

Genetic evaluation of male infertility

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Abstract: Men with severe oligospermia (<5 million sperm/mL ejaculate fluid) or azoospermia should receive genetic testing to clarify etiology of male infertility prior to treatment. Categorization by obstructive azoospermia (OA) or non-obstructive azoospermia (NOA) is critical since genetic testing differs for the former with normal testicular function, testicular volume (~20 mL), and follicle-stimulating hormone (FSH) (1-8 IU/mL) when compared to the latter with small, soft testes and increased FSH. History and physician examination along with laboratory testing (following appropriate genetic counseling) is critical to accurate selection of genetic testing appropriate for azoospermia due to primary testicular failure as compared with congenital hypogonadotropic hypogonadism (HH). Genetic testing options include cystic fibrosis transmembrane conductance regulator (CFTR) testing for men with congenital absence of the vas, while karyotype, Y chromosome microdeletions (YCMD), and other specific genetic tests may be warranted depending on the clinical context of severe oligospermia or NOA. The results of genetic testing guide management options. The most recent techniques for genetic analysis, including sperm microRNA (miRNA) and epigenetics, are forming the foundation for future genetic diagnosis and therapeutic targets in male infertility.

Keywords: Male infertility; genetics; oligospermia; azoospermia; spermatogenesis



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Introduction

Men with azoospermia or severe oligozoospermia (<5 million sperm/mL ejaculate fluid) are predisposed to genetic abnormalities. Genetic testing, including karyotype/cytogenetic testing, Y chromosome microdeletion (YCMD) testing, congenital hypogonadotropic hypogonadism (HH) mutation screening, and cystic fibrosis transmembrane conductance regulator (CFTR) gene screening, may reveal the etiology and likelihood of successful paternity, and potential risks to offspring (1). Informed discussion with the patient about the role of genetic testing, and the prognostic and psychological effects of genetic findings, should be completed prior to testing. Many genetic mutations and polymorphisms have been identified that have putative direct or indirect effects on spermatogenesis or yet unknown exact phenotypic consequences. Further the cause of infertility can be demonstrated in approximately 20-30%

of men with severe oligozoospermia or azoospermia, while up to 80% remain with an unknown genetic cause requiring additional research to identify candidate gene(s) (2).

Diagnosis

Differentiation between obstructive (physical blockage due to absence of parts of the male excurrent ductal system) and non-obstructive (spermatogenic failure without sperm in pellet of a centrifuged ejaculate) causes is of paramount importance in selection of appropriate genetic testing. History and physical examination including scrotal evaluation and serum lab tests are critical to this delineation to quantitate testis volume, signs of hypogonadism, and abnormal laboratory values especially serum follicle-stimulating hormone (FSH). In up to 70% of men, such preliminary workup will identify a cause for infertility (3).

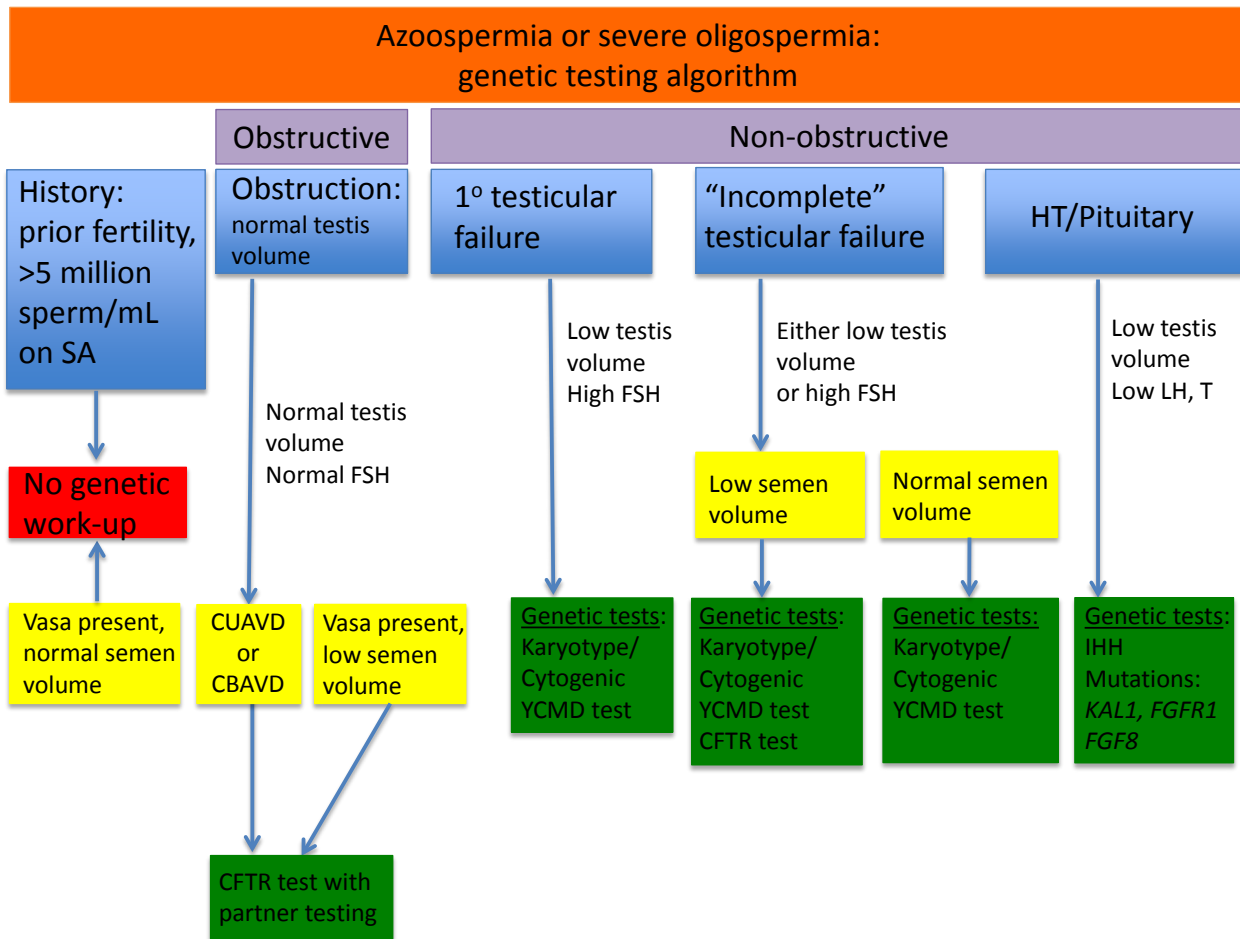


Figure 1 Azoospermia or severe oligospermia: genetic testing algorithm. CUAVD, congenital unilateral absence of the vas deferens; CBAVD, congenital bilateral absence of the vas deferens; YCMD, Y chromosome microdeletions; CFTR, cystic fibrosis transmembrane conductance regulator; FSH, follicle-stimulating hormone; SA, semen analysis; HT, hypothalamus; LH, luteinizing hormone.

Genetic testing: who should have it?

All genetic testing options should not be completed for every azoospermic patient. Testing should be considered given the clinical context for each patient (*Figure 1*). For men with normal testis volume, palpable vasa on physical examination, and strong suspicion for obstruction with normal serum FSH and normal semen volume, no genetic testing is indicated. Genetic testing is also not recommended for patients with known prior history of fertility or those patients with previously documented sperm concentration >5 million/mL of ejaculate fluid. Patients with known prior toxic exposure such as cytotoxic chemotherapy or radiation also do not require genetic testing (4). Patients who should be tested include those with

suspected congenital obstruction (normal testis volume and FSH), primary testicular failure (low testis volume and high FSH), “incomplete” testicular failure with either low testis volume or increased FSH, or HH (low testicular volume, low or low-normal LH, and low testosterone) (*Figure 1*).

Men with primary testicular failure and severe oligospermia or non-obstructive azoospermia (NOA) with impaired endocrine and exocrine function and sperm count lower than five million per mL of ejaculate fluid will classically have small, atrophic, soft testes, normal semen volume and seminal pH, and elevated FSH. These characteristics reflect poor spermatogenesis or reduction in seminiferous tubules. Decreased testicular endocrine and exocrine processes result in decreased testosterone and inhibin B negative feedback on the hypothalamus

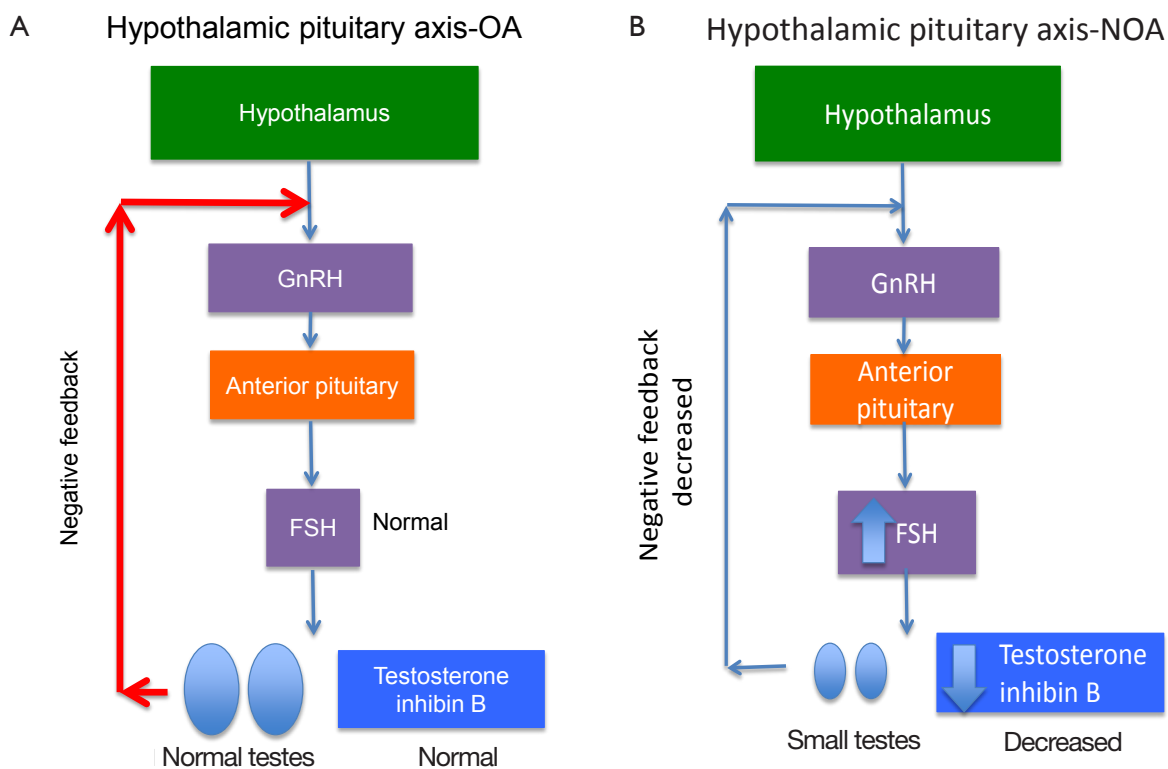


Figure 2 Hypothalamic pituitary axis in (A) OA with normal FSH, testosterone, testicular volume; (B) NOA with increased FSH, decreased testosterone, and small testes. OA, obstructive azoospermia; NOA, non-obstructive azoospermia; FSH, follicle-stimulating hormone.

and pituitary, resulting in increased FSH (and sometimes LH if testosterone is significantly decreased) (Figure 2). Inhibin B, a glycoprotein hormone produced by the testis, is inversely related to FSH and strongly correlated with testicular volume and sperm count. There are some cases, such as late maturation arrest, that may demonstrate normal FSH and inhibin B (5). Although FSH and inhibin B are most sensitive when obtained together, inhibin B does not provide valuable independent diagnostic information and is therefore not routinely measured in clinical practice at this time. Men with classic primary testicular failure should undergo karyotype and YCMD analysis (1,6). Patients with severe oligospermia or azoospermia along with testicular volume and serum FSH that have an incomplete primary testicular failure clinical picture (i.e., normal testis volume with elevated FSH) also require karyotyping and YCMD testing prior to any possible treatment (7). Men with normal FSH, normal testicular volume without evidence of obstruction on physical exam could have testicular maturation arrest, commonly associated with abnormalities of genes including MutL homolog 1 (*MLH1*) gene, MutS homolog 2 (*MSH2*), and excision repair cross-

complementation group 1 (*ERCC1*) (8-11). Additionally, CFTR testing should be completed in the setting of low semen volume due to association with seminal vesicle atresia (discussed further in cystic fibrosis section below).

Karyotype analysis of all numeric (gains/losses) and structural abnormalities (most frequently Robertsonian, but also reciprocal translocations, inversions) of entire chromosomes is associated with approximately 6% of all male infertility. Four percent of men receiving ICSI for male subfertility have chromosomal abnormalities, the majority of which involve sex chromosomes (12). Karyotypic abnormalities are identified in 3% to 5% of severely oligozoospermic (often translocations) and 14% to 19% of men with NOA (most frequently nonmosaic Klinefelter syndrome, 47,XXY) and are eight times more common in infertile than fertile men (13-15). Klinefelter syndrome (47,XXY) is identified in one in 600 males among the general population. Patients with Klinefelter syndrome present with a spectrum of phenotypes (dependent on testosterone level) but all have atrophic testes with characteristic abnormal exocrine and endocrine function (elevated LH, FSH, low testosterone) and azoospermia.

Reduced Leydig cell size along with germ cell loss, Sertoli cell loss, and tubular hyalinization contribute to testicular dysfunction. Deficient androgen production and excess estradiol from overexpressed CYP19 aromatase is manifested as delayed progression of puberty with decreased facial/body hair, decreased muscle development, bone density loss, and gynecomastia. This altered hormonal milieu may predispose to learning disabilities and social maldevelopment. Risk of sperm and subsequent embryo aneuploidy may be slightly increased in some studies (16), while others show no increased risk of aneuploidy (17). 46,XX is another possible but rare karyotype (1:20,000 live births) identified in azoospermic men, often resulting from translocation of the distal tip of Y chromosome short arm (containing *SRY* gene) to the distal tip of the X chromosome short arm. The remaining Y chromosome is not present, including AZF regions, and therefore spermatogenesis is absent making TESE not possible (18). Yq loss also occurs in isodicentric Y chromosome which may be unstable and also lack AZF regions. Additionally, abnormalities of X chromosome linked genes (i.e., androgen receptor Xq11.2-12) may exhibit a spectrum of androgen insensitivity based on specific mutation and CAG repeat length and can result in 46,XY azoospermic males (19,20).

Increasing deterioration of spermatogenesis (decreased sperm count) is associated with increased prevalence of karyotype abnormalities (for example increasing number of X chromosomes associated with increased clinical severity). Karyotype should be offered to all men with severe oligospermia or NOA prior to treatment. Following culture of leukocytes from the patient's blood, treatment with protease and characteristic staining (G banding after treatment with trypsin and Giemsa staining) is completed, and additional cytogenetic analysis including extended karyotyping or fluorescence in situ hybridization (FISH) is sometimes necessitated. Genetic counseling should outline risk of paternal transmission of chromosomal abnormalities (reciprocal more than Robertsonian translocations, inversions) and consideration should be given to preimplantation genetic diagnosis (PGD) since karyotypic abnormalities can translate to pregnancy loss and offspring risks (21).

YCMD, located on the long arm of the Y chromosome (Yq), involve three azoospermic factor (AZF) regions associated with spermatogenesis. YCMD are not typically identified on standard karyotypic analysis due to small size, but instead are detected by PCR amplification (using DNA extracted from peripheral blood) in 5-10% and up to 10-20% of men with severe oligospermia and NOA,

respectively. There is, however, variation in YCMD according to ethnic/geographic background (22-24). YCMD testing detects regions AZFa (USP9Y and DDX3Y), AZFb (including EIF1AY, HSFY, SMCY, RPS4Y2, PRY), and AZFc (including BPY2, CDY, DAZ) using sequence-tagged sites (STS). STS are pre-chosen unique DNA sequences sets which reflect presence or absence of the different Y chromosomal regions. YCMD testing has prognostic value and guides choice of microdissection TESE/ICSI *vs.* donor sperm/adoption. Sixty percent of YCMDs do not result in retrievable sperm with TESE, and these include all men with AZFa (Sertoli cell only syndrome), AZFb (maturation arrest, P5-proximal P1) and AZFb+c (maturation arrest, P5-distal P1) deletions, none of which have had sperm identified to date with TESE in the few available studies (25,26). Among infertile men, AZFc is the most frequent *de novo* microdeletion of the Y chromosome (1:4,000 men) presenting with severe oligospermia in two out of three cases, with remaining men being azoospermic (27). Men with AZFc microdeletions, which encompass seven groups of transcription units, have retrievable sperm 70% to 80% of the time and IVF/ICSI results are comparable to controls (28). However, it is important to note that AZFc microdeletions are transmitted from affected fathers to all sons resulting in compromised fertility potential (29,30). Additionally, partial AZFb and AZFc deletions (*gr/gr*, *b1/b3*, *b2/b3*) may exist that may be compatible with normal sperm count or reduced sperm count amenable to successful sperm retrieval (31,32).

Although men with NOA most often have primary testicular failure, it is also possible that there is secondary testicular failure (congenital or acquired) due to hypothalamic-pituitary axis deficiency resulting in decreased gonadotropin production and thereby decreased testosterone production. HH of congenital etiology is characterized by consistent hypogonadism throughout life that is, by definition, not acquired. This hypogonadism results from deficient gonadotropin production (LH, FSH) from the hypothalamus or anterior pituitary. The most common cause of congenital HH gonadotropin deficiency is Kallmann Syndrome, a developmental genetic disorder, affecting up to 1:10,000 men. Kallmann Syndrome includes a range of phenotypes including isolated congenital HH without other abnormalities or classic Kallmann's syndrome with olfactory abnormalities of anosmia or severe hyposmia. Anosmia or hyposmia are due to aberrant migration of GnRH-secreting neurons and olfactory axons during embryogenesis and may occur simultaneously with other phenotypic abnormalities. Multiple genetic etiologies

have been described including various *KAL1* mutations responsible for X-linked Kallmann syndrome (33,34). Autosomal genes *FGFR1*, *FGF8*, *PROKR2* and others have been linked to HH without olfactory abnormalities (35,36). Treatment includes hormone replacement therapy based on deficiency (36,37). Genetic tests are not yet readily available, however data about inheritance patterns (X-linked, autosomal dominant, autosomal recessive) may be useful for counseling patients regarding risks to offspring and need for PGD. More exhaustive search for a genetic source may be considered in familial cases.

Obstructive azoospermia (OA) occurs with normal sperm production but blocked flow in the male excurrent ductal system. With obstruction, testicular function, testicular volume (~20 mL), and FSH (1-8 IU/mL) are normal. FSH is normal due to Leydig and Sertoli cells' negative feedback (via testosterone, inhibin B respectively) on the hypothalamus and pituitary. In the case of congenital unilateral absence of the vas deferens (CUAVD) or congenital bilateral absence of the vas deferens (CBAVD), physical examination reveals unilateral or bilateral absence of the vas deferens and/or indurated, irregular epididymal segments or even partial absence of epididymal segments. The caput of the epididymis is always present due to different embryologic origin than the cauda or corpus. If CUAVD or CBAVD is likely, then genetic testing for *CFTR* gene mutation (chromosome 7q31.2) is performed with simultaneous renal ultrasound to rule out renal agenesis (38). Men with CBAVD exhibit two affected *CFTR* alleles (up to 80% of patients) with up to a 15% chance of additional abnormalities. Of the two *CFTR* alleles, there is most frequently one severe allele and one mild allele in 85% of patients (39). If renal agenesis occurs concomitantly with CBAVD, however, this is secondary to abnormal mesonephric duct formation unrelated to *CFTR* mutation genetics (40).

Cystic fibrosis is the most common autosomal recessive condition occurring clinically in 1:1,600 patients of Northern European descent/non-Hispanic white population with a progressive course adversely affecting lung, pancreas, and other parts of the gastrointestinal system (41). Cystic fibrosis results from abnormalities in the *CFTR* gene, which typically regulates exocrine epithelial cell tubal secretion consistency and increases sweat sodium chloride concentration via cyclic AMP pathways. The vast majority of males with CF demonstrate CBAVD (more than 95%) with resultant OA. Greater than 1,700 mutations and abnormalities have been demonstrated, with severity resulting from decreased or absent *CFTR* protein based on maternal and paternal allele

contributions. Phenotypes range from isolated CBAVD, vas without a true lumen, atrophic or absent epididymal corpus/cauda, to classic cystic fibrosis (42). The majority of CF screening panels include between 30 and 50 mutations (including the most common abnormality in CBAVD, F508del as well as 5T, 7T, and 9T variants) which are identified most commonly in patients with clinical symptoms of CF (43,44). Even with complete *CFTR* gene screening, mutations may still be undetectable in 25% of CBAVD patients. Comprehensive analysis of the *CFTR* gene with DNA sequencing is reserved for patients with CF history or CBAVD (45). In CBAVD, which is classified as surgically unreconstructable OA, microsurgical epididymal sperm aspiration (MESA) is the optimal treatment with subsequent assisted reproductive technology (ART) (46,47).

Patients with CUAVD/CBAVD classically have low semen volume, with low pH (<7) due to absent alkaline seminal vesicle secretions and fructose due to seminal vesicle atresia which is common in these patients (48). If vasa are palpable, but the patient has low semen volume (<1.5 mL), *CFTR* mutation testing is indicated since seminal vesicle atresia/obstruction could reflect partial absence of the vas with normal scrotal vas and missing retroperitoneal vas. In addition, CUAVD patients frequently have contralateral obstruction of the seminal vesicle and manifest *CFTR* mutations. If idiopathic epididymal obstruction is considered, then some groups recommend *CFTR* mutation testing since up to 50% of men may demonstrate mutations.

In rare cases, open testicular biopsy (optimally with microsurgical approach) may be indicated to differentiate OA from NOA in azoospermic men with normal volume testes, palpable vasa, normal FSH levels, and a negative serum anti-sperm antibody test (49). In men with CBAVD, testicular biopsy commonly shows active spermatogenesis and is not needed before definitive aspiration for IVF/ICSI. Female partner testing and genetic counseling is required prior to ART since up to 5% of partners may be heterozygotes among Caucasians. If the female partner is identified to be CF heterozygote, PGD selection of unaffected embryos may reduce the risk of having offspring with infertility or clinical CF.

Management and additional considerations

For all aforementioned genetic tests, genetic counseling should be provided for patients describing limits and benefits of genetic diagnosis prior to actual genetic testing (6). Management of oligospermia and azoospermia will be

discussed in further detail in this issue, but includes IVF/ICSI (50) in conjunction with microdissection TESE for men with NOA. Such treatment yields successful sperm retrieval in approximately 60% of patients (51). For men with severe oligospermia or OA, treatment with IVF/ICSI and with MESA/testicular tissue extraction with IVF/ICSI, respectively, results in very high success rates (52-55).

Genetic testing for male infertility may reveal more than only fertility risk. Men with CUAVD or CBAVD with CFTR abnormalities obviously must be monitored for sequelae associated with the spectrum of possible disorders. Men with NOA and oligospermia have been recognized in some studies to have defective DNA repair, with 2.9 fold and 1.4 fold respective increase in cancer risk (56) including testis, prostate, and hereditary nonpolyposis colorectal cancer (Lynch Syndrome) (57,58). Impaired systemic homologous recombination, DNA repair, microsatellite stability, and apoptotic response likely underlie this predisposition and necessitate consideration of an increased cancer risk on clinical examination.

Additional study, of sperm microRNA (miRNA) and small noncoding RNA which regulate transcriptional and post-transcriptional gene expression, has identified expression differences according to testicular histology which could enhance diagnosis and therapy in the future (59). Specifically, a number of miRNAs are produced by male germ cells throughout spermatogenesis and have been identified in testis tissue and semen (60,61). Differential miRNA expression has been noted using miRNA microarray assays with quantitative real-time PCR (qRT-PCR) confirmation. Two miRNA families, miR-34 and miR-449, have been identified to be significantly different between testis tissue of azoospermic men with SCO, MA (all with significant down-regulation of miRNA) when compared with normal spermatogenesis tissue (59). Additionally, differences in seminal miRNA expression profiles have been noted between oligoasthenozoospermic (including miR-34b, miR-34b*, miR-15b, miR-34c-5p) and asthenozoospermic (miR-34b, miR-122 and miR-1973) men and men with normal spermatogenesis (62,63). Additional miRNAs (miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p and miR-513a-5p) have been shown to be markedly decreased in azoospermia while increased in asthenozoospermia (63). The predictive value of the identified miRNA to differentiate control versus azoospermic cases and control versus asthenozoospermia cases had an AUC in ROC analysis ranging from 0.733-0.836 and 0.822-0.921, respectively (63). Late meiotic cells and

haploid germ cells are the main contributing source of miRNA during spermatogenesis, and miRNA aberrations may serve as modifiers early post-fertilization and persist in blastocysts (64,65).

Epigenetic studies have demonstrated differences in testicular DNA methylation (addition of methyl group to C5 on cytosine which may alter DNA expression) in azoospermic men using, for example, the Illumina Infinium™ Human Methylation27 BeadChip DNA methylation arrays. In one study of 94 azoospermic men, NOA and OA men were demonstrated to have significantly different DNA methylation profiles (assessed by CpG sites). Additionally, abnormal DNA methylation of imprinted genes in spermatozoa of oligozoospermic men have been identified (66). Histone/protamine modification profiles may also prove useful in identifying further genetic causes of oligospermia and azoospermia (67-69). Precursor (pre-mRNA) splicing regulators (studied in mice so far) (70), copy number variations (CNVs), and seminal mRNA transcript analyses may also prove helpful (71,72). Finally differences in sperm mitochondrial genome deletions may be an additional area for exploration (70). Lastly, work has proven promising in generating primordial germ cell-like cells for spermatogenesis from embryonic and induced pluripotent stem cells (from mice) (73) and human primordial germ cell reprogramming (74). With translation of such work to humans, profound characterization of the genetics of germ cells may provide additional treatment avenues for men with defects in spermatogenesis and future multi-gene predictive assays as have been formulated in prostate cancer (75) bladder cancer (76), and breast cancer prognosis (77).

Conclusions

Genetic testing is necessitated in all severely oligospermic and non-obstructive azoospermic men. Such men demonstrate small-to-atrophic, soft testes and increased FSH. Karyotype chromosome structural and numeric abnormalities, YCMD, and other genetic mutations have been implicated in male subfertility. Klinefelter syndrome (47,XXY) is the most common karyotypic abnormality, while AZFc microdeletion is the most common Y microdeletion. HH is diagnosed and may result from multiple genetic abnormalities with differing modes of inheritance. OA caused by CUAVD/CBAVD requires cystic fibrosis mutation testing (including less common variants identified specifically in CBAVD population) of the patient (who otherwise demonstrates normal volume testes

and normal FSH) and his partner. The above testing for severely oligospermic and azospermic men will provide prognostic information and guide medical management, sperm retrieval options, or in some cases alert the patient and clinician to a lack of any spermatogenesis in the case of 46,XX, and AZFa, AZFb, or AZFb+c microdeletions. Future study of sperm genetic expression profiles, stem cells, miRNA, and epigenetic modifications will optimize our genetic testing for male infertility and provide potential therapeutic targets.

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None.

Footnote

Conflicts of Interest: The author has no conflicts of interest to declare.

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