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BRIEF REPORT

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A novel stopgain mutation c.G992A (p.W331X) in *TACR3* gene was identified in nonobstructive azoospermia by targeted next-generation sequencing

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Background: Nonobstructive azoospermia (NOA) is one of the most severe forms of male infertility because of impaired spermatogenesis with the absence of spermatozoa in the ejaculate. The causes of this disease can be partly attributed to genetic factors. Some common structural variants and single nucleotide polymorphisms (SNPs) were reported to be associated with NOA. However, the underlying etiology and genetic mechanism(s) remain largely unclear. The aim of this study was to investigate the associated mutations of spermatogenic genes in Chinese infertile men with NOA.

Methods: The entire coding region of 25 genes associated with spermatogenesis was sequenced from 200 infertile men with NOA. Screening was carried out using the targeted exome sequencing to identify genetic variations and SNPs of the entire coding region of these genes.

Results: After the targeted exome sequencing data were filtered through several currently existing variation databases, a series of variations were found. In this paper, we report one novel stopgain variation c.G992A (p.W331X) in the exon 4 of *TACR3* gene. The variant was heterozygous and categorized as pathogenic.

Conclusion: In conclusion, our study revealed a novel stopgain mutation c.G992A (p.W331X) in *TACR3* which expanded the mutation spectrum of *TACR3* in Chinese NOA infertile men and advanced our understanding of the genetic susceptibility to NOA.

KEYWORDS
male infertility, mutation, next-generation sequencing, NOA, *TACR3*

1 | INTRODUCTION

Infertility affects approximately 15% of couples at childbearing age worldwide with equivalent male and female factor.[1] Male infertility accounts for almost half of all infertility cases.[2] Nonobstructive azoospermia (NOA) is one of the most severe forms of male infertility because of impaired spermatogenesis with the absence of spermatozoa in the ejaculate.[1] It occurs in approximately 1% of all adult men.[3] The causes of this disease can be partly attribute to genetic factors.[4] A series of studies have reported that genetic factors including karyotype abnormalities, Y-chromosome microdeletions, single-gene or polygenic defects including structural variants or SNPs in multiple biological pathways were involved in the development of NOA.[4,5] However, the underlying etiology and genetic mechanism(s) remain largely unclear.

In this study, we aimed to discover and investigate genetic mutations of a few spermatogenic genes in a population of infertile men

with NOA. Then we presented a novel stopgain mutation of *TACR3* gene in a patient with NOA which next-generation sequencing (NGS) indicated.

TACR3 gene encodes the receptor for the tachykinin neurokinin 3(NKR or known as NK3R), which is the endogenous receptor for neurokinin B (NKB), which is encoded by *TAC3*.[11] The receptors belonging to this family are characterized by interactions with G proteins and seven hydrophobic transmembrane regions. *TACR3* is expressed in uterus, placenta, skeletal muscle, liver, lung, intestinal tract, and widely expressed in the central nervous system, including hypothalamic nuclei involved in regulating GnRH release.[12,13]

In the recent years, missense loss-of-function mutations in*TAC3* and *TACR3* have been reported in cases with nonsyndromic normosmic congenital hypogonadotropic hypogonadism (nCHH),[14] the disease which result in azoospermia at the pre-testicular (NOA) level.[15] *TACR3* was considered to be one of the first two genes to be screened in a clinical setting for equivocal cases such as constitutional delay in puberty vs idiopathic HH.[16] NKB action *via* the NK3R plays a fundamental role in the physiology of human gonadotrope axis and this pathway is necessary for the central neuroendocrine control of human reproduction.[14,17,18]

2 | MATERIALS AND METHODS

This study was approved by the Ethics Committee of the First Hospital of Jilin University. All the participants were Han Chinese. Written informed consent was obtained after the nature of the procedures had been fully explained prior to the study. We recruited patients who consulted for sterility and diagnosed as nonobstructive azoospermia. By performing the comprehensive examinations including a detailed medical history, physical examination, chromosome analysis, Y-chromosome microdeletions, semen analysis, and hormone profiles, these patients were diagnosed as having nonobstructive azoospermia. If patients were detected with Y-chromosomal microdeletion, they were excluded by the study. Finally a total of 200 patients were recruited. They were subjected to sequencing analysis of the spermatogenesis associated genes.

Mutation screening of genes was performed by targeted exome sequencing. Genomic DNA was isolated from blood lymphocyte samples and subjected to exome capture using the in house Targeted genes Panel (Peking Medriv Academy of Genetics and Reproduction, Peking) followed by next-generation sequencing on the Illumina MiSeq sequencing platform including 25 spermatogenesis associated genes. Capture probes were prepared based on these spermatogenesis associated genes. Sequence reads were mapped to the human reference genome assembly (NCBI build 37/hg19) using the Burrow-Wheeler Aligner (BWA version 0.7.12). Duplicated reads from library and PCR preparation were removed with Picard tools. After alignment to the human reference genome (GRCh37/hg19), most likely nondeleterious variants were filtered as previously described.[19] Variants with minor allele frequencies greater than 1% in the databases including dbSNP, 1000 Genomes Project, Exome Aggregation Consortium, Exome Variant Server, were excluded because they were unlikely to be deleterious. Intronic variants and synonymous variants that were located within intronic regions, regulatory regions and nonregulatory intergenic regions were excluded. Nonsynonymous variants and splice site variants were remained. Pathogenicity of nonsynonymous variants was assessed using SIFT (https://sift.jcvi.org/) and PolyPhen2 (https://genetics.bwh.harvard.edu/pph2/). Pathogenicity of a splicing site mutation was assessed using Mutation Taster (https://www.mutationtaster.org/) and Human Splicing Finder (https://umd.be/HSF3/). The variant classification was determined followed the American College of Medical Genetics and Genomics (ACMG) standards and guidelines.[20]

The suspected pathogenic variant was confirmed by conventional PCR and Sanger sequencing (ABI 3730XL, BGI Genomics, Beijing Genomics Institute-Shenzhen, Shenzhen). Primers for the detected potential disease-causing mutation p.W331X in *TACR3* were 5′- TTTGTCCGTGTTTGAGT-3′ (forward) and 5′-TTGGAGGAAGAAGTTGG-3′ (reverse).

Multiple protein alignments were performed with BLASTP 2.8.0+ (blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequences of the TACR3 in humans, Orycteropus afer afer, Dasypus novemcinctus, Nannospalax galili, Acinonyx jubatus, Mus musculus, Bos Taurus, Myotis davidii, Propithecus coquereli, Felis catus, and Camelus ferus was determined. The identification numbers of TACR3 protein were as follows: Human, NP_001050.1; Orycteropus afer afer, XP_007957420.1; Dasypus novemcinctus, XP_012378583.1; Nannospalax galili, XP_008824373.1; Acinonyx jubatus, XP_014938373.1; Mus musculus, BAC32723.1; Bos Taurus, CAD67556.1; Myotis davidii, ELK33228.1; Propithecus coquereli, XP_012510544.1; Felis catus, XP_003985169.2; Camelus ferus, XP_014419730.1.

3 | RESULTS

After targeted exome sequencing was performed in 200 individuals with nonobstructive azoospermia and evaluated for mutations in the 25 spermatogenesis associated genes, we examined whether *TACR3* genetic defects were associated with nonobstructive azoospermia. As a result, the present study discovered a novel stopgain mutation c.G992A (p.W331X) in a patient, in whom no variations were found in other 24 spermatogenesis associated genes. The novel mutation, which was located in the exon 4 of *TACR3*, was heterozygous and categorized as pathogenic. We performed PCR and Sanger sequencing on this patient and confirmed the heterozygous stopgain mutation in the patient but not in the control (Figure 1A). Alignment of the amino acid sequence of *TACR3* to its orthologs in 10 different species demonstrated that the c.G992A (p.W331X) variant affected a highly conserved amino acid, as shown in Figure 1B. Scrotal color Doppler ultrasonography of this patient revealed that his testicular volume was 10.9 mL left and 11 mL right, with a homogeneous echotexture and wide hypoechogenicity. No solid or cystic lesions were observed. The clinical and hormone data of this patient were summarized in Table 1.

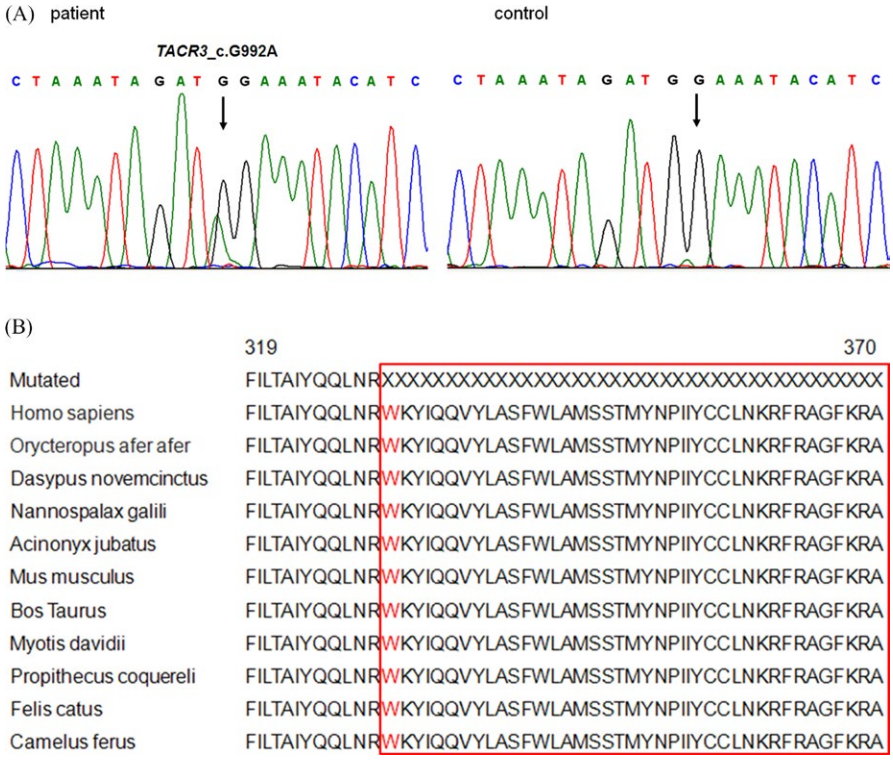

FIGURE 1 A, The *TACR3* C.G992A mutation in patient compared to the control was confirmed by Sanger sequencing. The position is indicated by an arrow. B, Multiple-sequence alignment in *TACR3* from different species reveals that codon 331, where the mutation(P. W167X) occurred, is highly conserved (highlighted in red box)

TABLE 1 Clinical and hormone profile of patient with nonobstructive azoospermia with novel *TACR3* stopgain mutation

Sample ID	Age, years	FSH, mIU/ml[a]	LH, mIU/ml[b]	Estradiol, pg/ml[c]	PRL, uIU/ml[d]	Testosterone, nmol/l[e]	Inhibin B, pg/ml[f]
2017A114	42	21.9	7.3	23.6	565	9.4	16.13

FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin.
Normal ranges are.
[a]1.5-12.4 mIU/mL.
[b]1.7-8.6 mIU/mL.
[c]25.8-60.7 pg/mL.
[d]86.0-324.0 uIU/mL.
[e]9.90-27.80 pg/mL.
[f]≥80 pg/mL.

4 | DISCUSSION

Congenital hypogonadotropic hypogonadism (CHH), which result in azoospermia at the pre-testicular (NOA) level,[15] has classically been categorized as a monogenic disorder in the past few years.[21] Lots of genes have been reported to be related with CHH, such as *GNRHR*, *KISS1*, *KISS1R*, *FSHB*, *DAX1*, *HESX-1*, *LHX3*, *PROP-1*, *SOX*, *POLR3A*, *POLR3B*, *PNPLA6*.[16] Missense loss-of-function mutations in *TAC3* and *TACR3* have also been reported in cases with nonsyndromic normosmic congenital hypogonadotropic hypogonadism (nCHH) from different families and population.[14] Although mutations in CHH gene identified so far account for <10% of cases,[21] *TACR3* was considered to be one of the first two genes to be screened in a clinical setting for equivocal cases such as constitutional delay in puberty versus CHH.[16]

Hypogonadotropic hypogonadism (HH) is characterized by low sex steroid and low gonadotropin levels resulting from a defect in the normal pulsatile secretion pattern of GnRH from the hypothalamus.[22] *TACR3* gene encodes the receptor for the tachykinin neurokinin 3(NKR or known as NK3R), which is a member of the rhodopsin family of G-protein-coupled receptors.[11] In rodents the receptor is expressed in GnRH neurons, which suggests it has a possible role in the regulation of GnRH secretion.[11,18] Topaloglu *et al* first confirmed the key role of loss-of-function mutations in *TACR3* (encoding NK3R) and *TAC3* (encoding NKB) in the pathogenesis of GnRH deficiency.[18] Other investigators have also reported additional loss-of-function mutations in the genes in the cases with GnRH deficiency subsequently, which confirmed the two genes' critical role in normal reproductive development and function.[13,14,17,23,24] These facts highlight the importance of TAC3 and *TACR3* in reproduction. However, the HH disease phenotype does not occur in the patient with the novel *TACR3* stopgain mutation c.G992A (p.W331X) in this study. We hypothesized that the less severe affected phenotype was due to the fact that the stopgain mutation was heterozygous. The patients with

heterozygous nonsense mutations in *TACR3* might be have less severe phenotypes than those bearing homozygous mutations. This hypothesis was also suggested by Gianetti *et al.*[23]However, it was also reported that both dominant and recessive forms of CHH transmission were present in patients with *TACR3* mutations and patients with heterozygous mutations were clinically similar to those with homozygous mutations.[12] But most researches suggested that *TACR3* was autosomal recessive inheritance, offsprings with heterozygous mutations were not affected.[13,17,18,21,25]

The pathogenicity of the variant may be due to the dominant negative effect. Noel et al reported the mechanisms of another heterozygous mutation in *TACR3* disrupting NK3R function in GnRH-deficient patients. This work found that R295S NK3R heterozygous mutation could interfere with dissociation of G proteins and IP signaling from wild-type NK3R, indicative of dominant negative effects.[24] This locus is R295S, which is close to W331X.

Our report has some limitations. We were not able to perform any functional study in order to demonstrate the pathogenicity of the variant. We will demonstrate the pathogenicity of the variant by proving the dominant negative effect of the *TACR3* mutation p.W331X according to the method described in Reference [[24]] in the future study.

Although we have not yet made a definite inference about the consequences of the stopgain mutation c.G992A (p.W331X) in *TACR3*, what is clear is that the mutation results in the loss of residues after 331st amino acids and the truncation of C terminal peptide. Therefore, the novel stopgain mutation was likely to affect the function of NK3R protein. In a word, our study revealed a novel stopgain mutation c.G992A (p.W331X) in *TACR3* which expanded the mutation spectrum of *TACR3* in Chinese NOA infertile men and advanced our understanding of the genetic susceptibility to NOA.

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AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: Ruizhi Liu, Dongfeng Geng. Performed the experiments: Xiao Yang, Ruixue Wang, Shu Deng. Analyzed the data: Leilei Li, Xiaonan Hu, Yuting Jiang. Wrote the paper: Dongfeng Geng, Xiao Yang.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of the First Hospital of Jilin University. All the participants were Han Chinese. Written informed consent was obtained after the nature of the procedures had been fully explained prior to the study.

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