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Genotype-phenotype correlations influence the response to angiotensin-targeting drugs in Japanese patients with male X-linked Alport syndrome

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Tomohiko Yamamura[1], Tomoko Horinouchi[1], China Nagano[1], Takashi Omori[2], Nana Sakakibara[1], Yuya Aoto[1], Shinya Ishiko[1], Koichi Nakanishi[3], Yuko Shima[4], Hiroaki Nagase[1], Hiroki Takeda[1], Rini Rossanti[1], Ming Juan Ye[1], Yoshimi Nozu[1], Shingo Ishimori[1], Takeshi Ninchoji[1], Hiroshi Kaito[1], Naoya Morisada[1], Kazumoto Iijima[1] and Kandai Nozu[1]

[1]Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan; [2]Clinical and Translational Research Center, Kobe University Hospital, Kobe, Hyogo, Japan; [3]Department of Child Health and Welfare (Pediatrics), Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan; and [4]Department of Pediatrics, Wakayama Medical University, Wakayama, Japan

Early kidney failure in the hereditary type IV collagen disease, Alport syndrome, can be delayed by renin-angiotensin inhibitors. However, whether all patients and all different genotypes respond equally well to this kidney-protective therapy remains unclear. Here, we performed a retrospective study on 430 patients with male X-linked Alport syndrome to examine the relationships among kidney prognosis, genotype, and treatment effect in a large cohort of Japanese patients. We analyzed the clinical features, genotype-phenotype correlation, and kidney survival period for patients treated with or without renin-angiotensin inhibitors. As a result, the median kidney survival period of patients in this cohort was found to be at 35 years with a strong genotype-phenotype correlation. The median age at the onset of end stage kidney disease (ESKD) significantly differed between patients treated with and without renin-angiotensin inhibitors (over 50 years *versus* 28 years, respectively). Moreover, these drugs delayed the onset of ESKD in patients with truncating variants for 12 years, extending the median age from 16 years to 28 years. Thus, our results confirmed a strong genotype-phenotype correlation in patients with male X-linked Alport syndrome. Additionally, it was suggested that renin-angiotensin inhibitors could significantly delay ESKD progression. Despite these therapies, patients with truncating variants developed ESKD at the median age of 28 years.

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KEYWORDS: ACE inhibitor; genotype-phenotype correlation; X-linked Alport syndrome

Correspondence: *Kandai Nozu, Department of Pediatrics, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo, Kobe, Hyogo 650-0017, Japan. E-mail: nozu@med.kobe-u.ac.jp*

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Alport syndrome is a hereditary kidney disease characterized by progressive renal dysfunction, sensory hearing loss, and ocular abnormalities.[1-3] Alport syndrome is caused by defects in the type IV collagen network, a major structural component of basement membranes in the kidney, inner ear, and eye. Six genetically distinct type IV collagen α-chains (α1–α6) have been identified. Pathogenic variants in the *COL4A5* gene, which encodes the type IV collagen α5 chain, are known to cause X-linked Alport syndrome (XLAS); XLAS is present in approximately 85% of patients with Alport syndrome.[4]

Because of the X-linked mode of inheritance, affected male patients with XLAS exhibited more severe phenotypes than the female ones; strong genotype-phenotype correlations in patients with male XLAS have been identified in populations from Europe and the United States.[5-7] In these studies, patients with missense mutations or small in-frame mutations showed less severe phenotypes, compared with patients who had truncating mutations (e.g., nonsense mutation, small insertion, or deletion leading to premature stop codon). In addition, patients with splice site mutations have shown intermediate severity, between the severities of nontruncating and truncating mutations. Our recent Japanese cohort study has focused on the differences between truncating and nontruncating variants at the transcript level in splice site mutations; the results of our study showed that renal prognosis significantly differed between patients with truncating and nontruncating splicing abnormalities (respective median renal survival periods of 20 and 29 years; $n = 21$ and $n = 25$).[8] On the basis of these findings, patients with male XLAS who have nontruncating variants (i.e., missense or in-frame variants) have exhibited less severe phenotypes, compared with patients who have truncating variants (i.e., nonsense or

Table 1 | Clinical characteristics of 282 male patients with X-linked Alport syndrome at the time of genetic diagnosis

Characteristics		
Median age (yr) at genetic diagnosis (range)	13 (0–73)	
Clinical features	*N* with data	*n* (%)
Hematuria	264	262 (99)
Proteinuria	262	238 (91)
End-stage renal disease	282	61 (22)
Hearing loss	238	77 (32)
Ocular changes	229	13 (6)

frameshift variants). These apparent differences in disease severity between patients with truncating variants and those with nontruncating variants are also determined in other inherited diseases (e.g., Duchenne muscular dystrophy and Becker muscular dystrophy).[9] Examining the genotype-phenotype correlation in patients with male XLAS is vital to estimate renal prognosis, perform genetic counseling, and guide the treatment of the affected patients.

Although there is no specific treatment for Alport syndrome, renal protective effects of treatment with angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB) have been demonstrated in several relatively large cohorts.[10-12] Therefore, experts in this field have recommended the treatment with ACEI/ARB after diagnosis in patients with male XLAS.[13,14] However, to the best of our knowledge, there has been no analysis yet examining the correlation between genotype and effects of ACEI/ARB treatment. Correlations have also been reported between clinical or pathogenic features (e.g., hearing loss or α5 expression on the glomerular basement membrane) and renal prognosis in patients with male XLAS (e.g., hearing loss or α5 expression on the glomerular basement membrane).[15,16] However, these studies were found to be relatively small, and further studies with larger cohorts should be performed to confirm these findings.

Here, we conducted a large-scale retrospective study of Japanese patients with male XLAS; this aims to evaluate the relationships between renal prognosis and genotype as well as between renal prognosis and various clinical features (e.g., use of ACEI and/or ARB, hearing loss, or α5 expression on the glomerular basement membrane). In addition, we performed a detailed analysis of the genotype-phenotype correlation based on gene transcripts and genotype-dependent differences in response to ACEI/ARB treatment.

RESULTS

General information

Mutation and clinical data were obtained from the total 430 participants (282 male patients and 148 affected male family members from 269 families). The clinical characteristics identified only in patients with genetic diagnoses (at the time of genetic diagnosis) are shown in Table 1. The median age at the time of genetic testing was determined to be at 13 years (range, 0–73 years). Proteinuria was detected in 238 patients (91%). Around 61 of the 282 patients and 110 of the 148 affected male family members were found to develop end-stage renal disease (ESRD). Specific ocular changes were only detected in 13 patients (6%), whereas hearing loss was detected in 77 patients (32%). The mutational characteristics for all 430 participants are presented in Table 2.

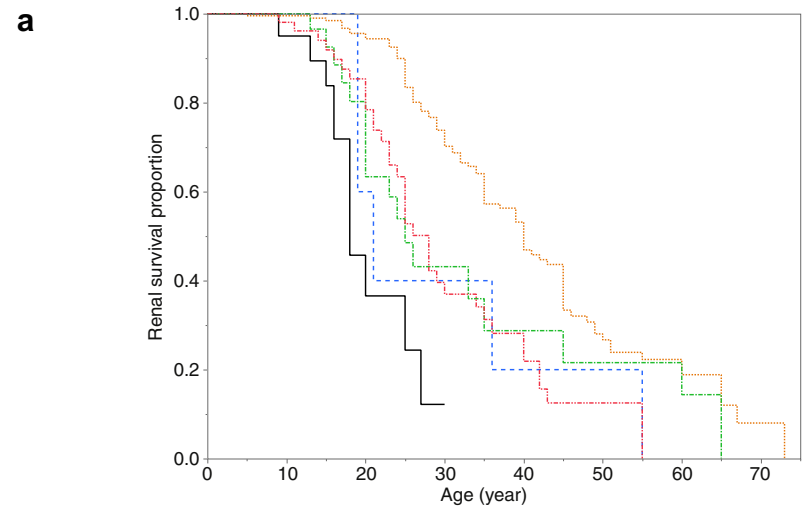
Genotype-phenotype correlation

Eight participants were excluded from further analyses because of missing data. The median renal survival period (age at the development of ESRD) of the remaining 422 participants with male XLAS was 35 years (95% confidence intervals: 32–40 years). Figure 1 presents the renal survival curves based on mutation type and transcript variants. The

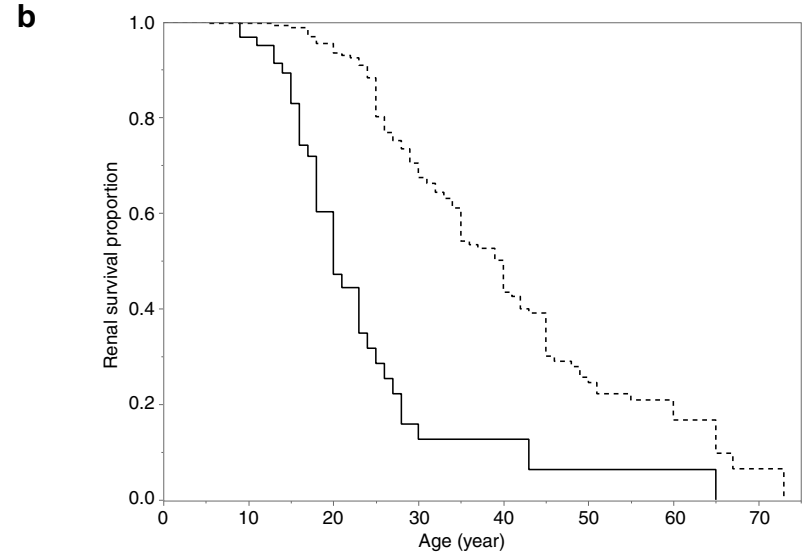
Table 2 | Mutational and clinical characteristics of 282 male patients with X-linked Alport syndrome and 148 affected male family members from 269 families

			Transcript type		Glomerular basement membrane		ACEI/ARB treatment	
Characteristics	*N* with data	*n* (%)	Truncating	Nontruncating	α5(IV)-positive	α5(IV)-negative	ACEI/ARB (+)	ACEI/ARB (−)
Mutation types in patients	430							
Nonsense mutation		30 (7.0)	30	0	1	11	12	7
Large rearrangement		14 (3.3)	–	–	3	5	6	5
Splicing variant		71 (16.5)	32	36	8	17	28	12
Small deletion/insertion/duplication		65 (15.1)	21	44	7	24	24	18
Missense mutation		250 (58.1)	0	250	45	25	62	44
Mutation types in families	269							
Nonsense mutation		19 (7.1)						
Large rearrangement		13 (4.8)						
Splicing variant		49 (18.2)						
Small deletion/insertion/duplication		44 (16.4)						
Missense mutation		144 (53.5)						

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.



Number of patients								
Nonsense mutation	30	19	5	1				
Large deletion/duplication	14	11	3	2	1			
Splicing variant	70	51	37	15	9	1		
Small deletion/insertion/duplication	64	35	19	6	4	3	3	
Missense mutation	244	195	159	102	51	21	13	3



Number of patients								
Nontruncating variants	296	237	193	117	60	23	15	3
Truncating variants	106	59	23	5	2	1	1	

Figure 1 | Renal survival proportion based on mutation type and transcript variant. (**a**) Renal survival curves for each genotype. From left: (i) solid line (black), nonsense mutation ($n = 30$); (ii) dashed line (blue), large rearrangement ($n = 14$); (iii) long dashed double-dotted line (red), splicing variant ($n = 70$); (iv) long dashed dotted line (green), small rearrangement ($n = 64$); (v) dotted line (orange), missense mutation ($n = 244$). There were significant differences between patients with missense mutation and all other groups ($P < 0.01$). The median renal survival periods of patients with each genotype are indicated in Table 3. (**b**) Renal survival curves based on transcript type. The solid line indicates patients with truncating variants ($n = 106$); the median age for the development of end-stage renal disease was 20 years in these patients. The dashed line indicates patients with nontruncating variants ($n = 296$); the median age for the development of end-stage renal disease was 40 years in these patients. There was a significant difference between 2 groups ($P < 0.001$) with the hazard ratio of 4.443 (95% confidence interval: 3.027, 6.525). Exact *P* values and hazard ratios are shown in Supplementary Table S4.

median age at the onset of ESRD was found to differ significantly among patients, based on genotype differences ($P < 0.01$) (Table 3). Specifically, differences between patients with missense mutation and all other genotypes were determined to be highly significant (Supplementary Table S1). However, no significant differences between patients with large

Table 3 | Comparison of renal prognosis between patients with truncating mutations and those with nontruncating mutations

	Median age for development of end-stage renal disease[a]			
Genotype (*n*)	All (95% CI)	Truncating (95% CI, *n*)	Nontruncating (95% CI, *n*)	*P* value
All	35 (32–40)	20 (18–23, 106)	40[e] (35–42, 296)	<0.01
Nonsense mutation (30)	18 (16–27)	18 (16–27, 30)	–	
Large deletion/duplication (14)	21 (19–55)	–	–	
Splicing variant (65[b])	25 (23–30)	23 (16–28, 32)	28 (25–36, 33)	<0.05
Small deletion/insertion/duplication (63[c])	26 (20–45)	20 (15–24, 44)	35 (25–65, 19)	<0.05
Missense mutation (244)	40 (35–45)	–	40 (35–45, 244)	
P value[d]	<0.01			

CI, confidence interval.
[a]The median age for developing of end-stage renal disease was determined using the Kaplan-Meier method.
[b]5 patients were excluded because 2 patients had somatic mosaicism, 1 patient showed both normal and aberrantly spliced transcript, and transcript was not analyzed in 2 patients.
[c]One patient was excluded because he was suspected as having aberrant splicing by small rearrangement but mRNA was not analyzed.
[d]There were significant differences between the patients with missense mutation and all other genotypes in the renal survival period.
[e]The median age for developing end-stage renal disease of patients with nontruncating variants was the same as patients with missense mutation because the number of patients with missense mutation was much greater than patients with other genotypes, as shown in the table.

rearrangement, splicing variant, and small rearrangement were noted. The median renal survival periods for patients with each genotype (95% confidence intervals, numbers of patients) were as follows: nonsense mutation, 18 years (16–27 years, $n = 30$); large rearrangement, 21 years (19–55 years; $n = 14$); splicing variant, 28 years (23–34 years, $n = 69$); small rearrangement, 25 years (20–45 years, $n = 64$); and missense mutation, 40 years (35–45 years, $n = 245$) (Figure 1a; Table 3).

The classification of splicing variants or small rearrangements as truncating or nontruncating variants revealed that, for each genotype, truncating variants were significantly more severe than nontruncating ones (Table 3). The median renal survival period for patients with truncating splicing variants was 23 years (16–28 years, $n = 32$); for patients with nontruncating splicing variants, the median renal survival period was 28 years (25–36 years, $n = 33$) ($P < 0.05$). For participants with small rearrangements (e.g., small deletions and insertions), the median renal survival period for patients with truncating small rearrangements was 20 years (15–24 years, $n = 44$); meanwhile for patients with nontruncating small rearrangements, the median renal survival period was 35 years (25–65 years, $n = 19$) ($P < 0.01$) (Supplementary Figures S1 and S2; Table 3). The classification of all variants as truncating or nontruncating revealed a significant difference in the median age at which ESRD developed between these 2 groups (20 years vs. 40 years; $n = 106$ and $n = 295$, respectively) ($P < 0.01$) (Figure 1b; Table 3).

Other clinical features and renal prognosis

A comparison of renal prognosis based on the presence or absence of hearing loss has revealed a significant difference in the median age at which ESRD developed between these 2 groups (28 years vs. 55 years; $n = 77$ and $n = 161$, respectively; $P < 0.01$) (Figure 2a). Immunostaining to determine the presence of α5 in the glomerular basement membrane was obtained from 146 patients. Among these patients, 64 patients showed to express α5, whereas 82 patients did not. The mutational characteristics of each group are shown in Supplementary Table S2. Genotype analysis revealed that 47 of the 82 (57.3%) patients in the α5-negative group had truncating mutations; in contrast, only 3 of the 64 (4.7%) patients in the α5-positive group had truncating mutations. In addition, renal survival curves based on the expression of α5 in the glomerular basement membrane revealed that the age at the onset of ESRD was significantly lower in the α5-negative group compared with the α5-positive group (29 years vs. >50 years, $n = 82$ and $n = 64$, respectively; $P < 0.01$) (Figure 2b).

Treatment effect of ACEI/ARB

A total of 207 patients were examined for the presence or absence of ACEI/ARB treatment. To assess the renal protective effects of these drugs, we compared renal survival curves for patients with ($n = 126$) and without ($n = 81$) these treatments. Our findings revealed that the renal survival period was significantly longer for patients with ACEI/ARB treatment than for patients without ACEI/ARB treatment (>50 years vs. 28 years, $P < 0.01$) (Figure 3). In addition, renal survival periods significantly differed between patients with nontruncating variants and those with truncating variants. Among patients with nontruncating mutations ($n = 132$), the median renal survival period of patients without ACEI/ARB treatment was 33 years (95% confidence interval: 28–73 years, $n = 56$). In contrast, more than half of patients with ACEI/ARB treatment did not develop ESRD until the age of 50 years ($n = 76$; $P < 0.05$) (Figure 4). For patients with truncating mutations ($n = 75$), the median renal survival periods were 16 years in patients without ACEI/ARB treatment (9–23 years, $n = 25$) and 28 years in patients with ACEI/ARB (20–43 years, $n = 50$; $P < 0.01$) (Figure 4). In addition, among patients with ACEI/ARB treatment, a significant difference between patients with truncating and nontruncating variants ($P < 0.001$) was noted (Supplementary Table S1).

DISCUSSION

In this study, we examined the genetic and clinical characteristics of a large cohort of Japanese patients with male XLAS

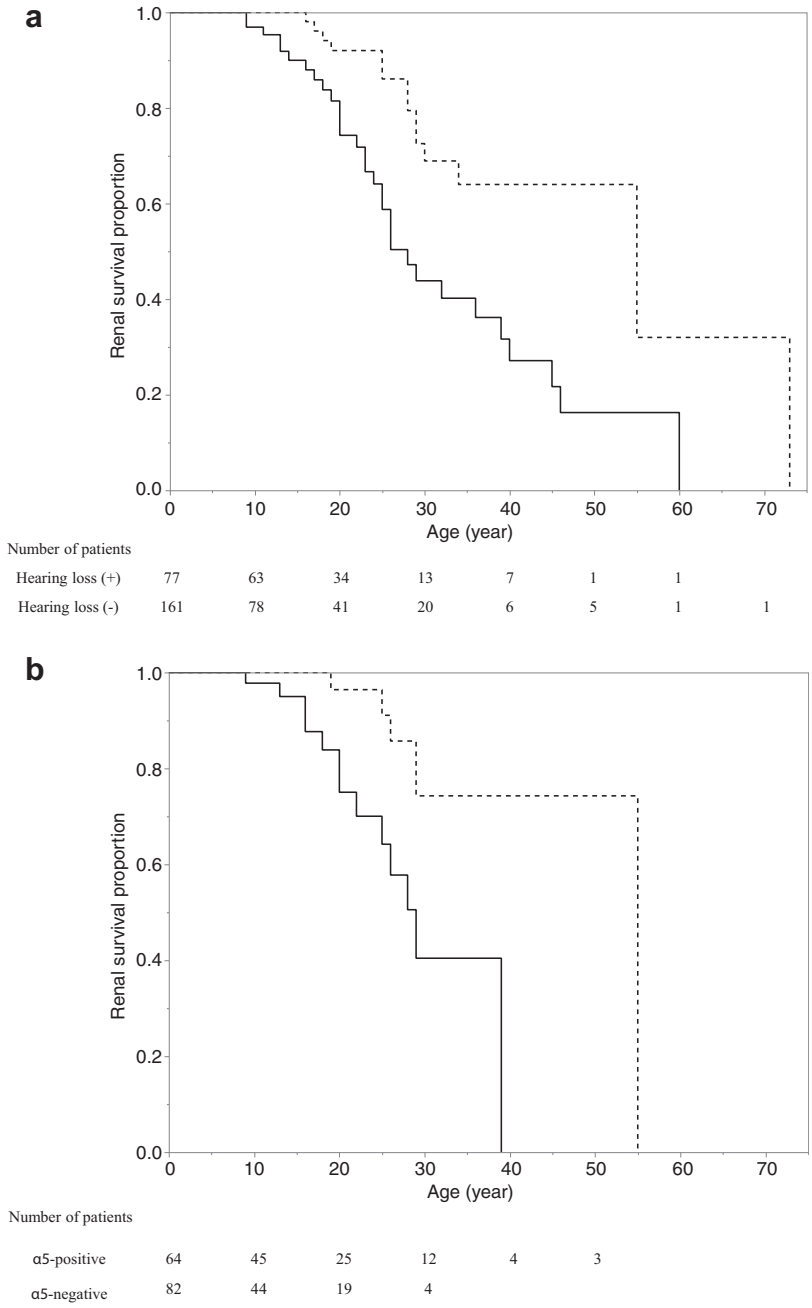


Figure 2 | Renal survival proportion based on other clinical features. (**a**) Correlation between the presence or absence of hearing loss and renal prognosis. The solid line indicates patients with hearing loss at the time of genetic diagnosis ($n = 77$); the median age for the development of end-stage renal disease (ESRD) was 28 years (95% confidence interval [CI]: 24–39 years) in these patients. The dashed line indicates patients without hearing loss ($n = 161$); the median age for the development of ESRD was 55 years (95% CI: 34–73 years) in these patients. There was a significant difference between 2 groups ($P = 0.0004$) with the hazard ratio of 3.037 (95% CI: 1.586–5.817, $P = 0.0008$). (**b**) Correlation between α5 expression in the glomerular basement membrane and renal prognosis. The solid line indicates α5-negative patients ($n = 82$); the median age for the development of ESRD was 29 years (95% CI: 22–39 years) in these patients. The dashed line indicates α5-positive patients ($n = 64$); the median age for the development of ESRD was 55 years (95% CI: 29–55 years). There was a significant difference between 2 groups ($P = 0.0035$) with the hazard ratio of 4.528 (95% CI: 1.588, 12.914, $P = 0.0047$).

who had proven *COL4A5* variants. Our results demonstrated a strong genotype-phenotype correlation, as previously observed in Western cohorts. In addition, our study replicated the improved outcome in male XLAS patients treated with ACEI and/or ARB. To the best of our knowledge, our study is the first to reveal a difference in the renal protective effects of these nephroprotective drugs between patients with non-truncating variants and patients with truncating variants (delay from 33 to >50 years vs. delay from 16 to 28 years). Jais *et al.*[6] reported that the median renal survival period of

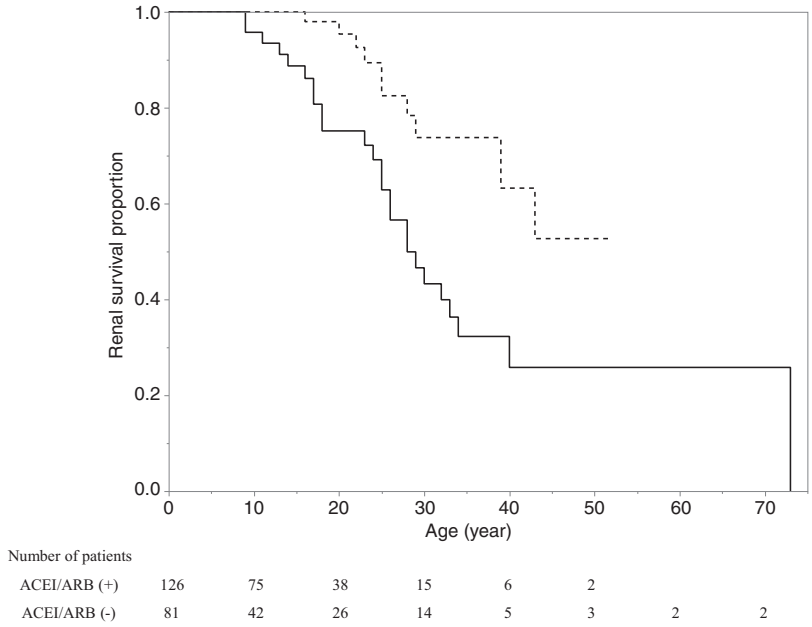
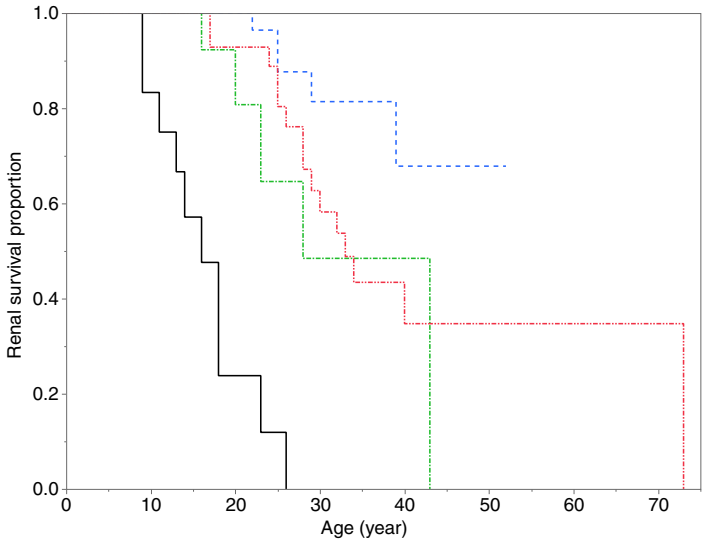


Figure 3 | Renal survival proportion based on the presence of angiotensin-converting enzyme inhibitor/angiotensin receptor blocker (ACEI/ARB) treatment. The solid line indicates patients not treated with ACEIs/ARBs at the time of genetic diagnosis ($n = 81$); the median age for the development of end-stage renal disease (ESRD) was 28 years (95% confidence interval [CI]: 25–34 years) in these patients. The dashed line indicates patients treated with ACEI/ARB at the time of genetic diagnosis ($n = 126$); most patients did not develop ESRD by the age of 50 years. There was a significant difference between 2 groups ($P = 0.0004$) with the hazard ratio of 3.099 (95% CI: 1.479, 6.491, $P = 0.0027$).

affected patients with male XLAS with any genotype was 25 years. In contrast, the total median renal survival period in our cohort was 35 years, which was considerably longer than in the previous report. This difference is presumably caused by renin-angiotensin system blockade; 48% of our cohort (207 of the 430 participants) received ACEI and/or ARB treatment at the time of genetic diagnosis.

A strong genotype-phenotype correlation has been previously reported in patients with male XLAS in a Western cohort; in particular, patients who had missense mutations showed a relatively mild phenotype, compared with patients who had truncating mutations (e.g., nonsense mutations and small deletions/insertions).[5-7] Although the renal prognosis of patients with splice site mutations in the prior studies showed intermittent severity (i.e., between the severities of missense and nonsense mutations), transcriptional analysis was not performed because of the difficulty of this technique. Recently, our group revealed that patients who had splicing variants that produce truncating transcripts have shown more severe renal phenotypes, compared with patients who had splicing variants that produce nontruncating transcripts; the median age for developing ESRD in the truncating group ($n = 21$) was 20 years, whereas it was 29 years in the nontruncating group ($n = 25$) ($P < 0.01$).[8] In this present study, which included a larger number of patients, the significant difference was confirmed between patients with truncating variants and those with nontruncating variants; the median renal survival period of patients with the truncating splicing variant ($n = 32$) was 23 years, whereas it was 28 years for patients with the nontruncating splicing variant ($n = 33$) ($P < 0.05$) (Table 3, Supplementary Figure S1). In addition, our study included an analysis of patients with small rearrangements, which revealed a significant difference of 15 years in median renal survival periods between patients with truncating variants and those with nontruncating variants (20 years vs. 35 years, $P < 0.01$) (Table 3, Supplementary Figure S2). Thus, there was a significant difference determined in the median renal survival periods between patients with truncating variants and those with nontruncating variants (20 years vs. 40 years, $P < 0.01$) (Figure 1). Therefore, it is important to determine whether a patient's mutation is a truncating variant for accurate estimation of renal prognosis.

Although there is no specific treatment for patients with Alport syndrome, the renal protective effects of ACEI/ARB treatment for these patients are already well established. A large-scale retrospective study conducted by Gross *et al.*[10] revealed that ACEI exhibit renal protective effects by delaying the progression to ESRD for patients with XLAS and autosomal recessive Alport syndrome, and 2 randomized controlled trials demonstrated that ACEI/ARB treatment can reduce the level of proteinuria in patients with Alport syndrome.[17,18] In addition, the latest randomized control trial of ACEI for patients with Alport syndrome revealed that preemptive ACEI treatment is safe and efficient.[19] Our current study showed additional evidence of renal protective effects and delayed development of ESRD by >22 years for patients treated with ACEI and/or ARB. Although many clinicians attributed ACEI/ARB treatment to the improvement of renal



Number of patients								
Truncating variants with ACEI/ARB	50	23	8	3	1			
Truncating variants without ACEI/ARB	25	10	2					
Nontruncating variants with ACEI/ARB	76	52	30	12	5	2		
Nontruncating variants without ACEI/ARB	56	32	24	14	5	3	2	2

Figure 4 | Renal survival proportion based on the presence of genotype-dependent angiotensin-converting enzyme inhibitor/angiotensin receptor blocker (ACEI/ARB) treatment. The long dashed double-dotted line (red) indicates patients with nontruncating mutations not treated with ACEIs/ARBs at the time of genetic diagnosis ($n = 56$); the median age for the development of end-stage renal disease (ESRD) was 33 years (95% confidence interval [CI]: 28–73 years) in these patients. The dashed line (blue) indicates patients with nontruncating mutations treated with ACEI/ARB at the time of genetic diagnosis ($n = 76$); most patients did not develop ESRD by the age of 50 years. The solid line (black) indicates patients not treated with ACEI/ARB at the time of genetic diagnosis ($n = 50$); the median age for the development of ESRD was 16 years (95% CI: 9–23 years) in these patients. The dashed line (green) indicates patients treated with ACEI/ARB at the time of genetic diagnosis ($n = 25$); the median age for the development of ESRD was 28 years (95% CI: 20–43 years) in these patients. There were significant differences between patients with treated and not treated ACEI/ARB in both transcript types ($P < 0.05$). Exact *P* values and hazard ratios are shown in Supplementary Table S1.

prognosis in patients with XLAS, a clear difference in renal protective effects depending on genotypes has not yet been determined. To the best of our knowledge, this study is the first to investigate genotype-dependent renal survival period with or without ACEI/ARB treatment. The results showed that the median renal survival period of patients treated with ACEI/ARB was significantly prolonged compared with untreated patients in both truncating and nontruncating group (Figure 4). Despite these therapies, among patients treated with ACEI/ARB, the median renal survival period prolonged by ACEI/ARB treatment was shorter in patients with truncating variants (12 years in truncating vs. >17 years in nontruncating), and the final renal prognosis of patients with treated truncating variants was found to be significantly worse compared with treated nontruncating variants (Figure 4). From these results, regarding patients with nontruncating variants, it was suggested that ACEI/ARB treatment has a big effect on their renal survival period. In addition, although the prolonged renal survival period for patients with truncating variants is shorter than nontruncating variants, it is considered to have a great significance for individuals' life in that they can avoid ESRD care from adolescence to young adulthood. In spite of these substantial effects on the renal survival period for both genotypes, renal prognosis of ACEI/ARB-treated patients with truncating variants is still determined to be shorter compared with untreated patients with nontruncating variants. Therefore, it is expected that a new treatment using ACEI/ARB can be developed. In regard to a strong genotype-phenotype correlation that patients with truncating variants show much severe renal phenotype than those with nontruncating variants, modifying the truncating variant into the nontruncating variant for XLAS is seen to be a very promising strategy.

Our study also investigated other factors related to renal prognosis. Like in our previous study, we confirmed that patients with male XLAS who demonstrated expression of α5 on the glomerular basement membrane exhibited a less severe clinical picture.[15] In this study, 42% of patients demonstrated expression of α5 on the glomerular basement membrane. This high percentage might be subject to sample bias because patients with atypical findings are more likely to undergo genetic testing. As shown in Supplementary Table S2, most of the α5-positive patients had nontruncating mutations; hence, the less severe phenotype of this group presumably reflects its genotype. Regarding the 3 patients with truncating mutations in the α5-positive group, 2 had truncating mutations in exon

51, which corresponds to the noncollagenous domain of the *COL4A5* gene. It has previously been reported that patients with mutations in the noncollagenous domain will show less severe renal phenotypes than those with mutations outside of the noncollagenous domain.[5] The remaining patient with a truncating mutation in the α5-positive group had a silent mutation in exon 29; exon 29 skipping (a 151-bp transcript) was detected by examining the patient's leukocyte mRNA. This case might have a small amount of normally spliced transcript in the kidney that might have led this case α5-positive and mild phenotype. These results suggested that the detection of α5 expression on the glomerular basement membrane could serve as a good prognostic marker for patients with male XLAS. Similarly, patients with hearing loss were found to exhibit a more severe renal phenotype than patients without hearing loss (Figure 2a).

Our study has its own limitations. First of all, our current study is retrospective without any randomization. Thus, we cannot exclude cofounders as described below. We could not also collect information regarding the dose and type of ACEI/ARB, timing of the initiation of ACEI/ARB administration, or the cumulative administration period. Therefore, renal survival curves stratified according to ACEI/ARB treatment were constructed solely on the basis of whether ACEI/ARB had been introduced for the treatment of this disease. Despite reports that suggest earlier diagnosis and earlier therapeutic intervention with ACEI lead to an improved or better prognosis by delaying renal failure,[10] our current study was not able to confirm this finding, because of the lack of data on the time point of start of therapy in our patients. In addition, clinical information was not collected regarding factors that might worsen renal function (e.g., poorly controlled hypertension, nonsteroidal anti-inflammatory drug use, or incidental glomerular disease); thus, we could not analyze the impacts of these factors.

In conclusion, phenotype was found to be strongly correlated with genotype in affected patients with male XLAS. In addition, the improvement of renal outcome in affected patients with male XLAS treated with ACEI/ARB was replicated by our large cohort study. Furthermore, our study revealed a remarkable difference in this effect between patients with truncating variants and patients with non-truncating variants. Our findings strongly support the use of genetic testing for patients with XLAS to facilitate further treatment decisions.

METHODS

Ethical consideration

All procedures were reviewed and approved by the Institutional Review Board of Kobe University School of Medicine. Informed consent was obtained from patients or their parents before participating in the study.

Patients

Patients enrolled in this study were referred to our hospital for clinical evaluation or genetic analysis, from 2006 to 2018. Most patients underwent follow-up in various local hospitals in Japan. Based on their pathologic findings or family history, they were suspected to have Alport syndrome. All the clinical and laboratory findings were obtained from the patients' medical records at the time of genetic analysis.

In this study, proteinuria was defined as the increased levels of protein in the urine, having a protein/creatinine ratio of >0.2 g/g Cre in the first morning urine, which persisted for >3 months. All patients have undergone ophthalmic evaluations for ocular lesions before they were permitted to apply for genetic analysis. In addition, audiometry screening was performed for all students at school in Japan (at the ages of 6, 7, 8, 10, 13, and 15 years); using this system, hearing loss can be detected.

Around 514 families received a genetic diagnosis of Alport syndrome from January 1, 2006, to December 31, 2018. Of these 514 families, 139 were excluded from this study because they had been diagnosed with autosomal dominant or autosomal recessive Alport syndrome with *COL4A3/COL4A4* mutations. Among the remaining 375 families with identified *COL4A5* mutations, 269 families have male patients or affected male family members. As a result, all 282 male patients and all 148 affected family male members were included in this study (Supplementary Figure S3). All patients and family members in this study were Japanese. In this study, affected male family members were identified as those who did not undergo genetic analysis but were diagnosed as XLAS based on their clinical symptoms (hematuria/proteinuria/renal failure/pathologic findings) and family pedigree.

Mutational analysis

Mutational analysis of *COL4A5* was performed using the following methods: (i) targeted next-generation sequencing using a custom disease panel; (ii) conventional direct sequencing using the Sanger method for all exons and exon-intron boundaries; (iii) multiplex ligation-dependent probe amplification to detect copy-number variations; and (iv) reverse transcription-polymerase chain reaction (PCR) analysis of mRNA and direct sequencing to detect abnormal splicing. We initially performed method (i) or (ii); if no mutations were detected, we then proceeded with methods (iii) and/or (iv). The detailed methods for each mutational analysis are described below.

Sanger sequencing

Sanger sequencing of *COL4A5* was performed using PCR and direct sequencing of genomic DNA for all exons and exon-intron boundaries. Blood samples were collected from patients and/or family members; genomic DNA was then isolated from peripheral blood leukocytes using the QuickGene Mini 80 system (Kurabo, Osaka, Japan), in accordance with the manufacturer's instructions. For genomic DNA analysis, 51 exons of *COL4A5* were amplified using PCR, as described previously.[5,6,15] The PCR-amplified products were then purified and subjected to direct sequencing using a Dye Terminator Sequencing Kit (Amersham Biosciences, Piscataway, NJ) with an automatic DNA sequencer (model ABI Prism 3130; PerkinElmer, Waltham, MA).

Targeted exome sequencing

A custom panel has been designed for targeted sequences; the gene list is provided in Supplementary Table S3. Next-generation sequencing samples were prepared using a HaloPlex Target Enrichment System Kit (Agilent Technologies, Santa Clara, CA), in accordance with the manufacturer's instructions. Amplified target libraries were then sequenced via MiSeq (Illumina, San Diego, CA) and

analyzed with SureCall (v.3.0; Agilent Technologies). Detected variants were confirmed by Sanger sequencing.

In addition, we used the analyzed next-generation sequencing data, combined with pair analysis using SureCall software, to screen for copy-number variations on the *COL4A5* genes. Briefly, pair analysis was used to compare the next-generation sequencing data of patients with suspected copy-number variations to reference data without copy-number variations, as previously described.[20] All copy-number variations detected by pair analysis were confirmed using the multiplex ligation-dependent probe amplification analysis.

Multiplex ligation-dependent probe amplification analysis and pair analysis

Multiplex ligation-dependent probe amplification was performed using SALSA P191/192 for the *COL4A5* gene, in accordance with the manufacturer's instructions (MRC-Holland, Amsterdam, the Netherlands). Briefly, 50 to 100 ng of genomic DNA in 5 μl of deionized water was denatured and hybridized overnight with the probe mix. Ligation was performed using the SALSA Ligase-65 enzyme, whereas PCR amplification was performed with the SALSA PCR primer mix. Amplification products and Size Standard 600 were mixed thoroughly; this mixture was later subjected to capillary electrophoresis using GeneMapper v.3.7 (Thermo Fisher, Waltham, MA).

RNA sequencing

Total RNA was extracted from blood leukocytes and/or urinary sediment. RNA from leukocytes was isolated using RNA*later* RNA Stabilization Reagent and RNA Blood Mini Kit (Qiagen Inc., Valencia, CA), and then, it was later reverse-transcribed into cDNA using random hexamers and the Superscript III Kit (Invitrogen, Carlsbad, CA), as previously described.[15] cDNA was amplified by nested PCR using primer pairs for *COL4A5*, as previously described with slight modifications (primer sequences are provided in Supplementary Table S4).[21] PCR-amplified products were purified and then subjected to Sanger sequencing.

Statistical analyses

All calculations were performed using standard statistical software (JMP for Windows, version 13; SAS Institute, Cary, NC). The occurrence of events (renal survival period) was examined using the Kaplan-Meier and Wilcoxon tests. Meanwhile, the hazard ratio was analyzed using the Cox proportional hazards model. Associations were considered to be statistically significant when P values were < 0.05.

DISCLOSURE

KI has received grant support from Daiichi Sankyo Co., Ltd., as well as consulting fees from Takeda Pharmaceutical Company and Kyowa Hakko Kirin Co., Ltd. KNo has received lecture fees from Novartis Pharmaceuticals Corporation. KI and KNo have filed a patent application regarding the development of antisense nucleotides for exon-skipping therapy in Alport syndrome. All the other authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

TY and KNa designed the study concept and wrote the manuscript. KNo interpreted the data and wrote the manuscript. TH, CN, NS, YA, SIshik, KNa, YS, RR, SIshim, TN, and NM collected and interpreted the data. TO interpreted the data and conducted the statistical analyses. HN, HT, HK, and KI critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

SUPPLEMENTAL MATERIAL

Supplementary File (PDF)

Table S1. Exact *P* values and hazard ratios of Figures 1a and 4.
Table S2. Mutation types in α5(IV)-positive and α5(IV)-negative patients.
Table S3. Targeted genes in the NGS custom panel.
Table S4. COL4A5 cDNA PCR primer list.
Figure S1. Renal survival proportion based on the transcripts of patients with splicing variants.
Figure S2. Renal survival proportion based on the transcripts of patients with small rearrangements.
Figure S3. Flowchart of patient enrollment.

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