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GENETICS

A novel *ADGRG2* truncating variant associated with X-linked obstructive azoospermia in a large Chinese pedigree

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Abstract

Purpose In this study, we aimed to identify sterility-related variants in a Chinese pedigree with male infertility and to reveal the different phenotypes and intracytoplasmic sperm injection (ICSI) outcomes of the affected members.

Methods Physical examinations were performed on male patients. G-band karyotype analysis, copy number variation sequencing, and quantitative fluorescent PCR were conducted to detect common chromosomal disorders in the probands. Whole-exome sequencing and Sanger sequencing were applied to identify the pathogenic genes and the protein expression changes caused by the very mutation were identified by Western Blot in vitro.

Results A novel nonsense mutation (c.908C > G: p.S303*) in the *ADGRG2* was identified in all infertile male patients of the pedigree, which was inherited from their mothers. This variant was absent from the human genome databases. This mutation was also unexpectedly found in a male member with normal reproductive capability. Members with the mutation had different genitalia phenotypes, ranging from normal to dilated phenotypes of the vas deferens, spermatic veins and epididymis. There was a truncated ADGRG2 protein in vitro after mutation. Of the three patients' wives treated with ICSI, only one successfully gave birth.

Conclusions Our study is the first to report the c.908C > G: p.S303* mutation in the *ADGRG2* in an X-linked azoospermia pedigree and is the first to report normal fertility in a member with this mutation, expanding the mutation spectrum and phenotype spectrum of this gene. In our study, ISCI had a success rate of only one-third in couples including men with azoospermia with this mutation.

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

Introduction

Infertility has become one of the most common public health concerns, affecting approximately 15% of couples worldwide [1]. Male factors are responsible for approximately half of infertility cases [2]. It is generally believed that defects in sperm production, decreased sperm motility and the inability of sperm to interact with oocytes are all reasons for male infertility [3, 4]. Azoospermia, defined as the complete absence of spermatozoa in ejaculate in two successive semen examinations, is one of the common reasons for male infertility [5–7]. Genetic diseases, hypogonadism, and varicocele are considered to be the main causes of azoospermia [8]. Nearly 50% of azoospermia cases are attributed to genetic defects, including chromosome number and structural aberrations, azoospermic factor (AZF) region microdeletions on the Y chromosome and cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations [9]. More than 15% of infertile men suffer from obstructive azoospermia (OA), which is caused by reproductive tract abnormalities, or nonobstructive azoospermia resulting from spermatogenic failure [10]. Obstructive azoospermia is classified

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according to the anatomical location of the obstruction (epididymal, vasal, intratesticular, ejaculatory duct, or distal functional obstruction of the ejaculatory duct) [11]. Congenital absence of vas deferens (CAVD), which can be divided into congenital bilateral absence of the vas deferens (CBAVD), congenital unilateral absence of vas deferens (CUAVD) and congenital bilateral partial hypoplasia of vas deferens (CPAVD), occasionally with renal abnormalities, is considered to be the main cause of obstructive azoospermia [12].

CBAVD occurs in up to 25% of men with obstructive azoospermia [10], and at least one *CFTR* mutation can be detected in men with obstructive azoospermia infertility caused by CBAVD [13, 14]. Previous studies have suggested that *CFTR* was the only identified gene whose mutations result in obstructive azoospermia in humans [1, 10]. The adhesion G-protein coupled receptor G2 (*ADGRG2*) gene located on the p22.13 region of the X chromosome, also called the human epididymal protein 6 (*HE6*) gene or G protein-coupled receptor 64 (*GPR64*), is a member of the adhesion-class G-protein-coupled receptors superfamily, the second largest human GPCR subfamily [15, 16]. Recently, *ADGRG2* was found to be a rare X-linked gene associated with male infertility, especially in those with CBAVD.

In this study, we applied whole-exome sequencing and Sanger sequencing to identify the cause of male infertility in a large Chinese pedigree, a novel hemizygous loss-of-function mutation, broadening the spectrum of *ADGRG2* mutations. Furthermore, we assessed the phenotypic differences of this mutation in males at different ages and evaluated the assisted reproductive outcomes in affected adults.

Materials and methods

Participants and clinical examination

A large four-generation Chinese pedigree that included four infertile males was studied. Routine clinical examinations, including biochemical, thyroid function and sex hormone (testosterone, oestradiol, follicle-stimulating hormone, luteinizing hormone, pituitary prolactin, progesterone) examinations, were performed on all family members who volunteered to participate in the study. Male members underwent additional palpation of genitourinary development and semen examination (including semen count, pH, volume, fructose, zinc, α-glycosidase, etc.). In addition, transabdominal ultrasonography was used to examine the vas deferens, testes, epididymis, prostate, spermatic vein, liver, gallbladder, pancreas, spleen, and kidneys. The ICSI outcomes of infertile male family members (III.3, III.6, III.12) were retrospectively reviewed. All participants (or their guardians) were fully aware of the study and provided signed informed consent.

Genetic analysis

Five millilitres of heparin anticoagulant blood was collected from two infertile cousins (III.12 and III.14), and G-banding karyotype analysis was performed according to standard operating procedures. EDTA anticoagulant blood samples were collected from III.12 and proband III.14, and Y chromosome microdeletion was detected by QF-PCR. Copy number variation sequencing (CNV-Seq) was performed on III.12. The two infertile members (III.12 and III.14) were subjected to whole-exome sequencing (WES) to identify candidate genes for male infertility, and the mutation point was verified by Sanger sequencing. Peripheral blood was collected from family members who volunteered to participate in this study, and target mutations were detected by Sanger sequencing.

Mutant transfection and Western Blot

By signing the contract, Nanjing Tengke Medical Research, Ltd was entrusted to complete the whole process of this experiment and promised to ensure that the data was true and reliable. The wild-type *ADGRG2* sequence was synthesized by total synthesis and PCR site-specific mutation, and primers were designed to amplify this segment (Takara, PrimeSTAR Max DNA Polymerase). The fragment was inserted into plasmid pcDNA3.1(+) to obtain the shuttle plasmid pcDna3.1-ADGRG2 expressing wild-type ADGRG2 protein by enzyme digestion binding (NEB, AbaSI). The plasmid was transformed into Tsingke (TF5α), which was selected as the receptive cell for monoclonal screening. Plasmid pcDNA3.1-ADGRG2 was extracted from the obtained monoclonal bacteria after shaking, and on this basis, mutant ADGRG2 plasmid pcDNA3.1-ADGRG2 mut was obtained by PCR point mutation technique. The mutant ADGRG2 plasmid pcDNA3.1-ADGRG2 mut was transformed into the transformed receptor cell DH5α (Tsingke, TF5α), and the monoclonal screening was performed (TIANGEN, TIANprep Rapid N96 Plasmid Kit). The obtained monoclonal bacteria were shaken to extract plasmid mutant ADGRG2 plasmid pcDNA3.1-ADGRG2 mut (TIANGEN, TIANprep Rapid N96 Plasmid Kit). The above two plasmids were transfected into 293T cells (Thermo, Lip3000), and the expression of ADGRG2 protein in 293T cells was detected by Western Blot (Elabscience, Western Blot Detection Kit).

Results

Clinical examination

No obvious abnormality was found in the clinical biochemical examination or thyroid function examination in any of the

participants. The findings of urogenital palpation by male physicians indicated that there were no obvious abnormalities in the development and distribution of pubic hair or in the penis, testicular epididymis, scrotum and spermatic vein; the vas deferens were palpable; and no morphological changes were found in members III.1, III.3, III.12 and III.14 (Table 1).

In semen examinations, no sperm was found in vitro in III.3, III.12 and III.14. Low ejaculation volume and acidic pH were exhibited in III.3 and III.12. Poor-quality sperm was shown in member III.1, who had normal fertility. The total amounts of a-glycosidase and fructose were significantly decreased in these four participants, especially in III.3. The presence of fructose in semen indicated that the ejaculatory duct and connected seminal vesicle were not occluded completely (Table 1).

The findings of transabdominal ultrasonography (Table 1) of the liver, kidneys, bile and pancreas in III.1, III.3, III.12, and III.14 were in the normal range. There were no abnormalities in their prostates, except for prostatic cysts in III.14 and small prostates in III.3. The bilateral vas deferens were dilated in III.3 and III.14, with varicocele on one side. Left epididymal dilatation was observed in III.12. The bilateral epididymis of III.1 was not clear, and an irregular fluid dark area was detected at the epididymis head (palpation showed a dilated duct nodule). The bilateral testicular network was dilated in III.1 and III.3, and the efferent ductules were dilated in III.3.

The wife of III.6 successfully gave birth to 2 healthy boys after the first transplantation within an egg collection cycle. The wife of III.3 successfully conceived two times out of three embryo transfers within two egg retrieval cycles; however, the outcomes of both conceptions ended with embryo abortion within approximately 1 month. The wife of III.12 had completed two ICSI treatments without a successful pregnancy (Table 2).

Genetic analysis

No special copy number variation was observed in CNV-Seq of III.12. The results of G-banding karyotype analysis of III.12 and proband III.14, were 46,XY, and no abnormalities were observed. No microdeletion or duplication was detected in the AZF region of the Y chromosome of the two infertile members. A novel hemizygous nonsense mutation, c.908C>G:p.S303* (NM_001079858.3) in the *ADGRG2* gene, was identified in both azoospermia members with the absence of mutations in the *CFTR* gene. The results were verified by Sanger sequencing. This mutation was not found in the Exome Aggregation Consortium (ExAC), Genome Aggregation Database (gnomAD) or other human genome databases. Sanger sequencing was applied to test the other members of the family, and this mutation was also identified in II.1, II.2, II.5, II.6, II.7, III.1, III.3, III.6, III.13, III.15, IV.21, and IV.22, as well as in the infertile members III.3 and III.6 (Table 3). That is, this X-linked *ADGRG2* mutation in members with azoospermia was inherited from their mothers. However, III.1, who showed normal reproductive capability, also carried this mutation (Figs. 1 and 2).

The ADGRG2 protein was truncated after the mutation in vitro

The results of the transfection fluorescence map of GFP cells showed that both the wild type and the nonsense mutation (c.908C > G: p.S303*) in the ADGRG2 gene were transfected successfully. Western Blot assay showed that both wild-type and mutant ADGRG2 could be expressed in 293T cells. But the molecular weight of wild-type ADGRG2 was found to be about 100 kD by SDS-PAGE electrophoresis and the mutant one was about 30 kD, that was, protein truncation occurred after mutation in vitro (Fig. 3).

Discussion

In our study, a new hemizygous nonsense mutation (c.908C > G: p.S303*) in *ADGRG2* was identified in all members of the family with azoospermia, and this mutation was inherited from their mothers. This mutation resulted in early

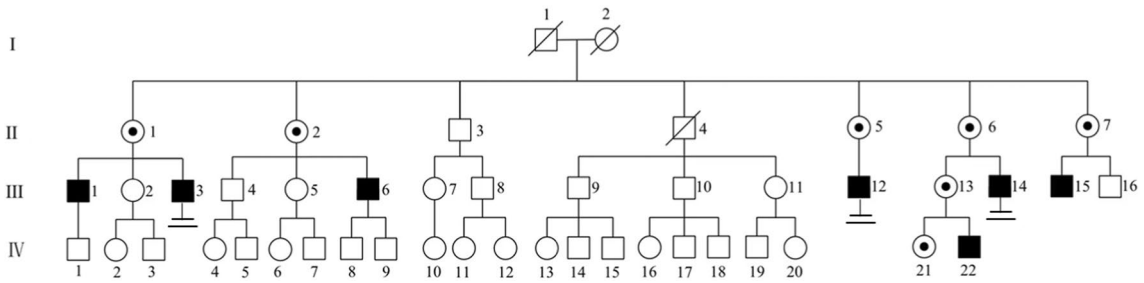

Fig. 1 Pedigree of a family with male infertility. Members III.3, III.6, III.12, and III.14 were diagnosed with azoospermia. Tested male members suffered from mutation (c.908C > G: p.S303*) in the *ADGRG2* gene are showed as black rectangles, and tested female carriers as white circles with a dot in the center.III.6 produced offspring after ICSI

Table 1 Clinical examination results of *ADGRG2* mutation male subjects

	Patient ID	III.1	III.3	III.12	III.14	IV.22
	Age(years)	59	52	35	35	8
Ultrasonography	Prostate size(mm)	37×27×30	22×18×20	NA	33×27×30(Cysts)	14×12×11
	Seminal vesicle size(mm)	15×8×12 (right) 16×8×12 (left)	11×7×10 (right) 12×8×10 (left)	NA	15×11×10 (right) 14×9×10 (left)	Not dilated
	Testes size(mm)	34×22×27 (right) 34×21×27 (left)	37×20×26 (right) 36×22×25 (left)	NA	34×19×26 (right) 34×18×25 (left)	17×7×11 (right) 17×7×10 (left)
	Vas deference	Not dilated	Bilateral dilated	Not dilated	Bilateral dilated	Not dilated
	Spermatic vein	Normal	Right side varicocele	Normal	Left side varicocele	Normal
	Epididymal duct	Bilateral dilated	Bilateral dilated	Left side varicocele	Normal	Normal
	Epididymal head	Dilated tubular nodules	Normal	Normal	Normal	Normal
	Rete testis	Bilateral dilated	Bilateral dilated	Normal	Normal	Normal
	Efferent ductules	Normal	Bilateral dilated	Normal	Normal	Normal
	Kidney	Normal	Normal	Normal	Normal	Normal
Sex hormone	T (nmol/L)	3.38	3.36	NA	4.10	<0.13
	FSH (mIU/mL)	8	4.36	NA	4.01	2.51
	LH (mIU/mL)	3.76	3.61	NA	4.94	0.51
	PRL (ng/mL)	11.76	13.25	NA	9.28	35.95
	E2	29	20	NA	27	15
Semen analysis	volume(mL)	1.6	0.2	1.2	1.5	NA
	pH	7.2	6.5	5.5	7.4	NA
	Progressive motility(PR,%)	4.19%	-	-	-	NA
	non-progressive motility(NP，%)	0.60%	-	-	-	NA
	Immobility (IM，%)	95.21%	-	-	-	NA
	Total motility(PR+NP)	4.79%	-	-	-	NA
	Sperm concentration (10^6/mL)	15.78	-	-	-	NA
	Total sperm number (10^6/ejaculate)	25.25	Azoospermia	Azoospermia	Azoospermia	NA
	Sperm viability(%)	15.00	-	-	-	NA
	Seminal zinc(μmol/ejaculate)	5.07	8.18	9.46	8.64	NA
	Zn-total(μmol/time)	8.11	1.64	11.35	12.96	NA
	Seminal fructose (μmol/ejaculate)	0.44	0.44	0.48	0.6	NA
	FRU-total(μmol/time)	0.7	0.09	0.58	0.9	NA
	NAG(μmol/ejaculate)	0.5	8.4	2.3	0.8	NA
	NAG- total(μmol/time)	0.8	1.68	2.76	1.2	NA

Abbreviations: *T*, testosterone; *FSH*, follicle stimulating hormone; *LH*, luteinizing hormone; *PRL*, prolactin; *E2*, estradiol; *NA*, not available

translation termination in the 16th exon of *ADGRG2* and was not found in the human genome databases. ADGRG2 is expressed within the efferent ducts specifically, where the majority of the testicular fluid carrying immature sperm is resorbed. The pathophysiological effects of targeted ADGRG2 deletion in mice strongly suggest that ADGRG2 is involved in regulating this fluid reabsorption process. Fluid accumulation in the testes and sperm stagnation within

Table 2 Clinical outcomes of the three *ADGRG2*-mutated azoospermia patients following ICSI

	III.3	III.6	III.12
Male age (years)	52	51	35
Female age (years)	50	41	35
No. of ICSI cycles	3	1	3
Male age in each ICSI cycles (years)	42/43/44	39	32/33/35
Female age in each ICSI cycles (years)	40/41/42	29	32/33/35
Clinical pregnancy	Y(3/3)	Y(1/1)	N(0/2)
Miscarriage	Y(3/3)	N	-

Abbreviations: *ICSI*, intracytoplasmic sperm injection; *Y*, yes, *N*, no

Table 3 Sanger sequencing results for all participants

ID	*ADGRG2*:c.908C>G
II.1	C/G
II.2	C/G
II.5	C/G
II.6	C/G
II.7	C/G
III.1	G/*
III.3	G/*
III.5	C/C
III.6	G/*
III.12	G/*
III.13	C/G
III.14	G/*
III.15	G/*
III.16	C/*
IV.6	C/C
IV.7	C/*
IV.21	C/G
IV.22	G/*

Abbreviations: *G/** hemizygotic mutation

the efferent ducts developed in mice carrying the mutation, resulting in an obstructive infertility phenotype [15, 17, 18]. Consistent with the results reported in mouse models, ADGRG2 may maintain electrolyte and fluid homeostasis in the human reproductive tract by interacting with CFTR [19]. *ADGRG2* mutation may disrupt the signalling pathway regulated by *CFTR* in electrolyte and fluid homeostasis maintenance in the efferent ducts [20]. Mutations in the *ADGRG2* and *CFTR* genes may lead to obstructive azoospermia through similar pathophysiological mechanisms [21].

ADGRG2 attracted substantial attention for the first time as an infertility-related gene in a study of 26 azoospermic men [21]. Then, a total of three missense mutations in the *ADGRG2* gene were found in two cohort studies of 38 and 18 Chinese men with CBAVD. These mutations are considered to be the cause of CBAVD [22, 23]. In studies of related azoospermia patients, Wu [20] reported that a new *ADGRG2* hemizygous mutation (c.g118t: p.glu40 *) can lead to CBAVD, resulting in infertility in two brothers born to consanguineous parents. Another nonsense *ADGRG2* mutation was identified in two brothers with obstructive azoospermia in a Pakistani pedigree [8]. In previous research, all patients bearing *ADGRG2* variants exhibited azoospermia caused by CBAVD. However, our study unexpectedly found that a male with normal fertility (member III.1) also inherited the maternal mutation (c.908C > G: p.S303*) in *ADGRG2*, which had not been previously reported in the literature. Although the sperm count and motility were slightly lower, and the contents of fructose and total α-glycosidase were both low, member III.1 impregnated his wife three times. The other results were generally within the normal range, and no other phenotype of obstructive azoospermia was observed, while the results of b-ultrasonography showed bilateral epididymis abnormalities, which may be caused by this mutation. Interestingly, unlike the other members with this mutation, the ultrasound findings of member III.1 revealed bilateral epididymal abnormalities, and when ultrasound was combined with palpation, dilated ductal nodules were confirmed in the epididymal duct. This may be a new phenotype of this gene mutation. Because men with this phenotype have normal fertility, they are difficult to detect in the clinic.

Knockout of the orphan receptor *ADGRG2* leads to male infertility due to dysregulation of fluid reabsorption in the efferent ductules, indicating active roles for this receptor in regulating these processes [18]. In our in vitro study, the protein was truncated after the (c.908C > G: p.S303*) mutation. Patat [21] reported three LoF mutations throughout the *ADGRG2* gene, predicting an identical pathophysiological impact of both C-terminal fragment (CTF) and N-terminal fragment (NTF) premature truncations of *ADGRG2*. Other *ADGRG2* truncating variants also found to be related to male infertility [21, 24]. Therefore, the hemizygous nonsense mutation identified in our study might share the same pathophysiological impact, presenting with infertility symptoms in affected male members (III.3, III.6, III.12 and III.14). However, the exact effect of the G protein subtypes on maintaining the epididymal or efferent ductule microenvironment is still unclear, as are the downstream effector factors involved in controlling ion/water homeostasis in these tissues [25]. In addition, it was suggested that aGPCRs had potential CTF-independent functions, as Gpr126st49 mutant zebrafish still expressed a functional NTF fragment but not a functional CTF fragment [26]. These might, to some extent, explain the presence of sperm in the semen of member III.1, and the different phenotypes of infertile members, but more research is needed.

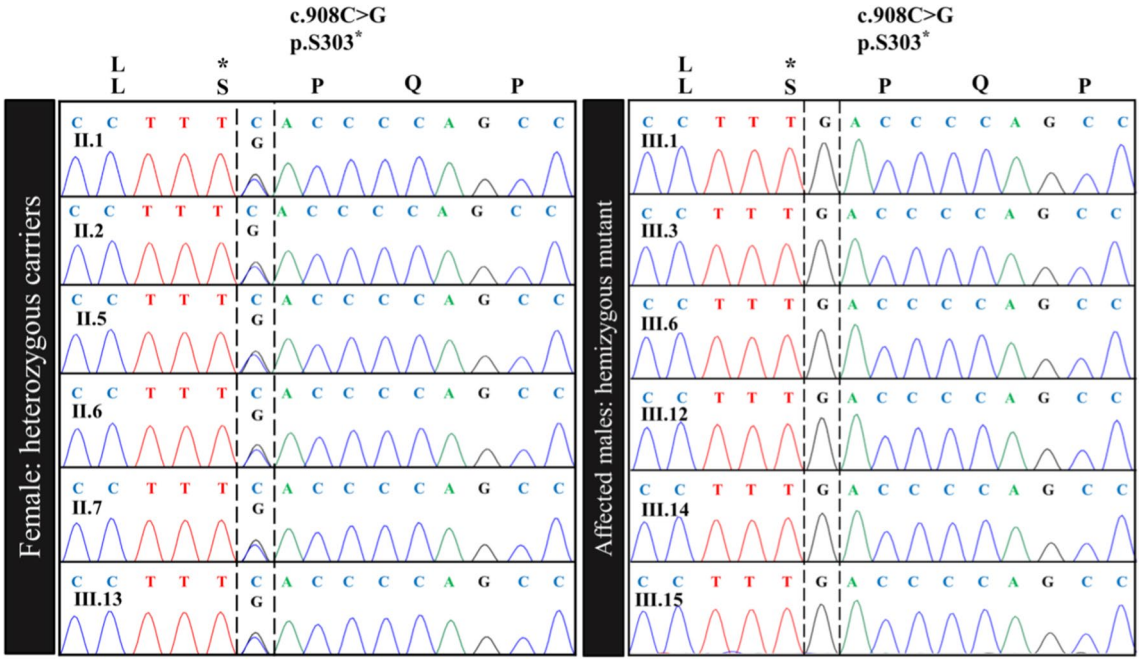

Fig. 2 Sanger sequencing validation of the *ADGRG2* truncating variants in the family members. The novel nonsense mutation (c.908C > G: p.S303*) in *ADGRG2* was verified by Sanger sequencing. This mutation presented in all male members (III.3, III.6, III.12 and III.14) diagnosed with azoospermia, which was inherited from their mothers, but member III.1 with normal reproductive capability also carried this mutation

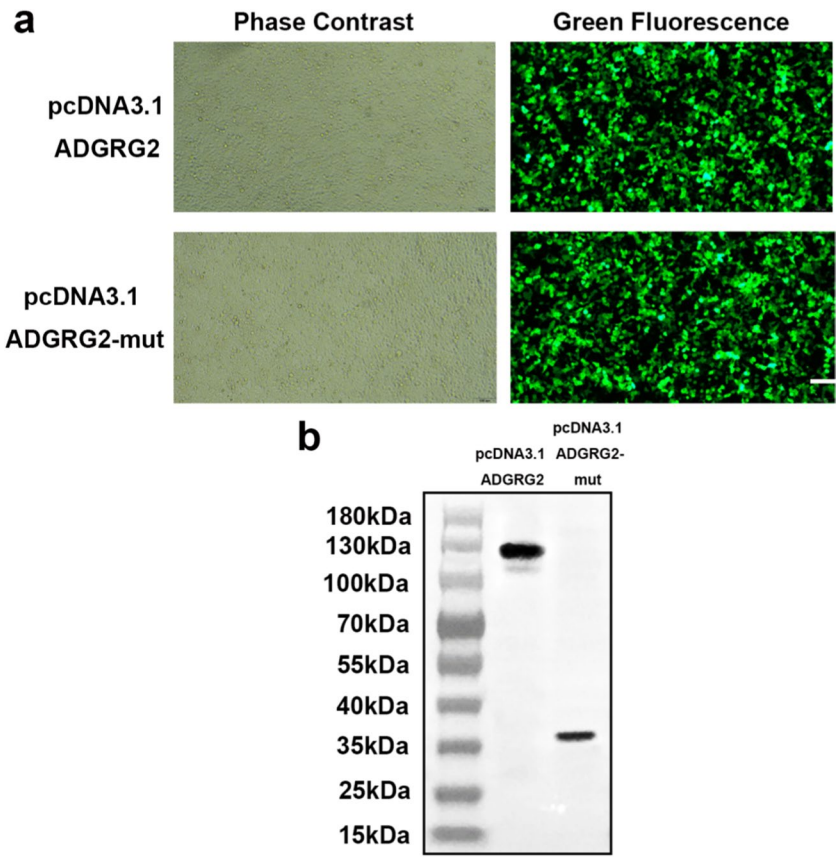

Fig. 3 Expression of ADGRG2 protein in 293T cells. **a** Transfection results of green fluorescent protein GFP cells. The cell density was maintained at a confluent rate of 70–90%. The cells were visible in the white light field of the microscope, the cell membrane was clear and in good condition. The green light emitted by GFP was clearly visible under the fluorescence microscope, and the fluorescence intensity was high. Bar = 100 μm. **b** Western Blot assay showed that both wild type and the mutant ADGRG2 could be expressed in 293T cells. But the molecular weight of wild-type ADGRG2 was found to be about 100 kD by SDS-PAGE electrophoresis and the mutant one was about 30 kD

Solitary kidney (URA) is found in approximately one-third of congenital unilateral absence of the vas deferens (CUAVD) patients [27, 28]. Therefore, for all men with isolated CAVD, the use of abdominal ultrasound for renal imaging is strongly recommended [29]. In our study, kidney abnormalities not found in any participant, as shown by transabdominal ultrasonography.

It was reported that varicocele was present in approximately 35–40% of infertile men [30]. Our participants with primary infertility (III.3 and III.14) also suffered from varicocele, but it was absent in members with normal fertility. Therefore, treatments for varicocele might help in obstructive azoospermia caused by this *ADGRG2* mutation.

Patients with OA usually have normal hormonal parameters and testicular size [11], as did our participants III.3, III.12, and III.14. Although there were no clinical manifestations of azoospermia, III.1 had dilated epididymal head ducts and dilated epididymal head duct nodules, which were similar to the underlying clinical features of OA [10, 31, 32]. In this study, the youngest participant, member IV.22 was 8 years old. Since he was a minor, his secondary sexual characteristics had not yet manifested, so no abnormalities were found in various examinations. In other words, the mutation may not affect the reproductive organs until adulthood.

There is growing evidence that infertile men are at higher risk of other comorbidities and cardiovascular death than fertile control participants [31–33]. Treatment options for OA are tailored to the cause: intratesticular obstruction requires testicular sperm extraction, and men with epididymal or vascular obstruction at the groin level may be offered microsurgical reconstruction or microsurgical epididymal sperm aspiration [8]. For *ADGRG2*-targeted treatment of male infertility, systematic screening of male infertility genes, genetic testing for variants in *CFTR* or *ADGRG2*, and early genetic or pharmacological intervention for male patients with the mutations can be considered [25]. Our study also found that the success rate of ICSI for men with azoospermia caused by this mutation was not as high as that reported in the literature [20]. Therefore, preimplantation genetic testing (PGT) may be an effective means to reduce infertility in patients' offspring with this mutation. In addition, the couple's age seemed to be a factor in our study; that is, the younger the couple, as long as the wife was healthy, the more likely they were to have a successful pregnancy, but more research is needed.

Smoking and drinking habits seemed to have little effect on pregnancy in this study in couples in which the male carried this mutation. III.1 had a history of smoking and drinking but had normal fertility, while patient III.3 had no smoking history and reported no interest in drinking, but ICSI failed many times.

To our knowledge, the effect of *ADGRG2* mutation on female fertility has not been reported; however, members III.15 (c.908C > G: p.S303* *ADGRG2* mutation carrier) and III.7 (untested) of the family in this study, who had had sex with their respective partners without contraception failed to achieve pregnancy for more than 2 years. More research is needed in the future to uncover the effect of this mutation on female infertility, its mechanism of action in the human body and the corresponding treatment.

Conclusion

In conclusion, our study is the first to describe a new *ADGRG2* truncating mutation in a large four-generation Chinese family including four azoospermia members and a normal fertile member with this mutation, expanding the mutation spectrum and phenotype spectrum of this gene. In our study, ISCI had a conception success rate of only two-thirds and a successful live birth rate of only one-third in conceiving azoospermia couples with this mutation.

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Authors' contribution YHL and YRQ conceived and designed the experiments. YHL, YLX wrote the manuscript. ML,YLX, BWL, NZ, SSN, MXN, JJS, YL performed the experiments. YHL and YRQ analyzed the data and wrote the paper. YHL, YLX and NZ revised the paper.

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Data availability The datasets and material used or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This study was approved by the medical ethics committee of Yulin Women and Children Care Hospital.

Consent to participate All subjects (or their guardians) were fully aware of the study and provided signed informed consent.

Conflict of interest The authors declare no competing interests.

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