Identification of KISS1R gene mutations in disorders of non-obstructive azoospermia in the northeast population of China

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Abstract

Background: Non-obstructive azoospermia (NOA), a serious phenotype of male spermatogenesis failure, is a multifactorial disease which is regulated by genetic, epigenetic, and environmental factors. Some gene structural variants have been demonstrated to be related to NOA. Loss-of-function mutations of KISS1R cause normosmic idiopathic hypogonadotropic hypogonadism (IHH) which result in azoospermia at the pre-testicular level. The objective of this research was to investigate genetic variants of KISS1R in NOA patients.

Methods: The entire coding region of 52 spermatogenesis-associated genes (KISS1R included) was sequenced from 200 NOA patients. Mutation screening was performed to identify genetic variations of these genes by targeted exome sequencing. Sequencing data analysis was carried out by a series of bioinformatics tools. Candidate variants confirmation was performed by Sanger sequencing. Functional analysis of candidate variants was evaluated using SIFT and PolyPhen-2.

Results: Three heterozygous missense variants in KISS1R were identified in three patients, respectively. No deleterious variations in other candidate genes were found in the three patients. Two of these three variants, p.A211T and p.G186E, had been reported in the ExAC and dbSNP database, respectively, while the other variant p.A301D was novel. These variants were all predicted to be likely pathogenic by in silico analysis.

Conclusion: Our study revealed three heterozygous missense variants in KISS1R which expanded the mutation spectrum of KISS1R in infertile men with NOA in the northeast of China.

Keywords

KISS1R, male infertility, mutation, NOA, targeted exome sequencing

1 | INTRODUCTION

Azoospermia, which affects up to 1% of men in the general population,1 is a type of male infertility with a lack of spermatozoa in the ejaculate.2 Types of azoospermia include obstructive azoospermia (OA) and non-obstructive azoospermia (NOA).2 Non-obstructive azoospermia, a serious phenotype of male spermatogenesis failure, is a multifactorial disease which is regulated by genetic, epigenetic, and environmental factors. The genetic causes of NOA including
Y-chromosome microdeletions, karyotype abnormality and autosomal single-gene or polygenic mutations or polymorphisms in multiple biological pathways are involved in the development of NOA.\(^3\) The prevalence of structural and numerical chromosomal abnormalities in the NOA population can up to 20%.\(^4\) Many other genetic causes of NOA remain unknown, although several genes have been reported based on an association between genetic variants of genes and NOA etiology— including PRM1, HSF2, KLHL10, SPATA16, AURKC, and ATM.\(^5\)

The original purpose of our study was to investigate the contribution of genetic variations in some known causative genes associated with spermatogenesis to the development of NOA. Then in this study, we discovered and clarified genetic mutations of KISS1R gene in a population of infertile men with NOA.

The human KISS1R gene, located at 19p13.3, was initially identified as an orphan G protein-coupled receptor (GPCR), and kisspeptin, a product of the KISS1 gene, was its endogenous ligand.\(^6\) Loss-of-function mutations of KISS1R cause normosmic idiopathic hypogonadotropic hypogonadism (IHH) which result in azoospermia at the pre-testicular level.\(^7\) Kisspeptin binds to KISS1R stimulating GnRH release by hypothalamic neurons, leading to secretion of sexual steroids and pituitary gonadotropins, which in turn will play roles in producing the gametes.\(^8\) In the immature animals, precocious activation of the gonadotropic axis and pubertal development was able to induce by intermittent kisspeptin administration.\(^9\) In addition, animal studies suggested the targeted deletion of KISS1R led to hypogonadotropic hypogonadism, abnormal sexual maturation, and infertility.\(^6,9\) Nevertheless, researches on kisspeptin and infertility are scarce.\(^9\) The understanding of genetic variations in KISS1R with NOA may lead to the use of KISS1R as a biomarker for diagnosis and treatments of male infertility.

2 | MATERIALS AND METHODS

Our study consisted of 200 Chinese patients with idiopathic NOA. Mean age was 30.25 ± 5.24 years. All patients were diagnosed with NOA based on the following examination, including a detailed medical history, physical examination, hormone evaluation, semen analysis, Y-chromosome microdeletions screening, and chromosome analysis. The exclusion criteria were as follows: (a) diagnosis with other clinical features; (b) chromosomal abnormalities that can be detected by cytogenetic analysis; (c) special diseases or history of chemical exposure that may affect spermatogenesis; and (d) Y-chromosome microdeletions.

Mutation screening of genes associated with spermatogenesis was carried out by targeted exome sequencing as described previously.\(^10\) Genomic DNA was isolated from peripheral blood samples of all patients and subjected to exome capture using the in-house targeted genes panel (Peking Medriv Academy of Genetics and Reproduction, Peking). Capture procedure was performed in solution that enriched the exonic sequences of 52 spermatogenesis-associated genes (Table 1, KISS1R gene included) which obtained by reviewing the literature. Next-generation sequencing was performed on the Illumina MiSeq sequencing platform (Illumina, Inc). Fastq sequence files were aligned against the human reference genome (NCBI build 37/ hg19) with the Burrow-Wheeler Aligner software (BWA version 0.7.12). Duplicated reads from the data sets were removed with Picard tools. Local realignment, recalibration, and variant calling were conducted with the Genome Analysis Tool Kit (https://software.broadinstitute.org/gatk/). Variants with minor allele frequencies >1% in the 1000 Genomes Project or in the dbSNP databases were excluded. Synonymous variants and variants in genes with unknown clinical phenotypes were filtered out. The remaining variants were evaluated for correlation with patient’s phenotype. As our research objective, pathogenicity of the candidate variants was evaluated using SIFT (https://sift.bii.a-star.edu.sg/) and PolyPhen-2 (https://genetics.bwh.harvard.edu/pph2/).

The candidate pathogenic variants were confirmed by conventional PCR and Sanger sequencing (ABI3730XL, BGI Genomics, Beijing Genomics Institute-Shenzhen). PCR amplification was performed using the following primers (5′-3′): exon4: TTTGCAGGGTGCTGGGTG (F) and GGGTGCCTGGAAGTGGTTAG (R); and exon5: GCCT TTCGCTAACCACCTTCA (F) and CACTGCTCCCTGGCTTCTGC (R).

3 | RESULTS

Targeted exome sequencing was carried out in 200 patients with NOA, and spermatogenesis-associated gene mutations were evaluated. To examine whether KISS1R genetic defects were associated with NOA, we focused on genetic variants in the exonic region of the KISS1R occurred in 200 patients with NOA. As a result, a total of 3 of 200 (1.50%) patients were found to have KISS1R variations, and we identified three KISS1R heterozygous missense variants in three cases, respectively (Table 2). No deleterious variations in other 51 candidate genes were found in the three patients. The p.A211T and p.G186E missense mutations had been reported in the ExAC and dbSNP database, respectively. The other variant p.A301D had never previously been reported, which was not found in the public databases including 1000 Genome Project, dbSNP, and ExAC database. According to SIFT software, the three variants were all predicted to be deleterious to the protein’s function. The p.A211T and p.A301D variants were both possibly damaging, and the p.G186E variant was probably damaging according to PolyPhen-2 (Table 3). We performed PCR and Sanger sequencing on the three patients and confirmed the three heterozygous missense mutations in these patients, respectively (Figure 1 and Figure 2). The relevant clinical and hormone data of these patients were summarized in Table 4. Color Doppler ultrasonography for scrotal of these three patients with missense variants revealed that Pat2 and Pat3 showed normal testicular volume, while Pat1 carrying p.A211T showed small testes in the scrotal sac. Hormone levels were normal or slightly lower than normal in the two patients with KISS1R mutations (Pat2 and Pat3). The hormonal level of the other one patient carrying p.A211T (Pat1) was obviously abnormal which exhibited high FSH level and a low T level.
In the present study, we have described three heterozygous missense mutations in the KISS1R gene in three patients with NOA. To our knowledge, this is the first reported heterozygous missense mutations in the KISS1R gene in the NOA population.

Idiopathic hypogonadotropic hypogonadism (IHH), which result in azoospermia at the pre-testicular level, is characterized by a defect in the onset or maintenance of puberty caused by hypothalamic-pituitary-gonadal axis dysfunction with the absence of an organic lesion, and its clinical manifestation depends on the onset time. Males can be clinically manifested as absent or incomplete puberty, small penis, cryptorchidism, and infertility. In the past few years, IHH has classically been categorized as a single-gene disease, but the phenotypic presentation of this disease and its genetic background are highly heterogeneous. A few genes that are involved in the pathogenesis of IHH have been identified at various sites, including TAC3, TACR3, GnRHR, FGFR1, GNRH1, FGF8, KISS1, and KISS1R. However, these genetic defects account for less than 30% of patients with IHH.

**TABLE 1** Fifty-two spermatogenesis-associated genes included in the study

| AR     | AURKC | CATSPER1 | CCDC39 | CFTR  | CHD7  | DNAAF1 | DNAAF2 |
|--------|-------|----------|--------|-------|-------|--------|--------|
| DNAF3  | DNAH1 | DNAH11   | DNAH5  | DNA1  | DNA12 | DPY19L2| DYX1C1 |
| ETV5   | FGF8  | FGF1     | GNRHR  | HEATR2| HSF2  | HYDIN  | KAL1   |
| KISS1R | LEP   | LEPR     | NANOS1 | NELF  | NR5A1 | PLCZ1  | PROK2  |
| PROKR2 | ROX1F | ROX2F    | RSPH1  | RSPH4A| RSPH9 | SEPT12 | SLC26A8|
| SOHLH2 | SPATA16| SUN5     | SYCE1  | SYCP3 | TAC3  | TACR3  | TEX11  |
| USP26  | WDR11 | ZMYDN15  | ZMYND10|       |       |        |        |

**TABLE 2** KISS1R variants identified in 200 patients diagnosed with NOA

| No. | Exon | Position | cDNA mutation | Protein mutation | dbSNP135 | Zygosity | Reported | Patients ID |
|-----|------|----------|---------------|------------------|----------|----------|----------|-------------|
| 1   | 4    | 919999   | c.G631A       | p.A211T          | rs76694658| Hetero   | Yes      | Pat1        |
| 2   | 5    | 920453   | c.C902A       | p.A301D          | /        | Hetero   | No       | Pat2        |
| 3   | 4    | 919925   | c.G557A       | p.G186E          | rs1281550153| Hetero   | Yes      | Pat3        |

Abbreviation: Hetero, heterozygous.

**TABLE 3** Functional analysis of missense variants by bioinformatics software

| No. | cDNA mutation | Protein mutation | Variant type | SIFT Score | Prediction | PolyPhen-2 Score | Prediction |
|-----|---------------|------------------|--------------|------------|------------|-----------------|------------|
| 1   | c.G631A       | p.A211T          | Missense     | 0.02       | Deleterious | 0.688           | Possibly damaging |
| 2   | c.C902A       | p.A301D          | Missense     | 0.02       | Deleterious | 0.599           | Possibly damaging |
| 3   | c.G557A       | p.G186E          | Missense     | 0.00       | Deleterious | 0.910           | Probably damaging |

Note: SIFT: cut-off score ≤0.05 for deleterious variants; PolyPhen-2: score <0.15 for benign, 0.15-0.85 for possibly damaging, and >0.85 for probably damaging.

**FIGURE 1** PCR amplification for the candidate pathogenic variants in the three patients. Lane 1: DNA marker is 5000, 3000, 2000, 1000, 750, 500, 250, and 100 bp from top to bottom. Lane 9 (A): Amplified products of Pat1. Lanes 6 and 7 (B): Amplified products of Pat2 and Pat3. Other lanes: Amplified products of other samples unrelated to this study.

4 | DISCUSSION

In the present study, we have described three heterozygous missense mutations in the KISS1R gene in three patients with NOA. To our knowledge, this is the first reported heterozygous missense mutations in the KISS1R gene in the NOA population.

Idiopathic hypogonadotropic hypogonadism (IHH), which result in azoospermia at the pre-testicular level, is characterized by a defect in the onset or maintenance of puberty caused by hypothalamic-pituitary-gonadal axis dysfunction with the absence of an organic lesion, and its clinical manifestation depends on the onset time. Males can be clinically manifested as absent or incomplete puberty, small penis, cryptorchidism, and infertility. In the past few years, IHH has classically been categorized as a single-gene disease, but the phenotypic presentation of this disease and its genetic background are highly heterogeneous. A few genes that are involved in the pathogenesis of IHH have been identified at various sites, including TAC3, TACR3, GnRHR, FGFR1, GNRH1, FGF8, KISS1, and KISS1R. However, these genetic defects account for less than 30% of patients with IHH.
In previous studies, KISS1R was one of the major genes that implicated in IHH in the autosomal-recessive form.\textsuperscript{16} Mutations of this gene have been identified in less than 5% of patients with normosmic IHH.\textsuperscript{12} Up to now, at least 20 different mutations have been described in the literature, most of which were loss-of-function mutations and were found to have variable clinical manifestation.\textsuperscript{12,17} KISS1R mutations which have been reported to be associated with IHH include point mutations, insertion, deletion, missense mutation, acceptor splice site mutation, and compound heterozygous mutation.\textsuperscript{17} According to the previous reports, there are no striking phenotype-genotype relationships for KISS1R mutations. The inadequacies of KISS1R can manifest itself in different clinical entities. Complete loss-of-function mutation does not necessarily cause complete gonadotropic deficiency, and variable phenotypes could be present in patients carrying the same mutation.\textsuperscript{18}

Current studies generally agree that IHH due to KISS1R mutations is transmitted as a recessive trait, although heterozygous mutations have been reported in the literature. Cerrato et al reported a heterozygous KISS1R mutation (c.A1079T/p.H360L) in a male IHH patient who had no pubertal development.\textsuperscript{19} Teles et al reported a heterozygous KISS1R variant (p.E252Q) in a male patient with sporadic normosmic IHH, who exhibited micropenis and cryptorchidism at birth, and no pubertal development.\textsuperscript{20} These studies indicated that heterozygous mutations in KISS1R could be also involved in the pathogenesis of IHH.

In the present study, we reported three heterozygous missense mutations in the KISS1R gene in three patients with NOA, in whom the typical IHH phenotype was not found but only infertility. The pathogenicity of these variants may be attributed to dominant-negative effects. Chevrier et al have reported a heterozygous insertion in the intracellular domain of KISS1R that may lead to hypogonadotropic hypogonadism. The mutation disturbed the normal expression of the receptor at cell surface. The KISS1R bearing the heterozygous insertion exerts a dominant-negative effect on the synthesis of the wild-type KISS1R.\textsuperscript{18}

There are some limitations in the report. Firstly, mutations of KISS1R, including heterozygous missense mutations, were reported in idiopathic hypogonadotropic hypogonadism, which results in NOA at pre-testicular level. However, no related phenotype was found in these three patients with KISS1R heterozygous missense mutations in this report. So what is the possible mechanism that KISS1R

\begin{figure}
\centering
\caption{The three KISS1R missense variants in these three patients with NOA were confirmed by Sanger sequencing. The base mutation position is indicated by a black arrow. A, Heterozygous KISS1R c.G631A mutation was detected in pat1; B, Heterozygous KISS1R c.C902A mutation was detected in pat2; C, Heterozygous KISS1R c.G557A mutation was detected in pat3.}
\end{figure}

\begin{table}
\centering
\caption{Clinical and hormone profiles of patients with NOA with KISS1R missense variants}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Patients ID & Age (y) & FSH (mIU/mL) & LH (mIU/mL) & T (nmol/L) & $E_2$ (pg/mL) & Testicular volume (Left/ Right, mL) \\
\hline
Pat1 & 42 & 21.9↑ & 7.3 & 9.4↓ & 23.6↓ & 11/11 \\
Pat2 & 45 & 7.21 & 5.08 & 13.59 & 17.5↓ & 15/15 \\
Pat3 & 21 & 2.95 & 3.09 & 17.21 & 37.4 & 15/15 \\
\hline
\end{tabular}
\end{table}

Abbreviations: ↑: elevated; ↓: decreased; E$_2$: estradiol (27.96-155.92 pg/mL); FSH: Follicle-stimulating hormone (1.5-12.4 mIU/mL); LH: luteinising hormone (1.7-8.6 mIU/mL); T: testosterone (9.9-27.8 nmol/L).
mutations lead to spermatogenesis failure? In the report, we only make theoretical conjectures briefly. Secondly, functional study was not performed to demonstrate the pathogenicity of the three KISS1R variants. It should be demonstrated by proving the dominant-negative effects of KISS1R mutations according to the method described in reference18 in our further study.

Although a definite inference about the consequences of the heterozygous KISS1R mutations has not yet made, we have presented three cases of NOA in three patients from northeast China due to three heterozygous missense mutations in the KISS1R gene. For better understanding of the correlations between KISS1R mutations and the relevant genetic background of patients with NOA, KISS1R gene detection should be recommended as a part of genetic screening of NOA patients. It is worth studying the related mechanism of spermatogenesis failure caused by KISS1R heterozygous mutation. We hope that our report will contribute to the ongoing genetic characterization of NOA.

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AUTHORS CONTRIBUTIONS

Ruixue Wang and Ruizhi Liu conceived and designed the experiments. Jia Fei, Yuting Jiang, and Guirong Zhang performed the experiments. Dongfeng Geng, Hongguo Zhang, Xiangyin Liu, and Yuting Jiang analyzed the data. Dongfeng Geng and Ruixue Wang wrote the paper.

ETHICAL APPROVAL

This study was approved by the Medical Ethics Committee of the First Hospital of Jilin University (No. 2017-404).

CONSENT TO PARTICIPATE

Performed after obtaining written informed consent from the participants.

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