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

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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).1–3 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.4,5 The currently known genetic causes of NOA include chromosome abnormalities, Y chromosome microdeletions, and defects in candidate genes such as nuclear receptor subfamily 5 group A member 1 (NR5A1), spermatogenesis and oogenesis-specific basic helix-loop-helix transcription factor 1 (SOHLH1), polo-like kinase 4 (PLK4), and ubiquitin-specific peptidase 26 (USP26).4–10 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.11–13 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.14–16 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.19,20 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,
requiring at least two semen samples with no sperm detected and no obstructive azoospermia or cryptoorchidism 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. 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 XhoI and SalI 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 SacI and XbaI 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% CO₂ 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.

**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) anti-mouse IgG (ZSGB-Bio, Beijing, China) for 1 h 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 variant 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).

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 | NOA | CHB | P | NOA | CHB | P |
|----------|----------|----------|-------------------|---------------------|--------|-----|-----|----|-----|-----|----|
| Exon 1   | CM107237 | 175      | c.191G>A          | Missense variant    | G      | 349(99.7) | –   | –  | 174(99.4) | –   | –  |
| Exon 1   | rs56166237 | 175      | c.99G>T           | Synonymous variant  | G      | 335(95.7) | 199(96.6) | 0.604 | 161(92.0) | 96(93.2) | 0.728 |
| Exon 1   | rs573733348 | 175      | c.1089C>A         | Synonymous variant  | C      | 349(99.7) | 206(100) | 1   | 174(99.4) | 103(100) | 1  |
| Exon 2   | rs1062215 | 183      | c.723C>T          | Synonymous variant  | C      | 364(99.9) | 205(99.5) | 1   | 181(98.9) | 102(99.0) | 1  |
| Exon 3   | rs201533584 | 184      | c.784-16G>A       | Intronic variant    | G      | 367(99.7) | 205(99.5) | 1   | 183(99.5) | 102(99.0) | 1  |
| Exon 4   | rs143010652 | 184      | c.910-58T>A       | Intronic variant    | T      | 360(97.8) | 204(99.0) | 0.507 | 176(95.7) | 101(98.1) | 0.504 |
| Exon 5   | rs200334160 | 183      | c.1146+4C>T       | Intronic variant    | -      | 365(99.7) | 205(99.5) | 1   | 182(98.9) | 99(96.1) | 0.089 |
| Exon 5   | rs141976814 | 183      | c.1146+24dup      | Intronic variant    | -      | 365(99.7) | 205(99.5) | 1   | 182(98.9) | 102(99.0) | 1  |
| Exon 5   | rs372062855 | 183      | c.998-36C>T       | Intronic variant    | C      | 365(99.7) | 206(100)  | 1   | 182(99.5) | 103(100)  | 1  |
| Exon 6   | rs1479512602 | 177      | c.84C>T           | 3’ UTR variant      | C      | 353(99.7) | –   | –  | 176(99.4) | –   | –  |

*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. 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. 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. 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.

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, and GATA4 is a master transcription factor essential for cardiac development and function. To date, more than 100 GATA4 mutations have been reported in CHD. 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. Gata4 deficiency in mice ovary results in depletion of follicular pool with sterility, as well as ovarian cysts. Higher expression of GATA4 is associated with more aggressive human ovarian granulosa cell tumors and higher rates of recurrence. However, to our knowledge, none of the GATA4 mutations have been reported in human female infertility or cardiomyopathy. The GATA4 p.G64E mutation was previously related to patients with nonobstructive azoospermia or male miscarriages. Since no significant difference in the expression of claudin-11 and Star promoters was observed between WT and mutant GATA4 proteins, the p.G64E mutation may have a less significant influence on transcriptional activity. Further studies are required to elucidate the potential role of the p.G64E mutation in the development of male infertility in this Chinese population.
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.
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