (25–37.5 mg/day; n = 4), and delapril (7.5–30 mg/day; n = 3). An ARB was prescribed in 36 patients: losartan (25–50 mg/day) in 16 patients and candesartan (2–8 mg/day) in 20 patients; 28 patients were given an ACE inhibitor and an ARB.

Determination of genotypes

Genomic DNA from the peripheral blood cells was isolated with an automatic DNA isolation system (NA-1000; Kurabo, Osaka, Japan). The *ACE* I/D, A-240T, and A2350G genotypes of the patients were determined in a double blind manner. The I/D polymorphism in intron 16 of the *ACE* gene was assessed by polymerase chain reaction (PCR), as previously described.[8] To avoid mistyping ID heterozygote as DD because of the preferential amplification of the D allele compared with the I allele, we amplified DNA from all participants with the DD genotype by using an I allele-specific primer (5′-TTTGAGACGGGAGTCTCGCTC-3′).[19] *ACE* A-240T and A2350G polymorphisms were assessed by allele specific PCR amplification. Fragments of DNA that contained the SNP region of the gene were amplified by PCR by using two allele specific primers and the biotin labelled antisense primers, or vice versa. The 5′-end of the allele specific primers was labelled with fluorescein isothiocyanate or Texas Red. For the A-240T polymorphism in the *ACE* gene, biotin-GTGGGCA GGCTCGGGTGTT-3′ was used as the forward primer and fluorescein isothiocyanate-AAAGGGCCTCCTCTCTTCAG-3′ and Texas Red-GAAAGGG CCTCCTCTCTTCTG-3′ as the reverse primers. For the A2350G polymorphism, fluorescein isothiocyanate-GACGAATGTGATGGCCAAGT-3′ and Texas Red-GACGAATGTGATGGCCAGAT-3′ were used as the forward primers and biotin-TTGATGAGTTCCACGTATTTCG-3′ as the reverse primer. The second base from the 3′-end of each allele specific primer corresponded to the nucleotide of each allele, and the artificial mismatch nucleotide was inserted at the third base (indicated by underlines) to obtain maximum specificity. The reaction mixture (25 μl) contained 0.02 μg of DNA, 5 pmol of each oligonucleotide primer, 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM magnesium chloride, and 1 unit of DNA polymerase (rTaq; Toyobo, Osaka, Japan) in rTaq buffer. The amplification protocol consisted of an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 62.5°C for 30 seconds, an extension at 72°C for 30 seconds; and a final extension at 72°C for 2 minutes.

Amplified DNA was mixed with streptoavidin conjugated magnetic beads in a 96 well plate, and after 15 minutes' incubation at room temperature, the magnetic beads were collected. The supernatant, which contained the primers not used in the PCR amplification, was taken out and placed into a new 96 well white plate that contained 0.01 N sodium hydroxide, and the samples were excited at 485 nm for fluorescein isothiocyanate and 584 nm for Texas Red. The fluorescence emissions for fluorescein isothiocyanate and Texas Red were measured with an automated chemiluminescent assay system (Toyobo).

Statistical analysis

We used χ^2 analysis to compare allele frequencies and categorical variables between the groups. We compared continuous variables with the Mann-Whitney U-test or Kruskal-Wallis analysis of variance, and we tested the Hardy-Weinberg equilibrium by a χ^2 test with one degree of freedom. We used the Kaplan-Meier method and Cox proportional hazards regression model to analyse the course from renal biopsy to endpoint. Variables that achieved statistical significance ($p<0.05$) in the univariate analysis subsequently were included in a multivariate analysis with a stepwise forward Cox regression procedure, and the effects of these covariates were expressed by a hazard ratio. Values of $p<0.05$ were considered statistically significant. Haplotype analysis, which was based on a maximum likelihood method, was performed with Arlequin (version 2.0; University of Geneva, Geneva, Switzerland). Pairwise linkage disequilibrium coefficients were calculated and expressed as $D' = D/D$ max or D/D min, according to Thompson et al.[20]

RESULTS

Clinical characteristics of the patients

Table 1 compares the clinical characteristics of patients who did and did not receive an ACE inhibitor or ARB. No differences were noted in sex, serum creatinine, or 24 hour creatinine clearance at the time of renal biopsy or duration of follow up between the groups. The age, amount of urinary protein excretion, and systolic blood pressure were significantly higher ($p = 0.0230$, $p = 0.0104$, and $p = 0.0099$, respectively) in patients who received ACE inhibitors or ARBs compared with those who did not. As the choice of antihypertensive agents was not controlled but was left up to each doctor, ACE inhibitors and ARBs tended to be given to patients with high grade proteinuria. The incidence of hypertension at the time of renal biopsy was significantly higher in patients who received ACE inhibitors or ARBs ($x^2 = 7.311$, $p = 0.0069$), and a larger number of the patients also received corticosteroid treatment ($x^2 = 15.574$, $p<0.0001$). Although the patients who received ACE inhibitors and ARBs had more severe clinical markers for the progression of renal dysfunction, the incidence of progressive renal disease during the observation period was significantly lower than in patients who did not receive ACE inhibitors or ARBs ($x^2 = 15.215$, $p<0.0001$). During follow up, the mean blood pressure and percentage of cases with blood pressure <140/90 mm Hg was not different between the groups.

Table 2 Genotype distributions and allele frequencies of polymorphisms of *ACE*

	ACE polymorphism					
Variable	A-240T		I/D		A2350G	
Genotype	AA	99	II	103	AA	87
	AT	128	ID	128	AG	142
	TT	40	DD	36	GG	38
	Total	267		267		267
Allele	A	0.610	I	0.625	A	0.592
	T	0.390	D	0.375	G	0.408

Table 3 Estimated frequencies of haplotypes of *ACE* polymorphisms

Haplotype			
Locus −240	Intron 16	Locus 2350	Frequency
A	D	A	0.0215
A	D	G	0.0020
A	I	A	0.5523
A	I	G	0.0347
T	D	A	0.0100
T	D	G	0.3415
T	I	A	0.0158
T	I	G	0.0226

Genotype, allele, and estimated haplotype frequencies of *ACE*

Table 2 gives the genotype distributions and allele frequencies of the *ACE* A-240T, I/D, and A2350G polymorphisms in patients with IgAN. The expected frequency of the genotypes, under the assumption of the Hardy-Weinberg equilibrium, did not differ from the observed genotype frequencies, and the genotype distribution of the I/D polymorphism was consistent with previous reports for the Japanese population.[21 22] Table 3 also shows estimated haplotype frequencies. The two most frequent haplotypes, A-I-A and T-D-G, complemented each other at all three loci and accounted for 89.6% of the total. These polymorphisms were in tight, but not complete, linkage disequilibrium. D' was 0.9017 between A-240T and I/D, 0.8957 between I/D and A2350G, and 0.8665 between A-240T and A2350G ($p<0.0001$ in each pair).

Effect of A2350G polymorphism of the *ACE* gene on clinical manifestations

Table 4 compares clinical manifestations among each genotype of *ACE* A2350G. The patients with the AA homozygote of the *ACE* A2350G polymorphism tended to have higher blood pressures and lower 24 hour creatinine clearance at the time of renal biopsy than those with heterozygotes or G allele homozygotes. Moreover, the incidence of progressive renal disease was higher in patients with the AA genotype during follow up ($\chi^2 = 7.970$, $p = 0.0053$). No remarkable difference was noted between patients with the AG genotype and the GG genotype at baseline with respect to urinary protein excretion, serum creatinine, 24 hour creatinine clearance, blood pressure, and incidence of hypertension. In survival analyses, we compared patients with the AA genotype and those who were heterozygous or homozygous for the G allele. No significant association was detected between the other *ACE* polymorphisms investigated (A-240T and I/D) and any clinical manifestations (data not shown).

Risk factors for progression to progressive renal disease

During follow up (mean 91.7 (SD 67.6) months), 83/267 (31.1%) patients progressed to progressive renal disease, while a substantial proportion of the patients had stable renal function. As a time to event analysis is favoured if a substantial proportion of patients have stable or slowly declining renal function, we used the Cox hazard regression

Table 4 Clinical manifestations in patients with immunoglobulin A nephropathy by genotype of *ACE* A2350G polymorphism. Values are mean (SD)

Clinical manifestations	Genotype of *ACE* A2350G polymorphism				
	AA (n=87)	AG (n=142)	GG (n=38)	p Value	χ^2
At the time of renal biopsy					
Age (year)	37.5 (SD 13.1)	37.4 (SD 14.0)	34.0 (SD 12.0)	0.3838	
Sex (male %)	50.8	43.7	44.7	0.5863	1.068
Urinary protein excretion (g/day)	1.47 (SD 1.32)	1.29 (SD 1.41)	1.26 (SD 1.07)	0.3869	
Cases with urinary protein >1.0 g/day (%)	37.9	36.6	34.2	0.4459	1.615
Serum creatinine (mg/dl)	1.09 (SD 0.69)	0.98 (SD 0.64)	0.87 (SD 0.27)	0.1176	
Creatinine clearance (ml/min)	83.3 (SD 36.2)	90.8 (SD 32.9)	96.6 (SD 26.5)	0.0247	
Blood pressure (mm Hg)					
Systolic	131.1 (SD 18.8)	127.2 (SD 18.5)	123.4 (SD 16.2)	0.0569	
Diastolic	79.2 (SD 14.0)	76.4 (SD 13.4)	76.7 (SD 12.5)	0.3615	
Incidence of hypertension (%)	47.1	31.9	31.6	0.0593	5.730
During observation					
Observed period (months)	93.7 (SD 62.8)	89.2 (SD 68.1)	96.4 (SD 77.5)	0.6756	
Incidence of PRD (%)	42.5	26.4	21.6	0.0159	8.286
Glucocorticoid (%)	29.0	28.9	28.9	0.4105	1.781
ACEi or ARB (%)	37.9	43.7	50.0	0.4295	1.690
Blood pressure (mm Hg)					
Systolic	132.0 (SD 17.0)	127.2 (SD 16.5)	126.0 (SD 15.2)	0.0479	
Diastolic	80.7 (SD 11.8)	75.7 (SD 11.5)	77.4 (SD 10.9)	0.0147	

PRD, progressive renal disease; ACEi, angiotensin I converting enzyme inhibitor; ARB, angiotensin receptor blocker.

model to investigate predictive risk factors for the progressive renal disease (table 4). In this analysis, a urinary protein excretion >1.0 g/day, hypertension, no ACE inhibitor or ARB treatment, and the AA genotype of the *ACE* A2350G polymorphism were all identified as independent risk factors for progression to progressive renal disease. After we adjusted for other prognostic factors (urinary protein excretion >1.0 g/day and hypertension), the risk of progressive renal disease was 3.1 times higher in patients who did not receive an ACE inhibitor or ARB than in those who did (hazard ratio 3.111 (95% confidence interval 1.797 to 5.385), p<0.0001) and 1.8 times higher in patients with the AA genotype of *ACE* A2350G than in those with other genotypes (hazard ratio 1.794 (1.112 to 2.894), p = 0.0166). None of the other clinical covariates, including age, sex, corticosteroid therapy, and other antihypertensive drugs, was a significant risk factor in the univariate analysis (table 4). No other polymorphisms in the *ACE* gene examined (I/D and A-240T) showed any significant association with the risk of progressive renal disease. As the A2350G polymorphism was associated most significantly with the progression to progressive renal disease, the A2350G genotype was included in the multivariate analysis.

Effect of ACE inhibitor, ARB, and *ACE* A2350G polymorphism on renal survival

Figure 1A shows renal survival in patients with IgAN who did and did not receive an ACE inhibitor or ARB. The therapeutic efficacy of ACE inhibitor or ARB on long term renal survival was observed by Kaplan-Meier analysis. The mean survivals of renal function in patients who did and did not receive an ACE inhibitor or ARB were 197.7 (SD 11.4) and 138.3 (SD 6.7) months, respectively (Figure 1A, Kaplan-Meier, log rank test $\chi^2 = 10.759$, p = 0.0010). Figure 1B shows that renal survival in patients with the AA genotype of *ACE* A2350G was worse than in patients with other genotypes, although this effect was marginal (log rank test $\chi^2 = 4.538$, p = 0.0332).

Interaction of genotype and treatment with ACE inhibitor or ARB on renal survival

We stratified patients according to the *ACE* genotypes to investigate the interaction between the *ACE* A2350G genotype and the renoprotective effect of ACE inhibitors or ARBs, and we investigated the effect of ACE inhibitor or ARB on the long term survival of renal function within each genotype. Renal survival within patients with the AA genotype of the *ACE* A2350G polymorphism was improved significantly by administration of ACE inhibitor or ARB (Figure 2A, log rank test $\chi^2 = 11.150$, p = 0.0008), while this effect was not statistically significant in patients with the AG or GG genotypes (Figure 2B, log rank test $\chi^2 = 2.266$, p = 0.1322). This was confirmed further by the Cox proportional hazard analysis performed on patients with each genotype (table 5). In patients with the AA genotype, no treatment of ACE inhibitor or ARB was a significant risk factor for progressive renal disease (hazard ratio 7.473 (2.899 to 19.265),

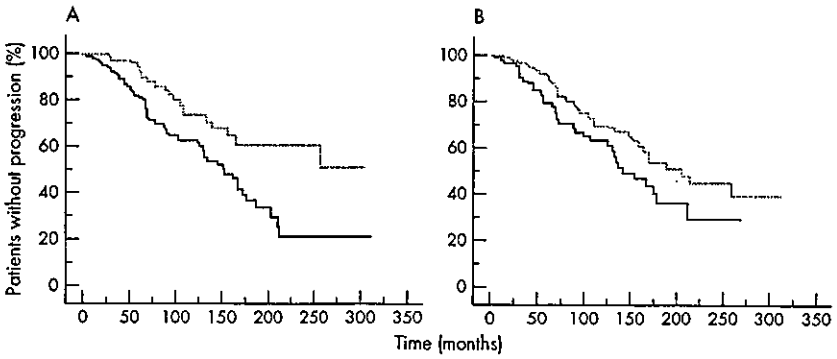

Figure 1 Renal survival in patients with IgAN who did (n = 114) or did not receive treatment with ACE inhibitor or ARB (n = 153) (Kaplan-Meier log rank test, p = 0.0010) (A) and in patients with IgAN with the AG/GG genotype (n = 180) or AA genotype (n = 87) of the *ACE* A2350G polymorphism who did receive treatment with ACE inhibitor or ARB (Kaplan-Meier log rank test, p = 0.0332) (B).

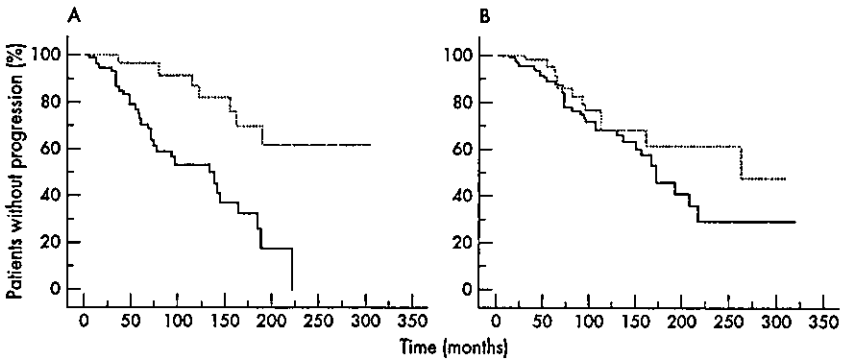

Figure 2 Renal survival curve for patients with IgAN with the AA genotype of *ACE* A2350G polymorphism that compares patients who did (n=33) and did not receive treatment with ACE inhibitor or ARB (n=54) (Kaplan-Meier log rank test p=0.0008) (A) and renal survival curve for patients with IgAN and the AG/GG genotype of *ACE* A2350G polymorphism that compares patients who did (n=81) and did not receive treatment with ACE inhibitor or ARB (n=99) (Kaplan-Meier log rank test, p=0.1322) (B).

p<0.0001), whereas this risk factor was not significant in those with other genotypes (1.767 (0.876 to 3.567), p=0.1119).

DISCUSSION

This study showed the therapeutic efficacy of inhibition of the renin–angiotensin system on the long term prognosis of renal function in IgAN independently of blood pressure control. The genetic polymorphisms of *ACE* A-240T and I/D were not associated with the renal prognosis, while A2350G was associated marginally. Many studies have reported conflicting results about the association between the *ACE* I/D polymorphism and renal prognosis in IgAN.[13] Although the effects of A-240T and A2350G polymorphisms in IgAN have not been investigated previously, the present data were in accordance with results from a large scale study on a Japanese population, which reported that the I/D polymorphism had no significant impact on the renal prognosis of IgAN.[22] The effect of the A2350G polymorphism on renal survival was statistically significant, but it was marginal compared with the strong impact of other clinical risk factors such as proteinuria, hypertension, and no treatment with ACE inhibitor or ARB.

Table 5 Cox proportional hazard model to test significance of clinical covariates and genotypes of *ACE* polymorphisms as predictors of renal survival

Variable	p Value	Hazard ratio (95% CI)
Univariate analysis		
Urinary protein excretion >1.0 g/day	<0.0001	2.961 (1.719 to 5.101)
Hypertension	0.0014	2.102 (1.334 to 3.312)
No administration of ACEi or ARB	0.0014	2.281 (1.373 to 3.789)
No glucocorticoid therapy	0.4817	0.831 (0.496 to 1.393)
Age	0.1023	1.015 (0.997 to 1.033)
Sex (male)	0.8028	1.057 (0.682 to 1.639)
Genotype		
ACE A2350G		
GG	Referent	1
AG	0.4277	1.363 (0.634 to 2.931)
AA	0.0352	1.599 (1.033 to 2.475)
ACE A-240T		
TT	Referent	1
AT	0.5490	1.239 (0.614 to 2.500)
AA	0.2755	1.479 (0.732 to 2.987)
ACE I/D		
II	Referent	1
ID	0.4007	0.732 (0.354 to 1.514)
DD	0.5332	1.163 (0.504 to 2.680)
Multivariate analysis		
Urinary protein excretion >1.0 g/day	<0.0001	3.102 (1.771 to 5.433)
Hypertension	0.0002	2.509 (1.537 to 4.097)
No administration of ACEi or ARB	<0.0001	3.111 (1.797 to 5.385)
AA genotype of *ACE* A2350G	0.0166	1.794 (1.112 to 2.894)

ACEi, angiotensin I converting enzyme inhibitor; ARB, angiotensin receptor blocker; CI, confidence interval.

To be able to predict the renoprotective effects of antihypertensive agents for individual patients with renal disease is important. Although it already has been reported that the DD genotype of the I/D polymorphism in the *ACE* gene is associated with the therapeutic efficacy of ACE inhibitors on proteinuria over a relatively short period of observation in patients with IgAN and diabetic nephropathy,[14 21] other studies have reported an opposite association in patients with diabetic and non-diabetic renal disease.[15–17 24] In the present study, whether the effect of ACE inhibitors and ARBs on long term renal survival (rather than on proteinuria) was affected by the *ACE* genotypes was evaluated, as this would have more important clinical implications and because consistent measurements of urinary protein excretion were not always available for each participant. In fact, we sometimes experienced fluctuations in urinary protein excretion of up to 50% or more, even in patients who had stable renal functions over a long time.

We could not completely deny the possibility that the observed renoprotective efficacy of ACE inhibitors and ARBs was secondary to the effect of corticosteroid therapy, because a larger proportion of patients was treated with corticosteroids in the group who received treatment with ACE inhibitors and ARBs. Although the efficacy of steroids on long term renal function is still a matter of controversy, its antiproteinuric effect has been confirmed by multiple randomised trials.[25–27] This study also could not detect an independent therapeutic effect of steroids on long term renal survival, and the results of the Cox proportional hazard regression analysis show that the efficacy of ACE inhibitors and ARBs was independent of steroid therapy. Moreover, the proportion of patients treated with corticosteroids, as well as those who received ACE inhibitors and ARBs was, at least, no different among groups with each genotype of the *ACE* A2350G polymorphism (table 3). This strongly suggests that differences in efficacy between genotype groups are not due to the effect of steroids.

Patients who received ACE inhibitors and ARBs had more proteinuria and a higher incidence of hypertension at the time of renal biopsy, which suggests that a substantial difference in the histological changes between the subgroups

Table 6 Significance of clinical covariates for patients with AA or with AG or GG genotype of *ACE* A2350G polymorphism by Cox proportional hazard model

Covariate	p Value	Hazard ratio (95% CI)
Patients with the AA genotype of *ACE* A2350G		
Urinary protein excretion>1.0 g/day	0.0147	2.620 (1.209 to 5.680)
Hypertension	0.0007	3.846 (1.766 to 8.377)
No administration of ACEi or ARB	<0.0001	7.473 (2.899 to 19.265)
Patients with the AG or GG genotype of *ACE* A2350G		
Urinary protein excretion >1.0 g/day	0.0059	3.307 (1.412 to 7.743)
Hypertension	0.0120	2.381 (1.210 to 4.689)
No administration of ACEi or ARB	0.1119	1.767 (0.876 to 3.567)

CI, confidence interval; ACEi, angiotensin I converting enzyme inhibitor; ARB, angiotensin receptor blocker.

with or without treatment with ACE inhibitors and ARBs may be detected.

The main purpose of this study was to investigate the possible role of the *ACE* gene polymorphisms in the prognosis of renal function and their interactions with efficacy of treatment with ACE inhibitors and ARBs. We chose covariates from clinical manifestations rather than histological changes to identify the prognostic factors. Histological changes correlate strongly with clinical findings, so the interaction between clinical and histological manifestations would reduce substantially their statistical significances in the multivariate analysis. Moreover, histological findings are much more complex and difficult to quantify and evaluate accurately.

Angiotensin converting enzyme inhibitors are currently the best documented inhibitors of the renin–angiotensin system and are reported to be renoprotective in diabetic and non-diabetic renal disease.[7 28] Angiotensin receptor blockers are the other inhibitors of the renin–angiotensin system and have pharmacological properties distinct from those of the ACE inhibitors. We are not aware of any comparisons of the long term renoprotective effects of ACE inhibitors and ARBs, or a combination of both.[7] Direct comparisons of the renal haemodynamic effects in patients with hypertension or renal diseases gave similar renal vasodilations in both classes of drugs.[29 30] In small studies of patients with IgAN, the combination of ARB and ACE inhibitor was at least additive in decreasing protein excretion,[31 32] whereas the results of larger trials still are controversial.[33 34] The numbers of participants who were treated with each drug were not large enough to analyse the data separately in the present study. Moreover, we could not completely deny the possibility of some bias as a result of this being a retrospective study. The tendency to use ACE inhibitor or ARB treatment in patients with glomerular diseases has grown stronger during the last decade. The duration of observation was not different between patients who did or did not receive ACE inhibitor or ARB, however, and patients who received treatment with ACE inhibitor or ARB were rather older than those who did not. Major potential clinical risk factors for progression of renal dysfunction, including no treatment with ACE inhibitor or ARB (which is assumed to be associated with chronologically older patients), were included as covariates in the Cox proportional hazard regression model. In addition, although other classes of antihypertensive drugs, such as diuretics, did not have any independent effect on the renal prognosis (data not shown), they may interact with the efficacy of ACE inhibitors or ARBs. Nonetheless, to confirm the results of the present study, a randomised, controlled, prospective study with a large scale population of patients on a fixed medication protocol is needed. To draw conclusions about the long term renal survival of patients with IgAN in a prospective study is difficult, because the actual prognosis of renal function in each case can only be determined after a long observation period. In fact, a substantial proportion of our patients had stable renal function, and only one third of them progressed to progressive renal disease during the mean observation period of 91.7 months. Even if the bias as a result of the retrospective nature of the study is taken into account, therefore, we believe that the Cox hazard regression model and time to event analysis with the past precise medical records is an adequate and feasible method for investigating the long term renal prognosis.

Another limitation of this study is that the association of the genotype and local activity or concentration of ACE was not provided. Although we have no data to investigate the association between *ACE* genotypes and circulating concentrations of ACE in our patients, the G allele at the A2350G polymorphism was associated most strongly with an increased concentration of ACE.[18] In addition, evidence for a linkage disequilibrium between the I/D and A2350G polymorphism in the *ACE* gene has been found,[35] although, as mentioned above, previous studies have produced conflicting evidence for the relation between genotype and therapeutic efficacy of ACE inhibitors. When we consider the strong linkage disequilibrium between I/D and A2350G loci, our results may support the results of van Essen *et al* and Parving *et al* and indicate that patients who have less circulating ACE tend to respond well to therapy with ACE inhibition in terms of glomerular injury.[15 16] At present, we have no data to explain the mechanism for the apparent dissociation between the genetic influence on concentrations of ACE on one hand and the response to ACE inhibition on the other. One possible explanation is that serum concentrations of ACE in healthy people may not necessarily reflect enhanced activity of ACE in local tissue, in particular of glomerular inflammatory injury. A second possibility is that the effect of the *ACE* genotype occurs through a mechanism other than its effect on serum concentrations of ACE. In support of this hypothesis is the finding from the EURODIAB controlled trial of lisinopril in insulin dependent diabetes that ACE inhibitors also have a beneficial effect on diabetic retinopathy,[36] even though retinopathy, unlikely nephropathy, is not associated with elevated concentrations of ACE.[37 38] As indicated by Ueda *et al*,[39] in pragmatic terms, relatively low doses of drugs that inhibit the renin–angiotensin system may be enough for patients with low concentrations of ACE and low levels of angiotensin II, but not enough for full and sustained inhibition in those with high concentrations of ACE.

Pairwise linkage disequilibrium between polymorphic loci in this gene and magnitude of the association between these markers and circulating concentrations of ACE have been reported to vary according to ethnicity,[40] whether the effect of the genetic variant investigated in this study is observed in other ethnic groups remains to be seen.

Conclusion

Polymorphisms in the *ACE* gene may be a significant genetic marker for predicting the renoprotective efficacy of renin–angiotensin inhibition on long term renal prognosis in Japanese patients with IgAN. Although patients with the AA genotype of the A2350G polymorphism may be at a higher risk for progressive renal dysfunction in the absence of treatment with ACE inhibitors or ARBs, they may also have a greater response to inhibition of the renin–angiotensin

system in terms of long term prognosis of renal function. These findings, if confirmed, may have important implications for clinical care, because ACE inhibitors and ARBs could be given more positively to patients with IgAN and the AA genotype at an early stage of the disease. Further study is needed to confirm our findings and to assess whether patients with the AG/GG genotype respond to high doses of inhibitors of the renin–angiotensin system.

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Gender specific association of aldosterone synthase gene polymorphism with renal survival in patients with IgA nephropathy

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LETTER TO JMG

Gender specific association of aldosterone synthase gene polymorphism with renal survival in patients with IgA nephropathy

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Immunoglobulin A nephropathy (IgAN), which is the most prevalent form of primary glomerulonephritis and one of the major causes of end stage renal disease (ESRD), has a variable clinical course.[1–3] Poor prognostic factors for the progression of renal dysfunction in IgAN have been identified as high blood pressure, heavy proteinuria, and a severe histopathological appearance of the renal biopsy.[4 5] In addition to these prognostic factors, it has been proposed that several genetic backgrounds are associated with a susceptibility to ESRD in patients with IgAN.[6 7]

The renin-angiotensin-aldosterone system is an important regulator of blood pressure and plays a central role in the development and progression of end organ damage. Polymorphisms in genes that encode components of this system have been reported to be associated with physiological risk factors for progressive renal dysfunction in IgAN. The most consistent of these is the angiotensinogen (*AGT*) gene, which is associated with essential hypertension and with an increased risk of cardiovascular diseases and renal failure.[8–10] A deletion polymorphism in the angiotensin converting enzyme (*ACE*) gene influences the circulating ACE levels, and although it has little effect on blood pressure, it has been associated with an increased risk of cardiovascular diseases in some but not all studies.[11–13]

Aldosterone is one of the main effectors of the renin-angiotensin system[14] and has classically been thought to act as a regulator for the absorption of Na and water, as well as the excretion of K in normal physiology, and as a mediator of oedema in numerous disease states. However, it is now well recognised that the actions of aldosterone are not limited to effects on ion transport in epithelial tissue, and its important role in cardiovascular disease involves non-epithelial tissues.[15 16] Recently, it has been shown that aldosterone has a number of deleterious effects on the cardiovascular system, including necrosis and fibrosis, vascular stiffening and injury, reduced fibrinolysis, endothelial dysfunction, and catecholamine release.

Aldosterone secretion is regulated largely by the expression level of the final enzyme required for its biosynthesis, aldosterone synthase (*CYP11B2*).[17] Expression of *CYP11B2* is regulated by angiotensin II through cAMP dependent modulation of the gene promoter region, which contains a variety of control factors.[18 19] Therefore, genetic variants in *CYP11B2*, which may be associated with the biosynthesis of aldosterone in local tissue, may also affect the progression of renal dysfunction in primary glomerulonephritis.

One potentially interesting variant of *CYP11B2* is located in the 5′ flanking region of the gene, with a C or T at 344 nucleotides upstream from the start of translation (C-344T) within a binding site for the transcription factor steroidgenic factor-1 (SF-1).[20] This genetic polymorphism has been reported to be associated with the serum level of aldosterone, as well as the left ventricular size, its function, and myocardial infarction independently of blood pressure,[21–23] In contrast, a previous study in patients with kidney disease, in which a variety of primary kidney diseases for ESRD were included in the analysis, did not show any significant effect of the *CYP11B2* polymorphism on the progression of renal dysfunction.[9] Thus, the possible role of the *CYP11B2* polymorphism on renal survival in patients with IgAN remains to be analysed fully.

In this study, we investigated the possible role of the *CYP11B2* C-344T polymorphism in renal prognosis in Japanese patients with IgAN.

Key points

- Aldosterone has a number of deleterious effects on the cardiovascular system, including necrosis and fibrosis, vascular stiffening and injury, reduced fibrinolysis, endothelial dysfunction, and catecholamine release. The C-344T polymorphism in the aldosterone synthase gene (*CYP11B2*), which is associated with circulating aldosterone concentration, has recently been proposed as a genetic candidate in cardiovascular disease such as hypertension. However, its role in the progression of renal dysfunction in IgA nephropathy (IgAN) remains to be investigated.
- We analysed the *CYP11B2* C-344T polymorphism in 271 patients with biopsy proven IgAN and investigated a possible association between the polymorphism and the prognosis of renal function using the Kaplan-Meier method and Cox proportional hazards regression model.
- At the time of renal biopsy there was no difference among the genotypes in any clinical manifestations, including age, gender, urinary protein excretion, and blood pressure. Predictive risk factors for progressive renal dysfunction were identified as hypertension, urinary protein of more than 1.0 g/day, and no administration of angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) in both female and male patients.
- Even after adjusting for these risk factors, the CC genotype of *CYP11B2* was a significant risk factor only in female patients with a hazard ratio (HR) of 4.284 (p=0.0022). In contrast, no effect at all was observed in male patients (HR=0.788, p=0.6269).
- Genetic variation in or near the *CYP11B2* gene is a possible genetic marker for the progression of renal dysfunction in females with IgAN but not males.

Table 1 Clinical characteristics of the patients and their comparison by each genotype in women and men

		Women (n=143)				Men (n=128)			
		Genotype of *CYP11B2*				Genotype of *CYP11B2*			
	Total n=271	TT n=64	CT n=63	CC n=16	P value	TT n=52	CT n=64	CC n=12	P value
At the time of renal biopsy									
Age (year)	37.0 (13.4)	38.9 (13.4)	36.0 (13.0)	35.6 (14.0)	NS	38.4 (12.4)	34.4 (14.3)	41.0 (12.7)	NS
Urinary protein excretion (g/day)	1.36 (1.35)	1.17 (1.20)	1.02 (1.17)	1.63 (1.39)	NS	1.62 (1.71)	1.56 (1.30)	1.53 (0.98)	NS
Serum creatinine (mg/dl)	0.99 (0.62)	0.84 (0.32)	0.77 (0.26)	0.91 (0.52)	NS	1.24 (0.75)	1.16 (0.87)	1.14 (0.27)	NS
Creatinine clearance (ml/min)	89.2 (33.1)	83.2 (25.6)	96.6 (33.5)	82.9 (33.4)	NS	88.2 (33.4)	92.0 (39.7)	82.5 (24.6)	NS
Blood pressure (mm Hg)					NS				NS
Systolic	127.6 (18.3)	126.6 (18.7)	123.6 (17.4)	130.8 (25.9)	NS	130.1 (16.0)	128.4 (18.1)	133.4 (19.5)	NS
Diastolic	77.1 (13.5)	76.4 (12.1)	74.8 (12.2)	76.9 (15.4)	NS	77.9 (15.0)	77.9 (14.4)	84.5 (13.2)	NS
Incidence of hypertension (%)	36.4	39.1	22.0	35.7	NS	41.2	37.5	66.7	NS
During observation									
Observed period (month)	92.0 (66.8)	102.1 (61.9)	86.9 (58.3)	61.1 (40.1)	0.0366	91.7 (80.6)	92.7 (74.1)	105.7 (48.3)	NS
Incidence of progressive renal disease (%)	31.0	29.0	25.8	50.0	NS	29.4	34.4	41.7	NS
Corticosteroid (%)	28.3	27.1	23.2	33.3	NS	32.7	28.3	33.3	NS
ACEI/ARB (%)	40.8	41.3	33.9	50.0	NS	54.9	33.3	41.7	NS
Blood pressure (mm Hg)									
Systolic	128.3 (16.7)	128.1 (17.3)	125.6 (17.8)	133.1 (22.7)	NS	127.6 (12.6)	129.5 (16.4)	132.8 (17.0)	NS
Diastolic	77.4 (11.9)	76.8 (10.5)	75.9 (12.2)	78.9 (13.1)	NS	76.4 (11.6)	78.3 (12.7)	84.6 (11.7)	NS

ACEI/ARB, angiotensin-I converting enzyme inhibitor and/or angiotensin receptor blocker; NS, not statistically significant. Values are mean (SD).

Table 2 Allele frequencies in hypertensive and normotensive subjects

	Allele	Hypertensives	Normotensives	p value	χ^2
Total	-344C	66	117		
	-344T	132	227	0.8723	0.026
Women	-344C	26	69		
	-344T	64	127	0.2891	1.124
Men	-344C	40	48		
	-344T	68	100	0.4436	0.587

METHODS

Study subjects

Written informed consent was obtained from all patients. The ethics committee of the institution approved the protocol for the genetic study. The study included 271 patients with biopsy proven IgAN. In all cases, the diagnosis of IgAN was based on the immunofluorescent microscopy of a biopsy specimen, which showed dominant or co-dominant deposition of IgA in the glomerular mesangium. Patients with Schönlein-Henoch purpura and secondary IgAN such as hepatic glomerulosclerosis or rheumatoid arthritis were excluded from the study. Clinical characteristics of the patients at the time of diagnosis including age, gender, urinary protein excretion (g/day), level of serum creatinine (sCr, mg/dl), and 24 hour creatinine clearance (ml/min) were investigated. 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. The primary end point (PRD, progressive renal disease) 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. The mean duration of observation was 92.0 (SD 66.8) months. The administration of glucocorticoids, antihypertensive agents, angiotensin converting enzyme inhibitors (ACEI), and angiotensin II receptor blocker (ARB) was also recorded for each patient.

DNA preparation and genotype determination

Genomic DNA was extracted from the peripheral blood cells of patients by an automatic DNA isolation system (NA-1000, Kurabo, Osaka, Japan). The genotype of *CYP11B2* C-344T was determined by the PCR-RFLP method using restriction endonuclease *Hae*III (Takara, Kyoto, Japan) as described previously.[21] Primers used for the PCR reaction were 5′-CAG GAG GAG ACC CCA TGT GAC-3′ (sense) and 5′-CCT CCA CCC TGT TCA GCC C-3′ (antisense). The reaction mixture contained 1 × PCR buffer, 1.5 mmol/l $MgCl_2$, 200 mmol/l deoxynucleotide triphosphates (dNTPs), 1 unit *Taq* DNA polymerase (Takara, Kyoto, Japan), 10 pmol of each primer, and 50-100 ng genomic DNA. The PCR amplification reaction consisted of a cycle at 95°C for five minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 67°C for 15 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72°C for five minutes. The 537 bp PCR products were digested with restriction endonuclease *Hae*III (Takara), and electrophoresed on a 2.5% agarose gel.

Statistical analysis

Statview 5.0 statistical software (Abacus Concepts Inc, Berkeley, CA, USA) was used for statistical analyses on a Macintosh G4 computer. Chi-square analysis was used when comparing allele frequencies and categorical variables between the groups. Continuous variables were compared using the Mann-Whitney U test or Kruskal-Wallis analysis of variance.

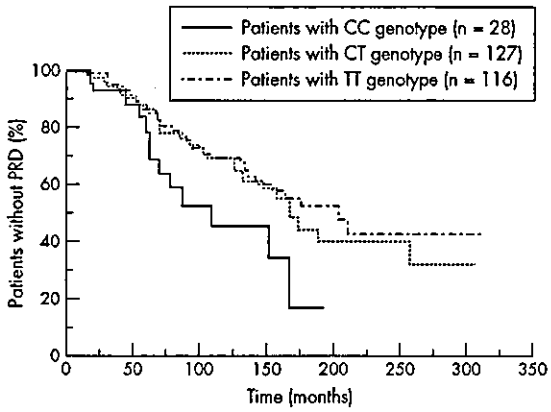

Figure 1 The renal survival rate in IgAN patients with each genotype of the *CYP11B2* C-344T polymorphism. The renal survival rate of patients with the CC genotype (solid line, n=28) was significantly worse than that in other genotypes (dotted line, CT, n=127 and broken line, TT, n=116). Kaplan-Meier log rank test, χ^2=5.208, p=0.0225.

Table 3 Cox proportional hazard model to test the significance of clinical covariates and genotypes of the *CYP11B2* polymorphism as predictors of renal survival

Variable	p value	HR	95% CI
Urinary protein excretion >1.0 g/day	<0.0001	3.362	1.891 to 5.977
Hypertension	0.0007	2.301	1.423 to 3.721
No ACEI/ARB administration	0.0002	2.779	1.629 to 4.740
CC genotype of *CYP11B2*	0.1667	1.584	0.825 to 3.042

HR, hazard ratio; CI, confidence interval; ACEI/ARB, angiotensin-I converting enzyme inhibitor and/or angiotensin receptor blocker.

The Hardy-Weinberg equilibrium was tested by a chi-square test with 1 df. The time from renal biopsy to end point (initiation of dialysis or a doubling of the sCr level after the time of diagnosis) was analysed by the Kaplan-Meier method and the Cox proportional hazards regression model. Covariates were selected by a stepwise backward method and their effects were expressed as a hazard ratio. A value of $p<0.05$ was considered statistically significant.

RESULTS

In total, 271 patients with IgAN were genotyped for the *CYP11B2* C-344T polymorphism. The frequencies of the genotypes TT, TC, and CC were as follows: males, 40.6%, 50.0%, and 9.4%, respectively, females 44.8%, 44.1%, and 11.2%, respectively, and overall, 42.8%, 46.9%, and 10.3%, respectively. These results are consistent with a previous report on a large population based sample of Japanese.[24] The observed genotype frequencies were in agreement with those expected under the assumption of Hardy-Weinberg equilibrium. Table 1 shows the clinical characteristics of the patients and their comparisons by each genotype in women (n=143) and men (n=128). There were no statistically significant differences among the three genotypes with regard to any clinical characteristics. Blood pressures both at the time of diagnosis and during observation, as well as the incidence of hypertension at the baseline, tended to be numerically higher in patients with the CC genotype both in men and women, but the differences were not statistically significant. Duration of observation was significantly shorter in female patients with the CC genotype than those with other genotypes, but not in males.

Table 2 shows the allele frequencies of *CYP11B2* C-344T in hypertensive and normotensive subjects. In both female and male patients, there was no difference in the allele frequencies between hypertensives and normotensives.

Of 271 patients, 84 (31.0%) progressed to the primary end point (PRD). The incidence of PRD was also numerically, but not significantly, higher in patients with the CC genotype. Figure 1 shows the length of time of renal survival in each genotype. The renal survival rate was significantly worse in the CC genotype than that in other genotypes (Kaplan-Meier, log rank test, χ^2=5.208, p=0.0225).

The Cox proportional hazard regression model was used to test further the significance of variates for progressive renal dysfunction. Although in univariate analysis the CC genotype of *CYP11B2* was a significant risk factor for PRD with a hazard ratio (HR) of 2.099 (p=0.0249, 95% confidence interval (CI) 1.098 to 4.013), after adjusting for other clinical risk factors, it was not recognised as an independent risk factor in the multivariate analysis. In this multivariate analysis, urinary protein of more than 1.0 g/day (HR=3.362, p<0.0001, 95% CI 1.891 to 5.977), hypertension (HR=2.301, p=0.0007, 95% CI 1.423 to 3.721), and no ACEI/ARB therapy (HR=2.779, p=0.0002, 95% CI 1.629 to 4.740) were identified as significant and independent risk factors for PRD (table 3). These covariates were selected by stepwise backward analysis. No other clinical variables, such as gender, age, glucocorticoids, antihypertensives other than ACEI/ARB, were selected as a significant prognostic factor by this analysis.

We next investigated the significance of these risk factors and the *CYP11B2* genotype within groups for each gender (table 4). In both female and male patients, urinary protein, hypertension, and no ACEI/ARB administration were significant risk factors. In female patients, the CC genotype of the *CYP11B2* C-344T polymorphism was also recognised as an independent risk factor (HR=4.284, p=0.0022, 95% CI 1.686

Table 4 Cox proportional hazard model to test the significance of clinical covariates and genotypes of the *CYP11B2* polymorphism as predictors of renal survival within groups for each gender

Variable	p value	HR	95% CI
Female patients (n=143)			
Urinary protein excretion >1.0 g/day	0.0141	2.505	1.203 to 5.217
Hypertension	0.0073	1.918	1.259 to 3.942
No ACEI/ARB administration	0.0074	3.225	1.368 to 7.599
CC genotype of *CYP11B2*	0.0022	4.284	1.686 to 10.881
Male patients (n=128)			
Urinary protein excretion >1.0 g/day	0.0036	4.768	1.664 to 13.666
Hypertension	0.0007	3.649	1.725 to 7.718
No ACEI/ARB administration	0.0214	2.299	1.131 to 4.672
CC genotype of *CYP11B2*	0.6269	0.788	0.301 to 2.061

HR, hazard ratio; CI, confidence interval; ACEI/ARB, angiotensin-I converting enzyme inhibitor and/or angiotensin receptor blocker.

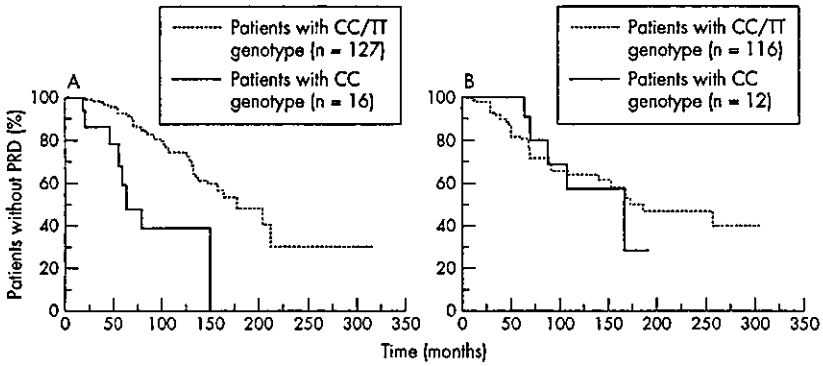

Figure 2 The renal survival rate in women (A) and men (B) with each genotype of the *CYP11B2* C-344T polymorphism. (A) Renal survival in female patients with the CC genotype (solid line, n=16) was significantly poorer than in those with other genotypes (dotted line, n=127). Log rank test, χ^2=13.748, p=0.0002. (B) In contrast, no effect of the *CYP11B2* genotype on the renal survival rate was observed in males (solid line, CC, n=12 and dotted line CT/TT genotype, n=116). Log rank test, χ^2=0.022, p=0.8832.

to 10.881). In contrast, its hazard ratio was much lower and was not significant in male patients (HR=0.788, p=0.6269, 95% CI 0.301 to 2.061). These gender specific effects of the *CYP11B2* genotype were further confirmed by the Kaplan-Meier analysis (fig 2). Although the *CYP11B2* genotype had no effect on the renal survival rate in males (fig 2B, log rank test, χ^2=0.022, p=0.8832), the renal survival in female patients with the CC genotype was significantly poorer than in those with other genotypes (fig 2A, log rank test, χ^2=13.748, p=0.0002).

DISCUSSION

This study clearly shows an association of genetic polymorphism in the promoter region of *CYP11B2* with the progression of renal dysfunction. Unexpectedly, the impact of the *CYP11B2* C-344T polymorphism was strikingly observed only in female patients, with no effect at all in males. The effect of this gene polymorphism was independent of other clinical risk factors including urinary protein excretion, hypertension, and no ACEI/ARB treatment.

We could not completely exclude the possibility that the *CYP11B2* polymorphism affected renal survival partly via an effect on the blood pressure, because, although the differences were not statistically significant, patients with the CC genotype of the *CYP11B2* polymorphism consistently tended to have higher blood pressures and a higher incidence of hypertension. However, this tendency for a higher blood pressure in the CC genotype was also observed in men, in whom no effect of the genetic polymorphism was detected. Moreover, the allele frequencies were no different between hypertensive and normotensive subjects at the time of diagnosis. These observations suggest that the *CYP11B2* C-344T polymorphism affected renal prognosis via a mechanism distinct from the regulation of blood pressure. The existence of mineralocorticoid receptors has been proven in non-epithelial tissue including vascular smooth muscle cells and endothelial cells both in experimental animals and in human.[25 26] In addition to its actions at the collecting tubule, aldosterone can also participate in pathophysiology via effects on the heart, vasculature, and kidney. Its most significant contributions to cardiovascular disease are the result of actions at these sites rather than those related to Na and water retention.[14 15 27] The role of aldosterone in the progression of renal injury has been shown,[28 29] and mineralocorticoid receptors are thought to mediate these actions including fibrogenesis/sclerosis in the glomerular mesangium.[30 31]

The mechanism by which the *CYP11B2* polymorphism affects renal survival only in female patients with IgAN is unknown. However, both in humans and animal models, it has been well documented that males have a higher blood pressure and prevalence of end stage renal disease than age matched females.[32] In the present study, a higher proportion of male patients were hypertensive as compared with female patients at the baseline, although gender was not identified as an independent risk factor for PRD and the difference was not statistically significant. The reason for this gender difference in humans is unknown, but animal models have provided insights into potential mechanisms. The sex hormones such as testosterone and androgens have been shown to have a direct effect on the renin-angiotensin-aldosterone system and on sodium reabsorption in the proximal tubule of the nephron.[33 34] Therefore, there is a possibility that the effect of male sex hormones reduces the sensitivity to the impact of the genetic polymorphism in *CYP11B2* in males. Conversely, the other possible explanation may be that female gender hormones such as oestrogen may be responsible for the increased sensitivity to the *CYP11B2* polymorphism in women. In fact, the increase in aldosterone level in response to angiotensin II infusion is greater in women than men.[35] Moreover, it has recently been reported that fluctuations in the ovarian hormones along the menstrual cycle are associated with significant variations in the homeostatic mechanisms regulating the cardiovascular system including aldosterone.[36] Unfortunately, the number of subjects in this study was insufficient to analyse female patients separately according to whether they were pre- or postmenopausal. Moreover, a substantial proportion of the female patients presumably experienced their climacteric phase during the observation, whereas the majority of them were estimated to be premenopausal, at least at the time of diagnosis, because the mean age of the patients was 37.0 (SD 13.4) years.

Recently, the aldosterone receptor antagonist, spironolactone, was reported to have a protective effect on cardiovascular diseases including glomerulonephritis.[28] The Randomized Aldactone Evaluation Study (RALES), in which a low dose of spironolactone was used as an adjuvant to conventional therapy with an ACEI, loop diuretic with or without digitalis, showed a 30% reduction in the overall risk of mortality that could not be accounted for by a blood pressure reduction or fluid loss.[37] The result of this study may suggest that the therapeutic efficacy of anti-aldosterone agents can be enhanced by more selective and active usage in female patients with the particular genotype of *CYP11B2* C-344T.

Although genetic polymorphism of *CYP11B2* C-344T has been reported to be associated with the serum level of aldosterone, as well as the left ventricular size, its function, and myocardial infarction independently of blood pressure,[21–23] the limitation of this study is that the association between the level of aldosterone and disease progression could not be

tested, because we have no data on the level of circulating or local tissue activity of aldosterone in each genotype group.

It also remains to be seen if the effect of the genetic variant investigated in the present study is observed in other ethnic groups. The allele frequency of *CYP11B2* C-344 in a white population was reported as 0.47 to 0.52.[22] This is higher than that in the Japanese population, which was 0.34 in this study. As it is well known that a polymorphism with a higher allele frequency has more statistical power in an association study,[38] investigations in other ethnic groups may provide further evidence for the role of this genetic polymorphism in the progression of renal dysfunction.

In conclusion, the present study provides the first evidence for a gender specific association between the *CYP11B2* C-344T polymorphism and the prognosis of renal function in Japanese patients with IgAN. Although the genotype has no influence on renal survival in men, the CC genotype of *CYP11B2* is a possible predictive genetic marker for progression of renal dysfunction in women.

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平成14年度～16年度　総合研究報告書（2/2冊）

主任研究者　林　松彦

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目次

Interaction Between *ACE* and *ADD1* Gene Polymorphisms in the Progression of IgA Nephropathy in Japanese Patients

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Abstract—An interaction effect between the angiotensin-converting enzyme insertion/deletion (*ACE I/D*) and α-adducin (*ADD1*) Gly460Trp polymorphisms (*G460W*) on blood pressure regulation has recently been suggested, although its significance in the prognosis of renal function in IgA nephropathy (IgAN) has not been fully investigated. Therefore, we evaluated the clinical manifestations and renal prognosis in 276 Japanese patients with histologically proven IgAN with respect to their *ACE I/D* and *ADD1 G460W* polymorphisms. The prognosis of renal function was analyzed by Kaplan-Meier survival curves and multivariate Cox proportional-hazards regression models. Baseline data, including blood pressures, proteinuria, renal function, and incidence of hypertension, were similar for the different genotypes of *ACE* and *ADD1*. The individual genotypes taken alone were not associated with the progression of renal dysfunction. However, renal survival of patients with the *460WW* polymorphism of *ADD1* was significantly worse within the group with the *II* genotype of *ACE* (Kaplan-Meier, log rank test; $\chi^2=6.062$, $P=0.0138$) but not for those with other *ACE* genotypes. In the Cox proportional-hazards regression model with adjustment for clinical risk factors, including hypertension, proteinuria, and no administration of an angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, the *460WW* variant of *ADD1* was a highly significant and independent risk factor only for patients with the *ACE II* genotype, with a hazard ratio of 3.65 ($P=0.0016$), but not for those with other *ACE* genotypes (hazard ratio=0.65, $P=0.2902$). These findings suggest an interaction between *ACE* and *ADD1* polymorphisms not only on blood pressure regulation but also on the progression of renal dysfunction in patients with IgAN. (***Hypertension.* 2003; 42:304-309.**)

Key Words: angiotensin-converting enzyme ■ blood pressure ■ hypertension, genetic ■ kidney failure ■ polymorphism ■ renal disease ■ renin-angiotensin system

Immunoglobulin A nephropathy (IgAN), the most prevalent form of primary glomerulonephritis, is one of the major causes of end-stage renal disease (ESRD).[1] IgAN has a variable clinical course,[2,3] and the mechanisms of interindividual differences in the rate of disease progression are still unclear. Poor prognostic factors for the progression of renal dysfunction in IgAN have been identified as high blood pressure, marked proteinuria, and a severe histopathologic appearance of the renal biopsy specimen.[4,5] In addition to these prognostic risk factors, several genetic backgrounds have been proposed to be associated with a susceptibility to ESRD in patients with IgAN.[6,7] Recently, interactions among multiple genetic variants of complex traits, including blood pressure regulation as well as the prognosis of kidney disease, have been suggested.

Among the genetic polymorphisms proposed to date, an insertion/deletion (*I/D*) polymorphism of the angiotensin-converting enzyme (*ACE*) gene has attracted much attention and is the most studied, because renin-angiotensin system (RAS) activity is an important regulator of blood pressure and plays a central role in cardiovascular and renal diseases. Many previous studies have suggested a significant association between the *D* allele and functional deterioration of the kidney.[8–10] However, these studies had common limitations, in that they only evaluated small numbers of patients. Larger-scale studies and a meta-analysis have reported rather negative results on the association between *ACE I/D* and progression of renal disease in both white and Japanese populations.[11–13]

The α-adducin gene, which encodes an actin accessory and calmodulin-binding protein, is another candidate that has been suggested to affect blood pressure by regulating renal sodium reabsorption.[14] In human α-adducin (*ADD1*), a single-nucleotide polymorphism accompanied by an amino acid substitution of tryptophan (W) in place of glycine (G) at residue 460 (*G460W*) has been implicated in the pathogenesis of salt-sensitive and low-renin hypertension.[15–17] Recently, an epistatic or synergistic interaction of the *ADD1 G460W*

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polymorphism with *ACE I/D* has been suggested for both blood pressure regulation and renal disease progression[18–20] in white populations. On the other hand, negative results on the significance of both the *ADD1 G460W* polymorphism alone and its interaction with *ACE I/D* on blood pressure and renal disease have been reported in multiple ethnic groups.[21–23]

Taken together, genetic polymorphisms of *ACE I/D* and *ADD1 G460W* have been suggested, but not confirmed, as candidate markers for the progression of renal disease, and their possible synergistic interaction also remains to be fully investigated in IgAN. Previous studies on this matter included various kidney diseases. Therefore, the aim of this study was to evaluate the role of the *ADD1 G460W* gene polymorphism, as well as its possible synergistic interaction with the *ACE I/D* gene polymorphism, on the prognosis of renal function in Japanese patients with histologically proven IgAN.

Methods

Subjects and Clinical Data

The ethics committee of our institution (Niigata University Graduate School of Medical and Dental Sciences) approved the protocol for the genetic study. Japanese 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 2001; (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 at our institute; and (4) written, informed consent for the genetic study was obtained. Patients who received immunosuppression therapy other than corticosteroids were excluded from the analysis. In total, 276 patients were analyzed. In all cases, the diagnosis of IgAN was based on immunofluorescence microscopy of a kidney biopsy specimen, which showed dominant or codominant 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/d), serum creatinine level (sCr, mg/dL), and 24-hour creatinine clearance (CCr, mL/min) were retrospectively investigated. Hypertension was defined by the use of 1 or more antihypertensive medications and/or a blood pressure ≥140 mm Hg systolic or 90 mm Hg diastolic. The primary end point (progressive renal disease) 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. The mean duration of observation was 93.0±67.3 months. Administrations of glucocorticoids, antihypertensive agents, angiotensin-converting enzyme inhibitors (ACEIs), and angiotensin II receptor blockers (ARBs) was also recorded for each patient.

DNA Isolation and Genotyping

Genomic DNA was isolated from peripheral blood cells by an automatic DNA isolation system (NA-1000, Kurabo). The *I/D* polymorphism in intron 16 of the *ACE* gene was assessed by polymerase chain reaction (PCR) under conditions that have been previously described.[24] Because of preferential amplification of the *D* allele compared with the *I* allele, DNA from subjects with the *DD* genotype was reexamined with an *I* allele–specific primer (5′-TTT GAG ACG GGA GTC TCG CTC-3′) to avoid mistyping *ID* heterozygotes as *DD* homozygotes.[25]

Genotyping of the *ADD1 G460W* polymorphism was determined by allele-specific oligonucleotide hybridization after PCR amplification. The forward primer and biotin-labeled reverse primer for PCR were 5′-AGA CAA GAT GGC TGA ACT CTG G-3′ and 5′-CAC ACC TTA GTC TTC GAC TTG G-3′, respectively. The PCR mixture (25 μL) contained 50 ng DNA, 5 pmol of each oligonucleotide primer, 0.2 mmol/L ddNTPs, 2.5 mmol/L $MgSO_4$, and 1 U DNA polymerase (KOD plus, Toyobo) in KOD buffer. The amplification protocol consisted of 1 cycle at 94°C for 5 minutes, followed by 40 cycles of denaturation at 93°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 2 minutes. The amplified 88-bp DNA fragment was denatured with NaOH and hybridized with allele-specific capture probes fixed to the bottom of a microtiter plate at 57.5°C for 30 minutes. Specific probes for each single-nucleotide polymorphism were 5′-GAA GGG CAG AAT GGA AGC A-3′ and 5′-GAA TGG CAG AAT GGA AGC A-3′. After being washed, alkaline phosphatase–conjugated streptavidin was added to each well, and the plate was incubated for 15 minutes at 37°C. Then 4-methoxy-4-(3-phosphatephenyl)-*spiro*-(1,2-dioxetan 3,2′-adamantane), a substrate for alkaline phosphatase, was added, and luminescence measured by an automated chemiluminescence assay system (Toyobo).

Statistical Analysis

Hardy-Weinberg equilibrium was tested by the χ^2 test with 1 *df*. χ^2 Analysis was also used when comparing allele frequencies and categorical variables between the groups. Continuous variables were expressed as mean±SD or percentage according to clinical features. When the baseline characteristic was continuous (eg, age, blood pressures, duration of observation, urinary protein, sCr, and CCr), the Kruskal-Wallis test and Mann-Whitney *U* test were used. The Kaplan-Meier method and the Cox proportional-hazards regression model were used to analyze the time course from renal biopsy to end point. When overall survival was significantly different in the Kaplan-Meier analysis, Greenwood's estimation was performed at every 12 months of observation to test at which time point the difference between groups become significant. In the Cox regression model, we tested covariates (age, sex, urinary protein, hypertension, steroid therapy, administration of ACEI and/or ARB, 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 (HR) and 95% confidence interval (CI). Statistical analysis was performed with Statview 5.0J software (SAS Institute, Inc) on a personal computer (Apple Macintosh G4). A value of $P<0.05$ was considered significant.

Results

Demographic Data at the Time of Diagnosis and During Observation

Two hundred seventy-six patients with histologically proven IgAN were all genotyped for *ADD1 G460W* and *ACE I/D* polymorphisms. The genotype frequencies of *ADD1* were *GG* (n=56), *GW* (n=150), and *WW* (n=70), and those of *ACE* were *II* (n=106), *ID* (n=135), and *DD* (n=35). The allele frequencies of *ADD1 460G* and *W* were 0.475 and 0.525, and those of *ACE I* and *D* were 0.629 and 0.371, respectively. These findings are compatible with previous reports of a general Japanese population.[26–28] The expected genotype frequencies of heterozygotes of *ADD1* and *ACE*, according to Hardy-Weinberg equilibrium, were no different from those observed in this study.

The clinical characteristics of the patients at the time of diagnosis and during the observation period are listed in Table 1. No difference was noted among patients with each genotype of *ADD1 G460W* with respect to gender, age, blood pressure, urinary protein excretion, sCr, and CCr. Systolic blood pressure and incidence of hypertension at baseline tended to be higher in patients with *ADD1 460WW* than in those with other genotypes, but the difference was not significant. During the observation period of 93.0±67.3 months, 31.2% (86 patients) reached the end point (progres-

TABLE 1. Clinical Data of the Subjects at the Time of Diagnosis and During Observation

		Genotype of *ADD1G460W*				Genotype of *ACE/D*			
	All Patients (N=276)	*GG* (n=56)	*GW* (n=150)	*WW* (n=70)	*P*	*II* (n=106)	*ID* (n=135)	*DD* (n=35)	*P*
At time of diagnosis									
Gender, male, %	46.7	33.9	50.7	48.6	0.0946	50.9	43.7	45.7	0.5307
Age, y	37.0±13.4	36.4±12.7	36.7±13.5	38.0±13.7	0.8001	37.9±13.6	36.7±13.5	34.8±12.2	0.4888
SBP, mm Hg	127.9±18.4	127.0±20.2	127.3±18.0	129.7±17.8	0.4644	129.6±18.4	127.7±18.8	123.3±16.2	0.2348
DBP, mm Hg	77.3±13.5	77.1±15.2	77.1±12.8	77.9±13.5	0.5972	78.5±13.4	76.7±13.8	75.7±12.1	0.6065
UP, g/d	1.3±1.3	1.2±1.2	1.4±1.4	1.3±1.2	0.8720	1.3±1.6	1.2±1.1	1.3±1.1	0.2443
sCr, mg/dL	1.0±0.6	1.0±0.9	1.0±0.4	1.0±0.7	0.1395	1.0±0.6	1.0±0.7	1.0±0.3	0.3470
CCr, mL/min per 1.73 m^2	88.9±33.1	88.4±32.4	91.1±35.0	84.8±29.3	0.4964	85.6±36.3	90.6±31.6	92.2±26.6	0.3778
Hypertension, %	35.9	30.4	34.7	42.9	0.3135	38.7	35.6	28.6	0.5543
During observation									
Observation, mo	93.0±67.3	90.2±65.1	94.0±70.6	92.8±62.3	0.9442	91.5±59.5	96.7±72.8	83.1±68.8	0.5013
Reached end point, %	31.2	26.8	32.7	31.4	0.7141	34.0	28.9	31.4	0.6998
Mean SBP, mm Hg	128.6±17.0	128.2±19.5	129.2±16.3	127.9±16.3	0.6650	131.0±16.51	127.6±17.61	125.6±15.4	0.1528
Mean DBP, mm Hg	77.5±11.8	78.3±13.5	77.9±11.1	76.3±12.0	0.7389	79.8±11.67	75.9±12.0	77.0±11.2	0.4285
Corticosteroids, %	28.3	30.3	28.0	27.1	0.7885	26.4	28.1	34.3	0.6684
ACEI/ARB, %	40.9	35.7	43.3	40.0	0.6001	37.7	40.0	54.3	0.2146

UP indicates urinary protein excretion. All other abbreviations are the same as in text.

sive renal disease). There was no difference for each genotype in observation duration, incidence of end point, mean blood pressure, incidence of corticosteroid treatment, and ACEI/ARB administration. As also shown in Table 1, among patients with each *ACE* genotype, no difference was noted in any clinical characteristic both at the time of diagnosis and during observation.

To investigate a possible interaction between the *ADD1* and *ACE* polymorphisms, analyses were performed by subdividing the patients according to *ACE* genotype. Because the *ACE I/D* polymorphism has been reported to be associated with some clinical phenotypes, such as hypertension and cardiovascular diseases, with an additive effect of the *D* allele[27] and because the frequency of the *ACE D* allele in our population was too low to separately analyze patients who were *DD* homozygotes, the patients were divided into 2 groups: patients who were *II* homozygotes and those who were heterozygous or homozygous for the *D* allele. Table 2 shows comparisons of clinical data between patients with each combined genotype. In patients with the *ACE II* genotype, hypertension at the time of diagnosis was more frequently observed (χ^2=4.350, *P*=0.0370), and observation duration was shorter (*P*=0.0412) in the *WW* genotype of *ADD1* than in other genotypes. However, these differences were not observed in patients with *ACE ID* or *DD* genotypes (χ^2=0.015, *P*=0.9024).

ADD1 Polymorphism as a Risk Factor for Progression of Renal Dysfunction

Figure 1A shows the renal survival rate of patients with each *ADD1* genotype for all patients studied. There was no difference between them. However, when the analysis was performed for a subgroup of patients with the *ACE II* genotype, the prognosis of renal function in patients with *ADD1 460WW* was significantly worse than in those with other genotypes (Figure 1B; Kaplan-Meier, log rank test, χ^2=6.062, *P*=0.0138). The difference in survival rate was statistically significant after 7 years of observation. The survival rates of patients with *ADD1 WW* and *GW/GG* at 84 months were 53.0±11.0% and 79.3±5.0%, respectively (*P*=0.0307 by Greenwood's estimation). In contrast, in patients with *ACE ID* or *DD*, patients with *460 WW* tended to have a better survival curve, but the difference was not significant (Figure 1C; χ^2=2.238, *P*=0.1386).

The prognostic significance of the *ADD1 460WW* genotype on the advance to the progressive renal disease end point was further evaluated after adjusting for other clinical risk factors by the multivariate Cox proportional-hazards regression model (Figure 2). In all patients, significant risk factors were identified, including urinary protein >1.0 g/d, hypertension, and no administration of ACEI/ARB, whereas the *460WW* variant of *ADD1* had no prognostic influence on renal survival (Figure 2A). These 3 clinical risk factors were significant in both groups with *ACE II* and those with *ID* or *DD* genotypes, with the exception that the significance of no administration of ACEI/ARB was much higher in patients with the *II* genotype. *ADD1 460WW* was found to be an independent risk factor only for the group with the *ACE II* genotype (Figure 2B; HR, 3.65; 95% CI, 1.63 to 8.20; *P*=0.0016). In contrast, in the group with the *ID* or *DD* variant of *ACE*, the hazard ratio of the *460WW* variant of *ADD1* was much lower and not significant (Figure 2C; HR, 0.65; 95% CI, 0.29 to 1.45; *P*=0.2902).

Discussion

This study shows the significance of the *ADD1 G460W* polymorphism on the progression of renal dysfunction in Japanese patients with IgAN, which was specific in patients