各遺伝子の平均カバレッジ
300
250
200
150
100
50
0
ABCG2
ACTN4
ADAMTS13
AGXT
APOE
APRT
AQP2
ATP6V0A4
ATP6V1B1
ATP7B
AVPR2
BSND
CA2
CASR
CD2AP
CEP290
CFH
CFI
CLCN5
CLCNKB
CLDN16
CLDN19
COQ2
CTNS
EGF
FGF23
FN1
FXYD2
GALNT3
NAT8
GLIS2
SLC2A2
GRHPR
HNF1B
HPRT1
IQCB1
ITGB4
KAL1
FGFR1
KCNA1
KCNJ1
KCNJ10
LMNB2
CAPG
CD46
NEK9
NPHP1
INVS
NPHP3
NPHP4
NPHS1
NPHS2
NR3C2
PAX2
PHEX
PKD1
PRKD2
PKHD1
PLCE1
ROBO2
RPGRIP1L
SCNN1A
SCNN1B
SCNN1G
SLC12A1
SLC12A3
SLC17A5
SLC22A12
SLC2A9
SLC3A1
SLC4A1
SLC4A4
SLC5A2
SLC7A9
TRPC6
TRPM2
TSC1
TSC2
UMOD
VHL
WNK1
WNK4
WT1
XDH

厚生労働科学研究費補助金(難治性疾患等克服研究事業)
腎・泌尿器系の希少難治性疾患群に関する調査研究
分担研究報告書

希少難治性腎疾患におけるiPS細胞作製・病態解析

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研究要旨
人工多能性幹細胞（iPS細胞）は、希少疾患やモデルマウスのない疾患の解析において有用であり、希少難治性腎・泌尿器疾患群においても重要な解析手段となると考えられる。iPS細胞の樹立過程においては患者の体細胞採取が必須であり、小児患者に最も負担が少ないものとして末梢血からの採取が挙げられる。我々は、Alport症候群、4p monosomy、シスチン尿症の患者から末梢血T細胞を採取し、センダイウイルスベクターを用いてOct3/4・Sox2・KLF4・c-Mycを導入し、iPS細胞を樹立した。
今回、多彩な症状をもつ希少難治性腎疾患患者を対象として、非侵襲的に血液細胞由来のiPS細胞を樹立し、病態解明および治療薬の開発を目指す。

A 研究目的

希少難治性腎疾患における iPS 細胞を作製し、病態解明および治療薬の開発を目指す。

B 研究方法

（iPS細胞作成）

以前、我々は Galloway-Mowat 症候群の皮膚線維芽細胞から人工多能性幹細胞（iPS 細胞）を樹立した。今回、より非侵襲的な方法として、患者の血液細胞由来の iPS 細胞作成を試みた。具体的には、まず患者から末梢血を採取し、当大学 発生医学研究所 幹細胞誘導分野(江良拓実 教授)にてTリンパ球の回収、培養を行った。次にセンダイウイルスをベクターとして山中4因子(Oct3/4, Sox2, KLF4, c-Myc)を導入し、iPS 細胞の樹立を試みた。

（倫理面への配慮）

患者とご両親に対し、説明文書を用い研究目的や研究方法等につき説明し同意を得、人権および利益の保護に十分配慮した。

C 研究結果

患者から末梢血を少量採取し、T リンパ球を回収、培養し、iPS 細胞を樹立した。

血液約 2ml から iPS 細胞樹立に十分な細胞数が得られた。血液採取から約4週間で iPS 細胞のコロニーが得られ、樹立効率は 4p monosomy 0.04%、Alport 症候群 0.09%であった。継代培養し、センダイウイルス除去を確認し、それぞれ 5 クローン、8 クローンを得た。ALP 染色で陽性を確認、免疫染色で NANOG・OCT・SSEA4・TRA1-60 の陽性を確認した。また得られたクローンの mRNA の PCR を行い、OCT4, SOX2, DNMT3b, REX1, c-MYC, GDF3, KLF4, SAL4f, NANOG の発現を確認した。

また、シスチン尿症患者の血液細胞と先天性ネフローゼ症候群患者の皮膚線維芽細胞より iPS 細胞を樹立した。なお、現在 6 疾患が iPS 細胞樹立準備中（細胞培養まで）である。

今後、得られた iPS 細胞を用いて、疾患解析を行っていく。

D 考察

iPS 細胞は、希少疾患やモデルマウスのない疾患の解析において有用と考えられている。小児の希少難治性腎・泌尿器疾患群は症例数も少なく、また小児という特殊性から、病態解析に十分な検体を得ることが困難である場合が少なくない。非侵襲的に iPS 細胞が樹立できれば、現時点で重要な解析手段となることはもちろん、将来的に新たな解析法が可能となるまでの患者情報の保存手段としての側面も挙げられる。

iPS 細胞を用いた疾患解析としては、疾患表現型を認める細胞へ分化誘導を行い、解析するのが一般的である。我々は、神経症状を有する Galloway-Mowat 症候群に対して、神経分化誘導を行い、その解析を行う方針を考えている。また、最近、間葉系細胞から腎臓構成細胞への分化誘導の報告や、腎臓の大部分が体軸幹細胞に由来し試験管内で三次元の腎臓組織を作成できたなどの報告がなされており、この方面からの解析も検討していく。

E 結論

① 4 疾患の iPS 細胞を樹立した。
- 血液細胞由来：Alport 症候群
  4p monosomy、シスチン尿症
- 皮膚線維芽細胞由来：
  先天性ネフローゼ症候群

② 6 疾患は iPS 細胞樹立準備中（細胞培養まで）である。

③ Galloway-Mowat 症候群の神経分化の研究を神戸大学、関西医科大学、慶應大学と共同研究中である。

④ 血液細胞由来の iPS 細胞樹立までの期間は皮膚線維芽細胞由来のそれより、２～３週間短い。

⑤ 血液細胞は採取が容易で、非侵襲的である（小児患者の病態解析に適する）。

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G 知的財産権の出願・登録状況

１）特許取得
なし

２）実用新案登録
なし

３）その他
なし

III. 研究成果の刊行に関する一覧表

IV. 研究成果の刊行物

研究成果の刊行に関する一覧表

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Mutational analyses of the *ATP6V1B1* and *ATP6V0A4* genes in patients with primary distal renal tubular acidosis

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Keywords: autosomal-recessive distal renal tubular acidosis, *ATP6V1B1*, *ATP6V0A4*, hyperammonemia, large deletions

ORIGINAL ARTICLE

ABSTRACT

Background. Mutations in the *ATP6V1B1* and the *ATP6V0A4* genes cause primary autosomal-recessive distal renal tubular acidosis (dRTA). Large deletions of either gene in patients with dRTA have not been described.

Methods. The *ATP6V1B1* and *ATP6V0A4* genes were directly sequenced in 11 Japanese patients with primary dRTA from nine unrelated kindreds. Large heterozygous deletions were analyzed by quantitative real-time polymerase chain reaction (PCR). The clinical features of the 11 patients were also investigated.

Results. Novel mutations in the *ATP6V1B1* gene were identified in two kindreds, including frameshift, in-frame insertion and nonsense mutations. Large deletions in the *ATP6V0A4* gene were identified in two kindreds. Exon 15 of *ATP6V0A4* was not amplified in one patient, with a long PCR confirming compound heterozygous deletions of 3.7- and 6.9-kb nucleotides, including all of exon 15. Direct DNA sequencing revealed a heterozygous frameshift mutation in *ATP6V0A4* in another patient, with quantitative real-time PCR indicating that all exons up to exon 8 were deleted in one allele. Clinical investigation showed that four of the six patients with available clinical data presented with hyperammonemia at onset.

Conclusions. To our knowledge, these dRTA patients are the first to show large deletions involving one or more entire exons of the *ATP6V0A4* gene. Quantitative PCR amplification may be useful in detecting heterozygous large deletions. These results expand the spectrum of mutations in the *ATP6V0A4* and *ATP6V1B1* genes associated with primary dRTA and provide insight into possible structure–function relationships.

INTRODUCTION

Primary distal renal tubular acidosis (dRTA) is a rare genetic disease caused by impaired excretion of hydrogen ions (H^+) by intercalated cells in the collecting ducts [1, 2]. Its clinical

features include hyperchloremic acidosis with inappropriately alkaline urine, hypokalemia, hypercalciuria, nephrocalcinosis and nephrolithiasis. Affected infants present with polyuria, dehydration and failure to thrive [3]. Both autosomal-dominant and autosomal-recessive forms of dRTA have been described. Mutations in the *ATP6V1B1* gene, which encodes the B1 subunit of the H^+-ATPase, have been associated with autosomal-recessive dRTA accompanied by severe sensorineural hearing loss (SNHL), whereas mutations in the *ATP6V0A4* gene, which encodes the a4 subunit of the H^+-ATPase, have been associated with autosomal-recessive dRTA without SNHL [1, 4, 5]. Recent genetic analyses, however, have revealed that some individuals with mutations in the *ATP6V0A4* gene also have early-onset severe SNHL [6, 7]. To expand knowledge of mutations in patients with dRTA, we analyzed the *ATP6V1B1* and *ATP6V0A4* genes in 11 Japanese patients from nine unrelated kindreds with dRTA.

MATERIALS AND METHODS

Patients

We analyzed 11 Japanese patients with dRTA belonging to nine unrelated kindreds; their clinical features and representative biochemical data are shown in Table 1. The diagnosis of dRTA was based on metabolic acidosis with a normal anion gap and overly high urinary pH, with or without nephrocalcinosis and SNHL. The parents of the patients in families 1 and 5 were consanguineous. Patients 1-1 and 1-2, and patients 7-1 and 7-2 were siblings. Detailed clinical data were not available for patient 5-1 at initial presentation, but she presented with full-blown symptoms of dRTA, including nephrocalcinosis and SNHL.

The study protocol was approved by the Ethics Committee of The University of Tokyo (Approval No. 2204). Informed consent was obtained from each patient and his or her parents.

Mutation analysis

Genomic DNA was extracted from the peripheral blood of the patients and their family members using QuickGene DNA whole-blood S kits (Fujifilm). Thirteen and 20 pairs of oligonucleotide primers were generated to amplify all exons of the *ATP6V1B1* and *ATP6V0A4* genes, respectively (Supplemental Tables 1 and 2). Polymerase chain reactions (PCRs) were performed in 20 μL of solution containing AmpliTaq Gold 360 Master Mix (Applied Biosystems), ~30-ng genomic DNA and 10 pmol of each primer. The amplification protocol consisted of an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at the temperatures shown in Supplemental Tables 1 and 2 for 30 s and elongation at 72°C for 30 s. PCR samples were subjected to bidirectional sequencing on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems).

Quantitative real-time PCR amplification

The heterozygous large deletion in the *ATP6V0A4* gene of patient 4-1 was analyzed by quantitative genomic real-time PCR using SYBR Green (Bio-Rad Laboratories, Hercules, CA) and the primer sequences shown in Supplemental Table 2. The standard curve method was used to calculate the target genome numbers in patient 4-1, with the target copy number normalized to normal human genomic DNA. Statistical significance was assessed using Student's *t*-tests.

RESULTS

Clinical findings

A total of 11 patients from nine unrelated kindreds were enrolled in this study (Table 1). Consanguinity was noted in two families. Among the 10 patients with available data at onset, all but 1 (patient 2-1) presented with metabolic acidosis with a normal anion gap and abnormally high urinary pH. Although patient 2-1 had a near normal serum bicarbonate concentration, she presented with hypokalemia, hypercalciuria, growth retardation and bilateral SNHL. Supplementation with bicarbonate improved her growth. Urine anion gap was positive or near-zero in all patients with available data, which is consistent with reduced NH4+ excretion as seen in dRTA [8].

Serum creatinine concentrations in all patients were within normal range throughout the follow-up period. Growth retardation in infancy occurred in 9 of the 11 patients, with alkali therapy improving their growth to almost normal range. Of the two patients without growth retardation at onset, one (patient 7-2) was diagnosed with dRTA by screening tests before presenting with any symptoms because her older sister had been diagnosed with dRTA. The other patient with normal growth during infancy (patient 9-1) presented with severe weight loss, metabolic acidosis, abnormally high urinary pH and hypercalciuria at age 7 years.

Abdominal ultrasonography showed nephrocalcinosis in nine patients. Unilateral or bilateral SNHL, as determined by audiograms, were observed in four patients. Hypokalemia was observed in six of the nine patients with available data and hypercalciuria in seven of nine. The chart of an additional patient (patient 3-1) mentioned hypercalciuria, although precise data were not available.

Hyperammonemia was observed in four of the six patients with available data, improving in all four after correction of metabolic acidosis. Of the two patients without hyperammonemia, one (patient 4-1) had high-normal (60 μg/dL) serum ammonia concentration, whereas the other (patient 2-1) had normal serum ammonia, a finding probably associated with the absence in this patient of profound metabolic acidosis at onset.

Patient 7-2, a younger sister of patient 7-1, was diagnosed with dRTA 10 days after birth by screening blood and urine tests. Since then, she has been treated with citrate and her growth is normal. At age 8 months, she presented with transient metabolic acidosis resulting from insufficient supplementation with citrate. A higher dosage of citrate improved her symptoms. Of note, she is free from nephrocalcinosis at age 2 years. In contrast, her older sister, patient 7-1, presented with prominent nephrocalcinosis at age 1 month.

Table 1. Clinical features of the patients

	Consanguinity	Age/sex	Age at onset	FTT	NC	SNHL	pH	HCO_3^- (19–23) mmoL/L	Potassium (3.5–6.0) mEq/L	Serum ammonia (12–66) μg/dL	Urine pH	Urine anion gap, mEq/L	Urine calcium excretion (<4 mg/kg/day, Ca/Cr <0.21)
Patients with mutations in the *ATP6V1B1* gene													
1-1	+	46 years/M	Infancy	+	+	+rt	7.230	11.8	3.4	NA	7.0	4	7.2 mg/kg/day
1-2	+	38 years/F	Infancy	+	+	–	7.215	12.4	1.8	NA	7.0	2	6.5 mg/kg/day
2-1	–	1 year/F	9 months	+	+	+bil	7.325	19.6	1.8	27	8.0	NA	Ca/Cr 0.42
Patients with mutations in the *ATP6V0A4* gene													
3-1	–	12 years/M	2 months	+	+	–	7.293	14.1	NA	NA	8.0	NA	(high)[a]
4-1	–	1 month/M	1 month	+	+	+rt	7.167	12.3	3.3	60	7.5	17	Ca/Cr 0.77
Patients with no mutations													
5-1	+	35 years/F	1 month	+	+	+bil	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
6-1	–	15 years/M	1 month	+	+	–	7.322	14.1	4.1	94	7.3	NA	Ca/Cr 0.19
7-1	–	6 years/F	1 month	+	+	–	7.220	11.2	4.1	97	7.0	NA	Ca/Cr 1.42

Continued

Table 1. Continued

	Consanguinity	Age/sex	Age at onset	FTT	NC	SNHL	pH	HCO_3^- (19–23) mmoL/L	Potassium (3.5–6.0) mEq/L	Serum ammonia (12–66) μg/dL	Urine pH	Urine anion gap, mEq/L	Urine calcium excretion (<4 mg/kg/day, Ca/Cr <0.21)
7-2	–	3 years/F	10 days	–[c]	–	–	7.354	14.1	3.8	144	8.0	28	Ca/Cr 0.27
8-1	–	3 years/F	1 year 0 month	+	+	–	6.999	9.2	2.2	NA	7.5	–2	Ca/Cr 0.06
9-1	–	7 years/F	7 years	–[d]	–	–	7.120	9.0	2.2	78	7.5	15	Ca/Cr 0.59

Normal values for infants are shown in parentheses.
FTT, failure to thrive; NC, nephrocalcinosis; SNHL, sensorineural hearing loss; rt, right; NA, not available; bil, bilateral; Ca/Cr, urine calcium to creatinine ratio (mg/mgCr).
[a]Although specific data were not available, hypercalciuria was noted in the chart.
[b]Detailed clinical data at initial presentation were not available in patient 5-1, but she presented with full-blown symptoms of dRTA, including nephrocalcinosis and SNHL.
[c]Patient 7-2 was diagnosed by screening tests when she had no symptoms.
[d]Although no growth retardation was noted in patient 9-1 in infancy, she presented with weight loss at the age of 7.

Genetic analysis

Three novel mutations in the *ATP6V1B1* gene were identified in two of the nine kindreds (Figure 1). Patients 1-1 and 1-2 (family 1) presented with novel homozygous single nucleotide deletions (c.33delG) in exon 1, causing a frameshift that resulted in the premature termination of the protein at codon 19 (L19X) (Figure 1A). In addition, patient 2-1 presented with novel compound heterozygous mutations, consisting of an in-frame insertion (c.978_979insGCC or p.A326insA) and a nonsense mutation (c.1251C>G or p.Y417X). Genotyping of family members of patient 2-1 revealed that this patient had inherited the p.Y417X mutated allele from her mother (Figure 1B).

We observed novel aberrations of the *ATP6V0A4* gene in two of the nine kindreds. In patient 3-1, the genomic PCR product of exon 15 was not amplified (Figure 2A), suggesting homozygous deletions that included exon 15. To confirm this finding, we performed long genomic PCR using primers for exons 14 (forward) and 16 (reverse), which amplified a 7.9-kb product derived from wild-type *ATP6V0A4* allele in the healthy control (Figure 2B). In patient 3-1, however, these primers amplified two PCR products, of 4.2 and 1.0 kb, suggesting that deletions involving exon 15 were responsible for the aberrant *ATP6V0A4* transcripts. Subsequent sequencing disclosed the presence of 3.7- and 6.9-kb deletions within introns 14 and 15, respectively, in each allele. These deletions caused a frameshift that resulted in premature termination of the protein at codon 536 (L536X) in exon 16 (Figure 2C).

We also observed a novel heterozygous large deletion of the *ATP6V0A4* gene in patient 4-1. This was accompanied by a novel heterozygous single nucleotide insertion (c.1185insC) causing a frameshift that resulted in premature termination of the protein at codon 429 (p.E429X) in exon 13, as shown by direct DNA sequencing (Figure 3A). The quantities of the PCR products up to exon 8 seemed to be reduced (data not shown), suggesting the presence of an interstitial heterozygous deletion of an *ATP6V0A4* allele in patient 4-1. To determine the *ATP6V0A4* gene copy numbers in this patient, we performed quantitative genomic PCR of exons 3, 5, 6, 7, 8, 9, 10 and 18 using the same amounts of genomic DNA (1 μg/tube) from the patient and a healthy control. We found that the signal intensities of exons 3, 5, 6, 7 and 8 were significantly reduced compared with those of exons 9, 10 and 18 ($P < 0.05$), indicating that the N-terminal region up to exon 8 was deleted in one of the *ATP6V0A4* alleles of patient 4-1 (Figure 3B).

Given that large deletions including one or more entire exons were frequently identified, multiplex ligation-dependent probe amplification analyses for both the *ATP6V0A4* and *ATP6V1B1* genes were performed in the five patients without any alterations of these two genes. No abnormalities in relative copy numbers were detected in these patients, suggesting that

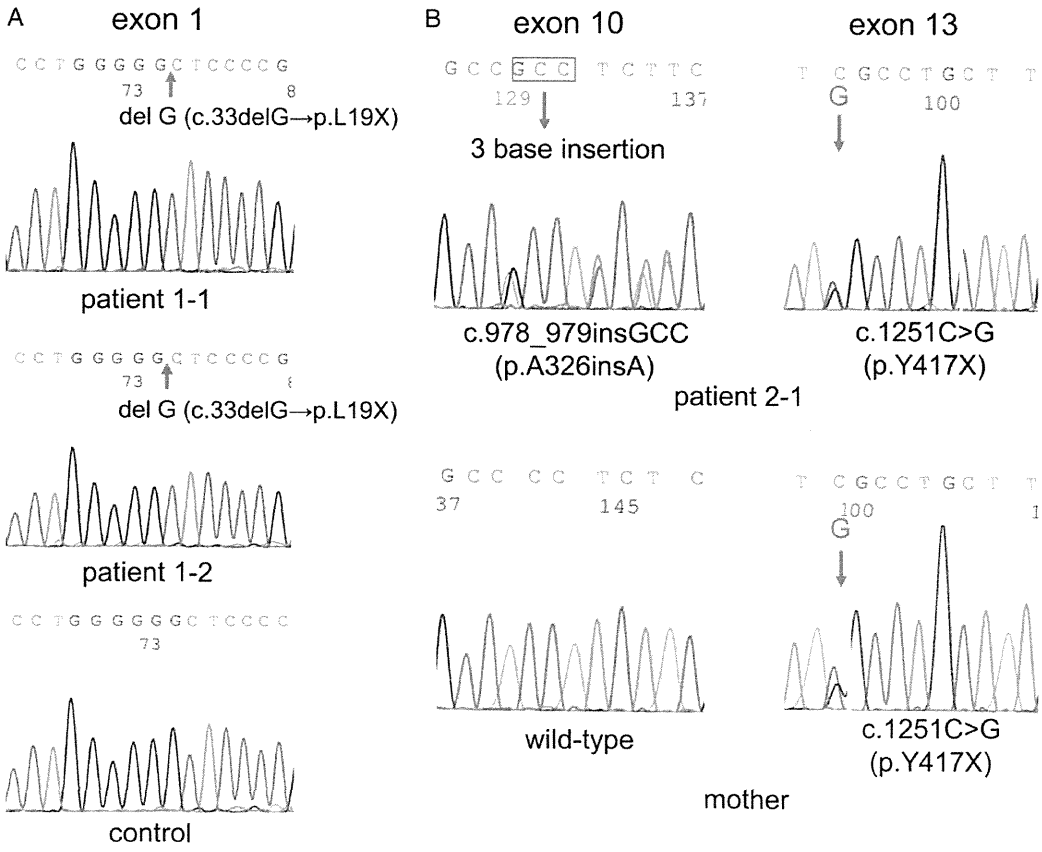

FIGURE 1: Novel mutations in the *ATP6V1B1* gene. (**A**) Genomic sequences of novel homozygous mutations in patients 1-1 and 1-2 (upper panels), as well as normal DNA from a healthy control (lower panel). (**B**) Novel compound heterozygous mutations in patient 2-1 (upper panels). Mother also carries a heterozygous mutation p.Y417X (lower panels). Mutated nucleotides are indicated by arrows.

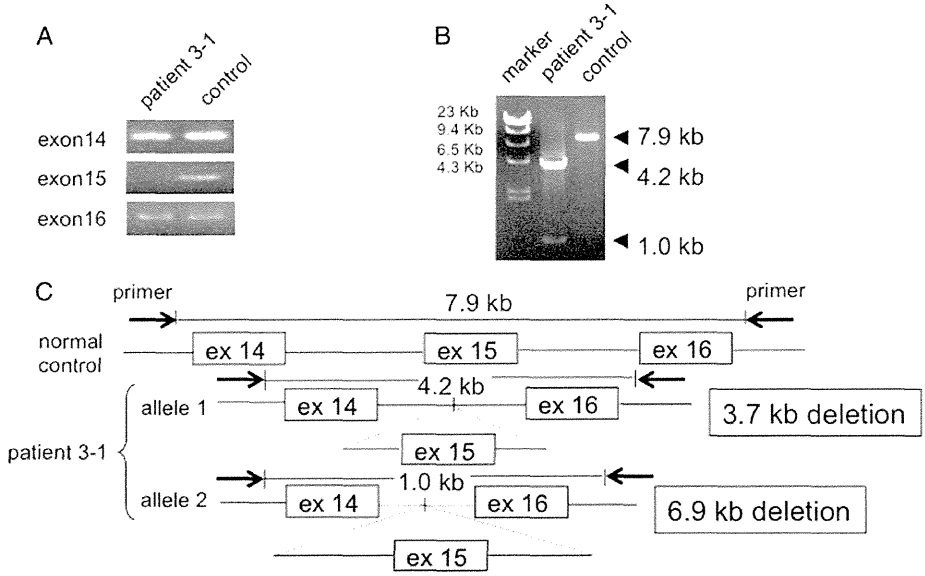

FIGURE 2: Novel large interstitial deletions in the *ATP6V0A4* gene in patient 3-1. (**A**) Genomic PCR product of exon 15 was absent, whereas DNA fragments of exons 14 and 16 were amplified in patient 3-1. (**B**) Long genomic PCR using primers for exon 14 (forward) and exon 16 (reverse) in patient 3-1. Two aberrant sizes of the PCR products, 4.2 and 1.0 kb, were observed in patient 3-1, while a 7.9-kb product was detected in the healthy control. (**C**) Schematic presentation of large deletions in the *ATP6V0A4* gene in patient 3-1. Deletions of 3.7- and 6.9-kb nucleotides including whole exon 15 were confirmed by subsequent sequencing analysis.

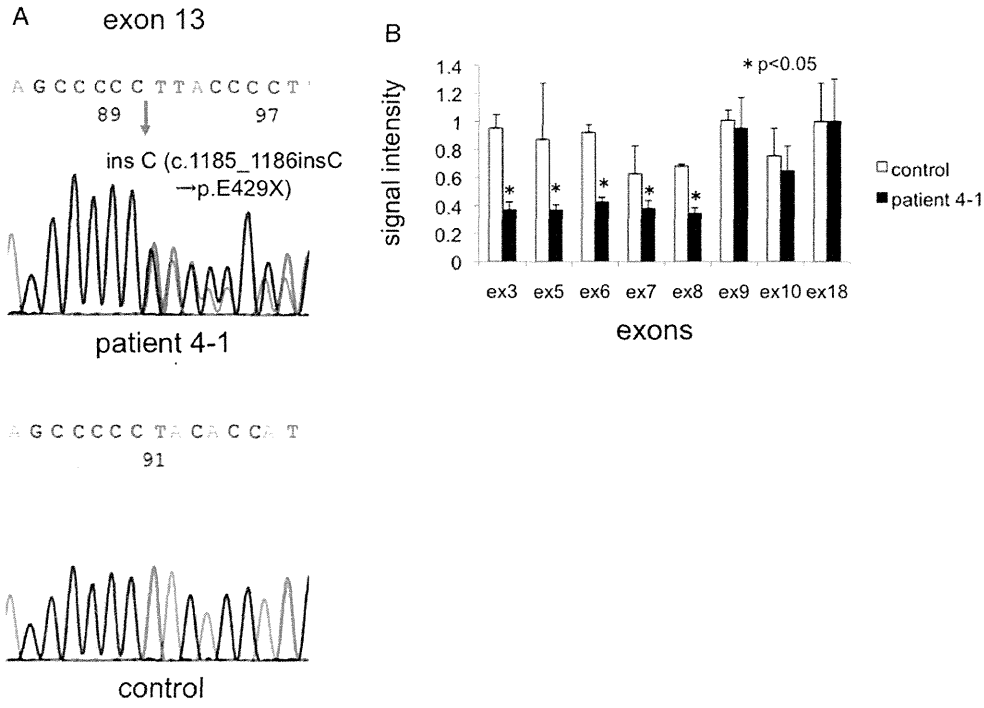

FIGURE 3: Novel compound heterozygous mutations comprising an insertion and a large deletion in the *ATP6V0A4* gene in patient 4-1. (**A**) Genomic sequences of a novel heterozygous mutation in patient 4-1 (upper panel) as well as normal DNA from a healthy control (lower panel) are shown. (**B**) Quantitative genomic PCR of the *ATP6V0A4* gene in patient 4-1. The signal intensity of exons 3, 5, 6, 7 and 8 was significantly reduced as compared with exons 9, 10 and 18 in patient 4-1, while the signal intensity was not significantly reduced in any exons in healthy control. The signal intensity of each exon was adjusted so that the intensity score was 1 in exon 18.*P < 0.05. P-value was assessed by Student's *t*-test. ex: exon.

they carry no homozygous or heterozygous large deletions of these two genes (data not shown).

Clinical features of the patients with mutations in the *ATP6V1B1* and *ATP6V0A4* genes

All the patients with mutations in the *ATP6V1B1* or *ATP6V0A4* gene presented with growth retardation in infancy and nephrocalcinosis accompanied by hypercalciuria. Three patients from three unrelated families presented with early-onset SNHL. Of these, two had mutations in the *ATP6V1B1* gene (patients 1-1 and 2-1), and the third had a truncating mutation in one allele of the *ATP6V0A4* gene and a large deletion that included several exons in the other allele (patient 4-1). Patients 1-1 and 1-2 are siblings and possess the same mutation in the *ATP6V1B1* gene. However, only the brother (patient 1-1) presented with early-onset unilateral SNHL, which was confirmed by audiogram at age 9 years, while his 38-year-old sister (patient 1-2) has had no audiographic evidence of SNHL to date.

DISCUSSION

We have identified three *ATP6V1B1* mutations (one deletion, one insertion and one nonsense mutation) and four *ATP6V0A4* mutations (one insertion and three large deletions involving one or more exons) in patients with primary dRTA. These findings expand the spectrum of mutations in the *ATP6V0A4* and *ATP6V1B1* genes associated with primary dRTA, and provide insight into possible structure–function relationships.

To date, about 30 mutations in the *ATP6V1B1* gene and about 40 in the *ATP6V0A4* gene have been identified, including missense, nonsense, frameshift and splicing site mutations [1, 3, 6, 7, 9–12]. Prior to this study, however, large interstitial deletions involving one or more exons in either gene had not been reported. Thus, to our knowledge, this is the first report of patients with large deletions in one or both alleles of the *ATP6V0A4* gene. Homozygous large interstitial deletions can be revealed by the absence of PCR products of particular exons. Although heterozygous large deletions are much harder to detect, quantitative real-time genomic PCR can be useful when PCR products of particular exons show relatively weak bands.

One of the compound heterozygous mutations in patient 2-1 was an in-frame insertion mutation (c.978_979insGCC/p.A326insA), the functional consequence of which should be addressed. A326 and T327 are highly conserved among species. Three-dimensional structural analysis shows that these amino acids are located near the hydrophobic residues at the interface between subunits A and B, which is critical for the catalytic activity [13].

In five of the nine kindreds, we observed no mutations in the *ATP6V1B1* and *ATP6V0A4* genes. In contrast, 80% of patients with dRTA were found to have a mutation in either gene [5, 14]. The low mutation frequency of these genes in our patients may be due to our small sample size and/or to differences in ethnicity, suggesting that dRTA is genetically heterogeneous. The clinical diagnosis seemed accurate in all of our patients, as all had metabolic acidosis with a normal anion gap and abnormally high urinary pH, accompanied by either nephrocalcinosis or hypercalciuria. Although hypokalemia was not noted at onset in three patients, it developed later in their clinical courses. H^+-ATPase is composed of at least 13 different subunits, including the B1 and a4 subunits. The expression of the isoforms C2, G3 and d2, which are encoded by the *ATP6V1C2*, *ATP6V1G3* and *ATP6V0D2* genes, respectively, is restricted to the kidneys and a few other tissues [2,15]. Although these genes were assessed as candidate causative genes in patients with recessive dRTA, no mutations were identified [15].

Our findings have important clinical implications. Administration of citrate salts is recommended for the prevention of nephrocalcinosis in dRTA, because they correct hypercalciuria and hypocitraturia [16]. Patient 7-2 was diagnosed with dRTA at age 10 days by screening blood samples, because her older sister, patient 7-1, had previously been diagnosed with dRTA. Alkali therapy was instituted immediately after diagnosis in patient 7-2, and this patient has shown no evidence of nephrocalcinosis at age 2 years. In contrast, her older sister, patient 7-1, presented with marked nephrocalcinosis at age 1 month. This difference in clinical manifestation between these two sisters, who are theorized to carry the same genetic abnormalities, suggests that nephrocalcinosis may be prevented by alkali therapy. As for early-onset SNHL, it was present in four patients, two with mutations in *ATP6V1B1*, one with a mutation in *ATP6V0A4*, and one without any mutations in either gene. This finding and the fact that only the brother (patient 1-1) in kindred one presented with unilateral SNHL confirm that intrafamilial variations in clinical manifestations can occur and that some individuals with mutations in the *ATP6V0A4* gene also have early-onset SNHL [6, 7]. The mechanism of intrafamilial variations in hearing disabilities remains unclear. Although the younger sister (patient 1-2) was diagnosed with dRTA earlier than her older brother (patient 1-1), earlier initiation of alkali therapy is reported to have no effect on hearing loss [17]. In regard to laterality in SNHL (patient 1-1), asymmetrical progression of hearing disabilities has been previously described, the mechanism of which is also unknown [18]. We found that four of six patients with available data presented with hyperammonemia. To our knowledge, a high frequency of hyperammonemia in patients with dRTA has not been previously described, although a few patients were found to have hyperammonemia secondary to dRTA [19–21]. Hyperammonemia may result from an imbalance between increased synthesis and reduced excretion of ammonia. Chronic metabolic acidosis and hypokalemia can increase renal ammoniagenesis. However, in our cases, hypokalemia was noted in only one of four patients presenting with hyperammonemia. The precise mechanism by which hyperammonemia develops in dRTA remains to be determined.

In conclusion, we have described patients with dRTA and large interstitial deletions involving one or more exons in the *ATP6V0A4* gene. Long genomic PCR as well as quantitative genomic PCR analyses can be useful in detecting large homozygous or heterozygous deletions. Clinically, nephrocalcinosis may be prevented by early and adequate alkali therapy.

Hyperammonemia is a frequent finding at onset in dRTA, suggesting that the latter condition be included as a miscellaneous cause of hyperammonemia.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

None declared.

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Milder clinical aspects of X-linked Alport syndrome in men positive for the collagen IV α5 chain

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X-linked Alport syndrome is caused by mutations in the *COL4A5* gene encoding the type IV collagen α5 chain (α5(IV)). Complete absence of α5(IV) in the renal basal membrane is considered a pathological characteristic in male patients; however, positive α5(IV) staining has been found in over 20% of patients. We retrospectively studied 52 genetically diagnosed male X-linked Alport syndrome patients to evaluate differences in clinical characteristics and renal outcomes between 15 α5(IV)-positive and 37 α5(IV)-negative patients. Thirteen patients in the α5(IV)-positive group had non-truncating mutations consisting of nine missense mutations, three in-frame deletions, and one splice-site mutation resulting in small in-frame deletions of transcripts. The remaining two showed somatic mutations with mosaicism. Missense mutations in the α5(IV)-positive group were more likely to be located before exon 25 compared with missense mutations in the α5(IV)-negative group. Furthermore, urinary protein levels were significantly lower and the age at onset of end-stage renal disease was significantly higher in the positive group than in the negative group. These results help to clarify the milder clinical manifestations and molecular characteristics of male X-linked Alport syndrome patients expressing the α5(IV) chain.

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KEYWORDS: Alport syndrome; *COL4A5*; genetic renal disease; somatic mosaic mutation

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Alport syndrome (AS) is a hereditary disorder of type IV collagen, characterized by chronic kidney disease progressing to end-stage renal disease (ESRD), sensorineural hearing loss, and ocular abnormalities.[1] Approximately 85% of AS patients show X-linked inheritance (XLAS), and mutations in the *COL4A5* gene, which encodes the type IV collagen α5 (α5(IV)) chain, can be detected.[1] Disease-causing mutations in *COL4A5* result in abnormal α5(IV) expression and typically in complete absence of α5(IV) in the glomerular basement membrane (GBM) and Bowman's capsule (BC). However, a previous review suggested that 20% of male XLAS sufferers showed complete or partial staining for this collagen chain,[2] although the genetic and clinical backgrounds of male XLAS patients presenting with such atypical immunohistological findings have not yet been elucidated. The aim of this study was to clarify the genetic and clinical backgrounds of XLAS patients with positive expression of α5(IV), and to determine if expression of this chain correlated with renal phenotype in men with XLAS.

RESULTS

Patient clinical features and pathological findings

A total of 139 patients were referred to our hospital for mutational analysis between January 2006 and January 2011. Among these, 63 female patients were excluded from this study. Of the remaining 76 male patients, 62 were genetically defined as male XLAS sufferers. α5(IV) Staining was not carried out in 10 patients, and 52 men with α5(IV) expression data were therefore included in this study (Figure 1). Thirty-seven (71%) patients showed no α5(IV) staining in kidney tissue (negative group), whereas the remaining 15 (29%) showed positive staining for α5(IV) (positive group). Thirteen patients in the positive group showed normal α5(IV) distribution, although some showed reduced expression levels, and two showed a mosaic expression pattern (Supplementary Table 2 online). The characteristics of the two groups are summarized in Table 1. Although the ages at kidney biopsy and mutational analysis were similar in both groups, the time between age at biopsy and age at mutational analysis was significantly shorter in the positive group. This

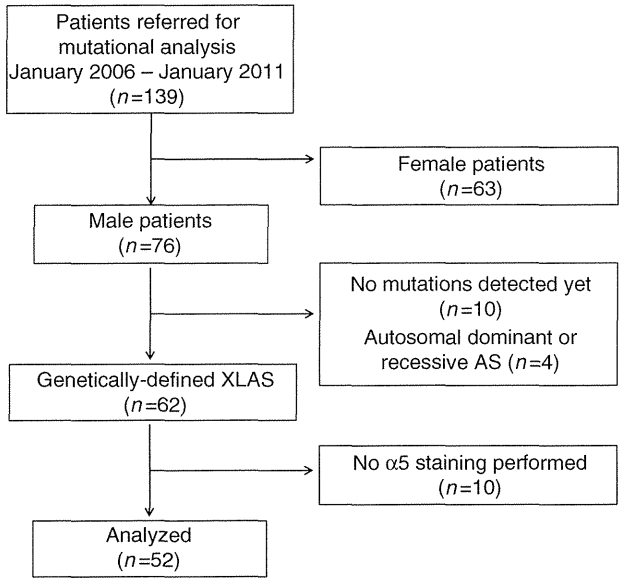


Figure 1 | Flow diagram showing patient recruitment and analysis. Fifty-two proven XLAS males were finally enrolled in this study. AS, Alport syndrome; XLAS, AS patients showing X-linked inheritance.

Table 1 | Clinical and laboratory data and treatments in α5(IV)-negative and α5(IV)-positive patients

	α5(IV)-Negative group (n = 37)	α5(IV)-Positive group (n = 15)	*P*-value
Age at analysis (years)	14.0 ± 9.2	12.8 ± 7.0	0.88
Age at kidney biopsy (years)	8.0 ± 6.2	10.5 ± 6.6	0.13
Time between kidney biopsy and mutational analysis (years)	5.8 ± 7.5	2.3 ± 3.0	0.041
Age at the detection of urinary protein (years)	4.0 ± 3.2	6.3 ± 4.8	0.036
Urinary protein/creatinine ratio (g/g Cr)[a]	1.7 ± 2.4	0.78 ± 1.0	0.027
Estimated GFR (ml/min per 1.73m^2)	111.2 ± 31.6	122.9 ± 20.6	0.43
Number of patients developing ESRD	5	1	0.66
Hearing loss (%)[b]	54.3	0	0.0002
Ocular abnormality (%)[c]	15.6	6.7	0.65
No medication	3	4	
ACEI/ARB only	29	11	
CyA only	0	0	
ACEI/ARB + CyA	5	0	

Abbreviations: ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CyA, cyclosporine A; ESRD, end-stage renal disease; GFR, glomerular filtration rate; α5(IV), type IV collagen α5.
[a]Values for patients with ESRD were excluded from this analysis.
[b]Hearing test was not conducted in one patient in the positive group.
[c]Ophthalmologic exams were not conducted in five patients in the positive group.

could be because atypical cases require genetic diagnosis, and doctors may tend to request mutational analysis sooner in these patients than in typical cases. The shorter duration of follow-up in positive patients could help to explain the smaller number of patients developing ESRD.

Table 2 | Mutation types in α5(IV)-negative and α5(IV)-positive patients

Genotype	α5(IV)-Negative group (n = 37)	α5(IV)-Positive group (n = 15)	Total
Missense	11	9	20
Nonsense	5	0	5
Insertion	7 (0)	0 (0)	7
Deletion	6 (0)	3 (3)	9
Splice site	8 (3)	1 (1)	9
Somatic mosaicism	0	2[a]	2
Total	37	15	52
Truncating mutation	23	0	
	62%	0%	

Abbreviation: α5(IV), type IV collagen α5.
Number of patients with in-frame mutation are shown in parenthesis.
[a]Both patients showed α5(IV) expression with mosaic pattern.

Five (13.5%) patients developed ESRD in the negative group, compared with one (6.7%) in the positive group. Three patients in the negative group and four in the positive group received no medications. Twenty-nine in the negative group and 11 in the positive group received angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers, whereas five in the negative group received angiotensin-converting enzyme inhibitor/angiotensin receptor blocker and cyclosporin treatment. There were no significant differences in medications between the two groups ($P = 0.091$).

Genotypes

All mutations detected in the positive and negative groups are shown in Supplementary Tables 1 and 2 online, respectively. Regarding *COL4A5* genotype, 23 (62%) of the 37 patients in the negative group had truncating mutations, including nonsense ($n = 5$), insertion ($n = 7$), deletion ($n = 6$), and splice-site mutations ($n = 5$). All these insertion, deletion, and splice-site mutations led to out-of-frame mutations. In contrast, no patients in the positive group had truncating mutations. Mutations in the positive group included missense ($n = 9$), large in-frame deletion ($n = 1$, exons 2–8, 384-bp deletion), small in-frame deletions ($n = 2$, 9-bp and 36-bp deletions), splice-site mutation ($n = 1$, leading to exon 9, 81-bp skipping), and somatic mosaic mutations ($n = 2$; Table 2). Twenty (38%) patients in this study had missense mutations in *COL4A5*; 11 in the negative and 9 in the positive groups. All the missense mutations in this study resulted in glycine substitutions. The positions of the missense mutations are shown in Figure 2. Receiver operating characteristic curve analysis of patients with missense mutations showed a cutoff value at exon 25 in both groups (sensitivity 0.778, specificity 0.8, Figure 2). Seven patients in the positive group and two in the negative group had missense mutations in the exons between 1 and 25, whereas two and nine patients, respectively, had mutations between exons 26 and 51 ($P = 0.02$). Missense mutations in the

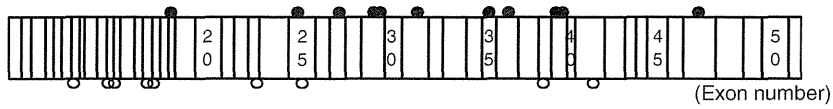


Figure 2 | Distribution map of *COL4A5* missense mutations. Black circles define the positions of missense mutations in the negative group, and open circles show the positions of missense mutations in the positive group.

positive group thus showed a significant tendency to be located before exon 25.

Phenotypes

Renal. Regarding phenotypes, the age at first proteinuria detection was significantly younger (4.0 ± 3.2 and 6.3 ± 4.8, respectively; $P = 0.036$, Table 1) and the urinary protein/creatinine ratio at the time of mutational analysis was significantly higher in the negative group than in the positive group (1.7 ± 2.4 g/g Cr and 0.78 ± 1.0 g/g Cr, respectively; $P = 0.027$, Table 1), although the ages at mutational analysis were similar in both groups. Only six patients developed ESRD, because of their relatively young age. We therefore compared the age at onset of ESRD in patients and their affected male family members. Five patients and 9 affected male family members in the negative group, and 2 patients and 13 affected male family members in the positive group developed ESRD (Supplementary Table 1 and 2 online). The age at onset of ESRD was significantly lower in the negative group than in the positive group (24.00 ± 14.2 and 37.53 ± 16.30 years, respectively; $P = 0.018$, Figure 3).

Two patients in the positive group demonstrated somatic mosaicism; both showed a mosaic pattern of α5(IV) expression in the GBM and BC, one of which we have recently reported on elsewhere.[3]

Cochlear. Previous reports have suggested a probability of hearing loss of 50% by age 15 in men with X-linked AS.[4] Our results showed that 54.3% of patients in the negative group developed hearing loss, with a median age of 13 years; however, no patients in the positive group developed hearing loss (Table 1 and Figure 4).

Ocular. It has also been reported that about 15% of X-linked AS men exhibit anterior lenticonus or other eye lesions.[4] However, there was no significant difference in ocular abnormalities between the two groups in this study (15.6% in the negative group and 6.7% in the positive group; $P = 0.65$; Table 1).

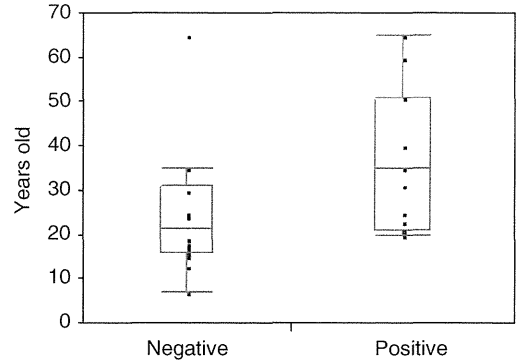
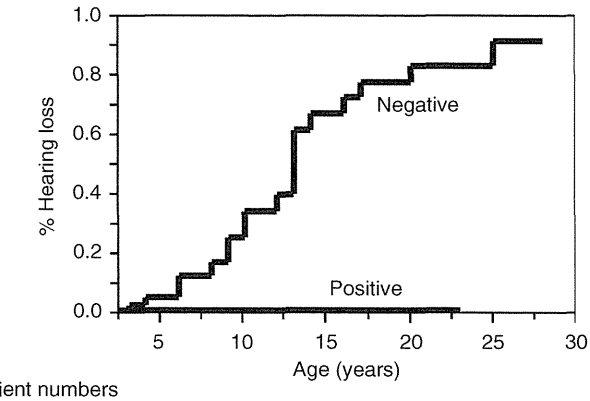


Figure 3 | Age at onset of end-stage renal disease (ESRD) in negative ($n = 14$) and positive group ($n = 15$) patients and their affected male family members. The onset age was significantly lower in negative group compared with positive group patients (24.00 ± 14.2 and 37.53 ± 16.30 years, respectively; $P = 0.018$).



Patient numbers						
Negative	2	9	15	18	19	19
Positive	0	0	0	0	0	0

Figure 4 | Probability of hearing loss in the type IV collagen α5 (α5(IV))-positive and -negative groups. The median age for developing hearing loss was 13 years in the negative group, whereas no patients in the positive group developed hearing loss.

DISCUSSION

This study provides the first report of the genetic and clinical backgrounds of male XLAS patients with atypical immunohistological findings of α5(IV) in the kidney. The results show valuable and novel information on the renal outcome and genetic background of these patients based on immunostaining patterns.

We determined the genotypes of patients with immunohistochemical evidence of α5(IV) expression, and confirmed that this staining pattern was associated with a milder renal course. We demonstrated that 29% of male XLAS patients were positive for α5(IV) staining; 60% of them had missense mutations, 27% had an in-frame deletions, and 13% had somatic mosaic mutations. In contrast, all patients with truncating mutations showed negative staining for α5(IV). Several groups have described genotype–phenotype correlations in XLAS.[5] Large rearrangements and all mutations that change the reading frame of the gene were associated with

severe types of AS,[6] whereas patients with in-frame mutations tended to have relatively mild phenotypes.[4,6,7] Jais *et al.*[4] reported normal GBM incorporation of defective α5(IV) and the related α3(IV) and α4(IV) chains in two patients, indicating that normal GBM expression of α5(IV) did not preclude a diagnosis of XLAS. Mazzucco *et al.*[8] also reported three female XLAS patients with normal α3(IV) and α5(IV) staining patterns and proven *COL4A5* mutations, including two patients with small in-frame mutations and one with a missense mutation. In addition, two patients showed positive α3(IV) staining patterns, despite negative staining for α5(IV) chain, and these authors hypothesized that very low levels of α5(IV) expression that were undetectable by immunohistochemical study may still be sufficient to allow the correct folding of the triple helix comprising the α3-α4-α5 chains.[9] Massella *et al.*[10] recently reported that 3 out of 22 patients (14%) showed α5(IV) positivity (one diffuse and two segmental), and 5 of 22 patients (23%) showed diffuse α3(IV) staining. Type IV collagen, which is a component of the GBM, is a triple helix composed of three α chains. We hypothesized that some missense and in-frame mutations might affect the structure of this triple helix, but its rate of degradation is low. The α3-α4-α5(IV) triple helix network in GBM may thus sometimes be present in reduced amounts, and low, rather than absent expression levels may lead to the milder phenotype of XLAS.[11] The apparent discrepancies in α5(IV) positivity between the current and previous reports may be the result of the use of different antibodies with different sensitivities, associated with the methods of antibody production.[12–14] These factors should be taken into account when interpreting the results of these studies.

We also examined the correlation between mutation positions and staining patterns in patients with missense mutations. Missense mutations located in exons 1–25 were more common in α5(IV)-positive patients. The three α-chains comprising the triple helix of type IV collagen consist of triple helical protomers with different compositions.[15] Each protomer has a 7S triple helical domain at the N-terminal, a collagenous domain in the middle of the molecule of Gly-X-Y repeats, and a non-collagenous trimer (NC1) at the C-terminal. The repetitive Gly sequence in the collagenous domain is required for proper assembly of the collagen triple helix and the amino-acid residues in the X–Y positions are located on the outside of the triple helix.[16] The NC1 domain has an important role in heterotrimer formation,[6,17] because the zipper-like folding mechanism of the triple helix of type IV collagen is believed to start from the C-terminal end.[7,18,19] We used receiver operating characteristic analysis to evaluate the distance from the NC domain of the missense mutations affecting α5(IV) expression, and found a cutoff point at exon 25 that distinguished between the two groups; mutations in the positive group were significantly more likely to be located before exon 25. A previous study analyzed the effect of mutation position on disease severity by comparing 98 glycine-substituting missense mutations between two groups with mutations located in exons 1–20 and 21–47 of *COL4A5*, respectively. They found that patients with mutations in exons 1–21 had less severe disease in terms of ESRD.[7] Dividing our patients into the same categories in terms of mutation locations showed that patients with missense mutations located in exons 1–21 were more likely to be positive ($P = 0.05$). The results of this study suggest that mutations located between exons 1 and 25 may lead to a less critical disruption of triple helix-forming process. Naito *et al.*[13] reported that a point mutation, such as a Gly substitution, within the collagenous domain had no effect on the construction of the NC1 domain. Predicted minimal changes in protein structure cause late onset of ESRD. Our study indicated that positivity was related to less severe effects on urinary protein levels and older age at onset of ESRD.

We also demonstrated the incidence of somatic mosaic mutations in male XLAS patients with mild phenotype. We previously reported a somatic mosaic mutation in *COL4A5* in a male XLAS sufferer,[3] and the current report showed two patients with this pattern, including a previously reported case, both of whom showed relatively mild phenotypes. This suggests that somatic mosaic mutations should be considered in male XLAS patients with mild phenotypes and mosaic α5(IV) expression.

None of the α5(IV)-positive patients developed hearing loss in this study, compared with more than half of the negative-group patients (54%). There was no difference in the incidence of ocular lesions between the two groups, although this could have been because of the relatively small number of patients in this study. These results suggest that α5(IV)-positive patients exhibit milder renal and cochlear phenotypes.

This study had several limitations related to its retrospective nature and the small number of patients who developed ESRD. A previous study reported a median renal survival rate of 25 years.[4] Patients in this study were too young to permit differences in clinical severity to be detected by estimated glomerular filtration rate. In addition, the sample size in this study was relatively small, and further studies with more patients should be conducted to confirm the current findings.

In conclusion, male XLAS patients with positive α5(IV) chain expression had milder clinical manifestations than those with no α5(IV) expression. All α5(IV)-positive patients had non-truncating or somatic mosaic mutations. Furthermore, the location of missense mutations was related to differences in α5(IV) expression.

MATERIALS AND METHODS

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

Inclusion criteria

Clinical and laboratory findings for patients with XLAS were obtained from their medical records. Patients were referred to our

hospital for clinical evaluation or genetic analysis. Most of the patients were followed in various local hospitals in Japan. DNA and data sheets were sent to our lab after acceptance of the request for mutational analysis. All patients in this study were identified with disease-causing mutations in the *COL4A5* gene and satisfied at least one of the following criteria: (1) male patients with proteinuria and hematuria or ESRD, whose renal pathology showed thickening and thinning with lamellation (basket-weave changes) or thin GBM by electron microscopy and total absence of α5(IV) in the GBM and BC. (2) Male patients with proteinuria and hematuria or ESRD whose renal pathology showed basket-weave changes or thin GBM by electron microscopy and positive α5(IV) expression in the GBM and BC.

The degree of urinary protein excretion was evaluated by the urinary protein/creatinine ratio. Estimated glomerular filtration rate was calculated using Schwartz's formula[20,21] or glomerular filtration rate-estimating equations for Japanese,[22] for patients under and over 21 years, respectively. All clinical, laboratory, and pathological data were collected when the request for mutational analysis was accepted. Images of kidney α5(IV) staining were sent to us for evaluation of the staining patterns and assessed by the same person (KN). Estimated glomerular filtration rate was measured based on the data in the data sheets. In Japan, mass urinary proteinuria screening is available for children aged 3 years, and every year from 6 to 18 years old. Information on the age at first detection of proteinuria is thus reliable. Hearing screening by audiometry is also available for children aged 6, 7, 8, 10, 12, 14, and 15 years, and information on age at detection of hearing loss is thus also very reliable.

Mutational analyses

Mutational analyses of *COL4A5* were carried out using the following methods: (1) PCR and direct sequencing of genomic DNA for all exons and exon–intron boundaries; (2) reverse transcription–polymerase chain reaction of mRNA and direct sequencing to detect abnormal splicing, and (3) multiplex ligation-dependent probe amplification to detect copy number variations. Genomic DNA was isolated from peripheral blood leukocytes from patients and family members using the Quick Gene Mini 80 system (Fujifilm Corporation, Tokyo, Japan) according to the manufacturer's instructions. For genomic DNA analysis, all specific 51 exons of *COL4A5* were amplified by PCR, as described previously.[23] The PCR-amplified products were then purified and subjected to direct sequencing using a Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) with an automatic DNA sequencer (model ABI Prism 3130; Perkin Elmer Applied Biosystems, Foster City, CA). Total RNA was extracted from blood leukocytes and/or urine sediments. RNA from leukocytes was isolated using a Paxgene Blood RNA Kit (Qiagen, Chatsworth, CA) and was then reverse-transcribed into complementary DNA using random hexamers and the Superscript III Kit (Invitrogen, Carlsbad, CA). RNA from urine sediment was isolated as described previously.[24] Complementary DNA was amplified by nested PCR using primer pairs for *COL4A5* as described previously[25,26] with slight modifications (sequences available on request). The PCR-amplified products were purified and subjected to direct sequencing.

Immunohistochemical analyses

Immunohistochemical analyses were performed using either frozen- or paraffin-embedded sections of kidney tissue. The immunohistochemical procedure has been described previously.[12,13,26] The mixture of fluorescein isothiocyanate-conjugated rat monoclonal antibody against human α5(IV) chain (H53) and Texas red-conjugated rat monoclonal antibody against human α2(IV) chain (H25) was purchased from Shigei Medical Research Institute (Okayama, Japan). Their epitopes were EAIQP at position 675–679 of the α2(IV) chain, and IDVEF at position 251–255 of the α5(IV) chain.[14] Patients showing complete negativity for α5(IV) staining were classified as the negative group; all other patients were classified as the positive group, including those with normal and mosaic expression patterns.

Statistical analyses

Data were expressed as mean ± s.d. All calculations were made using standard statistical software (JMP version 8 package for Windows, SAS, Cary, NC). The genetic and clinical backgrounds of patients in both groups were compared using Fisher's exact test, Wilcoxon's test, and receiver operating characteristic analysis. A P-value of <0.05 was considered statistically significant.

DISCLOSURE

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SUPPLEMENTARY MATERIAL

Table S1. COL4A5 mutations and family history in α5(IV)-negative group.

Table S2. COL4A5 mutations, α5 expression pattern, and family history in α5(IV)-positive group.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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