Mosaic Ring-like Small Supernumerary Marker Chromosome and Gene Mutation in a Male With Intermittent Azoospermia: A Rare Case Report

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Abstract

This study aimed to report a rare case of intermittent azoospermia and ring-like small supernumerary marker chromosomes (sSMCs). An infertile man was diagnosed with azoospermia presenting a normal male phenotype with complete masculinization. Karyotyping and polymerase chain reaction (PCR) were used to detect 16 sequence-tagged sites on the AZF subregions of the Y chromosome, and 115 candidate genes were screened for mutations. Mutations included single nucleotide variations, insertions, and deletions. Metaphase chromosomes were studied by standard trypsin-Giemsa banding; fluorescent in situ hybridization and PCR were performed to analyze specific Y chromosome regions; gene mutations were detected. Chromosomal analysis detected 117 metaphase cells; a mosaicism with marker 1 and marker 2 sSMCs in 2 metaphase cells (47, X, +mar1x2 karyotype), a mosaicism with marker 2 sSMCs in 14 metaphase cells (46, X, +mar2 karyotype), and a mosaicism with marker 1 sSMCs in 76 metaphase cells (46, X, +mar1 karyotype), coexisting with a 45,X cell line in the remaining 25 metaphase cells. PCR analysis showed the sY160 heterochromosome on the AZFc subregion was absent. Next-generation sequencing identified an asthenozoospermia-specific mutation in GAPDHS (rs2293681), and Sanger sequencing verified this mutation. This gene encodes a protein belonging to the glyceraldehyde-3-phosphate dehydrogenase family of enzymes that play an important role in carbohydrate metabolism. Like its somatic cell counterpart, this sperm-specific enzyme functions in a nicotinamide adenine dinucleotide-dependent manner to remove hydrogen and add phosphate to glyceraldehyde 3-phosphate to form 1,3-diphosphoglycerate. During spermiogenesis, this enzyme may play an important role in regulating the switch between different energy-producing pathways, and it is required for sperm motility and male fertility. A mosaic 46, X, +mar1[76]/45, X[25]/46, X, +mar2[14]/47, X, +mar1x2[2] karyotype could be the main explanation for the azoospermia/severe oligospermia, while the likely pathogenic GAPDHS intron mutation may contribute to the symptom of immotile sperms detected in the semen analysis.

Keywords

azoospermia, infertile male, AZF regions, ring Y chromosome, GAPDHS

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Infertility has a 15% incidence in married couples of child-bearing age, and male infertility contributes to approximately 50% of cases (Bhasin et al., 1997). Male infertility is a multifactor syndrome that represents one of the clear examples of a complex phenotype with a substantial genetic basis. The abnormal spermatogenesis caused by genetic factors is one of the most important infertility elements. In genetic factors, karyotypic abnormalities are detected in 5% of patients with fertility problems (Ferlin et al., 2007). Abnormal spermatogenesis causes severe oligozoospermia, azoospermia, or nonequilibrium changes in the sperm genome, which affects the normal fertilization process, and the prevalence increases to 13% when only considering men with azoospermia (Carrell et al., 2006).

Small supernumerary marker chromosomes (sSMCs) are relatively common in the field of clinical cytogenetics, and their prevalence is estimated to be 0.05% of unselected live-born infants in addition to Klinefelter syndrome (47, XXY), which is the most commonly detected karyotypic abnormality in infertile men (Manvelyan et al., 2008). According to the EAU Guidelines on Male Infertility (2017 version), idiopathic infertility contributed to approximately 30% of male infertility within unselected patients and approximately 13.3% male infertility within azoospermia patients. Infertile men who are labeled with idiopathic sperm abnormalities usually have azoospermia, asthenozoospermia, oligospermia, or teratospermia due to unknown causes.

Low semen quality is diagnosed when the number of sperm cells produced, their morphology, or their motility is below the World Health Organization (WHO) cutoffs for normal spermatogenesis. Reduced motility, that is, asthenozoospermia, oligospermia, or teratozoospermia (OAT), which required examination of genetic factors. Sixteen sequence-tagged sites (STSs) and the sY160 heterochromosome on the AZFa subregions on the Y chromosome were detected. Sixteen STSs on the AZFa, AZFb, and AZFc subregions were present, while the sY160 heterochromosome on the AZFc subregion was absent. In the current case with intermittent azoospermia, in addition to the mosaic 46, X, +mar1[76]/45, X[25]/46, X, +mar2[14]/47, X, +mar1x2[2] karyotype due to two ring-like Y sSMCs, a GAPDH gene mutation was also reported associated with another symptom of immotile sperms.

**Methods and Materials**

**Patient**

A 36-year-old patient with a 5-year infertility history after marriage was referred to Peking Union Medical College Hospital for infertility due to azoospermia. The patient was not focused on his infertility at the first 2 years. After being diagnosed with azoospermia at a local hospital by limited diagnostic methods, he sought Traditional Chinese Medicine at first, but the results of the treatment were dissatisfaction. He came to our hospital at last 1 year ago for the cause and further treatment of his infertility. Physical examination revealed a healthy-looking male with a normal male appearance. The patient had normal sexual function and could complete sexual intercourse normally. His wife had normal, regular menstruation and no obvious dysmenorrhea, and ultrasonography examination indicated bilateral tubal patency. Semen analyses were performed five times in our hospital with 3 months interval. In the first and fourth time, 1 or 2 immotile spermatozoa were found in every 200 high-power field under centrifugation. No sperm could be found without centrifugation. The patient was treated with levocarnitine, but there was no significant improvement in total sperm count or total motility.

**Andrology Examination**

A semen examination was performed. The semen volume was of 3 ml with pH 7.2 and a 30-min liquefication period. Examination of the external genitalia revealed both testes to be normally positioned in the scrotum, with normal consistency when palpated, and with a volume of 16 ml each. There were no abnormalities of the epididymis and the vas deferens, and degree I varicocele was detected in the testicle on the right side. A blood test for reproductive hormones and a genetic test were conducted. The serum follicle stimulating hormone level was 9.57 mIU/ml, the serum testosterone level was 7.68 ng/ml, the serum luteinizing hormone level was 10.78 mIU/ml, and the serum prolactin level was 8.68 ng/ml. The inhibitor B level was tested to determine the reproductive potential, and the patient’s serum inhibin- B was 128.65 pg/ml for the reference range of 94–327 pg/ml, which indicated a normal reproductive potential.

**Y Chromosome Microdeletion**

The preliminary diagnosis of this patient was severe oligo-astheno-teratozoospermia (OAT), which required examination of genetic factors. Sixteen sequence-tagged sites (STSs) and the sY160 heterochromosome on the AZF subregions on the Y chromosome were detected. Sixteen STSs on the AZFa, AZFb, and AZFc subregions were present, while the sY160 heterochromosome on the AZFc subregion was absent.

Terminate deletion of the sY160 heterochromosome is usually present in individuals in the chromosomal mosaic karyotype (46, XY/45, X; Kleiman et al., 2011). The 45 X cell line was indicated to be a negative contributor to testicular spermatogenesis (Jaruzelska et al., 2001).
Karyotype Analysis

Next, karyotyping was performed. Metaphase spreads from lymphocytes were prepared and trypsin-Giemsa banded according to standard techniques. Chromosomal analysis detected 117 metaphase cells; a mosaicism with marker 1 and marker 2 sSMCs in 2 metaphase cells (47, X, +mar1x2 karyotype), a mosaicism with marker 2 sSMCs in 14 metaphase cells (46, X, +mar2 karyotype), and a mosaicism with marker 1 sSMCs in 76 metaphase cells (46, X, +mar1 karyotype), coexisting with a 45, X cell line in the remaining 25 metaphase cells. The patient’s karyotype revealed 46, X +mar1 [76] / 45, X [25] / 46, X, +mar2[14]/47, X, +mar1x2[2] (Figures 1–3). As karyotype analysis cannot rule out diseases caused by microchromosomal abnormalities or gene mutations, peripheral blood samples of the patient were collected and sent to the Ardent Biomedical Detection Center for gene mutation detection.

Gene Mutation Detection and Analysis

Gene mutation detection was designed and completed by the Ardent Biomedical Detection Center (Guangzhou, China, and San Francisco, CA, USA). DNA was extracted from peripheral blood leucocytes according to standard procedures with the Ardent DNA Extraction Kit (Ardent, Guangzhou, China, and San Francisco, CA, USA). A targeted next-generation sequencing panel consisting of 115 genes related to male infertility disorders was developed (Table S1). Genes relevant to azoospermia, oligospermia, asthenozoospermia, teratospermia, Congenital bilateral absence of the vas deferens (CBAVD), idiopathic hypogonadotrophic hypogonadism, globozoospermia, cryptorchidism, androgen insensitivity, and primary ciliary dyskinesia were included based on relationships described in Online Mendelian Inheritance in Man (OMIM), Human Phenotype Ontology (HPO), Gene Reviews, and primary literature, in addition to the available genomic sequence information from the U.S. National Center for Biotechnology Information (NCBI). The panel included 1,500 reported mutation hotspots located in coding exons, splice sites, promoter regions, 5′ untranslated regions (UTRs), and 3′ UTRs among these genes. Clinically relevant noncoding (intronic) regions that contained previously described pathogenic variants, as reported in ClinVar, the NIH database, were included. All laboratory procedures were performed in a clinical gene amplification laboratory. DNA samples were

Figure 1. 46, X, +mar1 karyotype with karyotyping (trypsin-Giemsa-banding) in 76 metaphase cells; a ring-like small supernumerary marker chromosome labeled marker 1 was detected.

Figure 2. 46, X, +mar2 karyotype with karyotyping (trypsin-Giemsa-banding) in 14 metaphase cells; a ring-like small supernumerary marker chromosome labeled marker 2 was detected.

Figure 3. Karyotyping ((trypsin-Giemsa-banding) revealed two ring-like small supernumerary marker chromosomes in two metaphase cells of estimated Y chromosome origin of 47,X, +mar1x2 karyotype.
prepared for sequencing using NGS DNA Library Prepset (Guangdong Ardent Biomed, Guangzhou, China, and San Francisco, CA, USA) and sequenced on a personal genome machine (Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. All bioinformatics algorithms were implemented within the Life Technologies platform (Carlsbad, CA). After confirmation, each variant was classified as pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, or benign, following the American College of Medical Genetics (ACMG) guidelines (Richards et al., 2015). The likely pathogenic variant identified by NGS was confirmed by an orthogonal method (Sanger sequencing; Figure 4).

**Results**

In this case, a heterozygous variation in the GAPDHS gene coded c.660-22G>A (rs2293681) was detected. The frequency of the mutation in the ExAC population database was 1.98%; the clinical significance of the mutation was not included in ClinVar, and the Human Gene Mutation Database (HGMD) classified the mutation as “disease-causing mutation?”. The GAPDHS gene (MIM 609169), located at 19q13.12, encodes a sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDS), which is a glycolytic enzyme that is closely related to the fibrous sheath of sperm flagella. Dysplasia fibrous sheath (DFS) is a sperm defect observed in patients with severe asthenozoospermia, characterized by flagella with abnormally distorted fibrous sheath morphology. There was a statistically significant difference in GAPDS activity between DFS sperm and normal sperm.

According to the primary literature, in a study of sperm cells from five DFS patients and five healthy males, this heterozygous mutation on chromosome 35544923 (rs2293681), which changes guanine to adenine, was detected in all DFS patients but was not detected among healthy males. This mutation is located in the intron region between the sixth and seventh exons of GAPDHS. It is estimated that this variation may intervene in the regulation of GAPDS expression. The ultrastructure of a sperm flagellum with dysplasia of the fibrous sheath showed the absence of a central pair of microtubules, resulting in abnormal fibrous sheath morphology (Elkina et al., 2017).

Another study of 30 patients with severe azoospermia and 90 normal controls reported that 10 patients had c.660-22G>A (rs2293681) variation, 2 of which were homozygous mutations and others were heterozygous (Visser et al., 2011).
According to the ACMG guidelines, as this variation conforms to the PS4 evidence (the prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls) and the PM1 evidence (located in a mutational hot spot and/or critical and well-established functional domain such as active site of an enzyme), the Ardent Biomedical Detection Center reported this GAPDHS variation as a “likely pathogenic mutation” (Li et al., 2017).

Discussion

Assisted reproductive technology provides a possibility for patients with only one sperm to have their own child. However, the health and genetic problems of their children have raised concern. In the current case, although spermatozoa was found in one of the semen analyses, the existing severe genetic abnormalities cannot be ignored.

The karyotype of the patient is relatively rare, 46, X, +mar1[76]/45, X[25]/46, X, +mar2[14]/47, X, +mar1x2[2]. The karyotype confirmed that the sY160 heterochromosome deletion often exists in the mosaic 46, XX/45, X individuals. As 16 STSs on the Y chromosome AZF subregion were present in the Y chromosome microdeletion PCR examination while the Y chromosome was not present in any metaphase cell in the karyotyping, only ring-like marker 1 small supernumerary marker chromosome was detected in 76 metaphase cells, only ring-like marker 2 small supernumerary marker chromosome was detected in 14 metaphase cells, and both ring-like marker 1 and 2 small supernumerary marker chromosomes were detected in 2 metaphase cells. It is possible that the ring-like marker 1 and ring-like marker 2 originated from the Y chromosome, and the material of these two ring-like sSMCs contained almost the entire part of the Y chromosome except the sY160 heterochromosome. The 47,X,+mar1X2 cell line of the patient in this case, which is primarily estimated to contain almost the entire Y chromosome, has a very low ratio in the germ cells of the patients. Despite the PCR examination, which can detect the presence of all 16 STSs, the majority of the germ cells of the patients had a status of Y chromosome microdeletion, and it can be the reason for the symptom of azoospermia. Following experiments such as high depth genome sequencing of entire Y chromosome ought to be designed to verify this assumption of this patient.

In the detection and analyses of gene mutations, a GAPDHS intron variation was detected and reported to be a likely pathogenic variation. The GAPDHS gene encodes a glycolysis enzyme that is significant in sperm motility, and the patient’s heterozygous c.660-22G>A mutation was only detected in the asthenozoospermia group in previous studies. This gene mutation may classify the symptoms of immotile sperm in the semen analysis as this mutation intervenes in the regulation of GAPDS expression, resulting in the reduction in sperm motility. The following protein function verification experiments such as GAPDHS activity in peripheral blood and semen via enzyme linked immune sorbent assay (ELISA) required to verify of how this mutation influences sperm motility.

According to the EAU Guidelines on Male Infertility (2018 version) on the recommendation treatment for idiopathic male infertility, human menopausal gonadotropin (HMG) and human chorionic gonadotropin (HCG) combined therapy might be beneficial for pregnancy rates and live birth in idiopathic male factor subfertility and this is the only potential treatment recommended in the guidelines. Intramuscular injection HMG and HCG combination therapy (HCG: 3,000 UI + HMG: 150 UI) was used twice a week, in addition to Cardura XL tablet 4 mg QD (a type of alpha-blocker), which has been reported useful in patients with oligozoospermia by increasing sperm density and total motile sperm count (Yamamoto et al., 1986, 1995).

The patient came to the hospital for examination on January 9, March 24, May 28, July 3, and October 23 of 2019. During the first and fourth visits, 1 or 2 immotile spermatozoa were found in every 200 high-power field under centrifugation. No sperm could be found without centrifugation. Other semen examination indicated that there were still no mature sperm in the semen, while large amounts of germ cells, including spermatogonia and spermatocytes, were detected. The patient gave up and did not accept any further treatment because of the ineffectiveness of treatment with medicines. Assisted reproductive technology (ART) was not employed because of the extremely low quantity of sperm.

One possible reason for the spermatogenic failure in the patient is that a ring-like Y chromosome can interfere with X-Y bivalent formation and chromosomal separation during mitosis and lead to a breakdown in spermatogenesis (Odorisio et al., 1998), especially the Y chromosome of the patient estimated to be divided into two ring-like marker chromosomes. Chromosome conformation capture (3C technology) might be helpful for further research on how the ring-like Y chromosome influences the RNA expression associated with spermatogenesis and sperm motility.

There were several limitations of the current case report. First, the treatment was ineffective, and the patient gave up finally. Second, there were no further studies to demonstrate the potential mechanism of the gene mutation, ring-like chromosome, and intermittent azoospermia. Third, if enough motile sperm can be obtained by micro-cryopreservation, ART (mainly ICSI) can be suggested and performed. Before ART, there should be several essential steps including communication with the patient and his
wife about the risk and possible outcomes, the approval of the ethical committee, and monitoring the early embryos, pregnancy, and the development of the child.

In conclusion, a man with intermittent azoospermia with a rare karyotype of 46, X, +mar[1][76]/45, X [25]/46, X, +mar2[14]/47, X, +mar1x2[2] without AZF microdeletions was reported, in whom the sY160 heterochromosome was absent. His azoospermia might be caused by abnormal structure of the ring-like Y chromosome or even abnormal X-Y bivalent formation. The 45, X cell line and mosaicism of the marker chromosome would also be considered to be the reasons. The likely pathogenic variation in the GADPHS gene may be the reason for the immotile sperm in the semen analysis. Further RNA expression and the protein function experiment would be advised and should be performed for verification.

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Compliance With Medical Ethics
Written informed consent was obtained from the patient for receiving hormone therapy and publishing this case report in medical journals (supplemental material).

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Supplemental Material
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