Asian Journal of Andrology (2021) 23, 205–210
www.asiaandro.com; www.ajandrology.com

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ORIGINAL ARTICLE

Male Infertility

Mutational analysis of the *GATA4* gene in Chinese men with nonobstructive azoospermia

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As a crucial transcription factor for spermatogenesis, GATA-binding protein 4 (GATA4) plays important roles in the functioning of Sertoli and Leydig cells. Conditional knockout of *GATA4* in mice results in age-dependent testicular atrophy and loss of fertility. However, whether *GATA4* is associated with human azoospermia has not been reported. Herein, we analyzed the *GATA4* gene by direct sequencing of samples obtained from 184 Chinese men with idiopathic nonobstructive azoospermia (NOA). We identified a missense mutation (c.191G>A, p.G64E), nine single-nucleotide polymorphisms (SNPs), and one rare variant (c.*84C>T) in the 3′ untranslated region (UTR). Functional studies demonstrated that the p.G64E mutation did not affect transactivation ability of GATA4 for spermatogenesis-related genes (claudin-11 and steroidogenic acute regulatory protein, *Star*), and the 3′ UTR rare variant c.*84C>T did not generate microRNA-binding sites to repress GATA4 expression. To our knowledge, this is the first report to investigate the association between GATA4 and azoospermia; our results indicate that mutations in *GATA4* may not be pathogenic for NOA in Chinese men.

Asian Journal of Andrology (2021) **23**, 205–210; doi: 10.4103/aja.aja_33_20; published online: 28 August 2020

Keywords: biological function; GATA-binding protein 4 (*GATA4*) mutation; nonobstructive azoospermia

INTRODUCTION

Azoospermia is defined as the complete absence of spermatozoa in the seminal fluid and is classified as either obstructive (seminal tract obstruction) or nonobstructive (defective spermatogenesis).[1,2] As one of the major causes of human male infertility, azoospermia affects about 1% of men in the general population, and nonobstructive azoospermia (NOA) accounts for approximately 60% of these cases.[3,4] The currently known genetic causes of NOA include chromosome abnormalities, Y chromosome microdeletions, and defects in candidate genes such as Wilms' tumor gene (*WT1*), spermatogenesis and oogenesis-specific basic helix-loop-helix transcription factor 1 (*SOHLH1*), polo-like kinase 4 (*PLK4*), and ubiquitin-specific peptidase 26 (*USP26*).[5–10] However, the underlying mechanisms of azoospermia remain largely unknown in most cases.[6]

A member of the conserved GATA transcription factor family, GATA-binding protein 4 (GATA4) contains two zinc finger domains for binding consensus DNA sequences and interacting with other proteins.[11,12] The *GATA4* gene has been implicated in the development and function of mammalian testis with abundant expression in somatic cells (predominantly in Sertoli and Leydig cells) of embryonic and adult testes.[13–15] Mice with conditional deletions of *GATA4* in Sertoli cells exhibit age-dependent testicular atrophy and loss of fertility.[16] Likewise, mice with conditional fetal/adult Leydig cell *Gata4* knockout in the adrenogonadal primordium show small testes that lack mature sperm.[17,18] In addition, deletion of *Gata4/6* in adult Leydig cells causes an acute decline in testicular steroidogenesis as well as testicular atrophy and infertility.[17] Given the pivotal role of *GATA4* in adult testicular function, it might be a potential candidate gene for the dysfunction associated with NOA.

Increasing evidence suggests that human reproductive anomalies, such as NOA and disorders of sex development (DSD), can have common etiologies. For instance, variations in sex-determining genes such as nuclear receptor subfamily 5 group A member 1 (*NR5A1*) and *WT1* are reported to be associated with NOA and DSD.[7,19] Interestingly, GATA4 could cooperate with NR5A1 or WT1 in the regulation of transcription of genes required for testis determination and gonadal function.[20,21] In addition, a loss-of-function mutation in *GATA4* has been shown to be causative for DSD.[22] Herein, we sequenced the *GATA4* gene in 184 Chinese men from Northern China with idiopathic NOA and performed functional experiments to determine whether mutations in this gene contributed to NOA in these individuals.

PARTICIPANTS AND METHODS

Participants

In this study, 184 Chinese men with an age of 28 ± 3.9 (mean ± standard deviation [s.d.]) years with sporadic NOA and 197 unrelated Chinese normozoospermic men as controls were recruited from the Center for Reproductive Medicine of Shandong University (Jinan, China). All participants, including NOA cases and controls, were from Northern China. The NOA inclusion criteria were well-defined,

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Received: 07 October 2019; Accepted: 20 April 2020

requiring at least two semen samples with no sperm detected and no obstructive azoospermia or cryptorchidism present. Known causes of low sperm count, such as inflammation of the reproductive system, endocrinological defects, karyotypic abnormalities, or Y chromosome microdeletions, were excluded. Patients who had undergone chemotherapy and radiotherapy, testis trauma or immune diseases, hypogonadotropic hypogonadism, varicocele, inflammation, recurrent infections, or iatrogenic infertility were also excluded. None of the included patients were treated or had been treated with testosterone. According to the operation standards of the World Health Organization, testicular biopsies were conducted on azoospermic patients.[1,23] All participants showed normal karyotypes (46,XY). Informed consent was obtained from each participant. The study was approved by the Institutional Review Board of Reproductive Medicine of Shandong University in Jinan, China ([2016] IRB No. 12).

Mutational analysis of GATA4 and in silico prediction

The genomic DNA of patients and controls was extracted from peripheral blood samples using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany). The entire coding region and exon–intron boundaries of the human *GATA4* gene (NM_002052) were amplified by polymerase chain reaction (PCR) with six pairs of specific primers (**Table 1**). After purification, the PCR products were labeled using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems). The identified variants were confirmed in three independent PCR runs by sequencing in both the forward and reverse directions. Conservation analyses of amino acid sequences from human and other species were performed with the use of the ClustalW2 website (www.ebi.ac.uk/Tools/msa/clustalw2/). The online tool RegRNA (http://regrna.mbc.nctu.edu.tw/) was used to predict potential binding sites of microRNAs (miRNAs) in the 3′ untranslated region (UTR) of *GATA4*.

Plasmid construction, cell culture, and transfection

The full-length human *GATA4* cDNA sequence was obtained from the vector CH804895 (ViGene Biosciences, Rockville, MD, USA) and cloned into the pXF6F expression vector between *Xho*I and *Sal*I sites; directionality was confirmed by sequencing. A 307-bp fragment of the 3′ UTR sequence of *GATA4* containing the c.*84C>T site was cloned into pmirGLO Dual-Luciferase miRNA target expression vector (Promega, Madison, WI, USA) at *Sac*I and *Xba*I sites. The mutant vectors were constructed with the use of the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). For the promoter reporter constructs, the 5′ flanking region (nucleotides −1389 to +50) of the steroidogenic acute regulatory protein (*Star*) gene and the promoter region (nucleotides −367 to −1) of claudin-11 were inserted into the GLuc-ON promoter reporter construct (GeneCopoeia, Rockville, MD, USA) and the pGL3-basic vector, respectively. All plasmids were verified by sequencing before functional studies. The human embryonic kidney 293 cell line (HEK293) was cultured in Dulbecco's Modified Eagle Medium (Hyclone Laboratories/GE Healthcare, Chicago, IL, USA) with 10% fetal bovine serum (Hyclone Laboratories) and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The HEK293 cells were seeded onto a 12-well plate 1 day before transfection. The desired plasmids were transfected into cells using X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland). The transfected cells were then cultured for 48 h before use in functional assays.

Table 1: Primers for amplification of the coding region of the *GATA4* gene

Primer ID	*Sequence (5′–3′)*	*Product size (bp)*
GATA4-E1F	TTTAAGCGAGTTGGTTTTTTCCC	920
GATA4-E1R	TGGCTTCTGCCTTCCCTAGAAAC	
GATA4-E2F	CAGGGGCTGAAGTCAGAGTGAGG	509
GATA4-E2R	CATTCTGGTGGCTCCAGCTAACTC	
GATA4-E3F	CTGATTTATTCCTCGCAGTGGC	478
GATA4-E3R	GACGGAAGAGGCCAGCAAAGTAG	
GATA4-E4F	CATCACACAGGTGCTCGATAAGTTT	478
GATA4-E4R	TCCATGGCAATTGTTACTTTTTTGC	
GATA4-E5F	AGATAAGGACCTCTGCTGCTGTCC	428
GATA4-E5R	TTCCTAGCGCAGAGGGTAGCTCAC	
GATA4-E6F	TCAGGAGAAACAGAGAGAAGTGCTC	537
GATA4-E6R	CCATCAGCGTGTAAAGGCATCTG	

GATA4: GATA-binding protein 4; F: forward; R: reverse

Luciferase reporter assay, Western blot, and immunofluorescence staining

Luciferase assays were performed with the use of Dual-Luciferase Reporter Assay System (GeneCopoeia). The promoter constructs (0.5 μg) and *GATA4* expression vector (0.5 μg) were cotransfected into HEK293 cells, and the luciferase activities were determined 48 h posttransfection using a multimode plate reader (PerkinElmer, Waltham, MA, USA). The reporter genes (secreted alkaline phosphatase [SEAP] or Renilla luciferase) were used to normalize the efficiency of transfection, and the result of luciferase activity was expressed as a relative value. For Western blotting, total protein extracts from HEK293 cells were prepared in 1% sodium dodecyl sulfate (SDS) lysis buffer obtained from Beyotime Biotechnology (Shanghai, China), and a bicinchoninic acid (BCA) assay was performed to measure protein concentration by the use of a kit from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Protein extracts were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. After blocking with 5% nonfat milk in Tris-buffered saline-Tween (TBST) for 1 h at room temperature, the membrane was incubated with the primary antibody against GATA-4 (sc-25310, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight; after the overnight incubation, the corresponding secondary antibody from Wuhan Sanying Biotechnology Co. (Wuhan, China) was added and incubated for 1 h at room temperature. ECL Western blotting detection reagents (GE Healthcare) were used to detect protein bands with the ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA, USA). For immunofluorescence staining, HEK293 cells were cultured on coverslips in 12-well plates and transfected with the respective plasmids. At 48 h posttransfection, cells were fixed with 4% paraformaldehyde and permeabilized with the use of 0.3% Triton X-100. Cells were blocked with 10% normal goat serum purchased from Sangon Biotech Co. (Shanghai, China), followed by incubation with the GATA4 antibody (1:200 dilution; sc-25310; Santa Cruz Biotechnology) overnight at 4°C. Cells were incubated with fluorescein isothiocyanate (FITC)-goat anti-mouse IgG (ZSGB-Bio, Beijing, China) for 1 h. Finally, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and visualized under an Olympus BX53 fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analyses

SPSS version 21 software (IBM Corp., Chicago, IL, USA) was used for statistical analysis. All quantitative data were shown as

mean ± s.d. of duplicate assays in three independent experiments. Student's *t*-tests were performed to calculate the significance of the difference between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Mutational analysis of GATA4 in patients with NOA

Sequencing of all exons and exon–intron boundaries of the *GATA4* gene in 184 men with idiopathic NOA identified 11 genetic variants (**Table 2**). These variations included a missense mutation (CM107237, c.191G>A in exon 1), three synonymous variants (rs56166237 and rs573733348 in exon 1; rs1062215 in exon 2), and six intronic variants (rs201533584 in exon 3; rs143010652 with rs804280 in exon 4; and rs200334160, rs141976814, and rs372062855 in exon 5), as well as one rare variant (rs1479512602, c.*84C>T) with the highest population of minor allele frequency (MAF) <0.01 located in the 3′ UTR of *GATA4*. For allele/genotype frequencies of synonymous and intronic variants, we found no significant difference between NOA cases and population data from the 1000 Genomes-CHB Project database (http://asia.ensembl.org/index.html; **Table 2**). With respect to the missense mutation and the 3′ UTR rare variant, both were heterozygous and found to be absent in the 197 unrelated normozoospermic controls. These two variants are not novel; the p.G64E mutation has been previously identified in patients with congenital heart defects (CHD).[24,25]

Biological function of GATA4 mutations

The missense mutation (CM107237, c.191G>A) resulted in a p.Gly64Glu (p.G64E) substitution; the glycine residue is highly conserved among mammal species (**Figure 1**). This variant falls within the N-terminal transcription activation domain (TAD), which contributes to the interaction of *GATA4* with target DNA sequences or cofactors (**Figure 1a**). To further investigate whether the p.G64E mutation affected the biological function of GATA4, we performed a luciferase reporter assay to test the effect of the p.G64E mutation on the transactivation abilities of GATA4 on target gene promoters. HEK293 cells were transiently cotransfected with either an empty expression vector (serving as control) or expression vectors of wild-type or mutant *GATA4* along with the promoter constructs of two target genes, claudin-11 and *Star*. Overexpression of *GATA4* resulted in a three- to four-fold induction of

Table 2: Variants identified in *GATA4* gene in northern Chinese men with nonobstructive azoospermia

Location	*dbSNP ID*	*NOA case*	*Sequence variation*	*Amino acid variation*	*Allele*	*Allele frequency, n (%)*			*Genotype*	*Genotype frequency, n (%)*		
						NOA	*CHB*[a]	*P*		*NOA*	*CHB*[a]	*P*
Exon 1	CM107237	175	c.191G/A	Missense variant	G	349 (99.7)	–	–	GG	174 (99.4)	–	–
				p.Gly64Glu	A	1 (0.3)	–		GA	1 (0.6)	–	
									AA	0 (0)	–	
Exon 1	rs56166237	175	c.99G>T	Synonymous	G	335 (95.7)	199 (96.6)	0.604	GG	161 (92.0)	96 (93.2)	0.728
					T	15 (4.3)	7 (3.4)		GT	13 (7.4)	7 (6.8)	
									TT	1 (0.6)	0 (0)	
Exon 1	rs573733348	175	c.1089C/A	Synonymous	C	349 (99.7)	206 (100)	1	CC	174 (99.4)	103 (100)	1
					A	1 (0.3)	0 (0)		CA	1 (0.6)	0 (0)	
									AA	0 (0)	0 (0)	
Exon 2	rs1062215	183	c.723C>T	Synonymous	C	364 (99.5)	205 (99.5)	1	CC	181 (98.9)	102 (99.0)	1
					T	2 (0.5)	1 (0.5)		CT	2 (1.1)	1 (1.0)	
									TT	0 (0)	0 (0)	
Exon 3	rs201533584	184	c.784-16G>A	Intron variant	G	367 (99.7)	205 (99.5)	1	GG	183 (99.5)	102 (99.0)	1
					A	1 (0.3)	1 (0.5)		GA	1 (0.5)	1 (1.0)	
									AA	0 (0)	0 (0)	
Exon 4	rs143010652	184	c.910-58T>A	Intron variant	T	360 (97.8)	204 (99.0)	0.507	TT	176 (95.7)	101 (98.1)	0.504
					A	8 (2.2)	2 (1.0)		TA	8 (4.3)	2 (1.9)	
									AA	0 (0)	0 (0)	
Exon 4	rs804280	184	c.997+56C>A	Intron variant	A	365 (99.2)	202 (98.1)	0.257	AA	182 (98.9)	99 (96.1)	0.089
					C	3 (0.8)	4 (1.9)		AC	1 (0.5)	4 (3.9)	
									CC	1 (0.5)	0 (0)	
Exon 5	rs200334160	183	c.1146+4C>T	Intron variant	C	365 (99.7)	206 (100)	1	CC	182 (99.5)	103 (100)	1
					T	1 (0.3)	0 (0)		CT	1 (0.5)	0 (0)	
									TT	0 (0)	0 (0)	
Exon 5	rs141976814	183	c.1146+24dup	Intron variant	-	365 (99.7)	205 (99.5)	1	--	182 (99.5)	102 (99.0)	1
					A	1 (0.3)	1 (0.5)		-A	1 (0.5)	1 (1.0)	
									AA	0 (0)	0 (0)	
Exon 5	rs372062855	183	c.998-36C>T	Intron variant	C	365 (99.7)	206 (100)	1	CC	182 (99.5)	103 (100)	1
					T	1 (0.3)	0 (0)		CT	1 (0.5)	0 (0)	
									TT	0 (0)	0 (0)	
Exon 6	rs1479512602	177	c.*84C>T	3' UTR variant	C	353 (99.7)	–	–	CC	176 (99.4)	–	–
					T	1 (0.3)	–		CT	1 (0.6)	–	
									TT	0 (0)	–	

[a]The allele and genotype frequencies were obtained from 1000 Genomes-CHB Project database in Ensembl. *GATA4*: GATA-binding protein 4; NOA: nonobstructive azoospermia; SNP: single-nucleotide polymorphism; CHB: Han Chinese in Beijing, China; UTR: untranslated region; –: null

promoter activity in claudin-11 and *Star* compared with the control. As for the p.G64E mutant, no significant difference in transcriptional activity was observed using the two separate promoters, suggesting that the p.G64E mutation did not significantly affect the transactivation potential of GATA4 for the *Star* ($P = 0.495$) and claudin-11 ($P = 0.628$) promoters (**Figure 2a** and **2b**). In addition, we examined the expression and localization of mutant GATA4 in HEK293 cells by Western blotting and immunofluorescence staining, respectively. The results showed that the GATA4 p.G64E mutant and wild-type GATA4 had indistinguishable expression and both exhibited nuclear localization when expressed in HEK293 cells (**Figure 2c** and **2d**).

We next explored the effect of variant c.*84C>T on miRNA-regulated expression of *GATA4* (**Figure 3a**). Variations in the 3′ UTR may result in alterations of miRNA interactions by generating or destroying binding sites for miRNAs, and two human miRNAs (hsa-miR-3194 and hsa-miR-1225-3p) were predicted to potentially interact with the 3′ UTR of *GATA4* as a result of the c.*84C>T variation. To investigate the interaction between miRNAs and the possible binding site of the variant, a 307-bp fragment of the 3′ UTR sequence, containing either C or T at the variation site, was cloned into a luciferase reporter vector and cotransfected with hsa-miR-3194 or hsa-miR-1225-3p mimics into HEK293 cells. However, no significant difference in luciferase activity was observed in cells that were cotransfected with hsa-miR-3194 ($P = 0.300$) or hsa-miR-1225-3p ($P = 0.505$) and the GATA4-mutant 3′ UTR compared with those transfected with the wild-type GATA 3′ UTR (**Figure 3b** and **3c**). These results suggest that the c.*84C>T variation did not produce new binding sites for miRNAs and had no effect on the expression of *GATA4* via interactions with miRNA.

DISCUSSION

As a critical transcription factor for spermatogenesis, GATA4 is highly expressed and plays important roles in Sertoli and Leydig cells by regulating promoter activities of target genes.[16,17] Claudin-11 and *Star* are two factors essential for sperm cell development and male fertility in the mouse and both are transcriptionally activated by GATA4.[26,27] Claudin-11 is a key junction protein constituting inter-Sertoli tight junctions and is indispensable for the integrity of the blood–testis barrier; male mice lacking claudin-11 are sterile.[26,28] In Leydig cells, *Star* is pivotal in the rate-limiting step of steroidogenesis. Defects in *Star* expression lead to suboptimal production of androgen and impaired spermatogenesis.[29]

In our study, we screened a Chinese population with NOA for the *GATA4* gene and identified two heterozygous mutations: one missense mutation, p.G64E, and one 3′ UTR rare variant, c.*84C>T. These two mutations are not novel; the p.G64E mutation was previously reported to occur in individuals with ventricular septal defect, the most common type of CHD.[24,25] GATA4 is a master transcription factor essential for cardiac development and function.[30] To date, more than 100 *GATA4* mutations have been reported in CHD.[30] However, except for the p.G64E mutation, no other mutations previously found in patients with heart disease were present in our population. GATA4 is also required for follicular development and normal ovarian function.[31,32] *Gata4* deficiency in mice ovary results in depletion of follicular pool with sterility, as well as ovarian cysts.[33] Higher expression of *GATA4* is associated with more aggressive human ovarian granulosa cell tumors and higher rates of recurrence.[34,35] However, to our knowledge, none of the *GATA4* mutations have been reported in human female infertility or

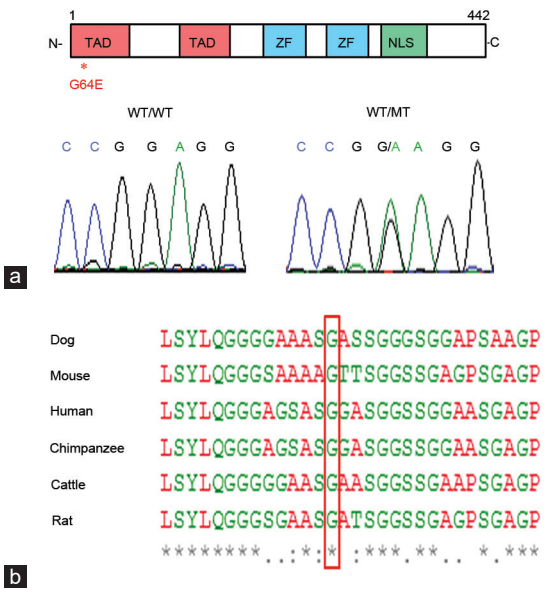

Figure 1: A missense mutation of the *GATA4* gene identified in patients with NOA. (**a**) Schematic diagram shows the functional domains of GATA4: N-terminal TADs, the DNA-binding domain-containing two ZFs, and the NLS. The location of the p.G64E heterozygous mutation in the first TAD is marked by an asterisk (*) and the Sanger sequence diagram is shown. (**b**) Sequence alignment surrounding the mutation site shows conservation of amino acid 64 in mammals (highlighted with a red box). GATA4: GATA-binding protein 4; WT: wild-type; MT: mutant; NOA: nonobstructive azoospermia; ZF: zinc-finger; TAD: transcription activation domain; NLS: nuclear localization signal.

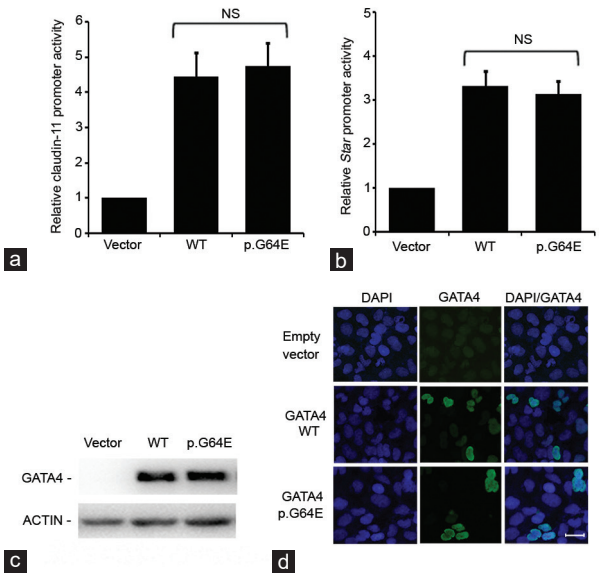

Figure 2: Functional analysis of the *GATA4* p.G64E mutant. (**a**) Transcriptional activation of claudin-11 promoters activated by GATA4. (**b**) Transcriptional activation of *Star* promoters activated by GATA4. HEK293 cells were transiently cotransfected with an empty vector (serving as control) or WT or mutant GATA4 (p.G64E) along with the promoter constructs of (**a**) claudin-11 and (**b**) *Star*. We observed no significant differences in transactivation potential between WT and mutant GATA4 proteins. All promoter activities are reported as fold activation relative to that of the control (vector). (**c**) HEK293 cells were transfected with WT or p.G64E mutant GATA4. Western blot analysis shows that WT and mutant protein were similarly expressed in total protein extracts. (**d**) Immunofluorescence staining demonstrated that both WT and mutant GATA4 (green fluorescence) showed strong nuclear localization when expressed in HEK293 cells. The nuclei were stained with DAPI. Scale bar = 25 μm. Results are the mean ± standard deviation of three independent experiments. NS: not significant between two groups. GATA4: GATA-binding protein 4; WT: wild-type; DAPI: 4′,6-diamidino-2-phenylindole.

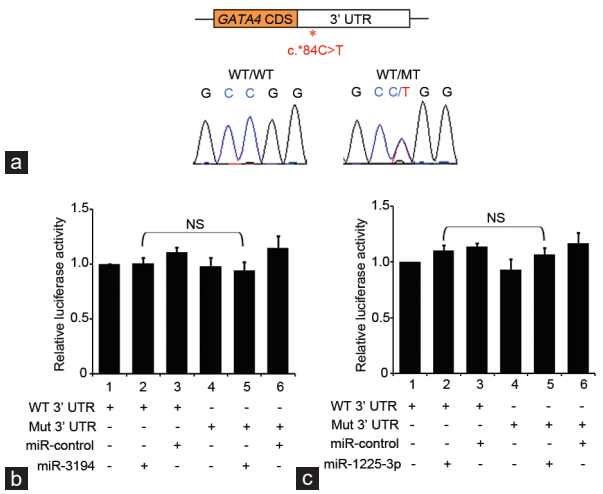

Figure 3: Functional analysis of the *GATA4* c.*84C>T variant located in the 3′ UTR. (**a**) The location and Sanger sequencing result of the 3′ UTR variant are shown in the diagrams. (**b**) In the dual-luciferase activity assay, HEK293 cells were cotransfected with luciferase reporters containing the 3′ UTR fragment along with hsa-miR-3194 mimics. (**c**) In the dual-luciferase activity assay, HEK293 cells were cotransfected with luciferase reporters containing the 3′ UTR fragment along with hsa-miR-1225-3p mimics. The luciferase reporter targets include two types of 3′ UTR fragments of *GATA4* with a 307-bp fragment of the 3′ UTR sequence containing either C or T at the variation site. They are wild-type 3′ UTR of *GATA4* with original base C (labeled WT 3′ UTR) and mutant 3′ UTR of *GATA4* with variation base T (labeled mut 3′ UTR), respectively. Cotransfection with miRNA mimics (**b**) hsa-miR-3194 or (**c**) hsa-miR-1225-3p resulted in no significant difference in luciferase activity between cells transfected with mut 3′ UTR and those with wt 3′ UTR. Results are the mean ± standard deviation of three independent experiments. NS: not significant between two groups. *GATA4*: GATA-binding protein 4; WT: wild-type; MT: mutant; UTR: untranslated region; CDS: coding sequence.

ovary-related diseases, such as premature primary ovarian insufficiency and granulosa cell tumors.[34–37]

We investigated the effect of the p.G64E mutation on the transcriptional activity of claudin-11 and *Star* to determine whether the *GATA4* mutation contributes to NOA. The glycine residue at position 64 (Gly-64) is highly conserved between mammals, and the variation site falls within one of the two important transcription activation domains of GATA4, suggesting a potential deleterious effect of the p.G64E mutation on GATA4 function. The same p.G64E mutation of *GATA4* is associated with CHD and results in impaired transcriptional activity of the heart-specific atrial natrium peptide (*ANP*) gene.[24,25] However, transient transfection assays in HEK293 cells showed no biological differences between mutant and wild-type proteins for the transactivation potential of GATA4 toward claudin-11 and *Star* promoters.

This discrepancy in transactivation ability elicited by the same gene mutation could be caused by differential promoter sensitivity.[38] A similar scenario was demonstrated with another CHD-associated *GATA4* mutation, p.Glu215Asp, which showed reduced transactivation potential for the heart-specific *ANF* promoter but not for gonadal promoters such as *Star* and cytochrome P450 family 19 subfamily A polypeptide 1 (*Cyp19a1*).[38] With respect to the p.G64E mutation, the difference in transactivation abilities of the mutant protein on distinct promoters suggests that the testes are less sensitive to the *GATA4* mutation than the heart. It is also possible that in the testes, the p.G64E mutation affects promoters other than those of claudin-11 and *Star*. In other words, expression of these two genes (claudin-11 and *Star*) is not definitive proof that GATA4 is not affected. Previous evidence suggests that differential promoter sensitivity occurs among distinct gonadal promoters; the *GATA4* p.Val266Met mutant had reduced transcriptional activity for the *Cyp19a1* and inhibin alpha (*Inha*) promoters but not the *Star* promoter.[38]

The expression and localization of GATA4 protein were not affected by the p.G64E mutation, suggesting that the DNA-binding capacity and physical or functional interactions with partners were more likely causes of mutation-induced alterations of GATA4 transcriptional activity. This scenario has also been shown to occur in human CHD and DSD.[22,38] In addition to the p.G64E mutation, we also identified a rare 3′ UTR variation (c.*84C>T) in *GATA4*. The binding of miRNAs to the 3′ UTR can regulate gene expression at the posttranscriptional level, and genetic mutations within the 3′ UTR can result in phenotypic variations by creating or destroying putative binding sites for miRNAs.[39,40] However, in our study, two predicted miRNAs (hsa-miR-3194 and has-miR-1225-3p) did not interact with the c.*84C>T mutant to affect *GATA4* expression posttranscriptionally, suggesting that the 3′ UTR rare variant is not a pathogenic variation for NOA.

To our knowledge, this study is the first to investigate *GATA4* gene mutations in men with idiopathic NOA. We identified one missense mutation (p.G64E) and a rare 3′ UTR variant in two NOA patients; neither mutation was present in the control group of 197 normozoospermic men. However, functional studies showed that the *GATA4* p.G64E mutation did not significantly alter GATA4 transactivation ability on two target promoters (claudin-11 and *Star*), in contrast to the distinct effect of the same mutation on the heart-specific *ANP* gene.[25] Unlike the deleterious effects of *GATA4* mutations on CHD,[38] mutations in *GATA4* may not be pathogenic for NOA in Chinese men. However, other potential *GATA4* alterations in the NOA patients, such as copy number variations of the *GATA4* gene or transcriptional changes of *GATA4* expression, have yet to be explored in our study. In addition to ethnic diversity, GATA4 cannot be ruled out as an important player in NOA. Further studies in other populations or of other distinct *GATA4* alteration mechanisms are needed to determine the exact role of *GATA4* in the pathogenesis of human NOA.

AUTHOR CONTRIBUTIONS

XZ and HBZ designed the study; XZ, YNN, YHB, and YZC performed the experiments and acquired, interpreted, and analyzed the data; HBZ, TJZ, WL, and JLM evaluated patients and collected patients' clinical data; and XZ, HBL, HBZ, and JLM wrote and revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (No. 81601337), the Fundamental Research Funds of Shandong University (No. 2016HW006), and the Key research and development program of Shandong Province (No. 2019GSF108237). The authors thank all of the participants involved in this study.

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