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TO EVALUATE NOVEL TAILORED NEXT-GENERATION SEQUENCING UNITFOR HEREDITARY ANALYSIS OF MALE INFERTILITY

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ABSTRACT

An NGS sample for the gene mutation of male infertility must be developed and evaluated. Sequencing of genes from the AR family and others from the FSHB, FSHR, KLHL10, NR5A1, NANOS1, SEPT12, SYCP3, and TEX11 families was done on 241 males who had idiopathic infertility. Minor allele frequencies were found to be less than 1% by Sanger sequencing. Among the 23 males with abnormal sperm counts, 19 missense variations were detected; however, no such variants were observed in the normal sperm counted males. Variations classified as pathogenic and of unclear significance (VUS) received the most attention (63.1% of all 19 observations). It was not possible to identify any missense variations in guys with normal seminal parameters (0/67). Patients with spermatogenic dysfunction had a greater prevalence of alterations (16/174 vs 0/67, p = 0.007). Prior studies have shown that NGS panels are more effective than traditional tests at uncovering new genomic variations associated with male infertility. The results of this investigation could be connected to quantitative spermatogenic impairment based on biological significance, general incidence, and clinical data collected from patients. However, more investigation is required.

Keywords:male infertility epilepsies, NGS, Gene sample, DNA sequencing

1. Introduction

Male infertility is a common yet difficult illness that tends to run in families. Male factor, alone or combined with female factor, is considered to be responsible for roughly half of the cases of Western women becoming infertile after a year of unprotected intercourses. A wide range of factors and conditions can affect male fertility, including poor lifestyle choices, thyroid

problems, testis trauma, congenital anomalies, varicocele, gastrointestinal diseases, autoimmune induces and surgical intervention, and metabolic disorders, but genetic diseases account for the vast majority of cases[1]. When it comes to male fertility, several genes are involved in spermatogenesis, testis development, and endocrine parameter, and their hereditary impact is significant. Genomic research has benefited greatly from microarray and next-generation sequencing (NGS) technology in recent years [2]. Even today's most often utilised genetic tests, such as karyotype, microdeletions, and mutations in CFTR, AR hypogonadotropic hypogonadism (HH), and other CFTR-related genes in chosen patients, is still quite limited in scope[3].

Ten to fifteen percent of males with the most severe forms of infertility have genetic defects, and the incidence is inversely proportional to the concentration of sperm. Infertile men are underdiagnosed in considerable numbers (between 30 and 60 percent). Idiopathic infertility is a common term, however there is a strong belief that hereditary factors are at play.

To screen gene variations, Sanger sequencing has long been preferred over karyotyping and other approaches that require the use of the Y-chromosome microdeletions. Keeping up-to-date knowledge on the ever-expanding number of male infertility genes is both time-consuming and expensive, but it's necessary[4].

Large cohort studies have been aided by new high-throughput technologies that allow for the simultaneous analysis of numerous DNA molecules. It is possible to find single nucleotide and copy number variations (CNVs) that may be linked to male infertility symptoms by using NGS,

Additionally, not all of the genome's sections have been sequenced equally, posing the risk of missing important genetic information. Instead, targeted sequencing allows for greater sequencing depth in only the sections that are of particular relevance. As a diagnostic tool, it is obvious that NGS can be used for the selection of genes linked with the phenotype (male infertility). There have been numerous calls in recent years for the creation of a gene panel to study the genetics of infertility [5].

As an alternative to traditional tests for the genetic screening of male infertility, this work sought to evaluate and create a novel target NGS panel. For this purpose, we built a bespoke geneticsampleacquire genes that have recently been linked to male infertility or that could be deemed instructive in preliminary research [6].

Routine genetic screening revealed no abnormalities in NGS panels of 241 infertile idiopathic men with sperm counts below 10 million (karyotype, microdeletions, and CFTR gene mutation analyses) [7].

2. Research Methodology

In May and June of 2018, the ethical committee approved the consent of 1100 patients' dependents. This meant that the university hospital could get their consent. It was necessary to rule out any form of cancer, orchitis (which included clinical varicocele), testicular torsion or trauma, obstructive azoospermia, mutations in the CFTR gene, use of gonadotoxic drugs, karyotic abnormalities (such as long-arm Y-chromosome microdeletions), and spermatogenic qualitative defects (such as hypospermia) to determine the cause.

Consequently, the studyinvolved a total of 241 people**.** According to their self-reports, all of the participants were Caucasian and of Italian ancestry. All patients had a thorough medical history and physical examination

2.1. Details of Respondents (Patients)

The results of the sperm analysis were in accordance with the recommendations of the (WHO, 2010) given in below table

S.no	Group	Total Sperm Count
1.	azoospermia	no sperm in the ejaculate after centrifugation
2.	oligozoospermia	total sperm count > 0 < 39 million/ejaculate
3.	normozoospermia	total sperm count ≥ 39 million/ejaculate

A commercial electrochemiluminescence immunoassay was used to measure the serum levels of FSH, LH, and testosterone. Men with less than 1 million sperm in their ejaculate were diagnosed with spermatogenic abnormalities using FNAC..

Genes that we typically analyse in our laboratory, as well as genes that have been significantly linked to male infertility, were included in our target gene panel to study the genetic aetiology of our cases' spermatogenic quantitative deficiencies.

Gene	Cytogenetic location	Broad category	Type of infertility	Reported inheritance	Clinical validity
AR	Xq12	Testicular (hormone resistance)	Reproductive system syndrome/isolated	XL	Definitive
FSHB	11p14.1	Pre-testicular	Endocrine disorder/ reproductive system syndrome	AR	Moderate
FSHR	2p16.3	Pre-testicular/testicular (hormone resistance)	Endocrine disorder/ reproductive system syndrome	AR	Moderate
KLHL10	17q21.2	Testicular	Isolated	AD	Moderate/definitive
NANOS1	10q26.11	Testicular	Isolated	AD	Unavailable
NR5A1	9q33.3	Pre-testicular/testicular	Endocrine disorder/ reproductive system syndrome	AD	Definitive/strong
SEPT12	16p13.3	Testicular	Isolated	AD	Unavailable
SYCP3	12q23.2	Testicular	Isolated	AD	Moderate
TEX11	Xp11	Testicular	Isolated	XL	Strong

Table 1 infertility, the heritagemodel, and hospitalauthority of each gene in the group

2.2 DNA extraction and evaluation by sequencing

DNA of individuals was extracted using the manufacturer's protocol for the QIAamp DNA Blood Mini Kit "(Qiagen Inc., Hilden, Germany)". Using a NanoDrop-1000 and a Qubit 2.0 fluorometer "(Thermo Fisher Scientific Inc., Waltham, MA, USA)", we assessed the DNA's purity "(Thermo Fisher Scientific Inc., Waltham, MA, USA)". Fluorescence measurements were made using the Qubit fluorometer and dsDNA Assay Kits, both of which have been approved by the manufacturer for use with the Qubit fluorometer. Genes that we typically analyse in our laboratory, as well as genes that have been significantly linked to male infertility, were included in our target gene panel to study the genetic aetiology of our cases' spermatogenic quantitative deficiencies. To summarise the panel's genes, look no further than Table 1.

Sequencing specimens were transcribed in accordance with the manufacturers ' instructions, using a low-input TSCA kit (Illumina, San Diego, CA, USA). Measurements were taken with the Agilent Tapestation System 4200 and the D1000 ScreenTape (Agilent, Santa Clara, CA, USA). Libraries (4 nM) were mixed, denatured, and diluted to 10 pM in accordance with the manufacturer's recommendations. It was processed using an Illumina MiSeq sequencer at 500 cycles (2x250paired-ends) using an Illumina MiSeq reagent cartridge (Illumina, San Diego, CA, USA). Each run had to have a minimum depth of coverage of at least 100% to discover all possible variants. BWA-MEM and GATK were used to align the sequenced reads to the GRCh37/hg19 reference genome. With the help of Sanger sequencing, an allelic frequency of less than 1% was discovered. Supplementary Table 2 includes a list of the primers that were utilised.

Phenotype	Age (year)	LH (IU/L)	FSH (IU/L)	T (nmol/L)	Mean testicular volume (mL)
Non-obstructive azoospermia (NOA) (*N* 67)	39.9 ± 8.6	10.7 ± 6.6*#	23.7 ± 14.3*#	14.4 ± 7.8	8.8 ± 2.6*#
Oligozoospermia (> 0 < 39 × 10^6 sperm/ejaculate) (*N* 107)	35.8 ± 8.2	7.3 ± 3.3*	12.8 ± 8.5*	14.0 ± 4.7	12.1 ± 3.4*
Normal sperm count (≥ 39 × 10^6 sperm/ejaculate) (*N* 67)	39.3 ± 11.5	4.8 ± 1.9	5.2 ± 2.6	15.7 ± 5.9	16.2 ± 3.8

Table 2 experimentalaspects of the 241Respondents (Patients)

Data are often given as a mean and standard deviation in statistical analyses (SD). The three hormones involved in ovulation are referred to as "LH," "FSH," "T," and "Testosterone." More than 39 106 sperm are considered unacceptable, as are LH, FSH, T, and T2 concentrations of less than 1–8 nmol/L, as well as testis volume of under 12 mL. When subjects with aberrant seminal parameters were compared to those with a normal sperm count, statistical significance was established.

3. Clinical significance filtration and interpretation (Data Analysis)

For analysis, Illumina's VariantStudio and the Integrated Genomics Viewer were employed (IGV). AR triplet repeats were counted using HipSTR, a bioinformatics approach. For the purpose of this study, the Genome Aggregation Database (gnomAD) allele frequencies were compared to the European non-Finnish population using Dataset v2.1.1 controls. In order to discover the position and frequency of these variations, we only used very uncommon non-synonymous mutations in our research (Minor Allele Frequency (MAF http://grch37.ensembl.org/index.html). Researchers employed PolyPhen2 and SIFT from the Broad Institute of MIT and Harvard (http://sift.jcvi.org) for missense prediction. The American College of Medical Genetics and Genomics (ACMG) established a classification system for variants..

3.1 Statistical analysis

SPSS 21.0 for Windows was used to perform statistical analysis on the data (SPSS, Chicago, IL). The results are expressed in terms of means and standard deviations (SD). To find out if there were any differences between the groups, we utilised a Student's t test. This method was employed when the expected number of participants was less than fivefisher's exact test.

4. Results

Seminal statistics for each patient are provided in Table 2, which includes age, reproductive hormones LH and FSH, and testosterone (T), as well as the mean testicular volume (MTV). A total of 93.5 percent of the targeted places were covered with an average reading depth of 351, thanks to at least ten individual readsonly two samples had to be re-sequenced due to insufficient coverage and were successfully sequenced a second time, making the first attempt to sequence nearly all samples a success.

The assay's analytical sensitivity and specificity were both greater than 99 percent as a result of the panel correctly sequencing positive controls with known missense mutations in AR and NR5A1 genes. A single nucleotide mutation was detected in 5396 target locations, with 2 821 of them occurring in noncoding regions. Coding regions included over 1300 distinct nucleotide

variants, with 1341 being synonymous and 1234 being nonsynonymous. Neither a false positive nor a false negative were discovered. In this analysis, only non-synonymous variants with a MAF of less than 1% were included.

Nineteen uncommon missense variations were discovered by our team. In the AR gene, Pro392Ser, Ala101Gln, Gly263Ser, Pro129Leu, Ala351Val, and the NR5A1 gene, Ala101Gln, Ala101Gln, Gly263Ser, Pro129Leu, and Ala351Val have all been previously described, whereas the remaining fourteen variants have not yet been studied. Variants found were categorised according to ACMG criteria in order to determine the clinical importance of the detected variants For a total of 26.3% of the variants, we discovered five that were classed as probably harmless (5/19), two that were classified as benign (10.5% of the variants, 2), eleven that were classified as VUS (11/19), and one variant that was classified as pathogenic (5.3 percent of the variants, 1/19).

Sixty-one percent (12/19) of patients (16/241) had at least one of the detrimental or VUS missense mutations, thus these were the first to be targeted

Patients with NOA had a missense variant rate of 11.9 percent (8/67) while those with oligozoospermia had a missense variant rate of 7.4 percent. Males with normal seminal parameters (0/67) had no missense variations observed. According to these findings, the number of variations found in patients with spermatogenic impairment was significantly larger than the number found in healthy individuals

5. Conclusion

A new NGS profile of nine transcripts was created and tested in this study. Seven of the genes studied had a strong or moderate connection to male infertility, however the causal association between male infertility and two other genes has not yet been confirmed (NANOS1 and SEPT12). Our panel included these genes despite this, because of the potential value they hold. Spermatogenic failure has been connected with SEPT12 and NANOS1 polymorphisms in some men with abnormally low amounts of male reproductive cells. The NANOS1 gene has been linked to infertility in SEPTEMBER 12 / SEPTEMBER 12+/+ male mice, with a high positive

missense Z score in ExAC browser. New genetic tests for male infertility were administered to all participants previously tested for common hereditary reasons of male infertility.

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