ORIGINAL ARTICLE: GENETICS

Copy number variation associated with meiotic arrest in idiopathic male infertility

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Objective: To assess the association between copy number variations (CNVs) and meiotic arrest and azoospermic men.
Design: Genetic association study.
Setting: University.
Patient(s): Australian men: 19 with histologically confirmed meiotic arrest, 110 men with azoospermia in the absence of histologic data, and 97 fertile men (controls).
Intervention(s): None.
Main Outcome Measure(s): The identification of CNV by microarray and/or multiplex ligation-dependent probe amplification (MLPA), and the localization of unique CNV encoded proteins to the human testis.
Result(s): Microarray identified two CNVs unique to meiosis arrest patients. One containing the *MYRIP* gene and a second containing *LRRC4C* and the long noncoding RNA *LOC100507205*. All three genes are transcribed in the human testis, and *MYRIP* and *LRRC4C* localize to meiotic cells. The reverse genetic screen for CNVs in meiosis genes identified in mouse models further identified CNVs including *HSPA2* as being associated with azoospermia.
Conclusion(s): These data raise the possibility that, while relatively rare, CNVs may contribute to human male infertility and that CNV screening should be incorporated into long-term plans for genome profiling as a diagnostic tool. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Azoospermia, copy number variation, genetics, human

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Male infertility is a common disorder that affects approximately 1 in 20 men of reproductive age (1). It is most commonly characterized by disorders of sperm function and/or numbers, and for the majority of cases a precise diagnosis cannot be ascribed (2, 3). Although there is no doubt that environmental factors play a critical role in defining human fertility, increasingly there is recognition that, like many complex traits, male infertility is often genetic in origin (2, 4). For many of these men, at least a few superficially functional sperm may be found within the ejaculate or after testicular biopsy, thereby allowing for the possibility of men fathering their own genetic offspring. For about 12% of these men however, complete germ cell arrest occurs at the spermatocyte period of germ cell development, so no sperm can be observed either within the ejaculate or testis biopsies (5). The chances of such men fathering their own children are extremely remote.

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Despite this, relatively little is known about the genetic origin of such developmental arrests in humans. As a male-infertility-causing mutation would by definition have a very limited penetration into the general population, it is not surprising that the unequivocal identification of monogenic causes of male infertility (outside of sex chromosome abnormalities) is rare.

Recent years have seen the emergence of copy number variation (CNV) as an important source of genetic and phenotypic diversity (6). Copy number variations are submicroscopic chromosomal duplications or deletions of at least 1 kb in length and are prevalent within the human genome (7). Although most CNVs are benign, alterations to the genomic architecture can have severe phenotypic implications through disruption of gene function and alteration to gene expression levels (8–10). A number of human conditions including epilepsy, autism, schizophrenia, and disorders of sex development have been associated with CNVs (11–15). More recently, evidence has emerged that CNVs may be an important determinant of male infertility (16, 17).

We have chosen to use both a forward genetic approach (single nucleotide polymorphism [SNP] microarrays) and a reverse genetic (candidate gene) approach to ascertain whether CNVs are associated with human male meiosis arrest. Meiosis arrest affects approximately 1 in 10 men presenting with azoospermia (zero sperm count) (5). Genetic factors including Yq microdeletions (18) and polymorphisms in the *SYCP3* (19), *MEI1* (20), and *ETV5* (21) genes have been associated with meiosis arrest in some patients, but for the vast majority the etiology is unknown.

MATERIALS AND METHODS

All human samples were obtained with the approval of the human research and ethics committees of Southern Health, Monash Day Surgery, Monash Medical Centre, and the Monash University. Informed consent was given by all participants involved in the study.

Patient Selection

For the forward genetic analysis, genomic DNA (gDNA) from 19 men with nonobstructive azoospermia (NOA) and histologically confirmed complete meiosis arrest (5) were obtained from the Monash Male Infertility Repository (22). For the reverse genetic analysis, gDNA samples from 110 azoospermic men with idiopathic primary spermatogenic failure were selected (Supplemental Table 1, available online). Patients with known causes of male infertility were excluded, including those with obstructive azoospermia, chromosomal abnormalities (numerical and structural), Y chromosome microdeletions, cryptorchidism, varicocele, and previous cancer treatment. For the control group, gDNA was obtained from 97 fertile men who had fathered children and had normal semen parameters (i.e., with average sperm concentration of $105 \pm 45 \times 10^6$/mL and >50% motility). Hormone, semen, and physical parameters are outlined in Supplemental Tables 1 and 2 (available online).

We extracted the gDNA from peripheral leucocytes using a standard phenol/chloroform technique (22). For multiplex ligation-dependent probe amplification (MLPA), the phenol/chloroform extracted gDNA was repurified using a standard ethanol/sodium acetate precipitation protocol to remove residual phenol.

Affymetrix Array

Hybridization of gDNA to the Affymetrix GeneChip Human Mapping 500K EA array was undertaken by the Australian Genome Research Facility (AGRF), according to the manufacturer's specifications, and the analysis was performed using AROMA (www.aroma-project.org/) (23). A minimum number of eight consecutive SNPs and log2-thresholds of at least +0.2 and −0.2 was used to define CNVs.

MLPA Analysis

The MLPA analysis was performed with synthetic oligonucleotides as previously described elsewhere (24). The MLPA reagents were purchased from Fisher Biotec (Australia). The oligonucleotides were purchased from Sigma-Aldrich (Australia), with the right-hand oligonucleotide of each MLPA probe phosphorylated at the 5′ end. Probe sequences are listed in Supplemental Table 3 (available online). We repeated the MLPA analysis at least twice per sample, and where polymerase chain reaction (PCR) product lengths were compatible they were combined into a single reaction.

Data analyses were performed as described elsewhere (25). Normalized ratios of at least 0.75 and 1.25 were used as thresholds to determine heterozygous deletions and duplications, respectively. For a CNV to be considered verified, it must have sat outside the defined normalized ratios and be confirmed by MLPA.

Immunohistochemistry

A testis biopsy was obtained from a healthy adult male with normal spermatogenesis after a vasectomy reversal, as described previously elsewhere (21). Immunohistochemistry was undertaken as described (26, 27) using a MYRIP goat polyclonal antibody (Santa Cruz Biotechnology), and a LRRC4C rabbit polyclonal antibody (Abcam).

Reverse-transcription PCR

The expression of candidate genes in the human testis was examined using reverse-transcription (RT) PCR. Total adult testis RNA (Origene) was converted to cDNA using Superscript III reverse transcriptase (Invitrogen) and oligo-dT primers, according to the manufacturer's instructions. Primers specific for human *LOC100507205* (GenBank accession number: NR_038309), *MYRIP* (GenBank accession number: NM_015460), and *LRRC4C* (GenBank accession number: NM_020929) cDNA sequences were designed using Primer 3 software (http://frodo.wi.mit.edu/) (Supplemental Table 4, available online).