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Exome Sequencing Analysis of Semen from Patient with Hyperspermia

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Abstract

Hyperspermia affects male reproductive health seriously. To date, the diagnosis of male infertility is usually based on standard semen analysis, but it is very limited in predicting sperm fertility. Moreover, it is well established that genetic causes account for 10%-15% of infertility cases, including chromosomal abnormalities and single-gene mutations that influence at different levels of many physiological processes involved in male reproduction. However, in many cases, the cause of infertility is not identified and little knowledge about the basic mechanism regulating spermatogenesis and sperm function.

In our previous work, we collected the semen samples from 44 patients with hyperspermia and 30 normal fertilizers to do exome sequencing analysis. Through exome sequencing, 87 genes with insertion or deletion mutations were found, of which 13 genes were involved in spermatogenesis and sperm function. Our results suggest that SPATA3, MICALCL, ALMS1, PPP2R2B, TBP, HTT, CELSR2, ADAMTS2, TCP11, ZAN, ODF1, REC8 and VCX3B are involved in the progression of hyperspermia and may be potential diagnostic targets for hyperspermia susceptibility.

Keywords: Hyperspermia; Exome sequencing analysis; Male infertility

Introduction

Infertility accounted for about 20% of childbearing couples, of which male factors lead to infertility accounted for 50% according to the World Health Organization statistics [1]. With the improvement of social industrialization, the accelerated pace of life, environmental pollution, bad habits and other factors, the quality of male semen decrease by degrees, azoospermia, oligospermia, teratozoospermia and other male infertility patients increased year by year. The issue of male reproductive health is concerned by the whole society increasingly [2].

The diagnosis of male infertility is mainly based on standard semen analysis currently, such as semen parameters analysis including sperm concentration, motility, morphology and vitality. But in many case, conventional semen analysis is very limited for predicting sperm fertility. It has been reported that chromosomal changes and Y chromosome microdeletions also can cause male infertility [2]. So, a better understanding of biomolecular genetic problems and mechanisms of injury to sperm can optimize the diagnosis and treatment of male infertility.

It is not sufficient to distinguish between fertile and infertile subjects by routine semen parameter analysis only. For example, normal sperm count is not synonymous with fertility. Assuming that there are abnormal sperm function or molecular defects, we must consider other sperm characteristics, such as DNA integrity, chromatin packaging defects, apoptosis, oxidative stress, DNA fragmentation and aneuploidy. Sperm DNA integrity is considered to be an important parameter increasingly in male infertility diagnosis.

High-throughput techniques, including genomics, epistemology, transcriptomics, proteomics and metabolomics can explore the molecular mechanisms and identify the pathogenesis of male infertility, can provide a theoretical basis of better diagnosis and treatment [3]. Sperm proteomics analysis found more than 6,000 non-redundant proteins [4], of which 898 were expressed in the sperm head only, which may be involved in DNA packaging, DNA replication, DNA repair, RNA metabolism and nuclear transport [5]; 984 were expressed in the sperm tail only, which may be involved in the production of energy and metabolite precursors, such as OXPHOS pathway, TCA cycle and so on [6]; 532 were expressed both in the head and the tail, which may be involved in sperm capacitation, acrosomal reaction, sperm-egg recognition, chromatin remodeling, the formation of sperm head and tail during spermatogenesis and ubiquitin-proteasome pathway [7].

Semen is mainly composed of sperm and seminal plasma. Seminal plasma is mainly composed of seminal vesicle fluid, prostatic fluid, and a small amount of liquid mixture secreted by testis, epididymis, vas deferens, urethral glands and urethral ball gland. Seminal plasma is the necessary medium for sperm activity, and can provide energy and nutrients to sperm, and can neutralize the vaginal acidic secretions, and is conducive to sperm motility and sperm-egg combination. The normal semen volume of human is 2-5 ml, and the volume of patient with hyperspermia is more than 7 ml. Semen reduction (seminal plasma deficiency) is not conducive to sperm through the vagina into the uterus and fallopian tubes, affecting fertilization. If the amount of semen more than 7 ml, the sperm is diluted, not conducive to fertility, because the sperm accounted for 5% of semen only, excessive semen is excessive secretion or exudation of seminal plasma actually, and the total number of sperm is not change, which will cause semen sperm density decreased naturally, thereby reducing the chance of conception. At the same time, excessive semen is easy to flow out of

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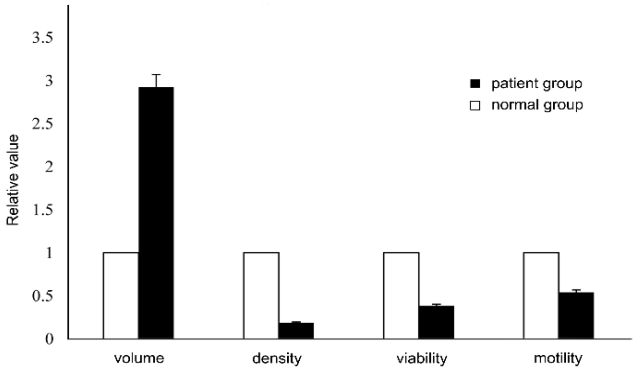

Figure 1: The semen volume, sperm density, viability and motility in patient with hyperspermia and normal fertile. The volume of semen in the patient group was higher than that in the normal group significantly, but the sperm density, viability and motility were lower in the patient group than that in the normal group significantly.

the reproductive tract leading to infertility.

We had collected 44 semen samples from patients with hyperspermia and 30 semen samples of fertile volunteers. Semen volume measurement analysis showed that the volume of semen in the patient group was higher than that in the normal group significantly, but the sperm density, viability and motility were lower in the patient group than in the normal group significantly. We analyzed the levels of citric acid, zinc, fructose and free L-carnitine in the semen of 44 patients with hyperspermia and 30 fertile volunteers, and found that the levels of these biochemical markers in the semen of the patient group is higher than those in the normal group significantly. Hyperspermia is different from other infertility, the seminal plasma biochemical indicators were higher than the normal group significantly, which not be used as an indicator of diagnosis. Thus, genomics analysis of this disease may explore the pathogenesis, which has not yet set foot in at home and abroad.

Materials and Methods

Ethical approval

This study was approved by the ethics committee of Shenzhen Second People's Hospital. The approval reference number is 20160039. The study was approved on April 11th, 2016, initiated on May 1st, 2016 and terminated on December 1st, 2018. Patients (average age 33.8) were recruited for this study from 2016 in Shenzhen Second People's Hospital. Fertile men (average age 35.2) from the Center of Physical Examination, Shenzhen Second People's Hospital were recruited as control: they had fathered at least one child without assisted reproductive techniques such as *In Vitro* Fertilization (IVF), Intracytoplasmic Sperm Injection (ICSI) and Intracytoplasmic Morphologically-Selected Sperm Injection (IMSI). Informed written consent was obtained from each subject. We have access to information that can identify individual participants during or after data collection.

Measurement of semen volume, density, viability and motility

The collected semen is poured into a graduated tube or cup to measure its volume. Inverted the semen 2 to 3 times to mix, sucked

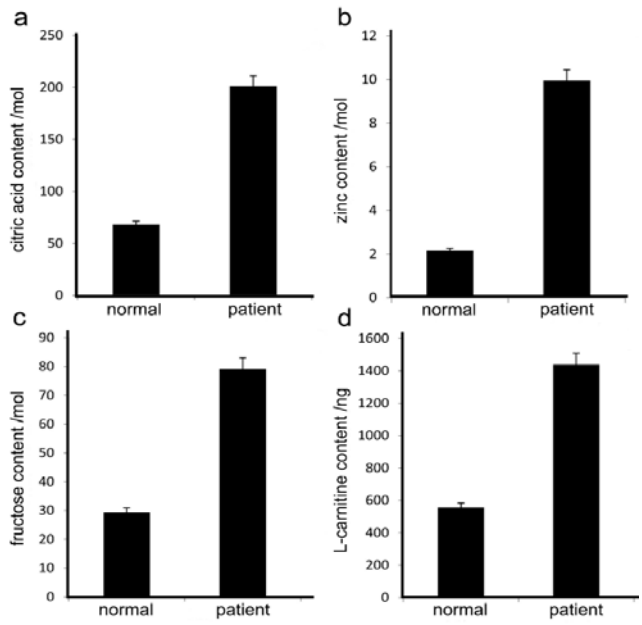

Figure 2: Analysis of biochemical indexes of citric acid, zinc, fructose and L-carnitine in semen of patients with hyperspermia. The contents of citric acid (a), zinc (b), fructose (c) and free L-carnitine (d) in the semen of 44 patients with hyperspermia were higher than those in the normal group significantly.

0.05 ml semen to standard erythrocyte dilution tube, dropped a small bubble in the tube and injected dilutions into a standard red blood cell dilution tube to 1.01mm mark. Placed in a cover glass on red blood cell count plate, the diluted sperm dripped into cover glass below. The counting plate should be allowed to stand for a few minutes before starting sperm counting, and sperm counts are calculated at room temperature. The number of cells in the red blood cell count plate is based on the concentration of semen and the distribution of spermatozoa on the slide. Calculate the total number of small squares 80 cells (1/20 × 1/20mm), the number of sperm obtained multiplied by 10,000 ml of sperm number. If the use of diluted semen rather than the original semen, the number of sperm counted must be multiplied by the dilution factor in order to obtain the correct number of sperm.

Dropped a small drop of semen into the center of a sterile cover glass. Applied a small amount of water around the pit, and then aligned the dimples with the semen on the coverslip and covered it with the coverslip to attach them together. Turned the slide upside down so that the sperm drop hangs under the cover slip and can be observed under a microscope. Observed the movement of sperm under the microscope, and distinguished between straight forward movement, rotary motion and swaying sperm motility situation.

Analysis of biochemical indexes of citric acid, zinc, fructose and L-carnitine in semen

We used kit for determination of the citric acid level in seminal plasma (Cat#BRED-005, BRED Life Science, China), seminal plasma fructose quantitative assay kit (Cat# BRED-010, BRED Life Science, China) and kit for determination of the zinc level in seminal plasma (BRED-004, BRED Life Science, China) to analyze the citric acid, fructose and zinc content in the seminal plasma. And we used

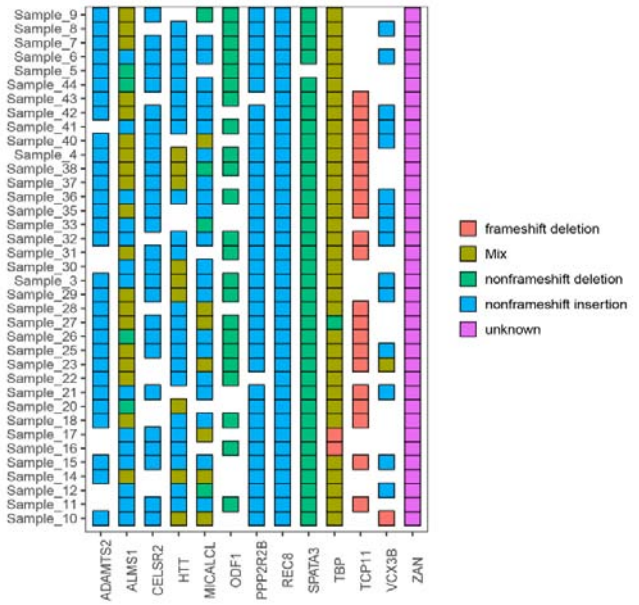

Figure 3: Mutation analysis of 13 mutant genes in each sample. Mutations of these genes include frameshift deletion, nonframeshift deletion and nonframeshift insertion.

L-Carnitine assay kit (Cat#MAK063, Sigma, America) to analyze the content in the seminal plasma.

DNA extraction, purification and quantification

For semen, DNA was extracted using DNeasy Blood & Tissue Kit (Cat#69506, QIAGEN, Germany), following the manufacturer's instructions. We used Qubit®2.0 Fluorometer (Cat#Q32866, Invitrogen, America) and 1% agarose gel electrophoresis to check the quantity and quality of purified DNA.

Library preparations

For each sample to be sequenced, individual library preparation, hybridizations, and captures were performed following the protocol of Sure Select XT Target Enrichment System for Illumina Paired-End Sequencing Library (Agilent Technologies, Inc. 5301 Stevens Creek Rd Santa Clara, CA 95051 USA).

Assess quality

Assess quantity of library with Qubit®2.0 Fluorometer. Use 2100 Bioanalyzer High Sensitivity DNA Assay to assess the quality and size range as instructed in the reagent kit guide.

Cluster generation and sequencing

TruSeq PE Cluster Kit (Illumina) was used for cluster generation in an Illumina cBOT instrument following the manufacturer's protocol (cBotTM User Guide). Libraries were loaded into each lane of flow cell. Sequencing was performed on an Illumina HiSeq2500 instrument (Illumina) by the manufacturer's protocol (HiSeq®2500 System User Guide). Multiplexed paired-reads run were carried out with 125 cycles.

Reverse-Transcription PCR (RT-PCR) analyses

Total RNA was extracted from human semen using TRIzol (Invitrogen, Carlsbad, CA), following the manufacturer's directions.

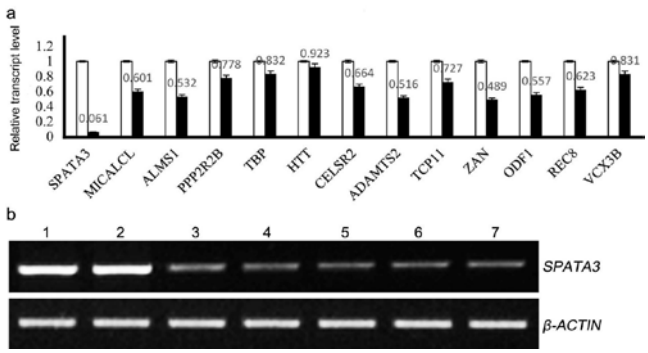

Figure 4: SPATA3 and other 12 genes were down-regulated expression in patients with hyperspermia. (a) SPATA3 and other 12 genes were down-regulated in patients with hyperspermia of which the expression of SPATA3 was the lowest by fluorescence quantitative PCR analysis. (b) SPATA3 was down-regulated in patients with hyperspermia through RT-PCR analysis. 1-2, normal fertile; 3-7, patients with hyperspermia.

cDNA synthesis was carried out with the Prime ScriptTM RT Master kit (Takara, Dalian, China). RT-PCR was performed using Takara Emerald Amp PCR Master Mix (Takara, Dalian, China), with primers specific for human SPATA3, MICALCL, ALMS1, PPP2R2B, TBP, HTT, CELSR2, ADAMTS2, TCP11, ZAN, ODF1, REC8 and VCX3B, and β-ACTIN was employed as an endogenous control. The program for all PCR assays was as follows: 98˚C for 10 s, 60˚C for 30 s, and 70˚C for 30 s, with a final extension at 72˚C for 5 min. The gene-specific primers were as described in supplementary data (Supplementary data Table 1).

Results

The semen volume of 44 patients with hyperspermia was higher than that of normal fertilizers significantly. We had collected 44 semen samples from patients with hyperspermia and 30 semen samples of fertile volunteers. Semen volume measurement analysis showed that the mean volume of semen in the patient group was 9.18 ml and the normal group was 3.14 ml, the volume of semen in the patient group was higher than that in the normal group significantly, but the sperm density, viability and motility were lower in the patient group than that in the normal group significantly (Figure 1).

Analysis of biochemical indexes of citric acid, zinc, fructose and L-carnitine in semen of patients with hyperspermia

Some biochemical markers in seminal plasma can reflect the function of accessory sexual gland. Such as citric acid and zinc can reflect the prostate function; fructose can reflect the seminal vesicle function; free L-carnitine can reflect the epididymis function. The level of total excretion of these specific markers can be used to assess the secretion function and potential lesion of accessory sexual gland. We analyzed the contents of citric acid, zinc, fructose and free L-carnitine in the semen of 44 patients with hyperspermia and 30 fertile volunteers, and found that the contents of these biochemical markers in the semen of the patient group is higher than those in the normal group significantly (Figure 2). Hyperspermia is different from other infertility, the seminal plasma biochemical indicators were higher than the normal group significantly, which not be used as an indicator of diagnosis. Thus, genomics analysis of this disease may explore the pathogenesis, which has not yet set foot in at home

Table 1: High frequency mutant genes in patients with hyperspermia.

Gene	Reference sequence	Sequencing result	Mutation	Location	frequency
SPATA3	TCAGCAGCCTAGCC CTGAATCCACACCA	T	deletion	exon	97.3%
MICALCL	GCTCCTC	GCTCCTCCTCCTCCTCCTC; GCTCCTCCTCCTCCTC; GCTC; GCTCCTCCTC; GCTCCTCCTCCTC; G	insertion/ deletion	exon	75.7%
ALMS1	TGGAGGA	T; TGGAGGAGGA; TGGAGGAGGAGGA; TGGA	insertion/ deletion	exon	100%
PPP2R2B	A	AGCTGCTGCT; AGCTGCTGCTGCTGCTGCT; AGCTGCTGCTGCT; AGCTGCTGCTGCTGC TGCTGCT; AGCTGCTGCTGCTGCT GCTGCTGCTGCTGCT	insertion	exon	94.6%
TBP	AGCAGCAGCAGC	ACAGCAGCAG; ACAGCAGCAGCAG; A	deletion	exon	100%
HTT	A	ACCGCCGCCG	insertion	exon	91.9%
CELSR2	A	ACGC	insertion	exon	72.9%
ADAMTS2	G	GGCA	insertion	exon	81.1%
TCP11	TC	T	deletion	exon	56.8%
ZAN	T	TG	insertion	exon	97.3%
ODF1	CTGCAACCCCTGCA GCCCCTGCAACCCG	C	deletion	exon	62.2%
REC8	G	GGAA	insertion	exon	100%
VX3B	C	CGAGATGGAAGAACCACTGAGTCAGGAGAGCGAGATGGAAGAACCACTGA GTCAGGAGAGT	insertion	exon	62.2%

and abroad.

Whole-exon sequencing analysis of 44 semen of patients with hyperspermia

High-throughput techniques, including genomics, epistemology, transcriptomics, proteomics, and metabolomics, can explore the molecular mechanisms of male infertility and identify the pathogenesis of male infertility, can provide a theoretical basis of better diagnosis and treatment. In order to clarify the molecular mechanism of the development and progression of hyperspermia, we performed whole-exon sequencing analysis of 44 semen of patients with hyperspermia. There are 37 samples that meet the sequencing requirement. Bioinformatics analysis of these sequencing results revealed 87 genes with high frequency mutations, including frameshift mutations, non-synonymous SNP, terminator mutations, non-synonymous insertion/deletion mutations and so on. These genes were analyzed by KEGG and GO, found that involving in important processes such as spermatogenesis, cell cycle regulation, chromatin remodeling, mitosis, meiosis, transcriptional regulation and so on.

Mutant genes involved in spermatogenesis or sperm development

Because the volume of semen in the patient group was higher than that in the normal group, the sperm density, viability and motility were lower in the patient group than that in the normal group significantly, therefore, we focused on the genes related to spermatogenesis and sperm development in 87 mutant genes from whole-exon sequencing analysis. Bioinformatics analysis found 13 high frequency mutant genes related to spermatogenesis or sperm development, including SPATA3, MICALCL, ALMS1, PPP2R2B, TBP, HTT, CELSR2, ADAMTS2, TCP11, ZAN, ODF1, REC8 and VCX3B. Mutations of these genes include shift-missing mutation, non-shift missing mutation, non-shift insert mutation, non-synonymous SNP, stop codon SNP, unknown SNP and unknown insert mutation. We studied these genes minutely and summarized the insertion/deletion of nucleotides in Table 1.

Mutation analysis of 13 mutant genes in each sample

A total of 93,000 mutations occurred in 37 samples through the whole-exon sequencing analysis, an average of 31 mutations occurring per Mb nucleotide, indicating that the frequency of DNA mutation was high in patient with hyperspermia. We screened 13 genes with high frequency insertion/deletion mutations by bioinformatics analysis and summarized in Figure 3.

SPATA3 and other 12 genes were down-regulated expression in patients with hyperspermia

We screened SPATA3 and other 12 genes by fluorescence quantitative PCR and found that these genes were down-regulated in patients with hyperspermia of which the expression of SPATA3 was the lowest (Figure 4A). In addition, we used RT-PCR to verify the expression of SPATA3 in 5 patients and found that the gene down-regulated indeed (Figure 4B).

Discussion

Hyperspermia affects male reproductive health seriously due to lower sperm density and excessive seminal plasma. The diagnosis of hyperspermia mainly depends on the measurement of semen volume and the biochemical index of seminal plasma. Hyperspermia has family history according to clinical data, so we want to explore the gene regulated hyperspermia development and pathogenesis through whole exome sequencing analysis.

We found an average of 31 mutations occurring per Mb nucleotide,

indicating that the frequency of DNA mutation was high in patient with hyperspermia and screened 13 genes with high frequency mutations including SPATA3, MICALCL, ALMS1, PPP2R2B, TBP, HTT, CELSR2, ADAMTS2, TCP11, ZAN, ODF1, REC8 and VCX3B. These genes were down-regulated *via* qRT-PCR analysis which may be the potential diagnostic targets for hyperspermia susceptibility.

Our results suggest that SPATA3, MICALCL, ALMS1, PPP2R2B, TBP, HTT, CELSR2, ADAMTS2, TCP11, ZAN, ODF1, REC8 and VCX3B are involved in the progression of hyperspermia and may be potential diagnostic targets for hyperspermia susceptibility.

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