Introduction

Infertility, commonly defined as inability to achieve pregnancy despite 1 year of unprotected intercourse, affects nearly 15% of couples (1), and a male factor contributes in up to 50% of these couples (2). The etiology of male infertility can be broadly divided into four major categories: hypothalamic-pituitary axis dysfunction, quantitative spermatogenic defects, qualitative spermatogenic defects, and ductal obstruction or dysfunction (3). Known genetic anomalies underlie ~15% of male infertility cases (4) and can cause abnormalities within any of these four etiologic categories. With significant advancement in our understanding of genetics came a concordant rise in the medical applications of genetic testing. Many of these tests now comprise essential components of the male infertility workup. This review will provide an overview of common genetic tests used for male infertility and the indications for each of these tests.

Initial workup of infertility

The American Urologic Association (AUA), European Association of Urology (EAU), and American Society of Reproductive Medicine (ASRM) agree that the initial workup of infertility should include at least a comprehensive medical history, physical examination with focus on male genitalia, and at least one (although some committees recommend two) semen analysis (5-7). If any part of this workup is abnormal, the man should be referred to a urologist or male reproductive specialist for a more thorough evaluation. In particular, the examiner should note the location of urethral meatus, size and consistency of testes, presence or absence of both vasa and epididymides, and secondary sex characteristics. Digital rectal examination may also be performed if there is concern for prostatic or seminal vesicle anomalies (7). Completion of this evaluation, in addition to serum FSH and testosterone levels, may identify the etiology of infertility in up to 70% of men (8).
Genetic testing is not indicated for all infertile men. Those men who have had previous fertility, known prior gonadotoxic (e.g., chemotherapeutic) exposure, or have an exam highly suspicious for ejaculatory duct dysfunction do not warrant a full genetic workup. Rather, genetic testing should be considered in men with nonobstructive azoospermia, severe oligospermia, or nonpalpable vasa (9). Genetic testing serves two major purposes in the setting of infertility: determination of heritable conditions that may be passed to offspring and evaluation for conditions which may impact the success of assisted reproductive techniques (10). Diagnosis of genetic problems causing infertility may also have implications for management of a patient’s overall health (e.g., consideration of testosterone therapy for skeletal and mood benefits in men with Klinefelter syndrome).

The most common genetic tests for male infertility used in clinical practice today are karyotyping, Y-chromosome microdeletion screening, and CFTR gene mutation testing. The AUA, EAU, and ASRM all have guidelines outlining the appropriate uses for each of these diagnostic techniques (Table 1). This review will primarily discuss the indications, rationale, and methodology of these three genetic tests. Other rarer genetic tests used for specific populations will also be described.

### Table 1 Guidelines for genetic testing in male infertility (5-7)

| Genetic test | AUA | EAU | ASRM |
|--------------|-----|-----|------|
| Karyotyping  | NOA or <5 million/mL | Sperm conc <10 million/mL | NOA or <5 million/mL |
| YCMD        | NOA or <5 million/mL | Sperm conc <5 million/mL | NOA or <5 million/mL |
| CFTR        | CBAVD | CBAVD or CUAVD without renal abnormalities | CBAVD, CUAVD without renal abnormalities, or bilateral epididymal obstruction |

Female partners should also be tested

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### Karyotype analysis (KA)

KA is a cytogenetic technique in which human chromosomes are visualized using light microscopy and subsequently analyzed for abnormalities in number or structure. To produce a karyotype, lymphocytes obtained from peripheral blood cultures are chemically arrested in metaphase with colcemid, a drug which depolymerizes microtubules and inhibits spindle formation (11). Conventional G-banded karyotype analysis, in which Giemsa stain is added to these arrested lymphocytes, produces characteristic banding patterns on each chromosome and allows for detection of abnormalities greater than 5 megabase (Mb) in size. Other staining patterns such as C (centromere)-banding and T (telomere)-banding exist but are less commonly used in the evaluation of infertility. Due to the relatively low resolution of KA, only large chromosomal abnormalities such as aneuploides, Robertsonian and balanced translocations, and inversions can be detected (12).

With over 2,000 genes thought to play a role in proper spermatogenesis (13), countless chromosomal anomalies could theoretically cause infertility. However, the most commonly detected etiologies on KA are Klinefelter syndrome, structural chromosomal aberrations, and 46, XX male syndrome. Klinefelter syndrome (47, XXY) and its variants (such as mosaic 47, XXY/46, XY) affect one in 660 men and are the most frequent genetic causes of nonobstructive azoospermia (13,14). Structural chromosome aberrations such as translocations and inversions are found in up to 10% of infertile men and are the most frequent genetic causes of nonobstructive azoospermia (13,14). Structural chromosome aberrations such as translocations and inversions are found in up to 10% of infertile men and are the most frequent causes of oligospermia (4,13). 46, XX male syndrome, also known as de la Chappelle syndrome, is a rare condition with a prevalence of one in 20,000 males and occurs when Y chromosomal material including the SRY gene is translocated onto another, usually autosomal, chromosome during paternal meiosis (15,16). Successful sperm retrieval rates vary widely among these populations, ranging from 0% in 46, XX males (due to an absent AZF region) to ~30% in males with mosaic Klinefelter syndrome (10).

There is robust evidence that karyotypic abnormalities are much more prevalent in oligospermic and azoospermic men compared to fertile controls (seen in ~0.4% of general population, ~3.6% of oligospermic men, and up to 15% of azoospermic men) (17-19). Overall, approximately 5% of male infertility cases can be attributed to chromosomal abnormalities identified on KA (20). Accordingly, both
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AUA and EAU guidelines recommend karyotype testing in all nonobstructive azoospermic or severely oligospermic men (AUA <5 million/mL, EAU <10 million/mL) (5,7). EAU guidelines additionally recommend karyotype analysis for infertile couples with family history of recurrent spontaneous abortion, malformation, or mental retardation (5).

Although commonly used, KA does have some important limitations. It has comparatively poor resolution relative to other genetic tests so point mutations, frameshift mutations, or microdeletions cannot be detected. Additionally, even in men with sperm concentration <10 million/mL, KA often does not produce a definitive diagnosis and may indeed find clinically insignificant chromosomal abnormalities. Ventimiglia et al. recognized this deficiency within the EAU guidelines and proposed a nomogram with parameters of sperm concentration, mean testis volume, and luteinizing hormone level to predict which infertile men would benefit from KA. Using this nomogram resulted in 94% sensitivity in detecting karyotype abnormalities compared to 80% when using EAU criteria, but there was no difference in the specificity (i.e., overtesting) (21). As with many laboratory tests for infertility, cost can be a limiting factor. Insurance coverage of KA varies widely based upon individual plans, and out-of-pocket expenses for these tests range from $700 to $1,200 (22,23) in the United States. Despite these drawbacks, KA remains an integral diagnostic test for the initial workup of many cases of infertility.

Fluorescence in-situ hybridization (FISH), a related microscopic cytogenetic technique in which specific DNA sequences are hybridized to collected samples and visualized under fluorescence microscopy, has a comparatively increased resolution of about 1 Mb (24) and is primarily used to analyze specific genomic regions. This technique is not typically used for routine KA but rather as an adjunct to further characterize specific cytogenetic anomalies, such as determining whether the sex-determining region of the Y chromosome (SRY) is present in patients with XX karyotype. Other important roles for FISH include analysis of spermatid chromosomal integrity and preimplantation genetic diagnosis (25). For those patients with an abnormal karyotype who are candidates for IVF/ICSI, FISH in retrieved sperm can be performed to assess for spermatid aneuploidy or structural defects. Males with Klinefelter syndrome and other sex chromosome aneuploidies such as 47, XYY have an increased incidence of aneuploid (e.g., hyperhaploid or diploid) sperm production, and men with other karyotype anomalies have similarly higher rates of spermatid chromosomal rearrangements (26). For this reason, it has been suggested that FISH analysis of spermatozoa may be a useful supplement to KA, as it may provide better prognostic data prior to assisted reproductive technology (27).

**Y-chromosome microdeletion testing**

The Y chromosome (Figure 1) is an acrocentric chromosome containing 60 Mb on a short arm (Yp) and long arm (Yq) separated by a centromere (28). Nearly 95% of its length is comprised of the male-specific region of the Y chromosome (MSY), a collection of ~80 genes which play an important role in male sex development and spermatogenesis (29). The MSY, unlike large segments of autosomes, does not recombine during meiosis as there is no homologous region to pair with on the X chromosome (30). Rather, the MSY contains eight repetitive, palindromic, and redundant segments which are highly susceptible to intru-
chromosomal rearrangement during meiosis—a process known as non-allelic homologous recombination (31). This process is relevant because ectopic reinsertion of these segments can result in deletions, duplications, or inversions within the Y chromosome, all of which can affect genes necessary for fertility (32-34).

Of particular clinical importance is the azoospermia factor region (AZF) found on Yq which has been rigorously studied due to its richness of genes implicated in spermatogenesis (35). First described in the 1970’s (36) and further characterized in the mid-1990’s to early 2000’s (29,37), AZF is subdivided into three sub-regions—AZFa, AZFb, and AZFc—with AZFb and AZFc overlapping by 1.5 Mb (32,35). Deletions in each of these regions vary in their clinical phenotypes. Deletions of the entire AZFa region are associated with Sertoli cell only syndrome, a histological diagnosis of germ cell aplasia in tissue obtained from testis biopsy that invariably results in nonobstructive azoospermia without the possibility of ART (38). Spermatocytes are also not present in patients with complete AZFb deletions due to absence of essential maturation factors (39) and consequently, ART is not offered in these men. Complete AZFc or partial AZFb+c deletions, however, do not preclude the presence of normal spermatozoa. Accordingly, normal sperm can be retrieved in up to 70% of cases (39,40) and assisted reproductive technology (ART) remains a viable option in this population.

As Y chromosome microdeletions (YCMDs) are too small to be detected with karyotype, polymerase chain reaction (PCR) amplification must be used. Primers specific to unique sequence-tagged sites (STS)—short DNA sequences with a single occurrence within the genome—on the MSY are then used to initiate PCR. Many STS primers have been described for use in the clinical setting but variability among these primers and protocols used by laboratories complicates the interpretation of test results and may reduce diagnostic accuracy (41). To remedy this, the European Academy of Andrology and the European Molecular Genetics Quality Network have released guidelines which detail a “basic” set of six STS primers, two on each of AZFa, AZFb, and AZFc, which have given robust and reproducible results across several laboratories and quality control trials (42). The ideal number of STS to maximize efficacy of PCR has not yet been elucidated (7).

Nearly 7.5% of all oligo- and azoospermic men are believed to harbor YCMDs, although this prevalence varies worldwide (35). In patients with nonobstructive azoospermia or severe oligospermia this prevalence rises to 10–15% (20). Due to the relatively high frequency of YCMD in oligo- and azoospermic patients, both the EAU and ASRM recommend offering YCMD testing to all men with sperm counts lower than 5 million/mL (5,6). Although this threshold has high sensitivity, multiple large retrospective studies have suggested that YCMDs are exceedingly rare in men with sperm counts greater than 1–2 million/mL (43-45). Kohn et al. recently reviewed 37 studies of oligospermic men (N=12,492) that identified 261 men with YCMD (46). Of these men with YCMD, 93% had sperm counts <1 million/mL and only 5% had sperm counts >1–5 million/mL. Consequently, they proposed lowering the testing threshold to from 5 to 1 million/mL given the rarity of YCMDs in men with sperm counts above this value.

Genetic counseling must also be considered for men who are found to have YCMD, as these deletions are necessarily transmitted to all male offspring (5). Although men with AZFc deletions have fathered sons via ICSI, the spermatogenic and reproductive capabilities of these children are currently unknown; however, there is thought to be a spectrum of infertility ranging from complete sterility to spermatogenic potential. Pan et al. described three options for fully informed couples who wished to proceed with ART: proceeding with ICSI and conceiving infertile sons, not using the retrieved sperm to conceive, or performing preimplantation genetic analysis and selecting only for 46, XX embryos (47). All couples should be aware of the risks and benefits for each of these options prior to make a decision.

**CFTR gene testing**

Congenital absence of the vas deferens (CAVD) can affect one (congenital unilateral absence of vas deferens, CUAVD) or both vasa deferentia (congenital bilateral absence of vasa deferentia, CBAVD). Bilateral absence occurs in 2–10% of all infertile men but accounts for up to 40% of obstructive azoospermia cases (48,49). Over 90% of men with CBAVD additionally have morphologically abnormal (absent, atrophic, hypotrophic, or cystic) seminal vesicles (50). Physiologically, this results in abnormal semen parameters characterized by low semen volume, acidic pH, and absence of spermatozoa. Nearly 80% of men with CBAVD have mutations within the cystic fibrosis transmembrane conductor (CFTR) gene (51,52), a finding which has prompted widespread adoption of CFTR genetic testing in infertile men with the aforementioned physical exam.
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*Cystic fibrosis* (CF) is a 250 Kb gene on chromosome 7 which encodes an ATP-dependent chloride channel found primarily in epithelial cells (53,54). To date, over 2,000 mutations have been described with varying phenotypic consequences from mild to severe (55). The prevalence of these mutations differs among different ethnicities and geographic regions (52,56). Cystic fibrosis (CF), an autosomal recessive disease which causes progressive respiratory and pancreatic failure, occurs when an individual has two severe mutations of *CFTR*, one on each homologous chromosome. Overall prevalence of CF in the United States ranges from 1 in 2,500 in Caucasians to 1 in 35,000 for Asian-Americans (57). Up to 80% of these cases are due to a deleterious mutation within the *CFTR* gene termed DeltaF508 which ultimately results in abnormal protein folding (58). Observed *CFTR* mutation carrier frequency is 1 in 38 Americans with significant variability based on race (59). While CF leads to pancreatic and pulmonary dysfunction, more mild mutations of the *CFTR* gene (or simply being a carrier) can result in either CBAVD or CUAVD as the sole clinical manifestation of the mutation (10). The precise mechanism for why *CFTR* mutations cause vasal agenesis is currently unknown; however, it has been proposed that epithelial secretory defects caused by these mutations disrupt proper Wolffian duct development in utero (60,61). Of note, men with *CFTR* mutations have normal renal anatomy because this disruption of Wolffian duct development occurs after kidney formation (60). The prevalence of unilateral renal agenesis has been reported as 25–85% in men with CUAVD and 10-15% in men with CBAVD (60,62-64). This abnormality is not associated with *CFTR* mutations, but rather may be explained by different genetic mutations which leads to anomalies within Wolffian duct structures (65).

Detection of *CFTR* mutations is usually accomplished using direct gene analysis techniques. As no standard protocol for gene analysis exists, these techniques are laboratory dependent. It is neither cost-effective nor practical to directly assess for all known *CFTR* mutations, so many commercial assays are available which test for ~20 to ~60 of the most common mutations (66). Different assays with alternate mutations can be tailored to the patient’s ethnicity and/or geographic location. Whole-gene sequencing, while the most thorough diagnostic method, was previously reserved for those patients with signs of *CFTR* dysfunction with normal mutation panels given the high cost. Recent advances in next-generation sequencing (NGS), however, have significantly improved diagnostic accuracy and reduced the cost of whole-gene sequencing which may make NGS a viable-first line test in the future (67,68).

Men with CBAVD typically have normal spermatogenic potential and are therefore excellent candidates for ART (69,70). Both the AUA and EAU recommend evaluating for *CFTR* mutations in men with CBAVD (5,7). Men with CUAVD should instead undergo renal imaging due to the high incidence of non-*CFTR*-associated ipsilateral renal agenesis (60). Partners of men with CBAVD should additionally be screened prior to attempts at ART in order to evaluate CF risk in offspring. If the female partner is also a *CFTR* mutation carrier, the risk of having a child with CF can rise to 50% depending on parental mutations. In these cases, pre-implantation diagnosis should be considered.

**Other genetic tests for infertility**

A comprehensive discussion of all genetic causes of male infertility is beyond the scope of this review, but it is worthwhile to note many of these disorders have known (or suspected) causative genetic alterations which can be detected using modern-day targeted tests (71,72). As a result, patients with clinical features suspicious for these disorders can undergo confirmatory testing to better predict their reproductive potential. For example, men with hypogonadotropic hypogonadism and anosmia suggestive of Kallmann syndrome can be screened for mutations in one of the known causative genes (e.g., *KAL1*, *FGFR1*, or *FGF8*) using a specialized assay (73), but given its rarity, routinely testing infertile men for this condition would have limited diagnostic utility. Accordingly, these tests should be reserved only for infertile men with symptoms consistent with a known syndrome associated with infertility.

**Novel techniques**

Despite significant advances in our understanding of clinical genetics, a diagnosis remains elusive in up to 80% of men with infertility (10), indicating that continued research must be performed. Unfortunately, due to the complexity of spermatogenesis and variable phenotypes of infertile men, it has been difficult to elucidate many treatable genetic targets. However, newer technologies and techniques have shown some promise in improving diagnosis, and possible treatment, of infertility.
Epigenetics

Epigenetics is the study of changes in gene regulation and function caused by mechanisms other than alterations in DNA sequence. The most common epigenetic regulators include DNA methylation, histone modification, and presence of various RNA transcripts (74). Multiple studies have demonstrated an association between abnormal DNA methylation and infertility, but whether this association is causative has not yet been determined (75-77). Histone modifications appear to play an important role in germ cell development, and abnormalities in histone function may decrease number of spermatids and alter sperm motility and morphology (78-80). Differences in spermatic micro RNA (miRNA) profiles have been demonstrated in oligo- and azoospermic men compared to fertile controls (81,82). One of the more intriguing applications of sperm epigenetics is discovery of environmental factors which cause infertility (83) and recent studies have linked diet, smoking, and stress to altered spermatic epigenetic regulation (84-87). Although epigenetic testing has not yet been validated in a clinical setting, it may help identify personalized modifiable risk factors for infertile men in the future.

Genomic microarrays and NGS

With improvements in genetic testing, new population-based experimental approaches have emerged which have furthered our understanding of the genetic basis of disease. Successful sequencing of the human genome in the early 2000s enabled the creation of large databases of common sequence variants known as single nucleotide polymorphisms (SNPs). Over the past 15 years, these databases have been used to conduct large genome-wide association studies (GWAS), observational studies which aim to detect genomic differences between populations (88). Candidate genes can then be selected from this data and further studied. Methods for characterizing the genome can be broadly classified into microarray and NGS approaches.

Genomic microarrays are tools used to detect the expression of many genes simultaneously by analyzing binding patterns of complimentary DNA strands from cells of interest to probes on the array. Both custom and commercially-made assays are available which can genotype thousands to millions of genomic regions simultaneously with high accuracy (89). These assays have already determined potentially causative genes for some etiologies of infertility. Globozoospermia, for one, was found to be associated with mutations in a gene called DPY19L2 using a 250 k SNP array (90) and this discovery prompted research into the role of this protein in sperm function (91).

Comparative genomic hybridization (CGH) is another research technique which utilizes fluorescently labeled DNA to study copy number variations (CNVs), segments of DNA with varying number of repeats among individuals. DNA samples from a case and control are hybridized with different colored labels then subsequently applied to a microarray. By visualizing the color of each probe in the array, it is possible to determine the relative quantity of complimentary DNA between the two subjects. CGH-based CNV has shown utility not only for discovering new causes of genetic conditions but also diagnosis of known etiologies. In 2014, Yuen et al. developed a custom CGH microarray which could reliably identify YCMD at higher resolutions than PCR but at nearly double the cost (92).

Next-generation sequencing (NGS) technology allows for accurate and rapid sequencing of large portions of the human genome at a cost far lower than prior techniques. The first NGS sequencing platform in the mid-2000s yielded a 50,000-fold decrease in cost compared to technologies used previously and efficiency continues to rise (93). Three applications of NGS are currently employed: targeted sequencing (TS), whole exome sequencing (WES), and whole genome sequencing (WGS). TS utilizes disease-specific gene panels to simultaneously sequence genes of interest. As these panels can assess for chromosomal abnormalities, microdeletions, and single-gene mutations, they have been a subject of particular interest in infertility. Recently, a gene panel was developed for diagnosing infertile men and women which had a >90% sensitivity for detecting sex chromosome aneuploidies and YCMDs at nearly one-fifth the cost of traditional testing ($599 vs. ~$3,300) (94). WES and WGS have also seen some success in identifying candidate genes such as CATSPER2, MNS1, CFAP65, and FANCM using GWAS (95-98). Over the past four years, these techniques have helped characterize seven novel genes accounting for nearly half of cases of multiple morphological abnormalities of the sperm flagella (MMAF) in a 78-patient cohort (99).

Both microarrays and NGS have enormous clinical potential to find possible causative genetic variants without prior hypotheses of genomic location (100). Unfortunately, these findings have not yet translated into meaningful clinical applications. Many of these studies are limited by small population sizes, unknown clinical relevance of identified genes, and inconsistent results in validation.
studies. In a large systematic review and clinical validity assessment of 521 gene-disease relationships by Oud et al., only 92 had at least moderate evidence for a role in male infertility, which highlights the need for confirmatory studies when new genes are identified (101). Nonetheless, further advances in genomics and bioinformatics will improve our ability to interpret future genetic research.

Conclusions

Genetic testing has an important and necessary clinical role in the diagnosis and treatment of infertility. Some genetic tests, such as karyotyping, YCMD screening, and CFTR sequencing, clearly benefit a subpopulation of infertile men by providing clearer prognoses and treatment options for future fertility. Still, many men have no known cause of their fertility. Recent advances in our ability to sequence the genome harbor the potential to improve diagnostic capabilities and develop novel treatments aimed at newly discovered genetic targets associated with male infertility.

Acknowledgments

Funding: None.

Footnote

Provenance and Peer Review: This article was commissioned by the Guest Editors (Keith Jarvi and Jared Bieniek) for the series “Genetic Causes and Management of Male Infertility” published in Translational Andrology and Urology. The article was sent for external peer review organized by the Guest Editors and the editorial office.

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tau-19-725). The series “Genetic Causes and Management of Male Infertility” was commissioned by the editorial office without any funding or sponsorship. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of this work are appropriately investigated and resolved.

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Cite this article as: Pelzman DL, Hwang K. Genetic testing for men with infertility: techniques and indications. Transl Androl Urol 2021;10(3):1354-1364. doi: 10.21037/tau-19-725