Somatic mosaicism for a mutation of the COL4A5 gene is a cause of mild phenotype male Alport syndrome*

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Abstract
Background. Alport syndrome is the most common form of hereditary nephritis and is mainly caused by mutations in the COL4A5 gene, which shows the X-linked form. It is well known that some male Alport syndrome cases show a relatively mild phenotype, but few molecular investigations have been conducted to clarify the mechanism of this phenotype.

Methods and Results. This report concerns an 8-year-old male sporadic Alport syndrome patient. While electron microscopy of the glomerular basement membrane showed typical findings for Alport syndrome, however, the immunohistochemical analysis of the glomerulus showed mosaic staining of the type IV collagen α5 chain. The mutational analysis of the COL4A5 gene unexpectedly disclosed two peaks at the intron 43 splicing acceptor site (c. 3998-2 a/t) with direct sequencing. Restriction enzyme analysis demonstrated that the presence of somatic mosaicism was responsible for this mutation. mRNA extracted from the urinary sediments was analysed by RT-PCR and two PCR fragments were amplified, one consisting of a normal sequence and one with skipping of exon 44.

Conclusions. Our findings indicate that somatic mosaicism for COL4A5 is responsible for male X-linked Alport syndrome with an α5 mosaic staining pattern. Several cases with somatic mosaicism have previously been reported, however, this is the first case where the presence of this mutation was proved with a comprehensive analysis of genomic DNA, mRNA and α5 expression in the tissues. Somatic mosaicism may thus be one of the causes of the mild phenotype in Alport syndrome.

Keywords: Alport syndrome; COL4A5; somatic mosaicism

Introduction

Alport syndrome (AS) is a hereditary disorder that generally runs a progressive course. It usually presents in children as haematuria and proteinuria associated with neurosensory deafness and progresses to end-stage renal failure (ESRF) [1]. The median renal survival rate for male X-linked AS (XLAS) cases is ~25 years [2]. Characteristic alterations of the glomerular basement membrane (GBM) are observable with electron microscopy (EM). AS is characterized by irregular thinning and thickening of the GBM as well as replication of the lamina densa, which produces a laminated appearance or basket-weave pattern. When these changes are diffuse, a diagnosis of AS can be made [3].

AS is genetically heterogeneous and X-linked, with both autosomal dominant and autosomal recessive modes of inheritance [1]. However, ~90% of AS cases show X-linked inheritance, caused by mutations in the COL4A5 gene, which encodes the α5 chain, a type IV collagen. The α5 chain is expressed in the GBM, Bowman’s capsule (BC) and epidermal basement membrane (EBM). In cases with typical male XLAS, immunohistochemical analysis shows a complete loss of the α5 chain in the GBM, BC and EBM, whereas typical female cases show segmental loss with a pattern known as mosaicism [1,4–7].

XLAS in males usually presents proteinuria from childhood and leads to terminal renal failure, particularly in children and young adults, while affected females typically display mild clinical symptoms [2,8]. It is well known among clinical nephrologists, however, that some male XLAS cases show a relatively mild phenotype, leading to, for example, the development of ESRF after the age of 60 or late-onset deafness [2]. However, few molecular investigations have been conducted to clarify the mechanism of this mild phenotype.
Our report concerns an 8-year-old male XLAS case with no family history who showed mild clinical symptoms: haematuria, only mild proteinuria and no deafness. He was diagnosed with AS because of renal EM findings such as diffuse basket-weave changes in the GBM. However, immunohistochemical examination indicated the presence of the α5 chain with mosaicism and genetic testing showed somatic mosaicism in a mutation of the COL4A5 gene.

In this paper we present our findings of both molecular and immunohistochemical investigations to clarify the mechanism of the relatively mild phenotype of XLAS.

Methods

All procedures were reviewed and approved by the ethics committees of Kobe University.

Patient

The patient was an 8-year-old boy when he underwent genetic examination at our hospital. Previously, haematuria was detected during a periodic medical check-up when he was 3 years old, and he underwent a kidney biopsy at the same age. His renal EM findings showed irregular thinning and thickening, as well as a diffuse basket-weave pattern that suggested the presence of AS. Since he still showed haematuria as well as slight proteinuria (urinary protein/creatinine ratio: 0.2–0.5) at age 4, lisinopril administration was started and proteinuria has been undetectable since age 6. He had no family history of renal disease, and neither his three siblings (two males and one female) nor his parents had any urinary abnormalities. The patient showed no hearing loss with an auditory test or ocular abnormalities on examination by an ophthalmologist. He showed no clinical features of Klinefelter syndrome, and karyotyping showed the normal male pattern, 46XY. His serum BUN was 11 mg/dl and creatinine 0.4 mg/dl at age 8.

Immunohistochemical analysis of kidney and skin specimens

The immunofluorescence and immunoperoxidase methods used for immunohistochemical analysis have been described previously [6,9,10]. Rat monoclonal antibodies that recognize the α2 and α5 chains (H22 and H52; Shigei Medical Research Institute, Okayama, Japan) were used [9]. Antigen retrieval was performed for immunoperoxidase methods using paraffin-embedded sections [10]. The α5 expression ratios of the GBM and the EBM were calculated with methods described elsewhere [6].

Genomic DNA analysis

Genomic DNA was isolated from peripheral blood leukocytes of the patient and his mother with the Qiagen kit (Qiagen Inc., Chatsworth, CA, USA), according to the manufacturer’s instructions. Specific exons of COL4A5 were amplified by the polymerase chain reaction (PCR), and the PCR-amplified products were purified and directly subjected to sequencing with a Dye Terminator Cycle Sequencing kit (Amersham Bioscience, Piscataway, NJ, USA) using an automatic DNA sequencer (model ABI Prism 310; Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Mosaicism analysis using genomic DNA extracted from leukocytes, urine sediments, hair roots and skin

Because the sequence peaks of the COL4A5 gene obtained from leukocyte DNA of the patient indicated that he was heterozygous for mutant and normal alleles, we performed a mutational analysis with the QIAamp DNA mini kit (Qiagen Inc.) of genomic DNA extracted from urine sediments, hair roots and skin. Because the mutation eliminates an HpyCH4 IV restriction enzyme recognition site, heterozygosity of the patient was confirmed by restriction enzyme analysis and semi-quantitative PCR amplification using capillary electrophoresis (Agilent 2001 Bioanalyzer with DNA 1000 Lab Chips; Agilent Technologies, Palo Alto, CA, USA). Each of the PCR products was quantified by measuring the peak area, using methods described elsewhere [11]. The proportions of the mutant and wild-type allele were calculated by using the ratio of the semi-quantitated value of the band of 360 bp and that of the band of 240 bp with the band of 120 bp added (Figure 5).

RNA expression analysis

Total RNA was extracted from urine sediments [12], which were obtained by centrifugation at 3000 rpm for 10 min from 50 ml of early morning urine. RNA was isolated by using Trizol (Invitrogen, Carlsbad, CA, USA) and was then reverse-transcribed onto cDNA by using random hexamers and the Superscript III kit (Invitrogen). cDNA was amplified by means of nested PCR with the first primers’ pair located in exons 37 and 46 and second primers’ pair complementary to exons 41 and 45 (first forward: CTGGATACAAAGGTCTGTG and second forward: CAAGGTCCTCTGGAGACCA; first reverse: ATCAAAGCCAGGGATCCAT and second reverse: GGTAAGCCTTGAGGTCCTTG). After 40 cycles of amplification, PCR products were separated on 2% agarose and also sequenced with a DNA sequencer. We used the Human Kidney cDNA Library (Invitrogen) to obtain normal control kidney cDNA.

Results

Pathological findings

Light microscopy showed no significant changes, but EM showed the typical GBM abnormalities for AS such as irregular thinning and thickening and the basket-weave pattern changes (Figure 1A and B). With immunohistochemical staining of the α2 and α5 chains of the type IV collagen, the mosaicism pattern of α5 staining in the GBM and BC was detected, which is usually seen in female X-linked AS cases (Figure 2A). In a paraffin-embedded tissue containing > 30 glomeruli, in 90% of them a mosaicism pattern in the GBM was observed and in 15% a mosaicism pattern was observed in the BC. α5 expression was detected in
Somatic mosaicism in Alport syndrome

Fig. 1. Electron micrography findings of the kidney biopsy specimen: (A) irregular thickening and basket-weave pattern of the glomerular basement membrane (GBM) and (B) thin GBM.

Fig. 2. Immunofluorescence staining for type IV collagen in the glomeruli and the epidermal basement membrane (EBM): (A) kidney tissue from the patient. GBM and Bowman’s capsule (BC) show mosaic staining (GBM: arrowheads; BC: arrows). (B) Skin tissue from the patient. EBM shows an interrupted linear pattern (arrowheads); however, the area negative for α5 staining was observed only at the bottom of the dermal papillary BM. (C) Kidney tissue from a female Alport syndrome case with a heterozygous mutation in the COL4A5 gene. α2 staining shows uninterrupted staining of the GBM and BC. (D) Kidney tissue from a female Alport syndrome case with a heterozygous mutation in the COL4A5 gene. α5 staining shows a mosaic pattern of the GBM and BC (GBM: arrowheads; BC: arrows). (E) Skin tissue from a normal control. EBM shows a linear, uninterrupted pattern. (F) Skin tissue from male Alport syndrome case with a mutation in the COL4A5 gene showing negative α5 staining in the EBM. (G) Skin tissue from a female Alport syndrome case with a mutation in the COL4A5 gene showing an interrupted linear pattern in the EBM.

85% of the total glomerular area. α5 staining of the EBM obtained with a skin biopsy conducted at the same time as the kidney biopsy (Figure 2B) and α5 positive staining was seen in 90% of the total length of the EBM.

Mutational analysis

To discover the reasons for this unusual staining pattern of the α5 chain, we conducted genetic testing. Direct nucleotide sequencing of the COL4A5 gene in the DNA from the leukocytes, urine sediments, hair roots and skin of the patient identified two peaks of a- and t-positioned 2 bp before exon 44 (c. 3998-2 a/t: Figure 3A–D). To confirm the inactivation of the splice-acceptor site, COL4A5 mRNA extracted from urinary sediments was used to amplify a fragment of exons 43–45 by RT-PCR with a pair of primers for recognition of exons 43 and 45. PCR products from the patient’s sample showed two bands, one the same size as the control and the other shorter (Figure 4). Sequencing of the shorter product disclosed the complete absence of...
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Discussion

Mosaicism analysis

Unexpectedly, our patient proved to be heterozygous for the mutant and wild-type alleles, whereas his mother did not carry this substitution. The restriction enzyme analysis confirmed heterozygosity for the mutant and normal alleles in all the tissues analysed, leukocytes, urine sediments, hair roots and skin (Figure 5). Because the mutation eliminates an HpyCH4V restriction enzyme recognition site, the wild-type allele shows 240 bp, 120 bp and 50 bp fragments whereas the mutant allele shows 360 bp and 50 bp fragments. In the case of heterozygosity, electrophoresis shows all fragments. Our findings therefore proved that our patient had the somatic mosaicism of the wild type and mutation of c. 3998-2 a/t of the COL4A5 gene. The semi-quantitative PCR analysis of his leukocyte DNA revealed that 63% had the mutant genotype and 37% the wild-type form. The same analysis performed on DNA from various tissues showed the mutant form in 29%, 85% and 68%, respectively, of the DNA extracted from urine sediments, hair roots and skin (Figure 5C, Table 1).

the exon 44 sequence since exon 43 was directly joined to exon 45 (Figure 4). Exon 44 consists of 73 base pairs and this transcript abnormality leads to out of frame in translating to proteins. The mother had only wild-type alleles (Figure 3E).

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Discussion

When using molecular analysis for diagnosis of AS, it is necessary to determine whether a case has the X-linked or autosomal form. Mutations in the COL4A5 gene, which represents coding for the α5 chain of type IV collagen, are responsible for XLAS whereas the COL4A3 and COL4A4 genes are involved in the less common autosomal dominant or recessive form of the disease. The most widely used method to determine the form of inheritance is the immunological analysis of type IV collagen chain staining. In typical male cases with the X-linked form, α5 is completely negative in the GBM, BC and EBM, whereas in typical female cases the mosaicism staining pattern is observed (Figure 2C–G). In cases with the autosomal form, on the other hand, α5 is not observed in the GBM but can be seen in the BC and EBM [1,7]. However, normal α5 staining can be seen in some of the male XLAS cases, so that in such cases the inheritance form cannot be determined by immunological analysis [4].

Our patient had persistent haematuria, mild proteinuria, no sensorineural deafness and no ocular abnormalities, thus making for a relatively mild phenotype of male XLAS. However, the EM findings of the GBM showed the typical abnormalities for AS such as irregular thinning and thickening and a diffuse basket-weave pattern, leading to a diagnosis of AS. The immunohistochemical analysis, on the other hand, showed α5 staining with mosaicism in the GBM and BC, which is consistent with typical female cases. Nevertheless, 90% of the EBM was positive for α5 staining, and α5 was absent only at the bottom of the dermal papillary BM as shown in Figure 2B, a pattern that has recently been observed in normal control [13]. This indicates that our case may be characterized by almost normal α5 expression in the EBM.

A similar mosaicism in a male with mild phenotype sporadic AS has been previously reported [14] although no genetic analysis of this case was conducted. The author of this report mentioned that the case might have a somatic mosaic mutation. In our case, the mutational analysis clearly showed the presence of a heterozygous mutation, a splice site mutation and a wild-type allele in the genomic DNA extracted from leukocytes, urine sediments, hair roots and skin. RT-PCR showed both a normal transcript and a transcript with abnormal splicing in the mRNA extracted from the urinary sediment cells. Although in a female heterozygote XLAS case and a male XLAS case with 47XXY, Klinefelter syndrome could account for aberrant mosaic expression [7,15], our patient proved to have the normal karyotype of 46XY. This proved that our patient possessed a somatic mosaicism for this gene. Although it was previously reported that the genetic analysis of several male XLAS cases with mild phenotype identified the presence of somatic mosaicism, no immunohistochemical analysis was performed in these studies [16,17]. Ours is therefore the first report of an XLAS case with somatic mosaicism that was identified and clarified with the results of a
Fig. 5. Restriction enzyme, capillary electrophoresis and semi-quantitative PCR analysis: (A) the resultant fragment length is shown schematically. (B) The restriction enzyme analysis confirmed heterozygosity for the mutant and normal alleles in the various tissues of the patient while his mother is negative for this mutation, semi-quantitation of the PCR products. The percentages of mutant and wild-type alleles were calculated by using the ratio of the semi-quantitated value of the band of 360 bp and that of the band of 240 bp with the band of 120 bp added. The percentages are shown in Table 1.

Although he is at present too young to be analysed, our patient may prove to have germ line mosaicism if the mutation has occurred during early embryogenesis before the commitment to germ cells. If that is the case and if he has a daughter, then she may well have female heterozygote XLAS, as has previously been reported by another

comprehensive analysis of the genomic DNA, mRNA and α5 expression in the kidney and skin.
The tissues that showed a mosaicism pattern included both mesoderm (leukocytes and skin cells) and ectoderm (hair roots), indicating that the mutation occurred at an early developmental stage, that is, post-zygote and pre-gastrulation.

We used the semi-quantitative PCR amplification method to determine the extent of mosaicism in various tissues. Leukocyte DNA contained ~60% mutant alleles; DNA extracted from the urine sediments was ~30% and from skin ~70%. Since the patient showed a relatively mild renal phenotype for male AS, it is not clear whether the low percentage of mutant alleles in the kidney cells was responsible for the mild symptoms. No correlation between the percentage of wild-type alleles and the degree of α5 expression was observed in either the GBM or EBM. This finding indicates that at the very last, there was no relationship between the various percentages of mutant alleles and the degree of α5 expression. In our study, we determined the extent of mosaicism using quantification of DNA rather than RNA because RNA could only be amplified with nested PCR, a method that cannot be used for RNA quantification.

We described a sporadic male XLAS patient with relatively mild clinical symptoms: haematuria and mild proteinuria but without sensorineural deafness that makes it impossible to decide whether he has the mild phenotype. However another group previously reported a mild phenotype case with somatic mosaic [16]. We can therefore assume that mosaicism may be one of the causes of the previously reported XLAS cases with mild phenotypes. Careful follow-up examination of our patient will therefore be important for clarification of the nature of XLAS.

To summarize, this report described a sporadic male XLAS patient with relatively mild clinical symptoms: haematuria, mild proteinuria and no deafness. Somatic mosaicism of the COL4A5 gene was proven for the first time with the comprehensive analysis of genomic DNA, mRNA and α5 expression in the tissues. Male probands of XLAS with a mild phenotype may possess this type of mutation, so that in such cases a further analysis for somatic mosaicism is clearly warranted.