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Mutational analysis of type IV collagen α5 chain, with respect to heterotrimer formation

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Abstract

Alport syndrome (AS) is caused by mutations in type IV collagen α3, α4, and α5 chains. The three chains form a heterotrimer. In this study, we introduced 12 kinds of missense and three kinds of nonsense mutations, corresponding to AS mutations, into the NC1 domain of α5(IV) and characterized the mutant chains. Nine α5(IV) chains with amino acid substitutions and all three truncated α5(IV) chains did not form a heterotrimer and were not secreted from cells. Three α5(IV) chains with amino acid substitutions did, however, form heterotrimers in cells, but these were not secreted from cells. These findings indicate that a defect in heterotrimer formation is the main molecular mechanism underlying the pathogenesis of AS caused by mutation in the NC1 domain. We also showed that even a single amino acid deletion in the carboxyl-terminal region markedly affected the heterotrimerization, indicating that the carboxyl-terminal end is indispensable for heterotrimer formation.

Keywords: Type IV collagen; NC1 domain; Heterotrimerization; Alport syndrome; Mutational analysis

Alport syndrome (AS) is a hereditary disorder characterized by hematuria and progressive renal failure and is frequently associated with sensorineural hearing loss and ocular abnormalities. Electron microscopic analysis of renal biopsies from AS patients shows typical ultrastructural changes such as regional thinning, thickening, and splitting in the glomerular basement membrane (GBM). Most cases of AS are caused by mutations in the type IV collagen α5 gene (*COL4A5*) on the X chromosome. The rarer autosomal forms are caused by mutations in the type IV collagen α3 and α4 genes (*COL4A3* and *COL4A4*) on the chromosome 2 [1].

Type IV collagens, containing six distinct α chains, α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), and α6(IV), are found in the basement membrane. Two α1(IV) chains and one α2(IV) chain form a heterotrimer, $[\alpha1(IV)]_2\alpha2(IV)$, and are ubiquitously present in basement membranes. In contrast, the other α chains are minor components in basement membranes with restricted tissue distributions. A heterotrimer, α3(IV)α4(IV)α5(IV), is present only in kidney, lung, testis, cochlea, and eye tissues. In the kidney, the heterotrimer is present in the GBM and in some tubular basement membranes [2]. In AS patients, mutations in one of the α3(IV), α4(IV), and α5(IV) chains usually leads to the absence of all three chains in the GBM [1]. This finding suggests that all three chains depend on each other for their incorporation into the GBM.

Each type IV collagen chain consists of a short 7S domain at the amino-terminus, a long collagenous domain of approximately 1400 residues with Gly-X-Y repeats and a non-collagenous (NC1) domain of about 230 residues at the carboxyl-terminus. Association of the three chains is initiated at NC1 domains, and folding of the collagenous domain into the triple helix occurs in a carboxyl-terminal to amino-terminal direction [1,3]. NC1 domains are considered to play an important role in the selection of α chains for assembly into heterotrimers [4].

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We previously established cell lines that expressed mouse α3(IV), α4(IV), and α5(IV) chains and confirmed that, using these cell lines, the three chains form a heterotrimer. We also found that the ability of the α5(IV) chain, with either a G1182R (Gly^{1182} to Arg) in the collagenous domain or a C1573R (Cys^{1573} to Arg) substitution in the NC1 domain, corresponding to previously reported mutations in AS patients, to form a complex with α3(IV) and α4(IV) chains was diminished [5].

This study aims to confirm whether other mutations of the *COL4A5* gene affect heterotrimer formation by α3(IV), α4(IV), and α5(IV) chains, focusing on the NC1 domain, and to establish that the defect in trimer formation is the main molecular mechanism underlying the pathogenesis of Alport syndrome caused by mutations in the NC1 domain.

Materials and methods

Plasmids and in vitro mutagenesis. The mouse *COL4A5* cDNA, including the entire open reading frame and an added FLAG epitope on the 3′ terminal region, was cloned into the expression vector pCAGGS, which contains a neo cassette. In vitro mutagenesis reactions were carried out on the plasmid using a PCR based method described in our previous report [5]. Briefly, oligonucleotide primers were designed in inverted tail-to-tail directions to amplify the cDNA together with the cloning vector. A point mutation for an amino acid substitution or a termination codon was introduced into the middle of one primer. After phosphorylation of the 5′ end of each primer, the expression plasmid was amplified using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). After digestion of the parental DNA with DpnI, the amplified DNA was self-ligated using a ligation-high kit (Toyobo) and transfected into DH5α-competent cells. Plasmids were purified, and DNA sequencing excluded additional mutations introduced elsewhere in the mutant *COL4A5* cDNA. Mutant *COL4A5* expression plasmids were constructed by replacement of a wild-type cDNA fragment with the corresponding mutant cDNA fragment using appropriate restriction endonucleases.

Cell culture and transfections. The human embryonic kidney cell line 293 (HEK293) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). α34 cells, previously established by us [5], express mouse α3(IV) with a myc epitope and mouse α4(IV) with a V5 epitope. α34 cells are resistant to hygromycin and blasticidin, due to transfected expression plasmids that express hygromycin or blasticidin resistant genes, and were cultured in medium containing 200 μg/mL hygromycin B (Invitrogen, Carlsbad, CA, USA) and 8 μg/mL blasticidin S (Funakoshi, Tokyo, Japan). α34 cells (5×10^4 cells) were transfected with 0.5 μg of the mutant *COL4A5* expression plasmid using Lipofectamine PLUS™ reagent (Invitrogen). Transfectants were selected in medium containing 400 μg/mL G418 (Invitrogen). Resistant clones were screened for protein expression from cell extracts by Western blot analysis using anti-FLAG monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA).

Immunopurification of α5(IV) protein and of associated protein. The clonal cell lines were grown to confluence on 100 mm petri dishes and extracted in 1 mL of NETN (50 mmol/L Tris–HCl, pH 7.8, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, 1 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL pepstatin A, and 1 μg/mL leupeptin). Confluent cells were also incubated for an additional 24 h in 3 mL of serum-free medium. The medium was collected and equilibrated to 1 mmol/L EDTA, 0.5 mmol/L PMSF, 1 μg/mL pepstatin A, and 1 μg/mL leupeptin. One milliliter of the cell extract or culture medium was then incubated with 30 μL anti-FLAG M2 affinity gel (Sigma Chemical Co.) overnight at 4 °C. The gel was washed five times with 50 volumes of ice-cold NETN. The bound proteins were extracted by boiling in sodium dodecyl sulfate (SDS) sample buffer containing 6% 2β-mercaptoethanol and then separated by 7.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then analyzed by immunoblotting with anti-myc (Cell Signaling Technology Inc., Danvers, MA, USA) for α3(IV), anti-V5 (Invitrogen) for α4(IV), and anti-FLAG for α5(IV) monoclonal antibodies and then with an alkaline phosphatase-conjugated horse anti-mouse IgG antibody. Color development was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Results

Recombinant production of mutant α5(IV)

In this study, 18 cell strains that express mutant forms of the mouse α5(IV) chain together with the mouse α3(IV) and α4(IV) chains were established. In a previous study we have confirmed the trimerization of α3(IV), α4(IV), α5(IV) chains, using HEK293 cells transfected with mouse *COL4A3*, *COL4A4*, and *COL4A5* cDNAs. In the present study we, therefore, used mouse cDNA to construct a series of mutant *COL4A5* cDNAs harboring point mutations in the NC1 domain.

The 11 amino acid substitutions that were introduced in the NC1 domain of mouse α5(IV) chain were: G1492A (Gly^{1492} to Ala), A1504D (Ala^{1504} to Asp), R1517H (Arg^{1517} to His), P1523T (Pro^{1523} to Thr), W1544R (Trp^{1544} to Arg), W1544S (Trp^{1544} to Ser), R1569Q (Arg^{1569} to Gln), C1570S (Cys^{1570} to Ser), C1592R (Cys^{1592} to Arg), L1655R (Leu^{1655} to Arg), and R1683Q (Arg^{1683} to Gln). The introduced amino acid substitutions, except W1544R, corresponded to those reported in Alport syndrome patients whose clinical phenotypes have been described previously [6–16]. Although the α5(IV) W1538R substitution in humans, which corresponds to W1544R in mice, was mentioned in the Human Gene Mutation Data Base (www.hgmd.cf.ac.uk/), the substitution was not described in the designated reference. These mutant *COL4A5* cDNAs were transfected into α34 cells. The cell strain that expressed the α3(IV) and α4(IV) chains, together with the mutant α5(IV) chain, containing the G1492A substitution, was named α345G1492A. Other cell strains were named in the same manner. The seven mutant α5(IV) chains with deletions were ΔC123, ΔC9, ΔC7, ΔC4, ΔC3, ΔC2, and ΔC1. These mutant α5(IV) chains lack carboxyl-terminal 123, 9, 7, 4, 3, 2, and 1 amino acid residues, respectively. ΔC123, ΔC9, and ΔC7 mutations have been reported in Alport syndrome patients [6,11,14,17]. Fig. 1 shows the expression of recombinant α3(IV), α4(IV), and α5(IV) chains in the cell extracts of newly established cell lines and in previously established α345 and α345C1573R cells [5], under reducing conditions. As all recombinant proteins were found at the expected sizes, the cell lines were used for further experiments.

Effect of amino acid substitution in α5(IV) on ternary complex formation of α3(IV), α4(IV), and α5(IV)

Whole-cell extracts or culture media of α345C1573R cells and of the 11 newly established cell strains that express

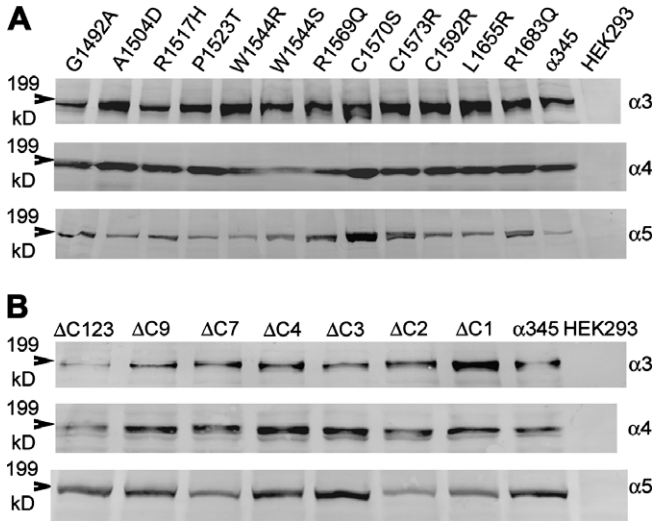

Fig. 1. Synthesis of recombinant α3(IV), α4(IV), and α5(IV) chains in transfected human embryonic kidney 293 (HEK293) cells. The α34 cell strain was established by transfection of α3(IV) and α4(IV) cDNAs into HEK293 cells. Immunoblot analyses of extracts from α34 cells transfected with α5(IV) containing amino acid substitutions (A) and extracts from α34 cells transfected with truncated α5(IV) (B) were performed. Extracts of these cells were subjected to 7.5% SDS–PAGE under reducing conditions, followed by immunoblot analyses using monoclonal antibodies against epitope tags, myc for α3(IV), V5 for α4(IV), and FLAG for α5(IV) chains.

mutant α5(IV) chains containing amino acid substitutions were immunoprecipitated with the anti-FLAG antibody. The immunoprecipitants were analyzed by SDS–PAGE, followed by immunoblotting with anti-myc, anti-V5, and anti-FLAG antibodies (Fig. 2). Based on complex formation and on secretion from cells, the mutant α5(IV) chains were divided into five groups. In the first group were A1504D, and P1523T α5(IV) chains, which formed a complex with α3(IV) and α4(IV) chains in the cells. Although the mutant-type α5(IV) chains were immunoprecipitated with the anti-FLAG antibody from the culture media, α3(IV) and α4(IV) chains were not co-immunoprecipitated with the α5(IV) chains. These findings indicate that the stability and/or the secretion of the formed complex in the cells might be diminished and the mutant-type α5(IV) chains may be secreted in monomeric form. In the second group, the R1683Q α5(IV) chain showed the same pattern as in the first group except that the chain was not secreted, even in monomeric form. In the third group, the W1544R and W1544S α5(IV) chains were considered unique. The mutant α5(IV) chains formed a complex with the α3(IV) chain, but not with the α4(IV) chain. The fourth group contained R1517H, R1569Q, and L1655R α5(IV) chains, which did not form a complex with the α3(IV) and α4(IV) chains and were secreted in monomeric form. In the fifth group, G1492A, C1570S, C1573R, and C1592R α5(IV) chains did not form a complex with α3(IV), and α4(IV) chains and were not secreted. These data are summarized in Table 1. Also listed in Table 1 are the corresponding amino acid substitutions in the human α5(IV) chain and the characteristic clinical manifestations of Alport syndrome, which include the age of onset of end-stage renal disease (ESRD), hearing loss, and ocular lesions. The patients whose age of onset of ESRD was before 31 years of age were classified as juveniles and those whose age of onset was later than 31 years were classified as adults [18]. There were no clear correlations between the abilities of the mutant-type α5(IV) chains and the clinical manifestations.

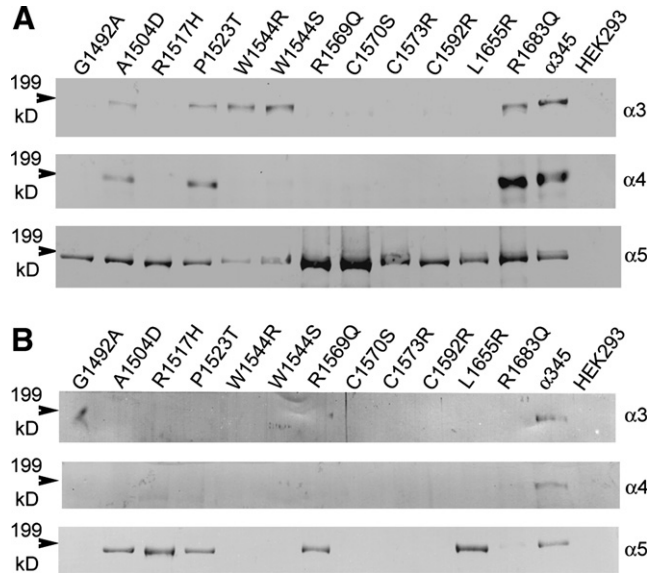

Fig. 2. Effect of amino acid substitution of α5(IV) on ternary complex formation of α3(IV), α4(IV), and α5(IV). Cell extracts (A) and culture media (B) of established cell strains with amino acid substitutions in α5(IV) were immunoprecipitated with an anti-FLAG monoclonal antibody and then subjected to 7.5% SDS–PAGE under reducing conditions, followed by immunoblot analyses with anti-myc, anti-V5, and anti-FLAG monoclonal antibodies.

Effect of carboxyl-terminal deletion of α5(IV) on ternary complex formation of α3(IV), α4(IV), and α5(IV)

The truncated α5(IV) chains ΔC123, ΔC9, and ΔC7, corresponding to the nonsense mutations found in AS patients, at codons 1563, 1677, and 1679, respectively, were analyzed. Each of the three truncated proteins did not form a complex with α3(IV), and α4(IV) chains and was not secreted from cells in monomeric form (Fig. 3).

Next, in order to determine how many α5(IV) chain carboxyl-terminal amino acid residue deletions influence the heterotrimerization of α3(IV), α4(IV), and α5(IV) chains, the cell extracts and culture media of α345ΔC4, α345ΔC3, α345ΔC2, and α345ΔC1 cells were immunoprecipitated with anti-FLAG antibody (Fig. 3). In each extract of α345ΔC4, α345ΔC3, and α345ΔC2 cells, the α3(IV) and α4(IV) chains were not co-immunoprecipitated with the mutant-type α5(IV) chains. On the other hand, the α3(IV) and α4(IV) chains were co-immunoprecipitated with the ΔC1 α5(IV) chain; however, the amounts of the co-immunoprecipitated α3(IV) and α4(IV) chains in α345ΔC1 cells were markedly reduced compared with the

Table 1
Fate of recombinant α5(IV) chain and AS clinical phenotypes

Group	Amino acid change in mouse α5(IV)	Function of recombinant α5(IV)			Homologous amino acid change in human α5(IV)	Clinical manifestations				Reference
		Heterotrimer formation		α5(IV) Secretion		Sex	ESRD (age)	Hearing loss	Ocular lesions	
		Cell extract	Culture medium							
1	A1504D	+	–	+	A1498D	Male	A (39)	ND	ND	[7]
	P1523T	+	–	+	P1517T	Male	J (16)	+	–	[9]
2	R1683Q	+	–	–	R1677Q	Male	A (40–62)	+	–	[16]
3	W1544R	–	–	–	W1538R[a]					
	W1544S	–	–	–	W1538S	Male	A (33)	–	–	[10]
4	R1517H	–	–	+	R1511H	Male	J (<30)	+	–	[8]
	R1569Q	–	–	+	R1563Q	Male	J (25–29)	+	–	[11]
	L1655R	–	–	+	L1649R	Male	A (>31)	+	ND	[15]
5	G1492A	–	–	–	G1486A	Male	–(49)	+	ND	[6]
	C1570S	–	–	–	C1564S	Male	A (33)	+	ND	[12]
	C1573R	–	–	–	C1567R	Male	J (16)	+	+	[13]
	C1592R	–	–	–	C1586R	Male	J (14)	+	ND	[14]
	R1569X (ΔC123)	–	–	–	R1563X (ΔC123)	Male	J (23)	+	–	[11]
	R1683X (ΔC9)	–	–	–	R1677X (ΔC9)	Male	J (13–25)	+	+	[6,14]
	Q1685X (ΔC7)	–	–	–	Q1679X (ΔC7)	Male	–(15)	–	ND	[17]

A, adult; J, juvenile; ESRD, end-stage renal disease (age at renal failure or at time reported, in years); ND, no data available.
[a] The W1538R substitution was mentioned in the Human Gene Mutation Data Base, but the substitution was not described in the designated reference.

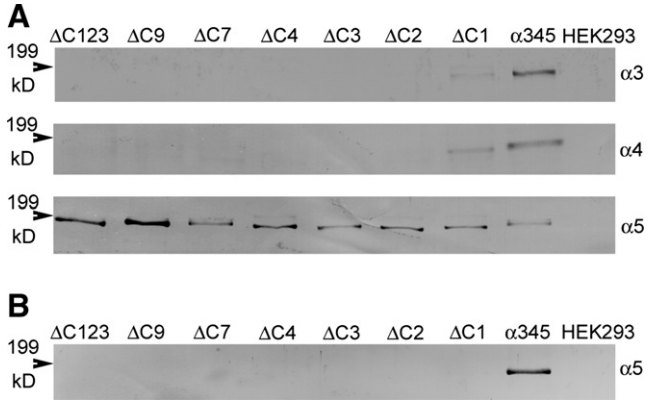

Fig. 3. Effect of deletion of α5(IV) on ternary complex formation of α3(IV), α4(IV), and α5(IV). Cell extracts (A) and culture media (B) of established cell strains with truncated α5(IV) were immunoprecipitated with an anti-FLAG monoclonal antibody. The immunoprecipitants were analyzed by 7.5% SDS–PAGE under reducing conditions and immunoblotting with anti-myc, anti-V5, and anti-FLAG monoclonal antibodies.

amounts of those chains in α345 cells. In culture medium of α345ΔC1 cells, the ΔC1 α5(IV) chain was not immunoprecipitated by the anti-FLAG antibody.

Discussion

In our previous study, we found that the ability of the α5(IV) chain, with either a G1182R or a C1573R substitution, to form a complex with α3(IV) and α4(IV) chains was diminished [5]. In this study, to elucidate how other amino acid substitutions and deletions in the α5(IV) chain, reported in AS patients, influence the heterotrimerization of α3(IV), α4(IV), and α5(IV) chains, we established cell lines that express wild-type α3(IV) and α4(IV) chains, together with mutant-type α5(IV) chains, containing amino acid substitutions or carboxyl-terminal deletions in the NC1 domain and analyzed complex formation between these three chains.

We focused on the NC1 domain for the following reasons. Firstly, the NC1 domains are supposed to contain molecular recognition sequences for α(IV) chain assembly and play an important role for heterotrimer formation [4]. Secondly, although the NC1 domain is much shorter than the collagenous domain, many kinds of amino acid substitutions in the NC1 domain of the α5(IV) chain have been reported in X-linked AS patients, whose clinical symptoms were varied.

We used mouse *COL4A5* cDNA in this study. Tissue distributions of type IV collagen α chains in mice are the same as those in humans [1,19]. The clinical and histological findings determined from *COL4A3* and *COL4A5* knockout mice were similar to those in AS patients [20,21]. These findings indicate that there may be no differences in the essential roles of the α3(IV), α4(IV), and α5(IV) chains in the GBM between mice and humans. In this study, we introduced single base substitutions, corresponding to mutations found in AS patients, in the NC1 domain of mouse *COL4A5* cDNA. Since the amino acid sequence of the mouse NC1 domain of α5(IV) chain shows 96% identity and 99% homology to the human counterpart, it is considered that the same amino acid substitution in the α5(IV) chain affects its function similarly in mice and humans.

In the process of formation of type IV collagen networks, the NC1 domains of the α3(IV), α4(IV), and

α5(IV) chains initially specifically interact with each other, and the three chains then assemble into triple-helical molecules called protomers. Two protomers associate head to head at the carboxyl termini (NC1– to –NC1) forming NC1 hexamers, and four protomers then associate on the 7S domain at the amino termini forming tetramers [22]. Mutations in the NC1 domain could prevent the assembly of the three chains or association of the protomers. Moreover, incompletely formed protomers or type IV collagen networks may be degraded rapidly. These defects can result in complete absence of these three chains in the GBM. In the present study, 12 out of 15 recombinant α5(IV) chains with mutations, which correspond to AS mutations, lost their ability to form the α3(IV)α4(IV)α5(IV) heterotrimer. Three mutated α5(IV) chains did, however, form heterotrimers in the cells, but the heterotrimers were not secreted from cells. Considering the fact that collagen chains associate together, are folded with the help of cytoplasmic chaperon proteins, and then a correctly formed heterotrimer is secreted [3], it is likely that the heterotrimer with each of the three mutated α5(IV) chains is formed incorrectly and is not secreted from the cells or is degraded rapidly after secretion from the cells. We suggest that a defect in heterotrimer formation by α3(IV), α4(IV), and α5(IV) chains is the main molecular mechanism underlying the pathogenesis of Alport syndrome caused by mutation in the NC1 domain of α(IV) chains.

Interestingly, deletion of even one carboxyl-terminal end amino acid residue of the α5(IV) chain, markedly affects trimer formation of the three chains. It means that the carboxyl-terminal end is indispensable for the trimerization of the α3(IV), α4(IV), and α5(IV) chains, and the introduction of a premature stop codon to any site on the three chains may result in AS phenotypes.

Almost all the missense mutations previously reported in Alport syndrome, other than conserved amino acids in the NC1 domain, were glycine substitutions in the collagenous domain. All mutated glycine residues correspond to Gly of Gly-X-Y repeats. Various phenotypes of the Alport syndrome with glycine substitutions have been reported [23]. In the mutated α(IV) chains with glycine substitutions in the collagenous domain, triple helix formation of the three chains, after initial interaction between the NC1 domains may be defective. The incorrectly folded heterotrimer might not be secreted, or the incomplete heterotrimer and resultant type IV collagen network may be degraded rapidly. In fact, an α5(IV) chain with a G1182R substitution, where glycine corresponds to the Gly-X-Y repeats in the collagenous domain, had the ability to form a heterotrimer in cells, to almost the same degree as that of the wild-type α5(IV) chain; however, in the culture medium the amount of the heterotrimer containing the mutant α5(IV) chain was much less than that containing the wild-type α5(IV) chain [5]. Further studies of various glycine substitutions in the α5(IV) collagenous domain using our assay system might provide a better understanding of the molecular pathogenesis of Alport syndrome.

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