Preimplantation Genetic Testing Prevented Intergenerational Transmission of X-Linked Alport Syndrome

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Keywords

Alport syndrome · Next-generation sequencing · Type IV collagen · Preimplantation genetic testing

Abstract

Background: Alport syndrome (AS) is a hereditary renal basement membrane disease that can lead to end-stage renal disease in young adults. It can be diagnosed by genetic analysis, being mostly caused by mutations in *COL4A3*, *COL4A4*, and *COL4A5*. To date, there is no radical cure for this disease. **Objectives:** The aim of this study was to avoid the transmission of AS within an affected family by selecting healthy embryos for uterine transfer. The embryos were identified by preimplantation genetic testing for monogenic disorders (PGT-M). **Methods:** We used next-generation sequencing (NGS) to identify mutations in the proband and his parents. The results of NGS were confirmed by Sanger sequencing. Targeted NGS combined with targeted single-nucleotide polymorphism haplotyping was used for the in vitro identification of *COL4A5* mutations in human embryos to prevent their intergenerational transmission. **Results:** The

c.349_359delGGACCTCAAGG and c.360_361insTGC mutations in *COL4A5* were identified in a family affected by X-linked AS. Whole-genome sequencing by NGS with targeted haplotyping was performed on biopsied trophoctoderm cells. A healthy baby was born after transfer of a single freeze-thawed blastocyst. **Conclusions:** The use of targeted NGS for identifying diagnostic markers combined with targeted haplotyping is an easy and efficient PGT-M method for preventing intergenerational transmission of AS.

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Introduction

Alport syndrome (AS) is a hereditary basement membrane disorder characterized by a family history of hematuria, progressive renal dysfunction, hearing loss, and ocular abnormalities [1, 2]. Although early interventions can suppress the progression of nephritis, patients with AS eventually develop end-stage renal disease (ESRD). There is no radical cure for AS. AS is caused by mutations in *COL4A3*, *COL4A4*, and *COL4A5* which encode the

Fig. 1. Workflow for PGT-M. PGT-M, preimplantation genetic testing for monogenic disorders; WGA, whole-genome amplified; SNP, single-nucleotide polymorphism.

$\alpha 3\alpha 4\alpha 5$ (IV) collagen heterotrimer [3]. Specifically, the $\alpha 3$ and $\alpha 4$ chains are encoded by *COL4A3* and *COL4A4*, which are located on chromosome 2, while the $\alpha 5$ chain is encoded by *COL4A5*, located on chromosome X [4, 5]. The type IV collagens are important for the structural integrity of basement membranes in the glomerulus, cochlea, and eye [6]. Mutant α chains cannot form the $\alpha 3\alpha 4\alpha 5$ (IV) trimer, which leads to abnormal glomerular basement membranes [7, 8]. There are 52, 48, and 51 coding exons for *COL4A3*, *COL4A4* and *COL4A5*, respectively. Approximately, 1,200 different mutations have been collectively reported for these 3 collagen IV genes (Human Gene Mutation Database, HGMD: <http://hgmd.cf.ac.uk>, 2019.4). The large size of these genes and the absence of mutational hot spots have hindered comprehensive genetic screening for large patient series [9]. According to which mutated gene and the pattern of inheritance, the 3 following classical models of Mendelian inheritance have been reported in patients with AS: semidominant X-linked (XL)AS due to mutations in *COL4A5*; autosomal recessive AS due to mutations in *COL4A3* or *COL4A4* and autosomal dominant AS [10].

Preimplantation genetic testing (PGT) is an in vitro fertilization (IVF) technique with the aim of assisting couples with heritable genetic disorders to avoid transmitting the genetic disorder to their offspring [11]. PGT encompasses testing for monogenic disorders (PGT-M), for structural rearrangements, and for aneuploidy [12]. Blastomeres or trophoblasts are taken from the preimplantation embryos and are tested for specific genetic changes. The normal embryos are transferred to the uterus to initiate a pregnancy. The main advantage of PGT is that it avoids the need for the therapeutic termination of a pregnancy, which can lead to complications. Since the first application of PGT in 1989 [13], it has been used for various single-gene disorders and for hereditary cancer predisposition syndromes [4, 14]. At present, >150 types of single-gene disorders can be detected through PGT-M [15]. The thousands of apparently healthy offspring suggest that PGT-M is a safe and reliable procedure without marked adverse effects. Genetic counseling for PGT-M has been recommended by the revised AS Guidelines [16]. Here, we report a family with AS who underwent PGT-M that used targeted next-generation sequencing (NGS) in combination with targeted single-nucleotide polymorphism (SNP) haplotyping. We found that the method was efficient and can be used for PGT-M for a family affected by AS.

Materials and Methods

A 32-year-old woman, her son (proband) affected by AS, and her 44-year-old husband were referred to the assisted reproductive unit of Women's Hospital, Zhejiang University School of Medicine, for IVF along with PGT-M for AS. The clinical examination found that the mother had microscopic hematuria (++) , but normal renal function (serum creatinine of 60 $\mu\text{mol/L}$) and normal blood pressure (105/77 mm Hg). Her body mass index was 24 kg/m^2 , and her ABO blood group was type O. Her husband's renal function and blood pressure were normal. Her son was a 10-year-old boy with a history of persistent hematuria. He did not have high-frequency sensorineural hearing loss or ocular abnormalities. When he was 5 years of age, genetic testing diagnosed his condition as XLAS. His mother had a subsequent natural pregnancy. Testing of an amniocentesis specimen identified the same pathogenic mutation as both mother and the proband. That pregnancy was terminated after informed consent was obtained. The study was approved by the Ethics Committee at Women's Hospital, Zhejiang University School of Medicine (Research license IRB- 20200290-R). Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

PGT-M was performed according to the workflow, as shown in Figure 1. To identify informative markers, approximately 5 mL of EDTA-anticoagulated peripheral blood was obtained from the proband (III-1), his mother (II-3), and his father (II-2). Genomic DNA specimens were extracted from the blood samples according

Fig. 2. Identification of a *COL4A5* mutation in an affected family. **a** Pedigree of the family affected by AS. (Arrow): affected proband with AS; (filled square, filled circle): male/female affected by AS. (blank square and blank circle): male/female without AS. **b** Whole-genome sequencing of the proband identified an INDEL (insertion and deletion: 349_359delGGACCTCAAGG, c.360_361insTGC) in *COL4A5*, visualized by Integrative Genomics Viewer software.

to standard procedures. NGS was used to detect mutations in the proband. Targeted regions of *COL4A5* were then amplified by specific primers (see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000517796), and were subsequently sequenced. The PCR conditions for the 50 μ L reaction mixture were as follows: (1) initial denaturation at 95°C for 10 min and (2) 35 thermal cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The primers were designed with the use of Primer 5 Web. Interpretation and classification of the identified variants were based on the guidelines of the American College of Medical Genetics and Genomics (ACMG) [17]. To validate the absence of contamination, blank controls were processed under identical conditions.

After establishment of the mutations and the provision of informed consent, the mother received controlled ovarian hyperstimulation. Intracytoplasmic sperm injection was used to inseminate mature oocytes. On day 5, a biopsy of the blastocyst was per-

formed by a noncontact laser (Hamilton Thorne, Beverly, MA, USA), which created a 30- μ m opening in the zona pellucida. A 35- μ m (inner diameter) biopsy micropipette (Origio, Charlottesville, VA, USA) was used to obtain 4–6 cells. A Sureplex DNA Amplification kit (Illumina, USA) was used for whole-genome amplification and an Ion Torrent PGM platform (ThermoFisher, USA) was used for whole-genome sequencing, based on the standard protocol. A vitrification kit (Kitazato, Fuji, Japan) was used to vitrify the blastocysts, as previously described for genome sequencing [18].

Results

AS was also diagnosed in the proband's grandfather and 1 granduncle. They both died of ESRD at about 40 years of age. The family tree (pedigree) of the described

Table 1. Fifty-five SNP markers for identifying disease-associated allele in embryos

N	Position	Mother		Father	Proband	E1	E2		E3	
		M1	M0	F1	M0	M1	M0	F1	M0	F1
1	105781740	G	T	G	T	G	T	G	T	G
2	105866473	A	G	A	G	A	G	A	?	?
3	106075674	A	G	A	G	A	G	A	?	?
4	106154233	C	T	C	T	C	T	C	T	C
5	106234294	C	T	C	T	C	T	C	?	?
6	106309583	C	T	C	T	C	T	C	T	C
7	107235134	G	A	G	A	G	?	?	A	G
8	107235173	A	G	A	G	A	G	A	G	A
9	107357441	C	T	C	T	C	?	?	T	C
10	107357464	G	A	G	A	G	?	?	A	G
11	107447234	A	G	A	G	A	?	?	G	A
12	107458970	C	G	C	G	?	?	?	G	C
13	107473447	C	T	C	T	C	T	C	T	C
14	107487527	T	C	T	C	T	C	T	C	T
15	107497330	T	C	T	C	T	C	T	C	T
16	107510821	T	G	T	G	T	G	T	G	T
17	107527106	A	G	A	G	A	G	A	G	A
18	107536830	A	G	A	G	A	G	A	G	A
19	107558125	G	C	C	C	G	C	C	C	C
20	107558154	C	A	C	A	C	A	C	A	C
21	107591481	T	C	T	C	?	?	?	C	T
22	107616156	C	T	C	T	C	?	?	T	C
23	107628092	T	A	T	A	T	A	T	A	T
24	107652710	G	A	G	A	G	A	G	A	G
25	107652712	G	A	G	A	G	A	G	A	G
26	107652788	C	T	C	T	C	T	C	T	C
27	107814732	G	T	G	T	G	T	G	T	G
28	107834453	T	G	T	G	T	G	T	G	T
29	107865895	G	A	A	A	G	?	?	A	A
30	107909784	A	G	A	G	A	G	A	G	A
31	107909866	T	C	T	C	T	?	?	C	T
32	107957748	G	A	G	A	G	A	G	A	G
33	107970869	G	A	G	A	G	A	G	A	G
34	108527429	G	A	A	A	G	A	A	A	A
35	108541570	T	A	A	A	T	A	A	?	?
36	108572955	G	A	A	A	G	A	A	A	A
37	108586511	G	A	A	A	G	A	A	A	A
38	108598964	A	G	G	G	A	G	G	G	G
39	108617053	A	C	C	C	A	C	C	C	C
40	108637996	G	A	A	A	G	A	A	A	A
41	108682301	A	G	A	G	A	G	A	G	A
42	108724593	T	C	T	C	T	C	T	C	T
43	108737288	C	A	C	A	C	A	C	A	C
44	108758257	C	T	C	T	C	T	C	T	C
45	108779743	C	T	C	T	C	T	C	T	C
46	108820506	G	A	G	A	G	A	G	A	G
47	108832955	T	A	T	A	T	A	T	?	?
48	108858672	C	T	C	T	C	T	C	T	C
49	109206317	G	A	G	A	G	A	G	A	G
50	109227951	T	C	T	C	T	C	T	C	T
51	109250572	A	G	A	G	A	G	A	G	A
52	109265534	A	T	A	T	A	T	A	T	A
53	109313740	G	A	G	A	?	A	G	A	G
54	109354937	T	A	T	A	T	A	T	A	T
55	109368709	G	A	G	A	G	A	G	A	G

Genetic results of the PGT-M cycle. Fifty-five SNP markers were identified and linked to the *COL4A5* site to identify the mutant haplotype and the normal haplotype in each embryo. “M1” represents “haplotype 1 of the mother,” “M0” represents “haplotype 2 of the mother,” “F1” represents “haplotype 1 of the father,” “E” represents “embryo,” “?” represents the undetected loci. Black bars indicate the maternal normal haplotype, gray bars indicate the maternal mutant haplotype, and light gray bars indicate the paternal normal haplotype. According to the SNP genotypes of E1, E2 and E3, we can infer that E1 inherited haplotype 1 of the mother because it carried the informative SNPs of M1. Similarly, we can infer that E2 and E3 inherited haplotype 2 of the mother because it carried the informative SNPs of M0. Therefore, we can define E1 as normal, and E2 and E3 as abnormal. SNP, single-nucleotide polymorphism; PGT-M, preimplantation genetic testing for monogenic disorders.

family (Fig. 2a) was based on the clinically affected individuals. Two novel adjacent variants in exon 6 of *COL4A5* existing on the same haplotype were identified by NGS of proband specimens. They were as follows: 1 deletion: c.349_359delGGACCTCAAGG and 1 insertion: c.360_361insTGC. These 2 variants result in a substitution of phenylalanine at residue 117 by leucine and led to a stop codon after 38 amino acids (p.phe117leufs*38) in the *COL4A5* protein. Based on the American College of Medical Genetics and Genomics guidelines, the mutated gene is “likely pathogenic.” These variants in the proband were inherited from his mother, who was confirmed to be heterozygous for the same mutations by Sanger sequencing. The father had wild-type *COL4A5* genes (Fig. 2b, c).

The PGT-M cycle retrieved 9 oocytes, of which 8 were fertilized. Three embryos were biopsied. Approximately 120 informative SNPs were found distributed upstream and downstream within 1 Mb of the *COL4A5* gene. To avoid the inevitable allele dropout (ADO), we selected 55 heterozygous SNP loci in the mother. The SNPs linked to the mutation site were used to determine the embryo haplotypes. Comprehensive direct detection of the mutations by targeted NGS was impossible because the primers did not encompass the mutation sites. Embryo 1 was normal and carried 51 informative SNP loci in haplotype 1 of the normal maternal haplotypes. Embryos 2 and 3 carried a mutated SNP haplotype that was inherited from the mother, as shown in Table 1. Embryo 1 was transferred into her uterus after preparation of the endometrium.

The mother’s serum β -hCG level was 132 IU/L 11 days after frozen-thawed embryo transfer. Confirmation of the clinical pregnancy was established by the presence of a gestational sac on ultrasound 35 days after frozen-thawed embryo transfer. She performed routine prenatal care in our hospital. Amniocentesis was performed on the mother at the 20th gestational week to confirm the genetic health of the baby. The result was consistent with the PGT-M result (shown in Fig. 2c III-3). At 38 gestational weeks, the mother underwent initiation of labor and gave birth to a healthy male infant weighing 3,150 g via cesarean section. During her pregnancy, her hematuria did not worsen, and her blood urea nitrogen level (4 mmol/L), serum creatinine level (53 μ mol/L), and blood pressure (122/74 mm Hg) remained within normal ranges. She gave birth to a healthy baby, and over a 2-year follow-up period, her hematuria still did not worsen, her proteinuria, creatinine level, and blood pressure remained normal.

Discussion

XLAS has been established to be associated with mutations in *COL4A5* [19], which has accounted for approximately 85% of patients with AS. Male patients with XLAS mutations are affected more severely than females [20], with about 70% of male patients developing ESRD before the age of 30 years [21]. In women with XLAS, the severity of the phenotype varies because of the 2 X chromosomes [22]. The initial manifestations that result in ESRD can occur at any time between the first and seventh decade of life in affected women [23]. In our case, the affected mother (II-3) did not have clinically detectable abnormalities, except for microscopic hematuria, even after she had given birth to 2 full-term babies. To date, 33 pregnancies have been reported in 23 women with different types of AS [24–29]. Among the affected women, 22 received a genetic diagnosis of AS, which included 16 women with XLAS, 4 with ARAS, and 2 patients with ADAS. According to these reports, the genotype was not predictive of the outcome of the pregnancy.

AS patients carry the risk of passing on the disease to future generations. Genetic testing is now the “gold standard” for the diagnosis of AS and for confirming the mode of inheritance [30]. Before the complete development and application of PGT-M, prenatal diagnosis was widely used for reducing the occurrence of inherited genetic disorders to avoid the transmission of pathogenic genetic variants [31]. The problem with prenatal diagnosis is that it occurs after conception, so if the fetus is affected, the parents must decide on whether or not to terminate the pregnancy [32]. The option of PGT-M for affected couples who want children enables prevention of the transmission of any identified single-gene disorder to their children and helps couples avoid the decision for terminating the pregnancy or having an affected child [33].

NGS-based PGT-M has been validated in multiple centers [34]. Couples are recommended to undergo pre-PGT-M testing before starting the IVF cycles in order to identify the mutated gene. This individualized and informative preliminary test, known as the PGT-M setup, requires a DNA sample from both partners and sometimes from other family members [35]. Once a mutation is identified in the DNA samples, PGT-M can be performed on an embryo biopsy specimen. In our study, DNA peripheral blood samples were obtained from each parent and their affected son. The mutation was confirmed before PGT-M testing. However, because the DNA sample is amplified, ADO might lead to the mis-

taken identification of a heterozygous locus as a homozygous locus, particularly when the starting sample is a single cell or a few cells [33]. Furthermore, ADO usually causes disastrous false-negative results [35]. NGS that is based on SNP markers has now been used to improve the accuracy of PGT-M [36]. The capture sequencing and linkage analysis of SNPs located near the gene of interest allow a convenient and efficient approach to the experimental design of PGT-M [35]. We mapped 55 informative SNPs that flanked the mutated region in *COL4A5* to confirm the haplotype linkage analyses of the parents and the embryos. At the PGT-M cycle, a single normal blastocyst was transferred, which was followed by pregnancy.

To avoid a misdiagnosis by PGT, prenatal genetic testing or postnatal confirmation has been recommended to confirm the diagnosis by PGT [37, 38]. In our study, prenatal diagnosis of a DNA sample from the amniotic fluid of the pregnancy was consistent with the PGT-M results, and a healthy baby boy was born at 38 gestational weeks. While NGS has been used in clinical practice for PGT-M, to the best of our knowledge, its detailed use for AS has only been described recently [24].

We reported on the use of PGT-M, which was based on an NGS-based haplotype linkage analysis of a family affected by XLAS mutations. The result was the successful birth of a healthy baby boy. Our results confirm the feasibility of PGT-M for a family affected by AS. There is no doubt that a sufficient number of embryos were needed to ensure transferable embryos for pregnancy. Since the number of collected oocytes and available embryos will decline with the increasing age of the mother, mothers undergoing this procedure should have adequate ovarian reserve.

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Statement of Ethics

The study was approved by the Ethics Committee at Women's Hospital, Zhejiang University School of Medicine (Research license IRB- 20200290-R). Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Xiaoling Hu, Jiahui Zhang, and Xijing Chen collected the data; and Xiaoling Hu, Jiahui Zhang, Yuan Lv, Guofang Feng, Liya Wang, and Yinghui Ye reviewed the literature. Yimin Zhu and Fan Jin provided valuable input for collecting the data and drafting the manuscript. All the authors read and approved the manuscript and met the criteria for authorship.

Availability of Data and Material

All the data generated or analyzed during this study are included in this manuscript and its online suppl. material files. Further inquiries can be directed to the corresponding author.

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