RESEARCH NOTE

Mutation analysis of *COL4A3* and *COL4A4* genes in a Chinese Autosomal-Dominant Alport Syndrome Family

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1. Introduction

Alport syndrome (AS) is a heterogeneous renal hereditary disease characterized byhematuria, proteinuria and progressive renal failure with structural defects of the glomerular basement membrane (GBM) and is often associated with sensorineural hearing loss and ocular lesions(Hertz, 2009; Savige et al., 2015). A distinctive trait of

AS is the predominant changes in the network α3/α4/α5 chains of type IV collagen in GBM. Any of the genes encoding these three chains could be involved in the pathogenesis of AS. Mutations in *COL4A5*, which encodes type IV collagen α5 chain, are generally believed to cause X-linked AS(XLAS,OMIM 301050), whereas mutations in *COL4A3* and *COL4A4* genes which encode type IV collagen α3 and α4 chainsrespectively can be associated with the autosomal-recessive AS(ARAS,OMIM 203780) and autosomal-dominant AS(ADAS,OMIM 104200).

While the XLAS and ARAS are well known, ADAS is a form of AS that has been described more recently. In 1997, this form of AS was linked to the *COL4A3*/*COL4A4* locus in a large family from Northern Ireland and 3 years later there came the first report of a *COL4A3* heterozygous mutation segregating in a family with ADAS(Jefferson et al., 1997; van der Loop et al., 2000). In 2004, Pescucci and colleagues investigated two ADAS families and confirmed that ADAS due to mutations either in the *COL4A4* or *COL4A3* gene (Pescucci et al., 2004). Till now, only a few proven *COL4A4* or *COL4A3* mutations have been reported. Both female and male patients showed a high clinical variability and a clear-cut genotype-phenotype correlation for ADAS is not available. Therefore, it is important to identify new mutations to clarify their clinical importance, to assess the prognosis of the disease, and to avoid renal biopsy for final diagnosis (Longo et al., 2002; Marcocci et al., 2009; Rosado et al., 2015).

In the present study, a novel exonic variantc.4195 A>T (p.Met1399Leu) at 44th exon of *COL4A4* gene was found, and this mutation showed heterozygous in all

patients of a Chinese ADAS family. Also a novel intronic nucleotide change(c.4090+11 C>T) was observed in *COL4A4* gene. Our results broadened the spectrum of mutations in *COL4A4* and had important implications in the diagnosis, prognosis, and genetic counselling of ADAS.

2. Subjects and Methods

2.1 Patients

The index patient was a 21-year-old man (Ⅲ5, supplementaryFigure.1) who was admitted to hospital with a month history of facial swelling and had a cough because of getting a cold half a month ago. The urinalysis revealed glomerular hematuria and phase contrast microscope showed the proportion of poikilocyte was more than 60%. The 24-hour urine protein excretion was 5.0 g, serum creatinine was 146 μmol/L and blood urea nitrogen was 8.64 mmol/L. B-ultrasonic showed diffuse renal injury. In addition, he was found to have moderate bilateral neurosensorial hearing loss. A renal biopsy revealed typical AS alteration, with irregular thickening, thinning, and splitting in the GBM (supplementary Figure.2a). Collagen IV immunofluorescence analysis exhibited negative collagen 3(IV) and 4(IV) immunostaining in Bowman's capsular basement membrane and the tubular basement membrane(supplementary Figure.2b).

His 45-year-old uncleand 26-year-old sister (Ⅱ7 and Ⅲ3, supplementary Figure.1) presented glomerular hematuria and proteinuria, and were in the state of renal compensatory. They also had mild sensorineural hearing loss. It was reported that the father (Ⅱ3) had undergone dialysis since the age of 60 years and had died of

kidney failure at the age of 62 years. Detailed information of the family was shown in supplementary Table 1. There was no consanguineous marriage in this family and pedigree analysis showed the inheritance pattern of this family was autosomal-dominant.

The study protocol was approved by the local Ethics Committee and followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects.

2.2 Mutation analysis

Genomic DNA was extracted using a QIAamp DNA Blood Maxi kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol. To amplify the different exons of the *COL4A3* and *COL4A4* genes, a standard PCR program was used and conditions were adapted to each exon. All PCR products were purified and bidirectionally sequenced using ABI BigDye Terminator v3.0 reaction cycle on ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, USA).

3. Results

DNA sequencing of the *COL4A3* and *COL4A4* genes in the index patient revealed the presence of eight nucleotide changes, including one exonic changec.4195 A>T, Met1399Leu at 44th exon of *COL4A4* gene (Table1, Figure.1), which showed heterozygous in all patients (Ⅱ7, Ⅲ3,Ⅲ5,Ⅳ2, supplementary Figure.1) of this family, but it was absent in the healthy members of the family.

We also found one new intronic change c.4090+11 C>T at 44th intron of *COL4A4* gene (Table 1, Figure.1), which was only present in the proband. None of

these variants were detected among 100 healthy individuals. Furthermore, six polymorphisms were identified, including c.1195 C>T, c.1223 G>A in *COL4A3* gene, and c.3011 C>T, c.4207 T>C, c.4548 A>G, c.4932C>T in *COL4A4* gene, they were also found in the control group, and all of them previously reported in other populations (Table1).

4. Discussion

Till now, approximately 25~50% mutations of AS have been reported to be glycine substitution (Ciccarese et al., 2001; Nabais Sa et al., 2015). Glycine is deemed as the only amino acid to fit into the center of the triple helix structure of type IV collagen, whose substitution mutation can interrupt the characteristic triple Gly-X-Y repeats in collagenous domain of the α3(IV) chain(Kharrat et al., 2006; Rosado et al., 2014). The missense mutation (Met1399Leu) identified in the present study was the first to report pathogenic methionine substitution. All patientsin this family were detected to be heterozygous in Met1399Leu mutation, however the proband's motherwho had no obvious clinical manifestation ,and other healthy members of the family, so as the 100 healthy individuals was absent in this mutation. Furthermore, we checked the conservation status of this exonic variant viz. Clustal X software in ten species including *Homo sapiens*, *Pan troglodytes*, *Cercocebus atys*,*Macaca nemestrina*, *Bubalus bubalis*, *Ovis aries musimon*, *Canis lupus familiaris*, *Oryctolagus cuniculus*, *Mus musculus* and *Rattus norvegicus*. Results showed that the novel exonic variant c.4195 A>T (p.Met1399Leu) was conservative in primates (Supplementary Figure.3a), suggesting the possible pathogenecity of this exonic

variant in Human beings.Also, *in silico* analysis was done viz. SIFT and Polyphen. Results showed that the exonic variant was predicted to "affect protein functions" (Supplementary Figure.3b). From this, we deduced that there was a high probability that the mutation Met1399Leu was the causative mutation of ADAS in this family, which was different from the common Glycine replace mutation.

Another novel change, c.4090+11 C>T, in the intron44 of *COL4A4* gene was found only in the proband of this ADAS family. Interestingly, the proband's condition was the most severe in the family and progressed to renal failure, we speculated that this mutation might be associated with different severity of clinical manifestations. Because it was not a splice site mutation, the association between the mutation and the severity of the illness was not clear.

Also some papers reported ADAS might associate with other genes except *COL4A3* and *COL4A4* genes (Rosado et al., 2015; Savige et al., 2015). In this family , Ⅱ7 · Ⅲ3 · Ⅲ5 and Ⅳ2 , who presented the same heterozygous mutation, had different severity of clinical manifestations, proteinuria and hematuria, it possibly due to combined effect with other genes to some extent. Still definite information about association between phenotype and genotype were needed in this family.

In conclusion, wedescribed a novel nucleotide change c.4195 A>T (p.Met1399Leu) in the *COL4A4* gene that might be responsible for a Chinese ADAS family.

Conflicts of interest

None

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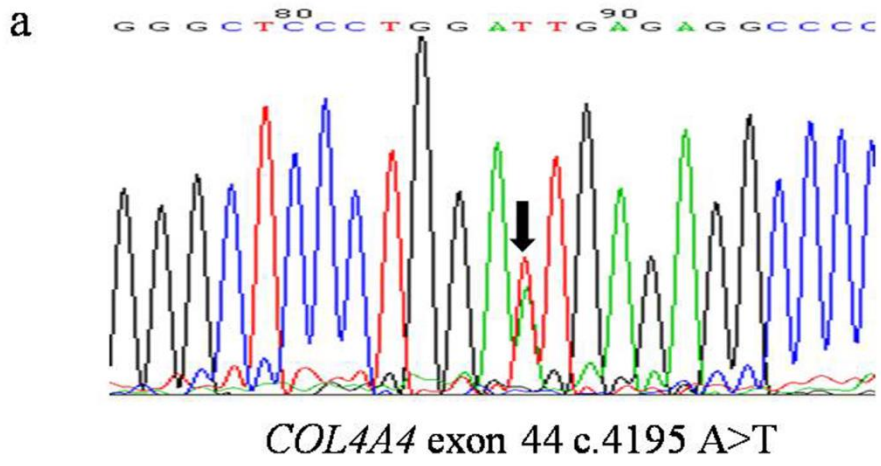
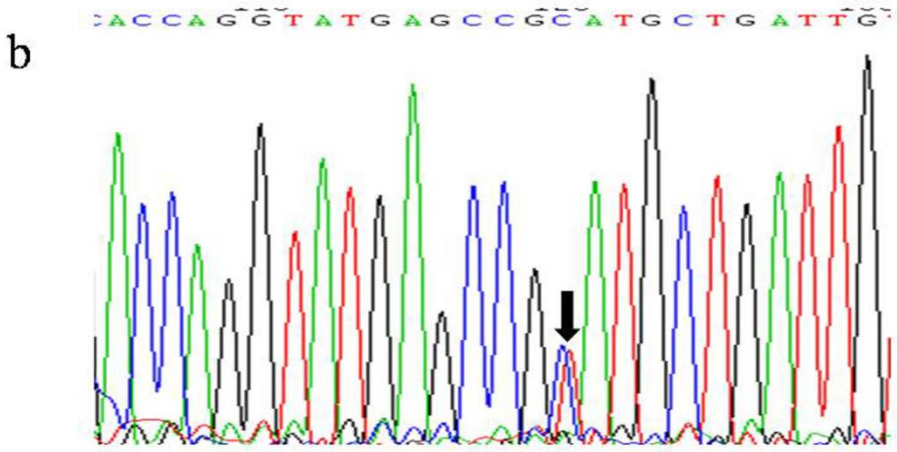
Table 1. Mutations of *COL4A3*/*COL4A4* genes found in the ADAS family

Gene	Site	gDNA change	rs numbers	Nucleotide change	Amino acid change	Mutation type	Reference
COL4A3	Exon 21	g.104260 C>T	rs772153778	c.1195 C>T	A399L	polymorphism	Gorinsek et al. 2007
	Exon 21	g.104288 G>A	rs536930700	c.1223 G>A	R408H	polymorphism	Papazachariou, Demosthenous et al. 2014
COL4A4	Exon 33	g.118444 C>T	rs1800517	c.3011 C>T	L1004P	polymorphism	Boye, Mollet et al. 1998
	Exon 44	**g.147491 A>T**	**rs149117087**	**c.4195 A>T**	**M1399L**	**missense**	**Novel**
	Exon 44	g.147503 T>C	rs375289	c.4207 T>C	S1403P	polymorphism	Boye, Mollet et al. 1998
	Intron 44	**g.147524 C>T**	**-**	**c.4090+11 C>T**		**intron mutation**	**Novel**
	Exon 47	g.161281 A>G	rs199517662	c.4548 A>G	V1516V	polymorphism	Gorinsek et al. 2007
	Exon 48	g.162094 C>T	rs2228557	c.4932C>T	F1644F	polymorphism	Longo, Porcedda et al. 2002

Figure 1. Sequencing results of the c.4195 A>T and c.4127+11 C>T mutation of *COL4A4* gene. (a) c.4195 A>T mutation at 44th exon of *COL4A4* gene. (b) c.4090+11 C>Tmutation in the intron44 of *COL4A4* gene.



COL4A4 exon 44 c.4195 A>T



COL4A4 intron 44 c.4090+11 C>T

Supplementary Tables

Supplementary Table 1. Clinical data of this ADAS family

Family members	Gender	age (yrs）	creatinine clearance rate （ml/min）	urine protein	urine erythrocyte
Ⅰ2	female	65	96	-	-
Ⅱ2	female	47	102	-	-
Ⅱ5	male	46	110	-	-

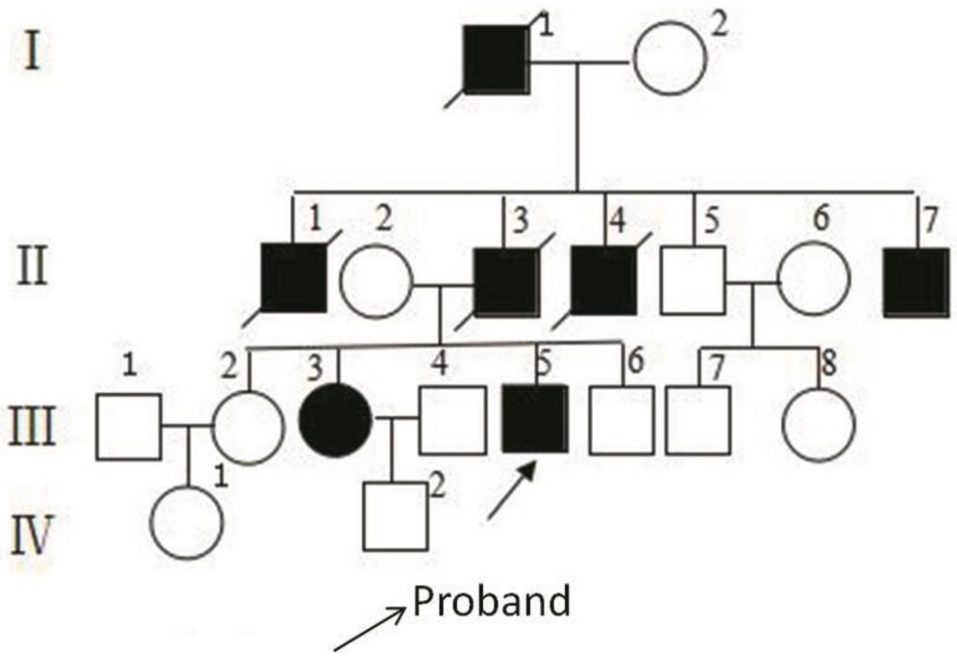
Ⅱ7	male	45	76	2+	+
Ⅲ1	male	27	118	-	-
Ⅲ2	female	25	110	-	-
Ⅲ3	female	26	73	+	2+
Ⅲ4	male	24	112	-	-
Ⅲ5	male	21	16	3+	2+
Ⅲ6	male	23	115	-	-
Ⅳ1	female	2	110	-	-
Ⅳ2	male	5	108	-	-

Note: normal range of creatinine clearance rate: 80-120 ml/min for adult.

urine protein<0.1g/24h: -, urine protein 0.2-1.0g/24:+, urine protein 1.0-2.0g/24h: 2+, urine protein 2.0-4.0g/24h: 3+.

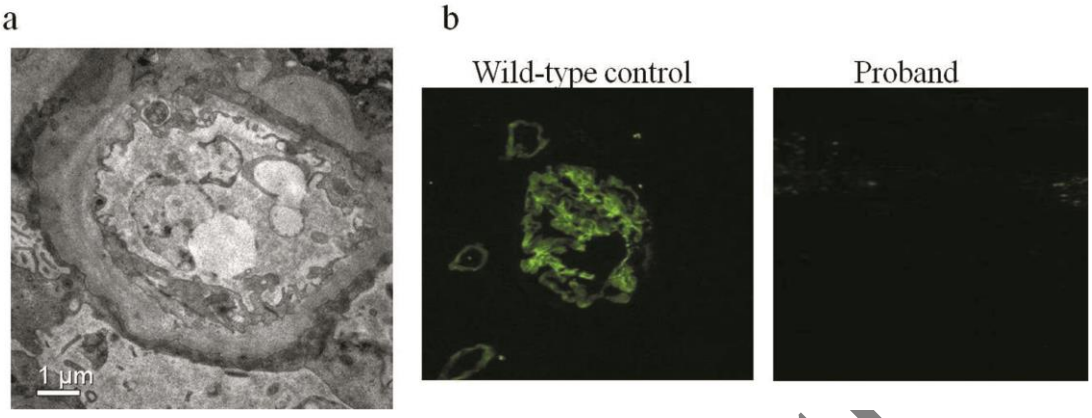
urine erythrocyte>10: +, urine erythrocyte>20: 2+.

Supplementary Figures

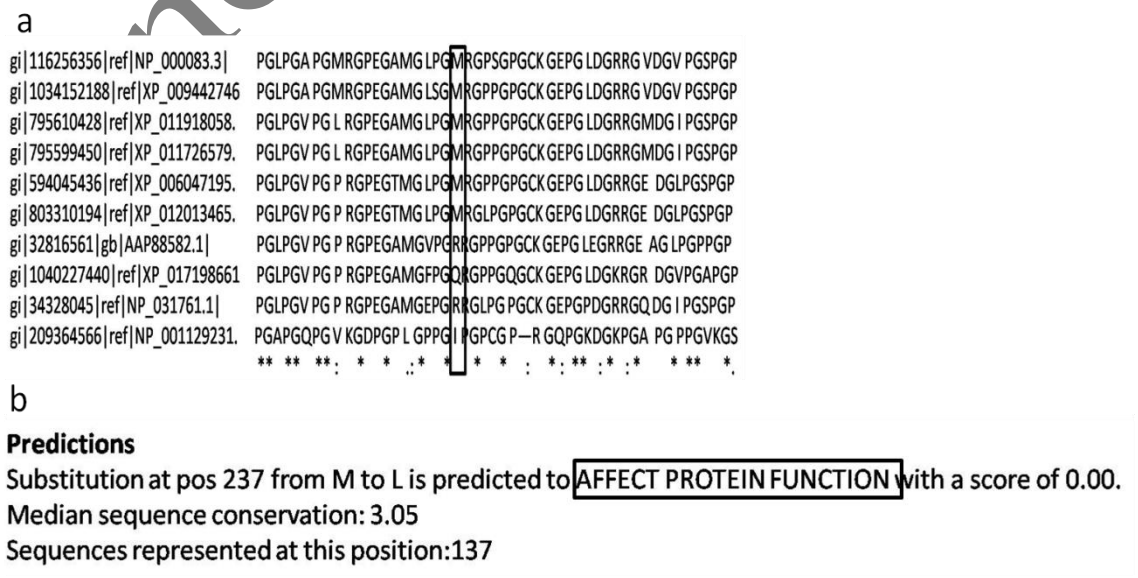


Supplementary Figure 1. Pedigree structure of the studied family. Proband is

arrowed. Squares represent males, circles represent females. Affected individuals are shaded.



Supplementary Figure 2. Renal biopsy EM image and collagen IV immunofluorescence analysis of the Proband. (a) Renal biopsy EM image from the proband showing thinning of the glomerular basement membrane. (b) Immunohistochemistry of collagen 3(IV) and 4(IV) in glomerular basement membrane. The expression of the collagen 3(IV) and 4(IV) exhibited normal in the Bowman's capsular basement membrane and tubular basement membrane of the wild-type control , and showed absent staining in the Proband of the ADAS family.



Supplementary Figure 3. Pathogenecity analysis of the exonic variant c.4195 A>T (p.Met1399Leu) *in silico*. (a) Conservation status of the exonic variant c.4195 A>T. Conservation status of this exonic variant was checked viz. Clustal X software in ten species. (b) *in silico* analysis of the pathogenecity of substitution at pos 1399 from M to L viz. SIFT.