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GENETICS

A novel hemizygous loss-of-function mutation in *ADGRG2* causes male infertility with congenital bilateral absence of the vas deferens

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Abstract

Purpose Cystic fibrosis transmembrane conductance regulator (*CFTR*) and adhesion G protein-coupled receptor G2 (*ADGRG2*) have been identified as the main pathogenic genes in congenital bilateral absence of the vas deferens (CBAVD), which is an important cause of obstructive azoospermia. This study aimed to identify the disease-causing gene in two brothers with CBAVD from a Chinese consanguineous family and reveal the intracytoplasmic sperm injection (ICSI) outcomes in these patients.

Methods Whole-exome sequencing and Sanger sequencing were used to identify the candidate pathogenic genes. Real-time polymerase chain reaction, immunohistochemistry, and immunofluorescence were used to assess the expression of the mutant gene. Moreover, the ICSI results from both patients were retrospectively reviewed.

Results A novel hemizygous loss-of-function mutation (c.G118T: p.Glu40*) in *ADGRG2* was identified in both patients with CBAVD. This mutation is absent from the human genome databases and causes an early translational termination in the third exon of *ADGRG2*. Expression analyses showed that both the *ADGRG2* mRNA and the corresponding protein were undetectable in the proximal epididymal tissue of *ADGRG2*-mutated patients. ADGRG2 expression was restricted to the apical membranes of non-ciliated epithelia in human efferent ducts, which was consistent with a previous report in mice. Both *ADGRG2*-mutated patients had normal spermatogenesis and had successful clinical outcomes following ICSI.

Conclusions Our study verifies the pathogenic role of *ADGRG2* in X-linked CBAVD and broadens the spectrum of *ADGRG2* mutations. In addition, we found positive ICSI outcomes in the two *ADGRG2*-mutated CBAVD patients.

Keywords *ADGRG2* · CBAVD · Obstructive azoospermia · ICSI · Male infertility

Huan Wu, Yang Gao and Cong Ma contributed equally to this work.

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Introduction

Congenital bilateral absence of the vas deferens (CBAVD; MIM: 277180) involves a complete or partial defect of the Wolffian duct derivatives and occurs in more than 25% of men with obstructive azoospermia (OA) [1]. Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*; MIM: 602421) gene are believed to be the main genetic contributor to CBAVD worldwide [2, 3], accounting for approximately 50% of affected individuals in mainland Chinese populations [4, 5]. CFTR is widely expressed on the epithelial surfaces of a wide range of organs, including the male reproductive tract [6]. This ATP-regulated Cl^- and HCO_3^- channel protein plays a vital role in maintaining electrolyte and fluid homeostasis in the male genital duct during development of the Wolffian duct [3]. In addition to *CFTR*, copy number variations in *PANK2* (MIM: 606157) and *SLC9A3* (MIM: 182307) were reported to be possibly related to CBAVD. However, these variants have only been identified in a small cohort of CBAVD patients from Taiwan [7, 8].

Recently, the X-linked adhesion G protein-coupled receptor G2 gene (*ADGRG2*; MIM: 300572) has been demonstrated to be associated with CBAVD-related OA among different ethnic groups, accounting for 3–15% of CBAVD cases [5, 9–12]. This new pathogenic gene exhibits epididymal duct-specific expression [13] and encodes a member of the adhesion-class of G protein-coupled receptors (adhesion-GPCRs), a superfamily with seven transmembrane domains [14]. *Adgrg2*-knockout (KO) male mice present sperm stasis and duct obstruction owing to dysregulation of fluid reabsorption in their efferent ducts [15, 16]. This phenotype is well matched with the clinical features of humans with *ADGRG2* mutations. Remarkably, in mice, ADGRG2 is co-localized with CFTR on the apical membranes of non-ciliated epithelia of the efferent duct, and ADGRG2 regulates epididymal fluid uptake by CFTR [16]. Intracytoplasmic sperm injection (ICSI), which has a limited success rate, is the only treatment to overcome CBAVD-associated male infertility [17, 18]; therefore, the ICSI outcomes of CBAVD patients with *ADGRG2* mutations need to be elucidated.

In this study, we identified a novel hemizygous loss-of-function (LOF) mutation in *ADGRG2* in two otherwise healthy brothers with CBAVD. Furthermore, we assessed the effect of this mutation on *ADGRG2* mRNA and protein expression and report on the outcomes of ICSI in both subjects who had normal spermatogenesis. Our report broadens the spectrum of *ADGRG2* mutations in CBAVD patients and further promotes our understanding of CBAVD and male infertility.

Material and methods

Patient and control populations

Two CBAVD-affected brothers (identified as IV-1 and IV-2) with consanguineous parents were recruited at the Reproductive Medicine Center of the First Affiliated Hospital of Anhui Medical University in May 2019. Both probands were healthy except for infertility owing to CBAVD. The diagnosis of CBAVD was made based on a physical examination, scrotal/rectal ultrasonography, hormonal testing, and semen analysis. Testicular and proximal epididymal tissues were obtained during testicular biopsies from the two probands. One patient with nonobstructive azoospermia (NOA) and one fertile prostate cancer patient with normal spermatogenesis were enrolled as controls. The ICSI outcomes of the two probands were retrospectively reviewed. This research was approved by the Ethical Review Board of The First Affiliated Hospital of Anhui Medical University. All subjects and the control individuals were well informed about this study and provided signed informed consent.

Genetic analysis and sanger sequencing

Whole-exome sequencing (WES) and bioinformatics analysis were performed according to our previously described protocols [19]. The identified mutations and their parental origins were verified using Sanger sequencing.

Testis histological analysis

Testicular tissue was fixed in modified Davidson's fluid (MDF; 50% diluted water, 30% formaldehyde, 15% ethanol, 5% glacial acetic acid) for over 48 h and then embedded in paraffin. Sections with 3 μm thickness were prepared and mounted on glass slides. After deparaffinization, the slides were stained with hematoxylin and eosin (H&E) for histological analysis.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the proximal epididymal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) was performed as described in our previous study [19]. The primers used in the qPCR experiments are listed in Table S1.

Immunohistochemistry

The 3-μm-thick sections of proximal epididymal tissue were deparaffinized and blocked with an immunohistochemistry kit (sp-9000, ZsBio, Beijing, China). Next, these sections were incubated with an anti-ADGRG2 rabbit polyclonal primary

antibody (HPA001478, Sigma, Darmstadt, Germany) at a 1:200 dilution followed by a peroxidase-labeled secondary antibody (sp-9000, ZsBio, Beijing, China). After being visualized with 3, 3′-diaminobenzidine (ZLI-9018, ZsBio, Beijing, China), the sections were counterstained with hematoxylin. Finally, the images were obtained using an Axio Scope.A1 microscope (Zeiss, Germany).

Immunofluorescence

After deparaffinizing, the 3-μm epididymal sections were incubated with different primary antibodies: a rabbit polyclonal anti-ADGRG2 antibody at 1:200 (HPA001478, Sigma, Darmstadt, Germany) and a mouse monoclonal anti-acetylated tubulin antibody at 1:1000 (5335S, Cell Signaling Technology, Boston, USA) at 4 °C overnight. Next, the sections were washed in PBS and incubated with the corresponding secondary antibodies (a rabbit polyclonal antibody conjugated with Alexa Fluor 594 for the anti-ADGRG2 antibody and a mouse monoclonal antibody conjugated with Alexa Fluor 488 for the anti-acetylated tubulin antibody) in the dark at room temperature for 2 h. Finally, all sections were coated with Hoechst (33342, Thermo Scientific, Wilmington, USA) for 5 min and then immediately visualized using an LSM 800 microscope (Zeiss, Germany).

Results

Clinical characteristics of the two CBAVD patients

The clinical and laboratory parameters of both the *ADGRG2*-mutated subjects are summarized in Table 1. Semen analyses revealed that spermatozoa were absent, and the semen volume was reduced to 0.6–0.7 mL with an acidic pH of 6.4. Abdominal/scrotal ultrasonography showed normal volume and blood supply in the bilateral kidneys and testicles. The bilateral epididymal caputs were dilated and the interior had a grid-like pattern. Moreover, the corpus and cauda of the epididymis were absent. Rectal ultrasonography indicated a bilateral absence of the vas deferens and the seminal vesicle. Serum sex hormone levels, the somatic karyotype, and the AZF gene were normal in both patients (Table 1).

A novel hemizygous nonsense mutation in *ADGRG2* identified by WES

Previous reports and pedigree analysis of the probands' family (Fig. 1a) suggested recessive inheritance of CBAVD. We focused on the homozygous or compound heterozygous variants having a minor allele frequency (< 1%) in the 1000 Genomes Project, the Exome Aggregation Consortium (ExAC) Browser and the Genome Aggregation Database (gnomAD), and on the genes with specific or preferential expression in male reproductive organs. In addition, null variants (such as stop-gain, frameshift, and splice-site mutations) and damaging missense variants predicted by SIFT, PolyPhen-2, and MutationTaster were prioritized [20–22]. The prioritized hemizygous and homozygous mutations in the two patients are presented in Tables S2 and S3, respectively. Intriguingly, one novel hemizygous nonsense mutation, c.G118T: p.Glu40* (NM_001079858.3, according to the NCBI database) in the X-linked *ADGRG2* gene was identified in both brothers, although no pathogenic *CFTR* mutations were found. Sanger sequencing verified the presence of this mutation and demonstrated that it had been inherited from the mother (Fig. 1a). This LOF mutation is located in the third of the 29 exons of *ADGRG2* and is absent from all three aforementioned genome databases (Table 1). The mutation site makes it clear that the mutation induces nonsense-mediated decay (NMD) of the transcript, resulting in a lack of ADGRG2 protein in the two subjects. The mutant amino acid is highly conserved in mammals (Fig. 1b).

ADGRG2 mRNA and its protein were absent from the efferent duct of *ADGRG2*-mutated patients

The mRNA expression levels of *ADGRG2* in proband IV-2's proximal epididymal tissue were dramatically reduced, to approximately zero. This NMD is likely triggered by the presence of the premature termination codon (Fig. 2b). The observation was confirmed at the protein level by immunohistochemistry (IHC) analysis. There was a high level of ADGRG2 apical expression in the efferent ducts of the two control subjects, but a complete lack of ADGRG2 staining in the dilated efferent ducts of proband IV-1 (Fig. 2a). Furthermore, an immunofluorescence (IF) analysis showed that ADGRG2-specific staining was restricted to the non-ciliated epithelial surfaces in the efferent ducts of the two control subjects but absolutely absent in the efferent ducts of proband IV-1 (Fig. 3).

Normal spermatogenesis was observed in an *ADGRG2*-mutant patient

The testicular spermatogenic function of *ADGRG2*-mutated patient IV-2 was evaluated with H&E staining. In patient IV-2, the seminiferous tubules and their components were similar to those in the fertile control who had normal spermatogenesis. The number and morphology of the spermatogonia, spermatocytes, and spermatids in the testis of patient IV-2 were normal (Fig. S1).

Successful ICSI outcomes using the testicular sperm from the two *ADGRG2*-mutated CBAVD patients

Both *ADGRG2*-mutated CBAVD patients had a positive outcome following ICSI using testicular sperm. After one frozen-

Table 1 Clinical laboratory evaluation for the CBAVD patients with *ADGRG2* mutation

		Patient	
		IV-1	IV-2
Gene		ADGRG2	
cDNA mutation [a]		c.G118T	
Amino acid alteration [b]		p.Glu40*	
Exon		Exon 3	
Allele frequency	1KGP	NA	
	ExAC	NA	
	gnomAD	NA	
Clinical parameters			
Ejaculated sperm volume (ml)		0.7	0.6
Ejaculated sperm count		0	0
Seminal PH		6.4	6.4
Ultrasonography	Kidney	107 × 53 mm (right)	106 × 55 mm (right)
		94 × 53 mm (left)	110 × 59 mm (left)
	Testes	40 × 20 × 29 mm (right)	37 × 20 × 26 mm (right)
		36 × 19 × 28mm (left)	38 × 18 × 27 mm (left)
	Epididymis caput	14 × 12 × 12 mm (right)	12 × 10 × 11 mm (right)
		11 × 9 × 10 mm (left)	12 × 8 × 12 mm (left)
	Epididymis corpus	Absence	Absence
	Epididymis cauda	Absence	Absence
	Vas deference	Absence	Absence
	Seminal vesicle	Absence	Absence
Sex hormone	T (nmol/L)	16.78	12.19
	FSH (mIU/mL)	5.40	4.28
	LH (mIU/mL)	3.35	4.49
	PRL (ng/mL)	7.03	7.94
Karyotype		46XY	46XY
AZF deletion		Undetectable	Undetectable

Abbreviations: *ExAC* Exome Aggregation Consortium, *NA* not available, *T* testosterone, *FSH* follicle stimulating hormone, *LH* luteinizing hormone, *PRL* prolactin, *AZF* azoospermia factor

[a] The GenBank accession numbers of *ADGRG2* is NM_001079858.3

[b] Full-length protein has 1017 amino acids

thawed embryo transfer cycle, one partner had a singleton pregnancy and the other had a twin pregnancy. The fertilization rate, embryo development rate (eight cells/blastocyst), and implantation rate of the two couples were 83.3%, 70.0%/60.0%, and 100%, respectively (Table 2).

Discussion

CFTR mutations are responsible for cystic fibrosis (CF; MIM: 219700) and have been demonstrated to play an essential physiological role in the development of CBAVD. Although almost all CF males have CBAVD [23] and many *CFTR* mutations have been identified in isolated CBAVD patients [2, 24], the underlying mechanisms of the genotype-phenotype correlations remain elusive. Based on a report that the vas deferens was properly formed in seven CF male fetuses aged up to 23 months and a 5-year-old CF boy [25], it has been suggested that the developmental abnormalities in the male genital tract in *CFTR*-mutated subjects should be defined as atrophy or inspissation rather than agenesis. The progressive atrophy of the vas deferens is presumably associated with secondary epididymal obstruction, which is caused by an abnormal accumulation of luminal mucus and proteins induced by CFTR dysfunction [26, 27]. This hypothesis is strongly supported by the detectable vas deferens that could be seen in male fetuses with biallelic *CFTR* mutations at 12 and 18 weeks of gestation [28].

High throughput sequencing is commonly employed as an effective genetic analysis method in *CFTR*-negative CBAVD patients to identify additional pathogenic genes. Remarkably, *ADGRG2* has been consistently demonstrated to be associated with X-linked CBAVD using WES since 2016, accounting for approximately 10% of studied cases worldwide [5, 9–12]. The

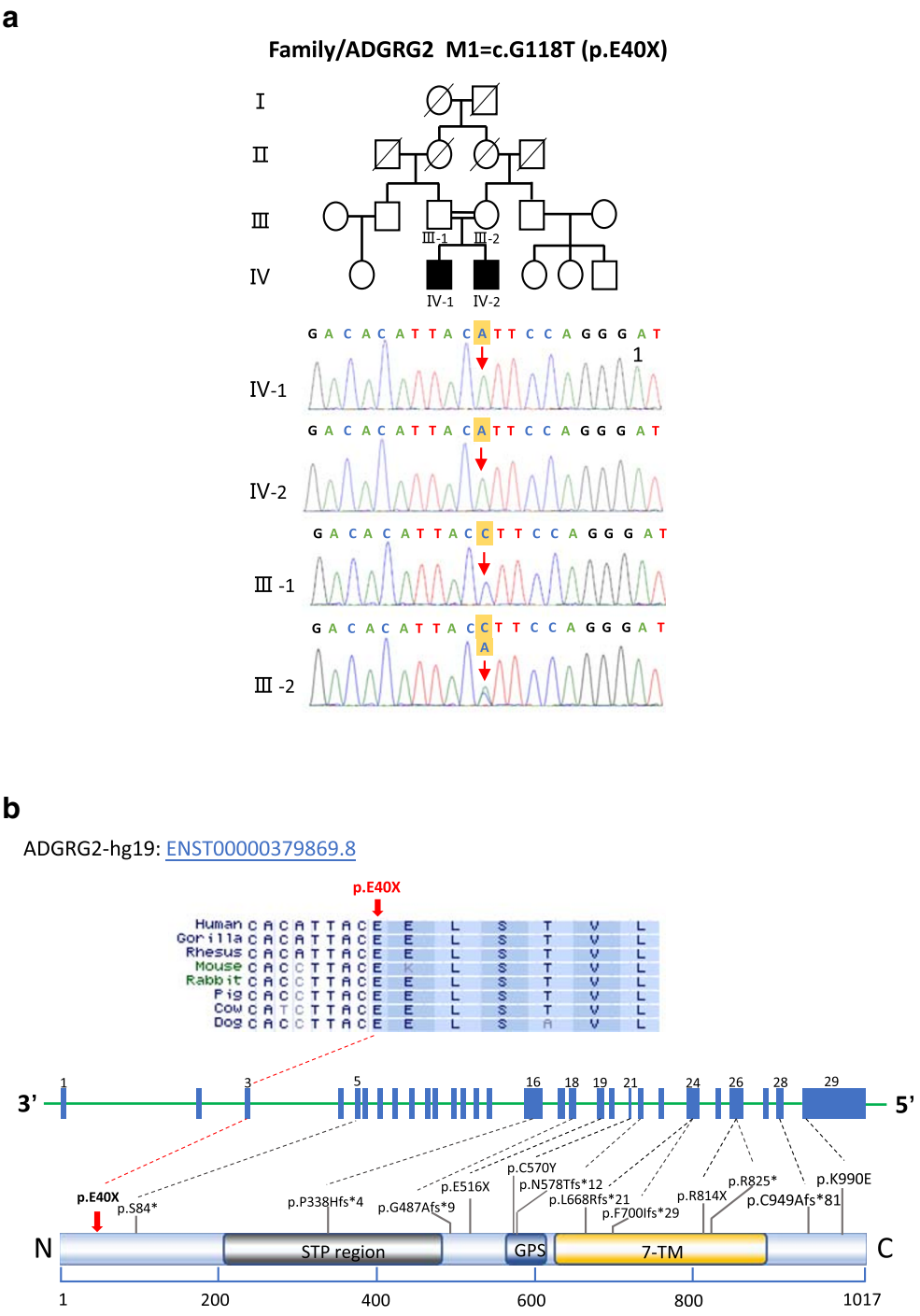

Fig. 1 A novel hemizygous loss-of-function (LOF) mutation in *ADGRG2* was identified in a Chinese consanguineous family. **a** Pedigree of the consanguineous family containing two brothers with CBAVD (IV-1, IV-2). Sanger sequencing verified the presence of this novel LOF mutation (c.G118T: p.Glu40*) in *ADGRG2* and confirmed that the hemizygous mutation was inherited from their mother. **b** Schematic representation of the *ADGRG2* gene and domain structure of its protein product. The position of the novel mutation identified in this study is indicated by a red arrow and a red line. The corresponding positions of previously reported *ADGRG2* mutations are indicated by gray lines. The gray box indicates the known serine-threonine-proline-rich (STP) region in the ADGRG2 protein, the blue box indicates the GPCR proteolysis site (GPS) domain, and the yellow box represents the seven transmembrane (7TM)-spanning domains. The sequence alignment shows the conservation of the affected amino acid in different mammals

ADGRG2 gene, also known as the human epididymal protein 6 (*HE6*) gene [13], is composed of 29 exons and is located on the p22.13 region of the X chromosome. As a member of the family of 33 adhesion-GPCRs [14], ADGRG2 is a highly conserved and epididymis-specific orphan receptor containing three functional domains [13, 29]. Targeted disruption of *Adgrg2* reveals that this epididymal heptahelical protein is primarily implicated in the control of electrolyte and water balance within the efferent ducts [15, 16]. Because of dysregulated fluid reabsorption in the efferent ducts, the mutant mice exhibit an obstructive infertility phenotype with sperm stasis in the efferent duct, matching the clinical manifestation of *ADGRG2*-mutated humans. It is noteworthy that the data from these mutant mice suggest that ADGRG2/CFTR coupling is mediated by Gq, and β-arrestin-1 and is crucial in regulating ion and fluid reabsorption in the male genital duct. Furthermore, constitutive ADGRG2 activity is necessary to maintain the function of CFTR in this process [16].

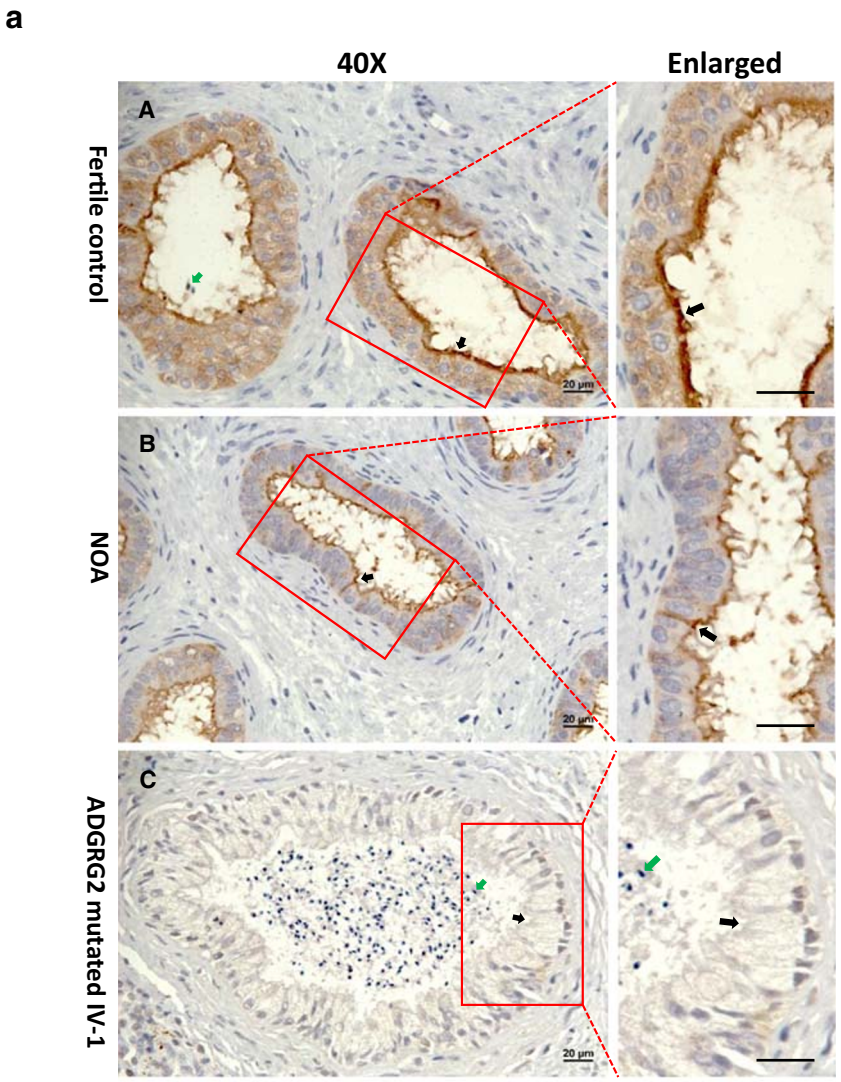

Fig. 2 Protein and mRNA expression levels of *ADGRG2* in proximal epididymal tissues from the probands and control men. **a** Immunohistochemical staining of the human efferent duct sections. In the efferent ducts of a fertile control with prostate cancer (A) and the nonobstructive azoospermia (NOA) patient (B), ADGRG2 expression was restricted to the apical part of the duct epithelia (black arrows). In contrast, ADGRG2 protein was completely absent in the apical membranes of proband IV-1's dilated efferent duct (C). Spermatozoa are present in the efferent ducts of the fertile control and the proband (green arrows). Scale bar, 20 μm. **b** Quantitative real-time PCR showed that the *ADGRG2* mRNA expression level was approximately zero in the proximal epididymal tissues of proband IV-2, whereas it was significantly higher in the non-*ADGRG2* mutated NOA patient ($^{***}p < 0.001$; Student's *t* test)

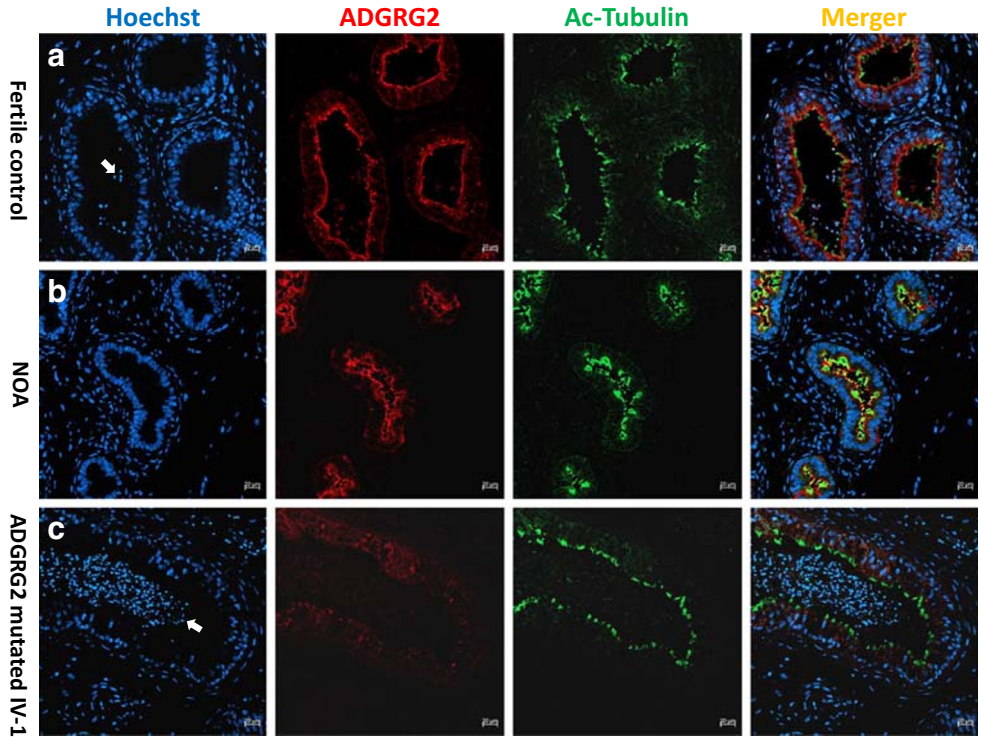

Fig. 3 Immunofluorescent staining of ADGRG2 and acetylated tubulin (Ac-tubulin) in the efferent ducts of one proband and the control men. Sections of the efferent ducts were stained with anti-ADGRG2 (red) and anti-Ac-tubulin (green) antibodies. Hoechst staining (blue) indicates the nuclei in the epithelia and spermatozoa (white arrows). In these sections, ciliated and non-ciliated cells are clearly identified by the immunofluorescence signal from Ac-tubulin. Notably, for the efferent ducts of both the fertile patient and the NOA patient, apical expression of ADGRG2 was restricted to non-ciliated cells (**a** and **b**), whereas the inter-tubular tissue showed an absence of staining. Compared with the control subjects, the ADGRG2 signal was completely absent in the apical membranes of the non-ciliated cells of proband IV-1's dilated efferent duct (**c**). Scale bar, 20 μm

Remarkably, a similar mechanism may be responsible for the etiology of OA in *Slc9a3*-KO mice. CFTR was drastically reduced in the male genital tract of mutant mice, suggesting interdependent roles of *SLC9A3* and *CFTR* in causing structural and functional abnormalities of epididymal ducts [30]. However, the role of *PANK2* in the development of the human reproductive tract remains unclear [7, 31]. Taking these facts together, it is therefore tempting to speculate that defects in ADGRG2 probably disrupt the signaling pathway conducted by CFTR in maintaining electrolyte and fluid homeostasis in the efferent ducts. These changes would ultimately cause secondary epididymal obstruction and result in progressive atrophy of the excurrent duct in *ADGRG2*-mutated patients.

Table 2 Clinical outcomes of the two *ADGRG2*-mutated CBAVD patients following ICSI

	IV-1	IV-2
Male age (years)	32	31
Female age (years)	28	23
No. of ICSI cycles	1	1
No. of oocytes injected	6	18
Fertilization rate (%)	4/6 (66.7)	16/18 (88.9)
Cleavage rate (%)	4/4 (100.0)	15/16 (93.8)
Eight cells embryo development rate (%)	3/4 (75.0)	11/16 (68.8)
Blastocyst development rate (%)	2/4 (50.0)	10/16 (62.5)
No. of frozen-thawed embryos transfer cycles	1	1
Number of embryos transferred	1	2
Implantation rate (%)	1/1 (100.0)	2/2 (100.0)
Clinical pregnancy	Y	Y
Miscarriage	N	N

Abbreviations: *CBAVD* congenital bilateral absence of the vas deferens, *ICSI* intracytoplasmic sperm injection, *Y* yes, *N* no

In this study, a novel hemizygous LOF mutation (c.G118T: p.Glu40*) in *ADGRG2* was identified in two CBAVD brothers from a Chinese consanguineous family. Neither of the otherwise healthy probands carried any CFTR pathogenic mutations, and Sanger sequencing demonstrated that the hemizygous mutation was inherited from their mother. This very rare *ADGRG2* mutation is in the third exon of *ADGRG2* and is absent from all three major public genome databases (1000 Genomes Project, ExAC Browser, and gnomAD). The variant may prevent ADRGG2 expression owing to NMD of the mRNA. Consistent with this notion, both the *ADGRG2* mRNA and ADGRG2 protein were undetectable in the probands' efferent ducts. These results confirm the pathogenicity

of this novel LOF mutation. Moreover, we demonstrated that ADGRG2 expression is restricted to the non-ciliated epithelial surface of the efferent ducts in CBAVD-negative humans. These non-ciliated cells contain many aquaporins and play a crucial role in luminal fluid reabsorption [32]. Similarly, CFTR is located on the apical membranes in the non-ciliated cells of the initial section of the human efferent duct [33]. Therefore, we speculate that ADGRG2 might interact with CFTR to maintain electrolyte and fluid homeostasis in the human reproductive tract, consistent with the conclusion reported for the mouse model [16]. In consequence, obstructive infertility, as well as the structural abnormalities of the male genital duct in *ADGRG2*-mutated patients, may result from an impaired signaling pathway mediated by CFTR in regulating ion and fluid uptake in the efferent ducts.

The ICSI outcomes of the two *ADGRG2*-mutated CBAVD patients were retrospectively reviewed in our reproductive center. Before ICSI, we provided genetic counseling to both couples; however, they refused to opt for a preimplantation genetic diagnosis that could prevent this X-linked *ADGRG2* mutation from being inherited by their offspring. Ultimately, both couples underwent one ICSI cycle with testicular sperm and got pregnant after one frozen-thawed embryo transfer cycle. Histological analysis of the testis showed normal spermatogenesis in the *ADGRG2*-mutant patient. These observations suggest that, unlike the occurrence of CBAVD, ADGRG2 defects might not directly impair spermatogenesis or disrupt the signaling pathways conducted by CFTR in spermatogenesis [34]. This hypothesis is supported by the epididymal duct-specific expression of *ADGRG2* and the different signaling pathways that are mediated by CFTR pertinent to the various reproductive processes, including spermatogenesis and the transmission and maturation of epididymal sperm [3, 35, 36]. Nevertheless, the probands are only 31 and 32 years old; thus, progressive hypospermatogenesis with age, caused by fluid back-up in the testis, as was observed in the *Adgrg2*-KO mouse [15] cannot be excluded. Beyond this, the sample size in our study is too small to draw definite conclusions.

In conclusion, our current investigation revealed a novel LOF mutation in *ADGRG2* in two Chinese brothers with *CFTR*-negative CBAVD. The results confirm the pathogenic role of *ADGRG2* in X-linked CBAVD and broaden the spectrum of *ADGRG2* mutations. Furthermore, ADGRG2 expression was restricted to non-ciliated cells in the human efferent duct. The positive ICSI outcomes in these two *ADGRG2*-mutant patients offer new insights into the relationship between *ADGRG2* and male infertility.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

Ethics approval This research was approved by the Ethical Review Board of The First Affiliated Hospital of Anhui Medical University.

Consent to participate All subjects participating in this study signed their informed consents.

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