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Early RAAS Blockade Exerts Renoprotective Effects in Autosomal Recessive Alport Syndrome

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Alport syndrome is a progressive renal disease caused by mutations in *COL4A3*, *COL4A4*, and *COL4A5* genes that encode collagen type IV alpha 3, alpha 4, and alpha 5 chains, respectively. Because of abnormal collagen chain, glomerular basement membrane becomes fragile and most of the patients progress to end-stage renal disease in early adulthood. *COL4A5* mutation causes X-linked form of Alport syndrome, and two mutations in either *COL4A3* or *COL4A4* causes an autosomal recessive Alport syndrome. Recently, renin-angiotensin-aldosterone system (RAAS) blockade has been shown to attenuate effectively disease progression in Alport syndrome. Here we present three Japanese siblings and their father all diagnosed with autosomal recessive Alport syndrome and with different clinical courses, suggesting the importance of the early initiation of RAAS blockade. The father was diagnosed with Alport syndrome. His consanguineous parents and his wife were healthy. All three siblings showed hematuria since infancy. Genetic analysis revealed that they shared the same gene mutations in *COL4A3* in a compound heterozygous state: c.2330G>A (p.Gly777Ala) from the mother and c.4354A>T (p.Ser1452Cys) from the father. Although RAAS blockade was initiated for the older sister and brother when their renal function was already impaired, it did not attenuate disease progression. In the youngest brother, RAAS blockade was initiated during normal renal function stage. After the initiation, his renal function has been normal with the very mild proteinuria to date at the age of 17 years. We propose that in Alport syndrome, RAAS blockade should be initiated earlier than renal function is impaired.

Keywords: autosomal recessive Alport syndrome; *COL4A3*; end-stage renal disease; proteinuria; renin–angiotensin–aldosterone system blockade
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Introduction

Alport syndrome (AS) is a progressive renal disease caused by mutations in *COL4A3*, *COL4A4*, and *COL4A5* genes that encode collagen type IV alpha 3, alpha 4, and alpha 5 chains, respectively. Because of abnormal collagen chain, glomerular basement membrane (GBM) becomes fragile and most patients progress to end-stage renal disease in early adulthood. Approximately 85% of cases were caused by *COL4A5* mutation transmitted in X-linked dominant form, whereas 15% of cases were caused by two pathogenic mutations in either *COL4A3* or *COL4A4* on chromosome 2q 35-37 in an autosomal recessive fashion. Rare cases have autosomal dominant inheritance of mutation in *COL4A3* or *COL4A4*.

No curative therapy for AS has been reported; thus, clinicians have been investigating therapies to delay AS progression. Callis et al. (1999) reported the beneficial effect of cyclosporine on AS progression, although recent reports suggest that the effect is limited and also associated with nephrotoxicity (Charbit et al. 2007; Massella et al. 2010). Recently, the renoprotective effect of renin-angiotensin-aldosterone system (RAAS) blockade by angiotensin-converting enzyme inhibition (ACEi) in AS has become evident (Gross et al. 2012), and there is increasing evidence that anigiotensin II receptor blocker (ARB) (Webb et al. 2011) and aldosterone inhibitor (Kaito et al. 2006) are antiproteinuric and renoprotective. Here we report the cases of three Japanese siblings and their father with autosomal recessive AS. The siblings shared the same genetic mutations in *COL4A3* in a compound heterozygous state and exhibited diverse clinical courses depending on the disease stage at which RAAS blockade was initiated.

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Methods

Calculation of estimated glomerular filtration rate (eGFR)

eGFR was calculated using the Shwartz Formula from the age of 4 to 21 years and the Japanese Equation for estimating GFR when they were older than 21 years (Matsuo et al. 2009). The formulae (Shwarts Formula) are as follows: eGFR (mL/min/1.73 m^2) = k* × body height (m) × 100/serum creatinine (mg/dL); *k = 0.55 (age 2-12), 0.70 (male, age 13-21), 0.55 (female, age 13-21); and (Japanese Equation for estimating GFR) eGFR (mL/min/1.73 m^2) = 194 × (serum creatinine)$^{-1.094}$ × (Age)$^{-0.287}$ (×0.739, if female).

Mutation analysis

The genetic analysis was performed as previously described (Oka et al. 2014). Genomic DNA was isolated from peripheral blood. All exons and exon boundaries of *COL4A3* and *COL4A4* were amplified using polymerase chain reaction (PCR). PCR products were purified and subjected to direct sequencing.

Immunofluorescent staining

Immunofluorescent staining for collagen IV α5 was performed as previously described (Oka et al. 2014). Rat monoclonal antibodies α5 (IV) chains were used that is already conjugated by secondary antibody (H52; Shigei Medical Research Institute, Okayama, Japan).

Ethics

This study was approved by the Ethics Committee of the Tohoku University School of Medicine and Kobe University Graduate School of Medicine. Informed consent was obtained from all patients and their mother.

Case Presentation

We report three Japanese siblings and their father with autosomal recessive AS (Fig. 1). The relevant data from the three siblings were published in a previous report (Oka et al. 2014).

Father

The father was diagnosed with AS and had impaired hearing. He underwent maintenance dialysis in his 30s and died at the age of 47 years (Fig. 1). His consanguineous parents and his wife (mother of the siblings) were healthy. Continuous hematuria was not evident in his parents and wife.

Patient 1 (Eldest sister)

At the age of 7 years, the eldest sister was referred to our hospital because of familial hematuria. Her laboratory findings were as follows: blood urea nitrogen (BUN), 8 mg/dL; serum creatinine (sCre), 0.3 mg/dL; total protein, 6.0 g/dL; albumin (ALB), 3.6 g/dL; uric acid (UA), 4.1 mg/dL; hematuria, 3+; and urine protein, negative. She showed neither edema nor hypertension. Her physical and mental developments were normal. Renal biopsy revealed irregular thickening and thinning of the glomerular basement membrane (GBM). The lamina densa was irregularly lamellated (Fig. 2A). According to the additional immuno-

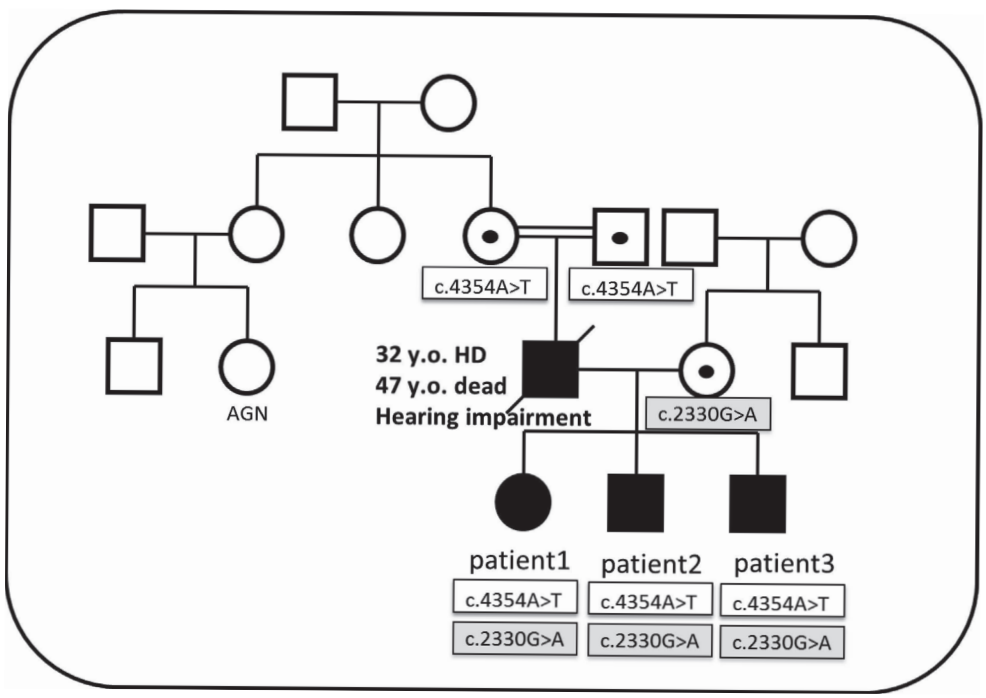

Fig. 1. Family tree showing the distribution of genetic mutations.
The father underwent hemodialysis (HD) at the age of 32 years and died at the age of 47 years. All three siblings share the same gene mutations c.2330G>A and c.4354A>T in *COL4A3* in a compound heterozygous state. c.2330G>A was transmitted from their mother. DNA from their deceased father was unavailable. Their paternal grandparents are expected to have c.4354A>T in a heterozygous state; hence, the father is expected to have c.4354A>T in the homozygous state. HD, hemodyalisis; AGN, Acute Glomeulonephritis.

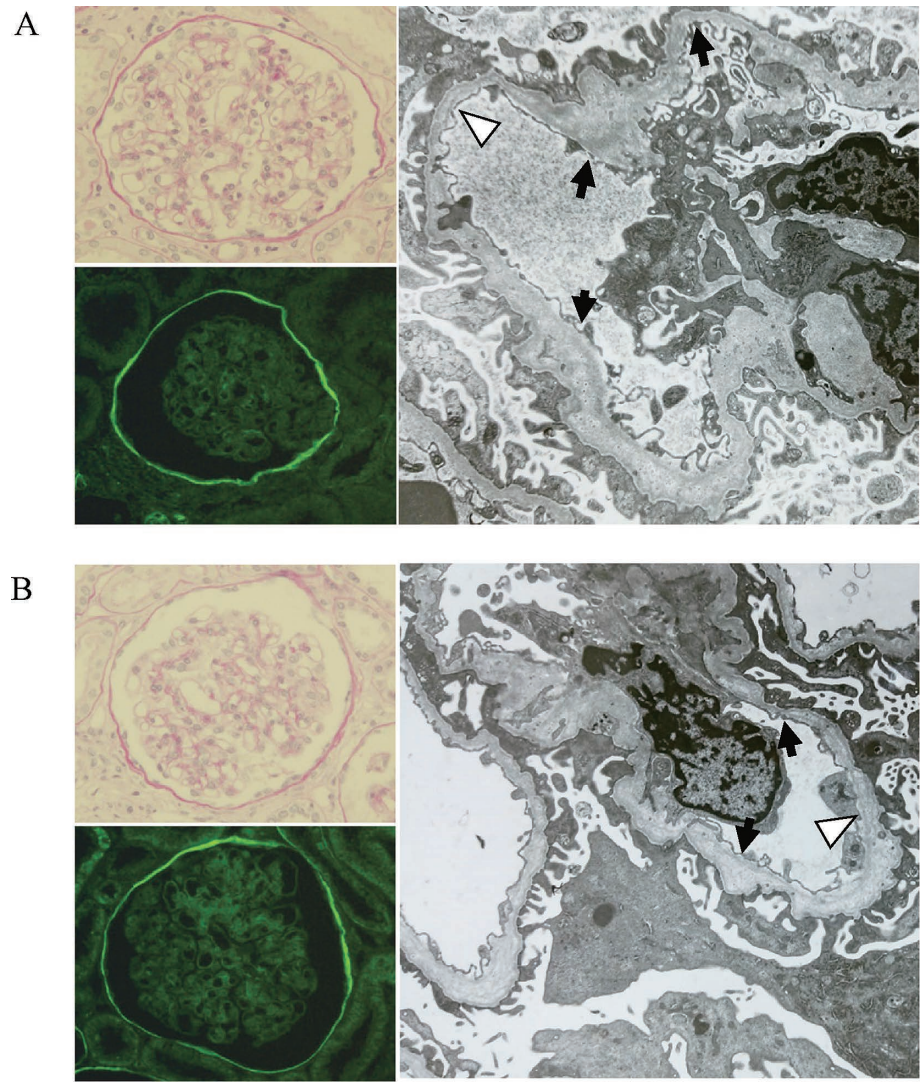

Fig. 2. Morphological analysis of the renal biopsy specimens.
The renal biopsy specimens were obtained from patient 1 (A) and patient 2 (B). Light microscopy with Periodic Acid-Schiff (PAS) stain (left upper) shows mild mesangial proliferation. Immunostaining for collagen 4α5 (left lower) reveals that collagen 4α5 is only distributed on the Bowman's capsule but absent in the GBM, suggesting the autosomal recessive AS. Electron microscopy (right) shows irregular thickening and thinning of GBM (black arrow). The lamina densa is irregularly lamellated (white arrow).

histochemistry performed 15 years later, collagen 4α5 was absent in the GBM and present only on the Bowman's capsule (Fig. 2A), indicating that she had autosomal recessive AS (Kashtan 2005). No effective AS treatment was available in the 1990s; hence, she was initially followed up annually without any therapy. Subsequently, cyclosporine was administered when she was at 12, 15, and 18 years of age, with the expectation of renoprotective effect. Trough value of cyclosporine was mostly kept within 50-70 ng/mL; maximum value was 83.5 ng/mL during follow up. It was partially effective in decreasing proteinuria; however, decline in renal function progressed. At the age of 17 years, her laboratory findings were as follows: BUN 17 mg/dL; sCre, 0.8 mg/dL; hematuria, 2+; eGFR, 86.9 mL/min/1.73 m^2; and urine protein creatinine ratio (Up/c), 1.8 g/gCre. At the age of 19 years (BUN, 33 mg/dL; sCre, 1.2 mg/dL; eGFR, 62.4 mL/min/1.73 m^2; hematuria, 2+; and Up/c, 1.9 g/gCre), RAAS blockade including lisinopril, candesartan, and aldactone was initiated. However, her renal function continued to decline. At the age of 27 years, she was diagnosed with stage 4 chronic kidney disease (Fig. 3A). Mild retinopathy was observed on follow up. Hearing impairment has not been observed.

Patient 2 (Elder brother)

The elder brother was referred to our hospital along with patient 1 for familial hematuria at the age of 6 years. His laboratory findings were as follows: BUN 13 mg/dL; sCre, 0.4 mg/dL; hematuria, 3+; eGFR, 107.2 mL/min/1.73 m^2; and urine protein, 1+. He had shown normal develop-

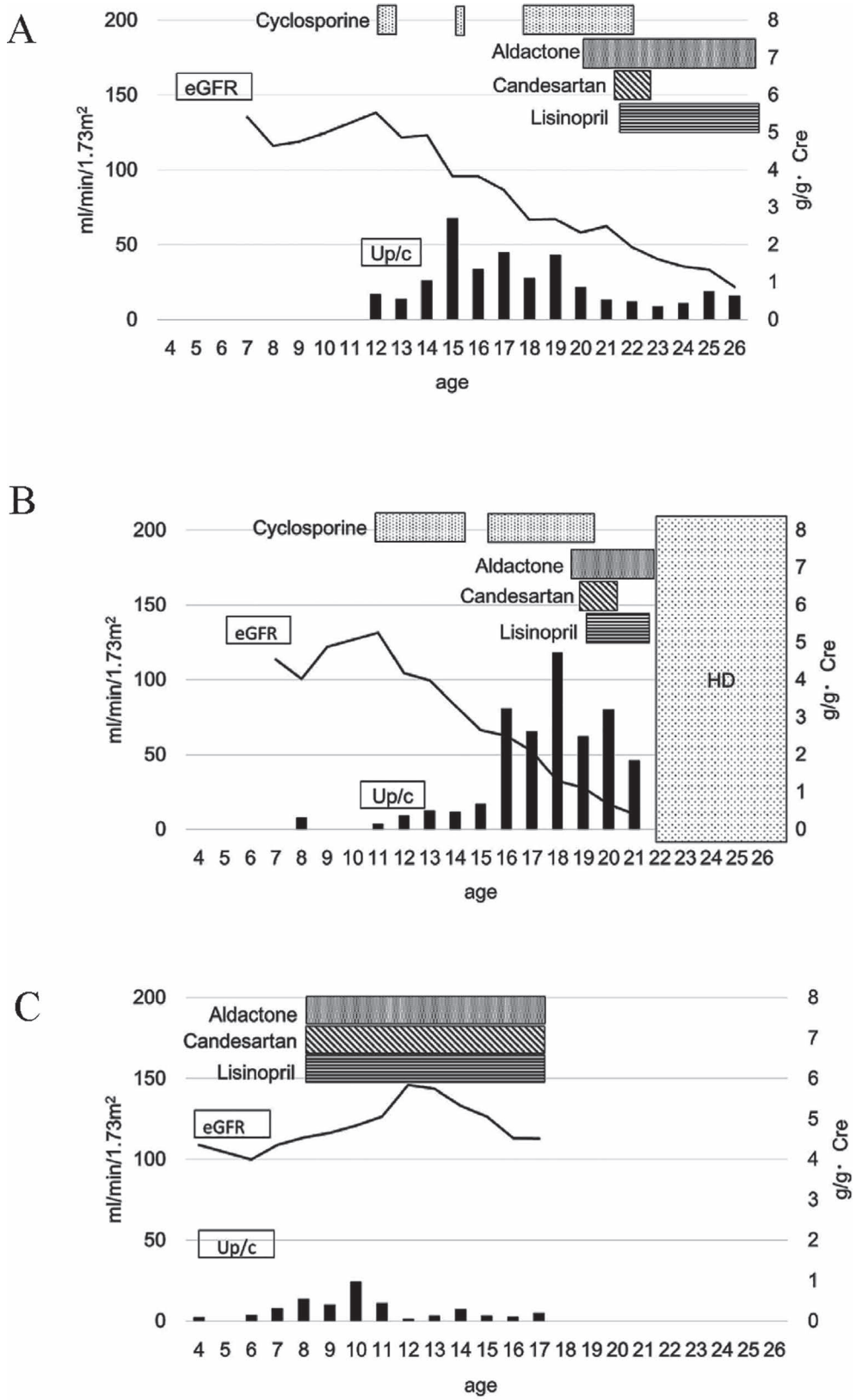

Fig. 3. Clinical courses of the three siblings.
A) Patient 1: Cyclosporine was administrated with the expectation of renoprotective effect, but could not attenuate disease progression. Despite the initiation of RAAS blockade including Aldactone, Candesartan and Lisinopril during the renal impairment stage, her renal function was deteriorated. B) Patient 2: Cyclosporine could not stop the deterioration of renal function. RAAS blockade was initiated during the renal impairment stage, but he has already progressed to end-stage renal disease. C) Patient 3: RAAS blockade was started earlier than the other siblings, before the onset of renal impairment, and he has normal eGFR. His urine protein level has been low after the initiation of RAAS blockade.

ment. His renal biopsy was also consistent with autosomal recessive AS (Fig. 2B). At the age of 11 and 15 years, cyclosporine was administered and seemed to be partially effective in reducing proteinuria, although the decline in renal function was not terminated. Trough value of cyclosporine was mostly kept within 50-70 ng/mL; maximum value was 84.4 ng/mL during follow up. At the age of 17 years, his laboratory findings were as follows: sCre, 1.6 mg/dL; hematuria, 2+; eGFR, 52.5 mL/min/1.73 m^2; and Up/c, 2.6 g/gCre. At the age of 19 years (sCre, 3.0 mg/dL; eGFR, 27.8 mL/min/1.73 m^2; hematuria, 2+; and Up/c, 4.7 g/gCre), RAAS blockade was initiated but was ineffective. At the age of 22 years, he underwent maintenance dialysis (Fig. 3B). Ocular and hearing impairment has not been observed thus far.

Patient 3 (Youngest brother)

Patient 3 was born 10 years after his siblings. Hematuria was noted from infancy. At the age of 9 years (with sCre, 0.4 mg/dL; eGFR 121 mL/min/1.73 m^2; hematuria, 3+; and Up/c, 0.98 g/gCre), RAAS blockade was initiated because proteinuria emerged. Renal biopsy was not performed, although genetic analysis revealed that he has the same mutations on *COL4A3* as his siblings, suggesting he has autosomal recessive AS. After treatment initiation, his urine protein levels returned to normal. At the age of 17 years, he has not yet shown any decline in renal function and his laboratory findings were as follows: sCre, 0.8 mg/dL; eGFR 112.9 mL/min/1.73 m^2; hematuria, 3+; and Up/c, 0.20 g/gCre (Fig. 3C) at the last follow-up. No ocular or hearing impairment has developed so far. No adverse event has been observed related to RAAS blockade, including hyperkalemia, hypotension, and dry cough.

Genetic analysis

Genetic analysis revealed that all three siblings shared the same gene mutation c.2330G>A (p.Gly777Ala) in exon 30 and c.4354A>T (p.Ser1452Cys) in exon 48 in *COL4A3* in a compound heterozygous state (Fig. 1). Both mutations were novel and were not single-nucleotide polymorphisms (Oka et al. 2014). c.2330G>A was transmitted from their mother. DNA from their deceased father was unavailable. Both of their paternal grandparents had c.4354A>T in the heterozygous state; hence, the father was expected to have c.4354A>T in the homozygous state.

Discussion

AS has been an untreatable disease for the past 90 years, and thus, effective therapies have been investigated. Although cyclosporine was reported to have an anti-proteinuric and renoprotective effect in AS patients during the late 1990s (Callis et al. 1999), recent reports showed that its anti-proteinuric effects are transient (Massella et al. 2010), and the prolonged use of cyclosporine results in nephrotoxicity (Charbit et al. 2007). At the beginning of the 2010s, RAAS blockade was shown to delay renal replacement therapy in AS patients (Gross et al. 2012). Our patients were representative of the transition in therapies for AS; there was no effective treatment when the first case (Father) was diagnosed with AS, and he started maintenance hemodialysis in his 30s and died in his 40s. When patients 1 and 2 were diagnosed, there was still no treatment available. Later, cyclosporine was administered to both of them with the expectation of renoprotective effect; however, its anti-proteinuric effect was transient, and it did not stop renal function deterioration. Although it was unclear how the administration of cyclosporine affected the decline of renal function in patients 1 and 2 (re-biopsy has not been performed), prolonged use of cyclosporine might affect their renal function. During the impaired renal function stage, patients 1 and 2 started RAAS blockade treatment, but they inevitably experienced renal failure. The effect of RAAS blockade on the reduction of proteinuria was also unclear. Patient 3 was born 10 years after his siblings. When his proteinuria emerged, the renoprotective effect of ACEi and ARB on AS progression had been verified by a number of studies (Webb et al. 2011; Gross et al. 2012). In addition, aldosterone inhibitor had been reported to effectively reduce proteinuria in AS patients who showed persistent proteinuria despite of initiation of ACEi and ARB (Kaito et al. 2006). RAAS blockade, including lisinopril, candesartan, and aldactone, was immediately started before his renal function deteriorated. To date, at the age of 17 years, his renal function is within the normal range with the very mild proteinuria. Oka et al. reported the median age of end-stage renal disease in autosomal recessive AS to be 21.0 years (Oka et al. 2014). In our cases, the older sister and brother progressed to stage 4 chronic kidney disease and end-stage renal disease at the ages of 26 and 22 years, respectively, which were consistent with those in the previous report. Compared with the siblings and the other patients with autosomal recessive AS, the youngest brother has shown a much better clinical course so far (Oka et al. 2014).

To evaluate the effectiveness of AS treatment is difficult because ESRD develops after 10-20 years of age, and the disease phenotype varies depending on the individuals' genotype. Because our cases were siblings sharing the same gene mutations, they should have developed the same phenotype. However, they showed diverse clinical courses depending on the time at which RAAS blockade was initiated which is consistent with the previous report (Gross et al. 2012).

Genetic analysis revealed our cases to have autosomal recessive AS. Autosomal recessive AS consecutively developed in the father and the three siblings, despite the father and his wife not being consanguineous (Fig. 1). To the best of our knowledge, there are no previous reports on such a hereditary form in autosomal recessive AS.

The three siblings shared compound heterozygous mutation, c.2330G>A in exon 30 and c.4354A>T in exon 48 on *COL4A3*. Collagen type IV alpha 3 has a 28-amino-acid leucine-rich signal peptide, followed by a 1,410-

amino-acid collagenous domain, and a 232-amino-acid C-terminal NC1 domain. GBM consists of triple helix of collagen type IV alpha 3, alpha 4, and alpha 5. C-terminal NC1 domain is involved in the alignment of individual alpha chains into a triple-helical structure. c.2330G>A from their mother encodes collagenous domain, and c.4354A>T from their father encodes C-terminal NC1 domain. Thus, the former mutation was expected to cause malformation of the collagen monomer itself and the later was expected to impair to form triple helix of collagen type IV. To confirm that these mutations cause the structural change, future study using in silico three-dimensional structural analysis would be needed.

Impaired hearing is a common extra-renal feature of AS. The rate of hearing impairment in autosomal recessive AS varies from 40 % to 100 % (Oka et al. 2014; Wang et al. 2014). In our cases, the father experienced hearing impairment, while none of the siblings have had difficulty in hearing so far. It was reported that the occurrence and progression of hearing impairment in AS varied depending on the mutations (Barker et al. 1996). It is possible that c.4354A>T mutation, which the father was expected to have in a homozygous form, was strongly related to hearing impairment but c.2330G>A was not. This might be the reason why the siblings who shared the mutations, c.4354A>T and c.2330G>A in a compound heterozygous form, have not developed hearing impairment.

Gross et al. (2012) reported that RAAS blockade with ACEi delayed dialysis in AS patients, regardless of disease stage at which RAAS blockade was initiated. However, the best effect was obtained when the treatment was initiated before proteinuria developed (Gross et al. 2012). Proteinuria is known to accelerate kidney disease progression (Abbate et al. 2006). Excessive protein in the glomerular filtrate activates proinflammatory and profibrotic signaling pathway in proximal tubular epithelial cells, leading to deterioration in renal function. Proteinuria is caused by alterations in glomerular membrane permeability and selectivity due to several mechanisms, including mechanical injury induced by glomerular hypertension (Remuzzi and Bertani 1990). ACEi reduces intra-glomerular pressure not only through inhibition of intra-renal angiotensin II generation but also through a bradykinin-mediated action (Salvetti et al. 1999). ARB blocks angiotensin II receptor and lowers intra-glomerular pressure. Reducing intra-glomerular pressure, RAAS blockade is expected to exert renoprotective effects by an anti-proteinuric pathway (Noone and Licht 2013). In addition, angiotensin II has been shown to be profibrotic, behaving as a cytokine, activating mononuclear cells, and increasing proinflammatory mediators (Mezzano et al. 2001). ACEi is expected to be renoprotective by blocking conversion of angiotensin I to angiotensin II (Gross et al. 2003). Consistent with the above findings, the youngest brother (patient 3), whose urine protein level remained to be low after the initiation of RAAS blockade, has not shown any deterioration of renal function so far.

Our cases suggest that the initiation of RAAS blockade earlier than decline of renal function is critical for instigating sufficient renoprotective effect of RAAS blockade. Nowadays, ACEi and ARB/aldosterone inhibitor are the first- and second-line agents for AS because the effectiveness of RAAS blockade has been strongly suggested by retrospective observational study (Gross et al. 2012). However, there remain some clinical questions, such as when RAAS blockade should be initiated and whether it could be done without severe adverse effect if initiated earlier. Ongoing large prospective study (The EARLY PROTECT STUDY) would answer some of these questions (Savva et al. 2016). We think our cases are clinically important because of the rareness of their hereditary form and the efficacy of RAAS blockade on the youngest brother despite of the severe clinical courses of his siblings. We hope that the initiation of RAAS blockade with no delay would improve the prognosis of AS by early diagnosis and careful monitoring in entire pediatric population with AS.

Conflict of Interest

The authors declare no conflict of interest.

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