Correlation Between mRNA Expression Level of the Mutant *COL4A5* Gene and Phenotypes of XLAS Females

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Alport syndrome (AS) is a progressive hereditary glomerulonephritis presented with hematuria, progressive renal failure, sensorineural deafness, and ocular lesions. Females with X-linked Alport syndrome (XLAS) have variable phenotypes, from asymptomatic hematuria to renal failure. In order to understand the possible mechanism of different phenotypes in female XLAS, we analyzed mRNA expression level of the mutant *COL4A5* gene in fibroblasts, the X-inactivation pattern in peripheral blood DNA, and the phenotype variability of XLAS females. Total RNA was isolated from cultured skin fibroblasts in five females with XLAS and confirmed deletion mutations of *COL4A5* mRNA. Reverse transcription–polymerase chain reaction (PCR) was performed to amplify the fragment, including the mutation sequences of the *COL4A5* gene. The PCR products were electrophoresed with 8% polyacrylamide gel. Messenger RNA expression level of the mutant *COL4A5* gene was analyzed with the optical density of PCR product revealed under polyacrylamide gel. The X-inactivation analysis was performed using *Hpa*II predigestion of peripheral blood DNA followed by PCR of the highly polymorphic CAG repeat of the androgen receptor *(AR)* gene. All patients in the study had persistent microscopic hematuria. Two of them had gross hematuria. Three cases had persistent and severe proteinuria of 2+∼3+, and the others had discontinuous and milder proteinuria of −∼+. The patients whose mRNA expression level of the mutant *COL4A5* gene was higher had persistent and more severe proteinuria ($r = 0.975$, $P = 0.005$). None of them had skewed X inactivation. Our preliminary results demonstrate that the quantity of mRNA expression level of the mutant *COL4A5* gene was correlated with the phenotypic severity of females with XLAS, and this could not be explained by X-inactivation pattern in peripheral blood leukocytes. Exp Biol Med 232:638–642, 2007

Key words: Alport syndrome; female; phenotype; messenger RNA; X-inactivation

Alport syndrome (AS) is a progressive hereditary glomerulonephritis presented with hematuria, sensorineural deafness, ocular lesions, and progressive renal failure (1). The pathogenesis of AS has been linked to defects of type IV collagen, a major structural component of basement membranes. Type IV collagen includes six distinct α-chains, named α1(IV) to α6(IV). The majority (about 85%) of AS cases are caused by mutations in the *COL4A5* gene on the X chromosome, which encodes the α5(IV) chain (X-linked AS, or XLAS; Ref. 2). As expected for an X-linked condition, the XLAS males have more severe phenotypes and usually progress to end-stage renal disease (ESRD), whereas the affected females are heterozygous for the *COL4A5* mutant gene and have more variable phenotypes, from microscopic hematuria to ESRD (3). In addition, the females with XLAS often lack the characteristic alterations in glomerular basement membrane (GBM) revealed on electron microscopy. The typical ultrastructural change of the Alport kidney is a thickened GBM that transforms over time into multilamellations surrounding lucent areas, which make up the so-called split GBM (2). This lesion can be found in most XLAS males. However, heterozygous females often display an attenuated GBM; some even have GBM of normal appearance, and only a few have the typical changes of GBM (4). The variability of clinical presentation and ultrastructural findings in XLAS females could not be understood solely by analyzing mutation types of the *COL4A5* gene. Because there are two X chromosomes in females, and one of the two genes in X chromosomes will be inactivated, we speculate that in XLAS females the mRNA expression level of the mutant gene is possibly different and caused by X inactivation, which correlates to the diversity of phenotypes.

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Table 1. Clinical Features and Genetic Diagnosis of Females with X-Linked Alport Syndrome[a]

Patient	Age (years)	Gross hematuria	Microscopic hematuria	Degree of proteinuria	EBM α5(IV) staining	*COL4A5* mutation	RNA level change	Deduced amino acid change
No. 1	33	Yes	+	++	Mosaic	g.2752_2775del	r.2752_2775del	p.P851_P858del
No. 2	33	No	+++	+++	Mosaic	g.4499+1G>A	r.4478_4495del	p.P1426_Q1431del
No. 3	34	No	+	−~+	Mosaic	g.2150+1G>A	r.1982_2150del	[p.G594_P649del; p. I659X]
No. 4[b]	27	Yes	++	+++	Mosaic	g.1718+1G>A	[r.1626_1718del, r. 1626_1718delins1625+?_1625+?+43]	[p.G475_P505del, p.G475_P505del; p.M556X]
No. 5	29	No	++	+	Mosaic	g.1718+1G>A	[r.1626_1718del, r. 1626_1718delins1625+?_1625+?+43]	[p.G475_P505del, p.G475_P505del; p.M556X]

[a] EBM, epidermal basement membrane.
[b] Patient No. 4 has hypertension and renal insufficiency, but the others do not. No one had deafness and ocular changes.

Materials and Methods

Patients. Five XLAS female patients from 27 to 34 years in age and from four families were included in the study. All patients were diagnosed according to the diagnostic criteria for AS proposed by Flinter *et al.* (5): the abnormal distributions of the α3(IV), α4(IV), and α5(IV) chains in the GBM as well as the abnormal distribution of the α5(IV) chain in the epidermal basement membrane (EBM) (6–8). Two kinds of *COL4A5* mRNA existed in each of the 5 females and were detected using reverse transcription–polymerase chain reaction (RT-PCR) and direct sequencing method with cultured skin fibroblasts (9): a normal sequence and a mutant sequence. The abnormal *COL4A5* mRNA that contained deletion mutations was induced either by deletion mutations or splicing mutations. The clinical features and genetic diagnosis of the patients are summarized in Table 1.

Skin Fibroblast Culture, RNA Isolation, and Reverse Transcription. Skin punch biopsy specimens (5 mm in diameter and 1 mm in depth) were obtained from the volar aspect of the forearm after informed consent. The skin specimens were washed in 0.01 *M* phosphate-buffered saline and Dulbecco modified Eagle medium (DMEM). Epithelium and fat were carefully trimmed away. Then the minced pieces of 1 to 2 mm in size were seeded in plastic flasks with DMEM containing 10% fetal bovine serum, L-glutamine (20 m*M*), penicillin (100 U/ml), and streptomycin (100 μg/ml) in an atmosphere of 95% air and 5% carbon dioxide at 37°C. As skin fibroblasts were grown to confluence in 25-cm^2 flasks, total RNA was extracted from cultured cells using Trizol reagent (Gibco, Grand Island, NY) according to the manufacturer's instructions, and was revere transcribed to first-strand cDNA by using random primers and Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI; Ref. 9).

PCR Amplification of the cDNA. Four pairs of primers were designed according to the sites of deletion mutations of *COL4A5* mRNA in every XLAS female as well as the published *COL4A5* sequence (10) to amplify a fragment of the coding sequence of *COL4A5* mRNA. The amplified fragments were 300 to 1017 bp in length (Table 2). The PCR mixture (per 25 μl) contained cDNA template 2 μl, 1× PCR buffer (10 m*M* Tris-HCl, pH 9.0), 1.5 m*M* $MgCl_2$, 0.2 m*M* dinucleotide triphosphates, 0.2 μ*M* of each primer, and 1.25 units of Taq polymerase (Promega Corp.). The PCR parameters were optimized as follows: an initial denaturation at 95°C for 5 mins, 35 cycles of denaturation (95°C for 30 secs), annealing (55°C for 30 secs, 45 secs, or 1 min, according to the size of amplified fragments), elongation (72°C for 1 or 2 mins, according to the size of amplified fragments), and a final elongation for 5 mins at 72°C. In order to avoid the selective amplification against the template of the normal sequence or the mutant sequence of *COL4A5* mRNA, each RNA sample was reverse transcribed twice, and each template was repeatedly

Table 2. Oligonucleotide Primers and the Size of PCR Products

Patient	Forward primer (5′-3′)	Reverse primer (5′-3′)	Tm (°C)	Size of normal fragment (bp)	Size of abnormal fragment that contained mutation (bp)
No. 1	ACCAAATGGACAACCTGGAC	CCCATCATACCCATTTCACC	55	300	276
No. 2	GCCTGGGCTAAAGGGTCTAC	CATTGACGGCAGCAGTAGTA	55	1017	999
No. 3	GGGTGACAAAGGAGAGTTGG	CCTGGAAGACCTACATCACCA	55	372	203
No. 4	CTGCAGTTATGGGTCCTCCT	AGTTGCACCAGCTTGTCCTT	55	401	352, 308
No. 5	CTGCAGTTATGGGTCCTCCT	AGTTGCACCAGCTTGTCCTT	55	401	352, 308

amplified approximately two to four times using PCR reaction.

Analysis of the Optical Density of PCR Products. The PCR products were checked with 8% polyacrylamide gel electrophoresis (PAGE) and stained with silver. The optical densities (ODs) of each fragment of PCR product revealed under polyacrylamide gel were determined using an AlphaImager 2200 UV-VIS recording spectrophotometer (Shimadzu Corp., Kyoto, Japan). The total OD of each PCR product (i.e., the OD of the band with an abnormal size that contained the deletion mutations together with the OD of the band with a normal size) was taken as the total mRNA of the *COL4A5* gene. The OD ratio of mutant to total mRNA of the *COL4A5* gene was calculated. In addition, the bands shown in 8% polyacrylamide gel all were subsequently sequenced with ABI PRISM 377 Automated DNA Sequencer (PerkinElmer, Waltham, MA) with both the forward and reverse primers to confirm the sequence.

X-Inactivation Analysis. The X-inactivation pattern was determined by PCR of the highly polymorphic CAG repeat in the first exon of the *AR* gene in peripheral blood DNA. Methylation of *Hpa*II sites in close proximity to this repeat correlates with X inactivation. The site is methylated on the inactive X chromosome and after digestion with *Hpa*II, and a PCR product is obtained from the inactive X chromosome only. The *AR* (CAG)n polymorphism method was performed according to a modified technique of Allen *et al.* (11). PCR products from both digested and undigested DNA were separated on an ABI 373 automated sequencer (PerkinElmer) and were analyzed by GeneScan software (version 9.1; Metairie, LA). In order to distinguish the mutant allele from the normal allele, one male patient from each family was analyzed at the same time. The X-inactivation ratios were calculated in heterozygotes by division of the allele product ratio generated from digested DNA by the allele product ratio from undigested DNA and subsequent conversion of this figure into percentages (12). An 80% or higher percentage for one allele is considered as indicative of skewed X inactivation (13, 14).

Statistical Analysis. Statistical analysis was performed using Stata statistical software (College Station, TX). The correlation between the degree of proteinuria and the OD ratio of mutant to total mRNA of the *COL4A5* gene was evaluated with nonparametric correlations. The correlation between the X inactivation and the OD ratio of mutant to total mRNA of the *COL4A5* gene was tested by the Pearson product moment correlation. P values of less than 0.05 were taken as indicating statistical significance.

Results

Correlations Between mRNA Expression Level of the Mutant *COL4A5* Gene and the Clinical Features. The nucleotide sequence of PCR products shown in 8% PAGE was confirmed for both the normal and the abnormal sequences using the direct sequencing method (9). The optical densities of PCR product shown in 8% PAGE were determined, and the ratios of mutant to total mRNA of the *COL4A5* gene were analyzed, as shown in Table 3.

All patients in the study had persistent microscopic hematuria. Two of them had gross hematuria. Three patients had persistent and more severe proteinuria of 2+∼3+: their OD ratios of mutant to total mRNA of the *COL4A5* gene were 0.85, 1.00, and 0.67, respectively. The others had discontinuous and milder proteinuria of −∼+: their OD ratios were 0.19 and 0.25, respectively. There was a significantly positive correlation between the degree of proteinuria and the ratio of mutant to total mRNA of the *COL4A5* gene ($r = 0.975$, $P = 0.005$; Fig. 1).

X-Inactivation Pattern and mRNA Expression Level of the Mutant *COL4A5* Gene. The rate of heterozygosity at the *AR* locus of the five female patients was 80.0% (four of five). Based on an X-inactivation ratio equal to or greater than 80:20 as a criterion for skewing, no one had skewed X inactivation, as shown in Table 3. Of the three patients who had persistent and more severe proteinuria, one was not informative for the *AR* (CAG)n

Table 3. Messenger RNA Expression Level of the Mutant *COL4A5* Gene and the X-Inactivation Patterns

Patient	Average mRNA expression ratio of mutant to total *COL4A5*	X-inactivation rate of the mutant allele
No. 1	0.67 ± 0.08	Unable to distinguish
No. 2	1.00 ± 0.00	54%
No. 3	0.19 ± 0.09	39%
No. 4	0.85 ± 0.03	49%
No. 5	0.25 ± 0.07	74%

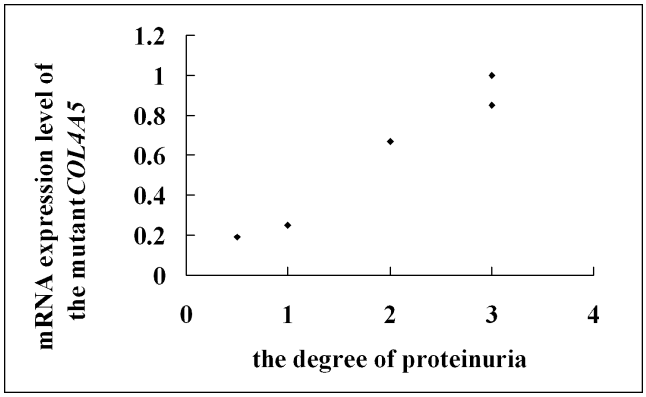

Figure 1. Graph showing the correlation between proteinuria and mRNA expression level of the mutant *COL4A5* gene. The patients whose OD ratios of mutant over total mRNA of *COL4A5* gene were higher had persistent and more severe proteinuria, whereas those whose OD ratios were lower had discontinuous and milder proteinuria. There was a significantly positive correlation between the degree of proteinuria and the ratio of mutant over total mRNA of *COL4A5* gene ($r = 0.975$, $P = 0.005$).

polymorphism, and the X-inactivation rates of the mutant allele of the two others were 54% and 49%, respectively. For the three who had discontinuous proteinuria and milder proteinuria, the X-inactivation rates of the mutant allele were 39% and 74%, respectively. There was no correlation between the X-inactivation rate and the mRNA expression level of the mutant *COL4A5* gene ($r = -0.115$, $P = 0.885$).

Discussion

Female patients with XLAS usually have variable manifestations, including clinical symptoms and ultrastructural changes in GBM, and the variabilities do not correlate with the mutation types. In the case of the five female patients with deletion mutations of the *COL4A5* gene in the present study, there also was no correlation found between the disease severity and the size of *COL4A5* deletion. Moreover, a peculiar feature of intrafamilial heterogeneity, which has been previously described by others (15), was also shown in this study. Patients 4 and 5, 27 and 29 years of age, respectively, were siblings with the same mutation, but one had had renal insufficiency, and the other had not. The molecular mechanism of the variable phenotype in XLAS females is unknown. Guo *et al*. reported one XLAS female patient whose severe phenotype was correlated with the majority (90%) of mRNA of the mutant *COL4A5* in her leukocytes and kidney (16). This might suggest that there exists a correlation between the mRNA expression level of the mutant *COL4A5* gene and the phenotype variability in XLAS females.

Our data showed that the patients in whom the mRNA expression level of mutant *COL4A5* gene was more than the normal had more severe proteinuria. Proteinuria was positively correlated with the expression ratio of mutant to total mRNA of the *COL4A5* gene in the XLAS females. Studies have shown that proteinuria was a significant risk factor for end-stage renal disease, and that monitoring the degree of proteinuria was valuable for estimation of the prognosis of female AS patients (15, 17). Taken together, we may speculate that the quantity of mutant mRNA expression of the *COL4A5* gene was related to the phenotype variability of XLAS females. However, the sample size was small and the measure of proteinuria was not qualitative in our study; it was a pitfall and a pity. Nevertheless, it did give some hints that there were some relationships between the mRNA expression level of the mutant *COL4A5* gene and the phenotypic severity of XLAS females. We hope this study and the preliminary results could give rise to more investigation and attention from more study groups, including ours. Although there is no evidence that the mRNA expression level of the mutant *COL4A5* gene in fibroblasts is similar to that in GBM, the preliminary result from our study did reveal that the mRNA expression level of the mutant *COL4A5* gene in fibroblasts, at least, correlated to qualitative level of proteinuria. In addition, since skin biopsy is simple and is easier than kidney biopsy, most AS patients could accept it, so fibroblasts were useful in analyzing the possible mechanism of different phenotypes in female XLAS.

In the case of X-linked disease, the different mRNA expression levels of mutant genes in carrier females may be attributable to the different X-inactivation patterns. Males normally have one copy of the X chromosome, whereas females normally have two copies. This potential imbalance of gene expressions from the two X chromosomes in females results from inactivating one X chromosome. This inactivation causes the transcriptional silencing of most or all genes on the inactive X chromosome. Studies from other groups have revealed that the phenotypic variations in carriers of some X-linked disorders were attributable to the variable patterns of X inactivation (13, 18). The X-inactivation pattern also was speculated to be responsible for the disease phenotype in female carriers with XLAS, but so far no definite correlation was demonstrated (15, 19).

In the present study, one subject was homozygous for the *AR* gene and was revealed to have the same number of CAG repeats in both alleles; therefore, X inactivation could not be assessed in this subject. The rate of heterozygosity for the *AR* gene was 80.0% in our study, which is quite similar to previous reports of 86.5%–90% (20, 21). None of the patients in our study had skewed X inactivation. There was no correlation between the mRNA expression level of the mutant *COL4A5* gene and the X-inactivation rate of the mutant allele in the peripheral blood cells, which is consistent with the result of Vetrie *et al*. (22). Vetrie *et al.* studied the X-inactivation patterns in peripheral blood leukocytes of XLAS females but did not find any correlation between the X-inactivation patterns and the disease severity (22). Previous studies from other groups have shown that X inactivation had tissue-specific variations (20, 23). Nevertheless, peripheral blood had a higher skewed X-inactivation pattern than other tissues, and the same X chromosome will

be predominantly inactivated in different tissues (20, 23). That is to say, in some ways the X-inactivation patterns in peripheral blood cells might reflect the X-inactivation patterns in other tissues, such as kidney or skin fibroblasts. Thus, the phenotype variation of female XLAS might not be explained by the X-inactivation patterns in peripheral blood cells. However, our sample size was small, and we may need to increase the sample number in future studies to further confirm the result. In addition, besides X inactivation there may be other mechanisms that impact the phenotype variations of female XLAS, such as DNA methylation, histone acetylation, noncoding RNAs, etc. (24, 25).

Our preliminary results demonstrated that the quantity of the mRNA expression level of mutant *COL4A5* gene was correlated with the phenotypic severity of XLAS females. We speculate that analyzing the ratio of mutant to total mRNA of the *COL4A5* gene might be applied to predict the outcome in female patients, but this needs further study. The X-inactivation pattern in peripheral blood cells had no correlation with the phenotypic severity of XLAS females, the mechanisms of which need further study.

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