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(Citation)
Kidney International Reports,7(1):108-116

(Issue Date)
2022-01

(Resource Type)
journal article

(Version)
Version of Record

KI REPORTS
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CLINICAL RESEARCH

Last Nucleotide Substitutions of *COL4A5* Exons Cause Aberrant Splicing

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Introduction: *COL4A5* is a causative gene of X-linked Alport syndrome (XLAS). Male patients with XLAS with nonsense variants have the most severe phenotypes of early onset end-stage kidney disease (ESKD); those with splicing variants have middle phenotypes and those with missense variants have the mildest phenotypes. Therefore, genotyping for male patients with XLAS can be used to predict kidney prognosis. Single-base substitutions at the last nucleotide position in each exon are known to affect splicing patterns and could be splicing variants. Nevertheless, in XLAS, these variants are generally considered to be missense variants, without conducting a transcript analysis, which underestimates some patients as having mild phenotypes. This study aimed to investigate whether single-base substitutions at the last nucleotide position of *COL4A5* exons cause aberrant splicing.

Methods: In total, 20 variants were found in the Human Gene Mutation Database ($n = 14$) and our cohort ($n = 6$). We performed functional splicing assays using a hybrid minigene analysis and *in vivo* transcript analyses of patients' samples when available. Then, we investigated genotype–phenotype correlations for patients with splicing variants detected in this study by comparing data from our previous studies.

Results: Among the 20 variants, 17 (85%) caused aberrant splicing. Male patients with splicing variants had more severe phenotypes when compared with those with missense variants. Findings from the *in vivo* analyses for 3 variants were identical to those from the minigene assay.

Conclusion: Our study revealed that most single-base substitutions at the last nucleotide position of *COL4A5* exons result in splicing variants, rather than missense variants, thereby leading to more severe phenotypes.

Kidney Int Rep (2022) **7,** 108–116; https://doi.org/10.1016/j.ekir.2021.10.012
KEYWORDS: *COL4A5*; genotype–phenotype correlation; last nucleotide position; missense variants; single-base substitutions; splicing

COL4A5 (NM: 000495.4) encodes type IV collagen α5 chain and is a causative gene of XLAS. XLAS is a hereditary kidney disease that causes heterogeneous renal manifestations, from hematuria alone to ESKD accompanied by sensorineural deafness and ocular abnormalities.[1,2] Male patients with XLAS exhibit proteinuria and hematuria in the early stage of childhood and develop ESKD at a median age of 25 to 35 years.[3,4] In male patients with XLAS, genotype–phenotype correlation is evident; patients with nonsense variants have the most severe phenotypes of early onset ESKD, whereas patients with splicing variants have moderate phenotypes, and patients with missense variants have mild phenotypes.[3–6] Patients with nonsense variants, splicing variants, and missense variants developed ESKD at a median age of 18, 28, and 40 years, respectively, in our cohort.[4] In contrast, the genotype–phenotype correlation does not exist in female patients with XLAS.[7,8] Renin–angiotensin–aldosterone system

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Received 14 July 2021; revised 8 September 2021; accepted 11 October 2021; published online 21 October 2021

inhibitor treatment can improve kidney prognosis, especially when patients have missense variants, for which this treatment has been found to work more effectively.[4,9,10] Therefore, genotyping for male cases is important in the prediction of kidney prognosis.

Type IV collagen α3, α4, and α5 chains form the triple-helix structure in the glomerular basement membrane. Type IV collagen α5 chain consists of the N-terminal domain, collagenous domain, and C-terminal noncollagenous (NC1) domain. The collagenous domain consisted of the nonhelical region (NC2) and triple-helical region.[11] In the triple-helical region, the amino acid sequence strictly repeats glycine (Gly) on every third position (Gly-Xaa-Yaa)n, and Gly in every third position contributes to the stability of the triple-helical structure.[12,13] In contrary, Gly missense variants in the triple-helical region have pathogenicity owing to disruption of the normal folding of the triple-helical structure[14] and reduce protein secretion.[15–17] For this reason, most Gly missense variants in the *COL4A5* triple-helical region are caused by Gly substitutions regardless of the position of the single-base substitutions, and male patients with XLAS with these pathogenic variants have mild phenotypes.[18,19] Nevertheless, single-base substitutions, especially located at the last nucleotide position of each exon, sometimes affect splicing patterns.[20–23] In fact, in the *COL4A5* gene, several single-base substitutions at the last nucleotide position in each exon have been reported and interpreted as missense variants in the Human Gene Mutation Database (Cardiff, United Kingdom). Nevertheless, these variants may be correlated with splicing variants, not missense variants, and the kidney prognosis of these variants may be more severe than the expected conventional missense variants.

The aim of this current study is to investigate the possibility that single-base substitutions at the last nucleotide position of exons in *COL4A5* gene cause aberrant splicing. To predict the kidney prognosis, we need to determine which variants lead to missense variants and which variants lead to splicing abnormalities.

METHODS

Editorial Policies and Ethical Considerations

The study was conducted in accordance with the Declaration of Helsinki and the ethical guidelines issued by the Ministry of Health, Labor, and Welfare of Japan (2017). Moreover, it was approved by the Institutional Research Committee of Kobe University Graduate School of Medicine. Informed consent was obtained from all participants included in the study, which was in accordance with the guidelines for the patients' benefit. Therefore, patients could refuse to participate in this study.

Analyzed Variants

We identified all reported missense variants in *COL4A5* from Human Gene Mutation Database (professional release 2021.1). Among 578 missense variants, 14 (2.4%) pathogenic variants caused by single-base substitutions at the last nucleotide positions in exons were included in this study (Figure 1 and Table 1).[5,24–36] In addition, 6 novel variants in our cohort were included. Finally, 20 variants were included in this study.

In Vitro Splicing Assay

Minigene Analysis

Genomic DNA of all wild-type (WT) samples and 8 patient samples (numbers [Nos.] 1, 4, 10, 11, 13, 16, 18, 19) in our cohort were extracted from whole blood using QuickGene DNA Whole Blood Kit S (Kurabo Industrial Ltd., Osaka, Japan) or QuickGene-Auto S DNA Blood Kit (Kurabo Industrial Ltd.), in accordance with the manufacturer's instructions. Fragment primers were designed to contain 1 exon and >100-base pair upstream and downstream flanking introns to this exon (Supplementary Table S1). Nevertheless, when the intron was small, primers were sometimes designed to contain <100-base pair flanking introns. Because the length of intron 15 was short, the fragment for the exon 15 variant was designed containing introns 14 to 16 in *COL4A5*. When the minigene results revealed a normal splicing pattern, we re-examined them and prepared a new fragment, which contained both exons with the variant and its downstream exons. To create hybrid minigene constructs, we used the H492 vector that we modified previously, which is based on the pcDNA 3.0 mammalian expression vector and contains a

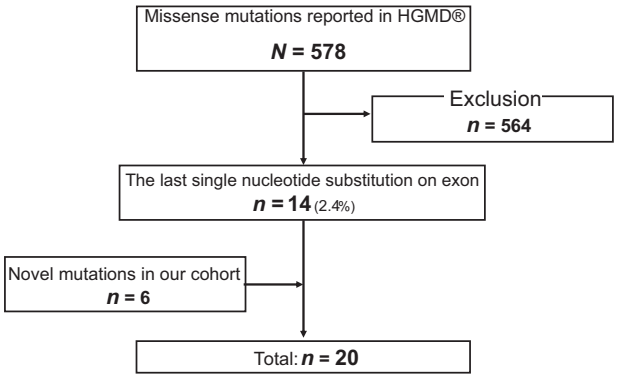


Figure 1. Flow diagram of variant selection. In total, 578 missense variants in *COL4A5* were identified from HGMD and 14 missense variants caused by single-base substitutions at the last nucleotide position in each exon were included. There were 6 novel variants that were included in our cohort. Finally, 20 variants were included in this study. HGMD, Human Gene Mutation Database.

Table 1. *In vitro* (minigene) and *in vivo* results and patient characteristics

No.	Exon	Variant		*In vitro*	*In vivo*	Pathogenicity	Sex	Age, yr	ESKD, yr	Deafness	Ocular abnormality	ID
		Nucleotide	Amino acid	Transcription	cDNA							
1	15	c.891A>T	Arg297Ser	Normal	NA	Unknown	Female	54	(−)	(−)	(−)	A917
2	19	c.1165G>A	Gly389Arg	Exon 19 deletion	ND	Splicing	Female	16	(+)	(+)	(−)	Weber *et al.*[33]
3	21	c.1423G>A	Gly475Ser	36 bp deletion	ND	Splicing	Male Male	ND ND	27 (+)	(+) (−)	(+) (−)	Bekheirnia *et al.*[5]
4	25	c.1948G>T	Gly650Cys	Exon 25 skipping	Exon 25 skipping	Splicing	Male Female Female Female Female Female	31 34 58 46 67 ND	11 (−) 30 27 35 68	(+) (−) (−) (−) (+) (+)	(−) (−) (−) (−) (−) (−)	A375
5		c.1948G>A	Gly650Ser	Exon 25 skipping	ND	Splicing			ND			Wang *et al.*[30]
6	28	c.2244G>T	Lys748Asn	Exon 28 skipping	ND	Splicing			ND			Hertz *et al.*[27]
7	30	c.2509G>A	Gly837Ser	Exon 30 skipping	ND	Splicing	Male			ND		Zhang *et al.*[36]
8	31	c.2677G>C c.2677G>C (+c.384+1G>A)	Gly893Arg Gly893Arg (+c.384+1G>A)	18 bp deletion	ND	Splicing	Male Male Female Female	44 43 16 20	37 28 (−) (−)	(+) (+) (−) (−)	(+) (+) (−) (−)	Mohammad *et al.*[28]
9		c.2677G>A	Gly893Ser	18 bp deletion	ND	Splicing			ND			Hanson *et al.*[29]
10	32	c.2767G>C	Gly923Arg	Exon 32 skipping	NA	Splicing	Female Female Female Female	2 34 8 31	(−) (−) (−) (−)	(−) (−) (−) ND	(−) (−) (−) ND	A268 Abe *et al.*[32] A906
11	35	c.3106G>A	Gly1036Arg	Normal	NA	Missense	Female Female	11 yr Adult	(−) (−)	(−) ND	(−) ND	A604
12	37	c.3373G>A	Gly1125Arg	Exon 37 skipping	ND	Splicing	Female Female Female	30 31 12	(−) (−) (−)	ND	ND	Bullich *et al.*[34]
13	41	c.3790G>A	Gly1264Arg	Exon 41 skipping	Exon 41 skipping	Splicing	Male Female Male	53 49 21	26 yr (−) (−)	(+) (−) ND	(−) (−) ND	A21/A399
14	42	c.3924G>C	Gln1308His	Exon 42 skipping	ND	Splicing	Male	ND	13 yr	(+)	(+)	Bekheirnia *et al.*[5]
15	43	c.3997G>A	Gly1333Ser	Exon 43 skipping	ND	Splicing	Male			ND		Plant *et al.*[25]
16	44	c.4069G>C	Gly1357Arg	Exon 44 skipping	NA	Splicing	Female	38	(−)	(−)	(−)	A685
17		c.4069G>A	Gly1357Ser	Exon 44 skipping	ND	Splicing	Male	ND	22	ND	ND	Plant *et al.*[25]
18	46	c.4297G>A	Gly1433Ser	Normal	NA	Missense	Male	21	(−)	(−)	(−)	A771
19	48	c.4688G>A	Arg1563Glu	Exon 48 skipping	Exon 48 skipping	Splicing	Male Female Female Male Female Male Female Female Female Male Female Male Male Male Male	16 48 25 21 44 36 ND 53 ND Dead 11 26 ND	(−) 16 (−) (−) (−) 29 (−) (−) (−) 27 (−) (−) Average 34.5	(+) (−) (−) (+) (−) (+) ND (+) ND (+) (−) (+) ND	(−) (−) (−) (−) (−) (−) ND ND ND (+) (+) (−) ND	A582 A910 Zhou *et al.*[24] Han *et al.*[35] Gross *et al.*[26] Pont-Kingdon *et al.*[28]
20	50	c.4976G>A	Ser1659Asn	Exon 50 skipping	ND	Splicing			ND			Wang *et al.*[30]

bp, base pair; ESKD, end-stage kidney disease; ID, identification; NA, not available; ND, no data; No., number.

multicloning site (Invitrogen, Carlsbad, CA) (Supplementary Figure S1).[37]

Infusion cloning for 8 variants (Nos. 1, 4, 10, 11, 13, 16, 18, 19) and all WT samples was reacted using Infusion HD Cloning Kit (Takara Bio Inc., Tokyo, Japan) according to the manufacturer's instructions. For the reported 12 variants in Human Gene Mutation Database (Nos. 2, 3, 5–9, 12, 14, 15, 17, 20) that were not observed in our cohort, variations were introduced by site-directed mutagenesis using Prime STAR Mutagenesis Basal Kit (Takara Bio Inc.), following the manufacturer's instructions.

Plasmid DNA was confirmed by sequencing using YH303 and YH304 primers (Supplementary Table S1) and transfected into HeLa and HEK293T cells using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Total RNA was extracted from these cells 24 hours after transfection using the RNeasy Plus Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using RNA to cDNA EcoDry Premix (Double Primed) (Takara Bio Inc.) following the manufacturer's instructions. Polymerase chain reaction of reverse-transcribed cDNA was performed using YH307 and YH308 primers. Polymerase chain reaction products were analyzed by electrophoresis on a 1.5% agarose gel using an $\phi\times174$-Hae III digest marker, and direct sequencing was performed.

Aberrant Splicing Evaluation Criteria

Abnormal splicing patterns were determined according to the following criteria. Results of electrophoresis reveal the following: (i) WT with only normal splicing and the variant with only aberrant splicing; (ii) WT with only normal splicing and the variant with both normal and aberrant splicing; (iii) WT with both normal and aberrant splicing and the variant with only aberrant splicing; and (iv) normal and aberrant splicing in each WT and variant, with a higher proportion of aberrant splicing in the mutant than in WT.

In vivo Splicing Analysis

When patients' samples were available, the total mRNA was isolated from peripheral leukocytes using RiboPure-Blood (Thermo Fisher Scientific) and then reverse-transcribed into cDNA using RNA to cDNA EcoDry Premix (Double Primed) (Takara Bio Inc.) following the manufacturer's instructions. Polymerase chain reaction and direct sequencing were performed using relevant primer pairs (Supplementary Table S1).

In silico Analysis

The pathogenicity of each missense variant was evaluated using SIFT, PolyPhen-2, Mutation Taster, and Align GVGD. We predicted aberrant splicing in each variant using Human Splicing Finder Professional (https://hsf.genomnis.com/home), SD-SCORE,[23] and EX-SKIP (https://ex-skip.img.cas.cz). As for the splice site score in both original and variant sequences, MaxEntScan scores were obtained from Alamut Visual (Sophia Genetics Company, Boston, MA). In both original and variant sequences, the numbers of exonic splicing enhancers and exonic splicing silencers were obtained from EX-SKIP.

Genotype–Phenotype Correlation

We conducted a genotype–phenotype correlation analysis of splicing variants in this study compared with modified data from our previous report of missense variants, intronic splicing variants, and nonsense variants.[4] Given that Ile194Val is not considered pathogenic because of its high allele frequency among healthy Japanese people (2.1%) and because an *in vitro* analysis revealed that Glu633Lys causes aberrant splicing, we decided not to classify these variants as missense and recognized the latter variant as splicing in the modified data from our previous report.

Statistical Analyses

JMP software version 14 (SAS Institute Inc., Raleigh, NC) was used for data analysis. Continuous and categorical data were compared using Pearson's χ^2 test and Fisher's exact test. Cumulative event rates were calculated using the Kaplan–Meier method. Two-tailed $P<0.05$ were considered statistically significant.

RESULTS

Existence of Aberrant Splicing Based on Single-Base Substitutions at the Last Nucleotide Position in Exons

In 20 variants, 17 (85%) exhibited splicing abnormalities that were detected by minigene analysis (Supplementary Figure S2 and Table 1). All 17 variants fulfilled our aberrant splicing evaluation criteria compared with the WT results. In addition, in *in vivo* analysis, aberrant splicing patterns exhibited identical patterns to minigene results in all 3 variants (nos. 4, 13, 19) (Supplementary Figure S3). Therefore, we concluded that the pathogenicity of these variants was caused by aberrant splicing.

In contrast, 3 of 20 variants were concluded as not causing aberrant splicing. Of these 3 variants, 2 (nos. 11, 18) were Gly missense variants in the triple-helical regions. *In silico* analysis revealed pathogenic activities

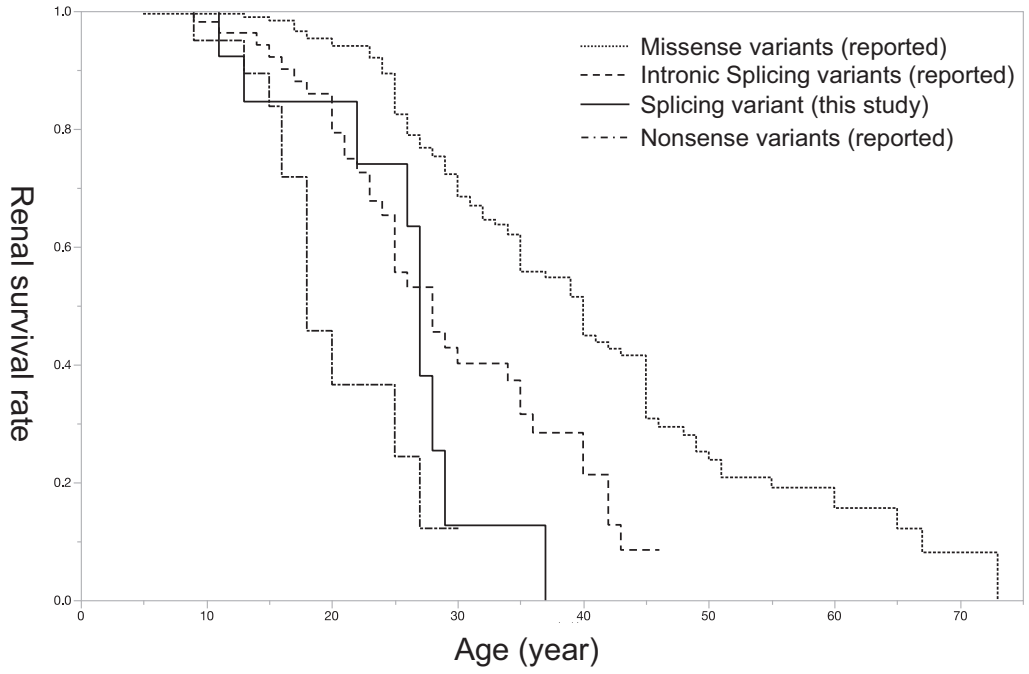
for these variants (Table 2). Therefore, we concluded that these 2 variants were Gly missense variants. The other variant (no. 1) was a non-Gly missense variant (p.[Arg297Ser]) in the collagenous domain, and *in silico* analysis of SIFT and Mutation Taster evaluated this variant as pathogenic. Nevertheless, the mechanism of pathogenicity in this variant could not be determined.

Genotype–Phenotype Correlation

Among all 20 variants, clinical features of male patients were reported in 7 variants with aberrant splicing and 1 variant with normal splicing (Table 1). Nevertheless, we could not obtain clinical information on the age of ESKD development in 1 male patient with the no. 3 variant[5] and 5 male patients with the no. 19 variant.[28] Therefore, these patients were excluded from the genotype–phenotype correlation analysis. Finally, we could evaluate 13 male patients with XLAS with aberrant splicing in 6 variants and 1 patient with normal splicing in 1 variant.

In patients with splicing variants, ESKD, hearing loss, and ocular abnormality were observed in 9, 11, and 5 cases, respectively. Kaplan–Meier analysis revealed that the median age of developing ESKD in these 13 male cases was significantly worse than those with missense variants in our previously reported cohort[4] (27 years of age vs. 40 years of age, $P < 0.01$) (Figure 2). In addition, no significant differences were detected when comparing their data against that from patients with intronic splicing variants (27 years of age vs. 28 years of age, $P = 0.72$) or nonsense variants (27 years of age vs. 18 years of age, $P = 0.09$).

In contrast, the clinical features of only 1 male patient who was found to have only mild phenotypes without aberrant splicing (no. 18) were available: proteinuria was detected at the age of 19 years, and his kidney function was within the normal range at age 21 years.



Number of patients								
Missense variants (reported)	235	186	150	95	47	18	11	4
Intronic splicing variants (reported)	71	54	39	16	8			
Splicing variants (this study)	13	13	12	2				
Nonsense variants (reported)	30	19	5	1				

Figure 2. Kidney survival rate in male patients with XLAS. The solid line represents splicing variants detected in this study, and the dotted, dashed, and dot-dashed lines represent missense variants, intronic splicing variants, and nonsense variants reported in our previous study, respectively. The Kaplan–Meier kidney survival analysis results revealed that the median age for developing end-stage kidney disease was significantly lower for patients with splicing variants in our present study compared with those with missense variants in our previous study (27 years of age, 95% CI: 22–29 vs. 40 years of age, 95% CI: 35–45; Wilcoxon: $P < 0.01$). Nevertheless, there was no significant difference in the median age for developing end-stage kidney disease between patients with splicing variants in this study and those in our previous study with intronic splicing variants (27 years of age, 95% CI: 22–29 vs. 28 years of age, 95% CI: 24–35; $P = 0.72$) or nonsense variants (27 years of age, 95% CI: 22–29 vs. 18 years of age, 95% CI: 16–27; $P = 0.09$).

Table 2. Results of *in silico* analysis

In vitro			Variant		*In silico* (missense)				*In silico*				*In silico* (splicing)		
									MaxEntScan		ESS/ESE (count)				
Splicing	No.	Exon	Nucleotide	Amino acid	SIFT	PolyPhen-2	Mutation taster	Align GVGD	WT	Mut	WT	Mut	HSF	EX-SKIP	SD-SCORE
Aberrant	2	19	c.1165G>A	Gly389Arg	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C25 (GV: 00.00–GD: 55.27)	8.76	4.44	10/23	10/23	Aberrant	Normal	Aberrant
	3	21	c.1423G>A	Gly475Ser	D (score: 0.01)	Pro (score: 1.000)	DC (prob: 1)	C65 (GV: 60.00–GD: 97.30)	5.13	−3.12	2/62	2/61	Aberrant	Normal	Aberrant
	4	25	c.1948G>T	Gly650Cys	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C65 (GV: 0.00–GD: 158.23)	10.86	8.31	0/25	0/25	Aberrant	Normal	Aberrant
	5		c.1948G>A	Gly650Ser	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C55 (GV: 0.00–GD: 55.27)	10.86	10.08	0/25	0/25	Aberrant	Normal	Normal
	6	28	c.2244G>T	Lys748Asn	D (score: 0.04)	Pro (score: 0.999)	DC (prob: 1)	C0 (GV: 109.22–GD: 46.95)	3.93	0	13/16	13/16	Aberrant	Normal	Aberrant
	7	30	c.2509G>A	Gly837Ser	D (score: 0)	Pro (score: 0.993)	DC (prob: 1)	C55 (GV: 0.00–GD: 55.27)	9.45	5.2	16/13	16/11	Aberrant	Aberrant	Aberrant
	8	31	c.2677G>C	Gly893Arg	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C65 (GV: 0.00–GD: 128.13)	8.55	4.34	4/30	4/30	Aberrant	Normal	Aberrant
	9		c.2677G>A	Gly893Ser	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C55 (GV: 0.00–GD: 55.27)	8.55	3.86	4/30	4/30	Aberrant	Normal	Aberrant
	10	32	c.2767G>C	Gly923Arg	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C65 (GV: 0.00–GD: 125.13)	8.49	2.1	8/18	8/17	Aberrant	Aberrant	Aberrant
	12	37	c.3373G>A	Gly1125Arg	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C65 (GV: 0.00–GD: 125.13)	8.59	1.89	10/29	10/29	Aberrant	Normal	Aberrant
	13	41	c.3790G>A	Gly1264Arg	Tole (score: 0.15)	Pro (score: 1.000)	DC (prob: 1)	C0 (GV: 170.55–GD: 2.75)	9.8	7.44	19/19	19/19	Aberrant	Normal	Aberrant
	14	42	c.3924G>C	Gln1308His	D (score: 0.02)	Pos (score: 0.560)	DC (prob: 1)	C0 (GV: 75.14–GD: 11.15)	9.79	3.74	0/55	0/55	Aberrant	Normal	Aberrant
	15	43	c.3997G>A	Gly1333Ser	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C55 (GV: 0.00–GD: 55.27)	7.51	1.24	11/14	11/14	Aberrant	Normal	Aberrant
	16	44	c.4069G>C	Gly1357Arg	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C65 (GV: 0.00–GD: 125.13)	10.77	7.45	18/21	18/21	Aberrant	Normal	Aberrant
	17		c.4069G>A	Gly1357Ser	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C55 (GV: 0.00–GD: 55.27)	10.77	7.61	18/21	18/21	Aberrant	Normal	Aberrant
	19	48	c.4688G>A	Arg1563Glu	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C35 (GV: 0.00–GD: 42.81)	10.48	5.2	7/12	7/12	Aberrant	Normal	Aberrant
	20	50	c.4976G>A	Ser1659Asn	Tole (score: 0.65)	Pos (score: 0.953)	DC (prob: 1)	C0 (GV: 118.33–GD: 0.00)	9.65	2.69	14/19	14/19	Aberrant	Normal	Aberrant
Normal	1	15	c.891A>T	Arg297Ser	D (score: 0.04)	Pos (score: 0.669)	DC (prob: 1)	C15 (GV: 97.59–GD: 95.93)	9.35	8.46	5/31	5/29	Aberrant	Aberrant	Normal
	11	35	c.3106G>A	Gly1036Arg	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C65 (GV: 0.00–GD: 125.13)	10.67	9.1	22/24	22/24	Aberrant	Normal	Aberrant
	18	46	c.4297G>A	Gly1433Ser	D (score: 0)	Pro (score: 1.000)	DC (prob: 1)	C55 (GV: 0.00–GD: 55.27)	10.77	7.61	1/33	1/34	Aberrant	Normal	Aberrant

D, deleterious; DC, disease casing; ESS, exonic splicing silencer; ESE, exonic splicing enhancer; HSF, human splicing finder; Mut, mutant; No., number; Prob, probably damaging; Pos, possibly damaging; SD-SCORE, SD-SCORE algorithm; Tole, tolerated; WT, wild type.

In Silico Analysis

The 5′ splice site scores of all variants were lower than the WT scores, including 3 variants with normal splicing (Table 2). When the 5′ splice site scores by MaxEntScan were compared, the mutant scores were significantly lower in the variants with aberrant splicing than those without aberrant splicing (4.34 ± 0.81 vs. 8.46 ± 0.43, $P = 0.03$).

The sensitivity/specificity of the HSF, SD-SCORE, and EX-SKIP in our study was 100%/0%, 94.1%/33.3%, and 11.1%/50%, respectively. The numbers of exonic splicing enhancers decreased in 4 variants (nos. 1, 3, 7, 10) and increased in 1 variant (no. 18) (Table 2). Nevertheless, the exonic splicing silencers revealed no change among all 20 variants in this study.

DISCUSSION

In the *COL4A5* triple-helical region, the Gly missense variants are known to be pathogenic.[14–17,38] Therefore, these single-base substitutions have been considered to be missense variants. Nevertheless, 15% to 50% of single-base substitutions in exons can cause splicing abnormalities; in particular, canonical sequences in exons at the 5′ splice site have a high potential of disrupting normal splicing.[14,20–23,39] We revealed that 85% (17 of 20) of the single-base substitutions at the last nucleotide position on the exons in *COL4A5* led to aberrant splicing (Supplementary Figure S2 and Table 1). In addition, all *in vivo* transcript analyses for 3 variants (nos. 4, 13, 19) using patient samples exhibited the same splicing pattern with minigene analysis results (Supplementary Figure S3). These results revealed that single-base substitutions at the last nucleotide position of *COL4A5* exons are likely to cause aberrant splicing. In our study, *in silico* tools were found to have high sensitivities but low specificities, so variants with aberrant splicing could not be detected by *in silico* tools alone. Because male patients with XLAS have a strong genotype–phenotype correlation, transcript analysis is important to reveal whether these variants lead to missense variants or splicing variants.

Male patients with XLAS with nonsense variants have the most severe phenotypes of early onset ESKD; those with splicing variants have middle phenotypes, and those with missense variants have mild phenotypes.[3–6] Our results revealed that those with variants with aberrant splicing exhibited a more severe kidney prognosis than those with previously reported missense variants (Figure 2). Compared with data from our previous reports on intronic splicing and nonsense variants, this study found no significant differences among variants with aberrant splicing. These results support the necessity of investigating splicing patterns to understand kidney prognosis correctly.

In vertebrates, pre-mRNA splicing is caused by U2-dependent spliceosome, which consists of a complex of the following 5 uridine-rich small nuclear ribonucleoproteins (snRNPs): U1, U2, U4, U5, and U6 snRNP proteins and numerous non-snRNP proteins.[40] In the first step of spliceosome formation, U1 snRNP recognizes and combines with the exon-intron boundary at the 5′ splice site,[41] which includes the last 3 bases of exons and the first 6 nucleotides of introns; its consensus sequence is [(C/A)AG|GT(A/G)AGT].[42] Single-base substitutions at the last nucleotide position in each exon lead to a weaker 5′ splice site, which depresses the removal of the upstream intron.[43–46] This mechanism causes changes of specificity or fidelity at the 5′ splice site that suppress the recognition and connection of U1 snRNPs at exon-intron boundaries, and these suppressions reduce upstream 3′ splice site recognition and lead to aberrant splicing.[47,48] Nevertheless, this mechanism of aberrant splicing owing to single-base substitutions at the last nucleotide position in exons is still unclear. Actually, in this study, all variants decreased the 5′ splice site scores (Table 2); however, 3 of 20 variants did not have aberrant splicing. In addition, the number of exonic splicing enhancers changes was not characteristic.

This study had several limitations. First, only a small number of variants were included, and our results were mostly achieved through *in vitro* analysis. Because minigene contains only exons and a portion of flanking introns, *in vitro* results from the minigene analysis may not always be consistent with the *in vivo* results. Second, some patients were excluded from the genotype–phenotype correlation analysis owing to a lack of qualifying clinical characteristics. In addition, we excluded 5 male patients with aberrant splicing (no. 19) who had a relatively mild kidney prognosis because we lacked their individual data,[28] although this might have distorted the findings from our kidney survival analysis. Third, we did not evaluate synonymous variants resulting from substitution of the last nucleotide position in exons because they have not been considered pathogenic thus far and, therefore, are not registered in the Human Gene Mutation Database. Nevertheless, they may have the potential to cause aberrant splicing. We recognize that single-nucleotide substitutions at the second position from the last nucleotide may also cause aberrant splicing. Both these points warrant further investigation in the future.

In conclusion, we revealed that most single-base substitutions at the last nucleotide of exons did not cause missense variants, but aberrant splicing led to more severe phenotypes. Therefore, when the single-base substitution at the last nucleotide position in each exon in *COL4A5* is detected, mRNA analysis should be performed to confirm whether these variants are causing XLAS by missense or splicing variants to accurately predict kidney prognosis.

DISCLOSURE

KI reports receiving grant support from Daiichi Sankyo Co. Ltd.; consulting fees from Kyowa Kirin Co. Ltd. and Boehringer Ingelheim; and lecture fees from Kyowa Kirin Co. Ltd., Chugai Pharmaceutical Co. Ltd., Takeda Pharmaceutical Company, Integrated Development Associates, and Novartis Pharmaceuticals Corporation. KNo reports receiving consulting fees from Kyowa Kirin Co. Ltd. and lecture fees from Kyowa Kirin Co. Ltd., Novartis Pharmaceuticals Corporation, and Chugai Pharmaceutical Co. Ltd. All the other authors declared no competing interests.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (subject identifications: 16K19642 to TY, 26293203 and 17H04189 to KI, and 19K08726 to KNo); by the Japan Agency for Medical Research and Development (grant number JP19ek0109231h0003 to KI and 19ek0109231s0103 to KNo); and by The Japan Foundation for Pediatric Research (grant no. 19-002 to TH). YA conceived of and directed the study with support from TH, TY, and KNo. YA and KNo wrote the manuscript. AK, SN, SI, EO, RR, NS, and CN established and conducted molecular analysis and interpreted the data. MM established the minigene assay. The manuscript was critically reviewed by all the authors. The authors thank Enago (www.enago.jp) for the English language review of this manuscript. The authors thank all study participants and their families. We are immensely grateful to Mrs. Yoshimi Nozu, Ming Juan Ye, and Yuko Noguchi (Kobe University) for their excellent technical assistance.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Schematic for the hybrid minigene.

Figure S2. Fragment component of minigene and the results of electrophoresis and schematic for transcript analysis from the minigene assay.

Figure S3. Results of electrophoresis and direct sequencing of reverse-transcriptase polymerase chain reaction from patient's blood.

Table S1. Primer list in this study.

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