Male Infertility in Humans: An Update on Non-obstructive Azoospermia (NOA) and Obstructive Azoospermia (OA)

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Introduction

Infertility is an emerging global health issue facing married couples [1, 2]. More than 10% of couples are unable to conceive their own child(ren), and approximately half of these cases are contributed by men [3, 4]. Azoospermia, defined as the complete absence of sperm from the ejaculate, is a major factor in male infertility. Azoospermia can be categorized into obstructive azoospermia (OA), comprising about 40% of the cases, and non-obstructive azoospermia (NOA), constitutes the other 60%. Obstruction in the ductal system is the cause of OA [5]. For NOA, the cause is the failure of the testicles to produce mature sperm so that no sperm are found in the ejaculate. NOA can be classified clinically into four types: NOA-I, no spermatozoa; NOA-II, no spermatids; NOA-III, no spermatocytes; and NOA-SCO (Sertoli cell-only), no spermatogenic cells of any types; in the ejaculate [6]. The definition of these four types of NOA is based on diagnostic analysis of the testes, hormonal analysis (e.g., FSH, testosterone) in plasma or serum, and physical examination [7]. OA patients are characterized by an obstructed flow of spermatozoa along the male genital tract. However, OA patients have normal spermatogenesis in the testis. The etiology of NOA is more complex, which can be divided into primary and secondary testicular failure. The primary testicular failure refers to pathology localized to the testis, including chromosomal/genetic abnormalities, Klinefelter's

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syndrome, testicular tumor, undescended testes, and others. The secondary testis failure is caused by the abnormal secretion of gonadotropins from the pituitary gland, which contribute to insufficient stimulation of Leydig cells and Sertoli cells in the testis to maintain normal spermatogenesis. As a result, Sertoli cells are unable to secrete adequate factors including hormones (e.g., estradiol-17ß), and Leydig cells fail to provide enough local testosterone to support normal spermatogenesis [8, 9].

Genetic Mutation(s)

The diagnostic tools of male infertility are limited, and clinicians are racing to identify more predictive biomarkers. Several crucial fundamental laboratory analyses, including semen analysis, anti-sperm antibodies, Y-microdeletion analysis, Karyotype analysis, microarray technologies, and endocrine-based laboratory investigations are currently being used to support clinicians in diagnosing, categorizing, and treating male factor infertility [10]. To date, seminal fluid, containing the highest concentration of biomolecules, is often used as a standard biomarker for the evaluation of male infertility, including DNA fragmentation index, anti-sperm antibody and sperm fluorescence in situ hybridization (Table 1) [16]. Azoospermia often involves gene mutations. However, it is difficult to determine the genetic component of male infertility because more than 2000 genes have been shown to be involved in human spermatogenesis [17]. In recent years, novel high-throughput approaches have developed to study pertinent genes that are mutated in azoospermic men, including genomic hybridization-arrays (arrays-CGH), genome wide association studies (GWAS) and next generation sequencing (NGS). Whole genome sequencing (WGS), an advanced technology, has brought an unprecedented opportunity to explore the genetic basis of infertility, which also makes it possible to perform studies of large cohorts of patients [18]. Whole exomes sequencing is also widely used to identify potential pathogenic and novel genes pertinent to infertile

Table 1 Current basic research biomarkers to identify OA and NOA

Methods	Assay content	Reference(s)
Semen analysis	Coagulation, color, viscosity, pH, volume, sperm agglutination, sperm counts, sperm concentration, sperm motility, sperm morphology, and sperm viability	[10, 11]
Antisperm antibodies	Sperm agglutination and cervical mucus penetration	[12, 13]
Acrosome reaction	Sperm penetration assay (Spa)	[14, 15]
Karyotype analysis	Genetic composition of Turner Syndrome	[10]
Y-microdeletion analysis	Light microscopy to evaluate the appearance of chromosomes	
Microarray technologies	Copy number variations, gene expression levels, and Single Nucleotide Polymorphisms (SNPs)	

males. Copy number variation (CNV) and single nucleotide polymorphisms are also the risk factors associated with male infertility [19]. Nonetheless, relatively few studies have provided functional and biological evidence to validate the variants as pathogenic genes.

Accumulating evidence has shown that *TEX11* and *TEX15* are mutated in infertile men of NOA and also meiotic arrest, analogous to the mutation mouse model [20, 21]. In one study of 40 Japanese patients with idiopathic NOA by conducting sequence analysis of 25 known disease-associated genes using next-generation sequencing and genome-wide copy-number analysis. The results revealed that oligogenic mutations, including *SOHLH1* and *TEX11* and monogenic mutation, which accounted for more than 10% of cases of idiopathic NOA (Table 2).

Table 2 A summary of selected mutated genes involved in male infertility

Gene	Name	Phenotype of infertility	Reference(s)
TEX11	Testis expressed 11	Meiotic arrest	[20–23]
TEX15	Testis expressed 15	NOA	[20, 21]
SOHLH1	Spermatogenesis and oogenesis specific basic helix-loop-helix 1	NOA	[22]
TAF4B	TATA-box binding protein associated factor 4b	NOA	[24]
ZMYND15	Zinc finger MYND-type containing 1	NOA	[24]
SYCE1	Synaptonemal complex central element protein 1	Meiotic arrest	[25, 26]
DMC1	DNA meiotic recombinase 1	NOA	[27]
CFTR	CF transmembrane conductance regulator	Maturation arrest	[28]
TDRD9	Tudor domain containing 9	Apoptosis of hSSC	[29]
FOXP3	Forkhead box P3	Apoptosis of hSSC	[30]
PAK1	P21 (RAC1) activated kinase 1	Meiotic arrest	[31]
STAG3	Stromal antigen 3	NOA	[32, 33]
PABPC1	Poly(A) binding protein cytoplasmic 1	NOA	[34]
PABPC3	Poly(A) binding protein cytoplasmic 3	NOA	[34]
EPAB	Poly(A) binding protein cytoplasmic 1 like	NOA	[34]
ADGRG2	Adhesion G protein-coupled receptor G2	Meiotic arrest	[35]
MEIOB	Meiosis specific with OB-fold	Meiotic arrest	[36]
TEX14	Testis expressed 14	Meiotic arrest	[36]
DNAH6	Dynein axonemal heavy chain 6	NOA	[36]
TDRD7	Tudor domain containing 7	NOA	[37]
WT1	WT1 transcription factor	NOA	[38]
USF1	Upstream transcription factor 1	NOA	[39]
SYCP3	Synaptonemal complex protein 3	NOA	[40]
SPINK2	Serine peptidase inhibitor Kazal type 2	NOA	[41]
USP26	Ubiquitin specific peptidase 26	NOA	[42]
BCORL1	BCL6 corepressor like 1	NOA	[43]
DZIP1	DAZ interacting zinc finger protein 1	NOA	[44]
SYCP2	Synaptonemal complex protein 2	NOA	[45]
CFAP65	Cilia and flagella associated protein 65	NOA	[46]
RNF212	Ring finger protein 212	NOA	[33]

Furthermore, submicroscopic copy-number variations (CNVs) on the autosomes and X chromosome may contribute to NOA, which require additional validation [22]. In 2014, truncating mutations in *TAF4B* and *ZMYND15* were reported in the azoospermic brothers of two families by exome sequencing. The two genes were shown to have an important role in spermatogenesis in mice, and they were also the first genes identified in the azoospermic men [24]. Also, using exome analysis and Sanger sequencing, a splice site mutation in SYCE1 was found in two NOA patients in a consanguineous Iranian Jewish family [25]. SYCE1, a component of the central element of the synaptonemal complex, was shown to be a crucial interacting/regulatory protein between proteins SYCE2 and RAD51, and its deletion led to meiosis arrest in *SYCE1* null mouse [26]. He et al. discovered *DMC1*, a meiosis-related gene, was crucial to support meiosis since its missense mutation led to meiotic arrest at the zygotene stage during human spermatogenesis. This *DMC1* mutation was identified from the male patient's family by whole-exome sequencing [27]. Cystic fibrosis transmembrane conductance regulator (CFTR), a crucial gene in supporting spermatogenesis, was recently found to be involved in NOA [28]. In another study of five azoospermic infertile NOA patients, recessive deleterious mutation in *TDRD9* was identified which contributed to sperm maturation arrest. Similar clinical phenotype was also observed in the *Tdrd9* global knockout mice [29]. Additionally, mutations and polymorphisms in *HIWI2* were detected in idiopathic NOA, which were crucial for piRNA biogenesis and function in supporting spermatogenesis [47]. piRNA pathway is a fundamental component of spermatogenesis which ensures male fertility and genome integrity [48]. Furthermore, *PABPC1*, *PABPC3*, and *EPAB*, the poly(A)-binding protein genes, are differentially expressed in NOA patients when compared to normal men, implying their involvement in NOA [34]. It is known that development of the spermatogonial stem cells (hSSC) is essential for human spermatogenesis, and *FOXP3* pathogenic variants affected the proliferation and apoptosis of hSSC, causing male infertility [30]. Similar to *FOXP3*, a reduced expression of *PAK* was noted in NOA patients, which thus inhibited apoptosis and promoted proliferation of hSSC through PDK1/KDR/ZNF367 and ERK1/2 and AKT pathways [31]. Also, genetic variants in STAG3, a meiosis-specific protein, has been reported to cause meiotic arrest in both male and female mice; however, its genetic variants in humans led to premature ovarian failure in women, but not in infertility in men [49] (Table 2).

DNA Methylation

Emerging evidence has shown that DNA methylation, sperm-borne and epigenetic abnormalities in chromatin dynamics, may contribute to male infertility [50]. Epigenetic modifications take place frequently during spermatogenesis, including large-scale demethylation of the genome to allow for sex-specific resetting by DNA methylation and histone modifications. DNA methylation, a heritable epigenetic modification, and a widely investigated epigenetic marker plays an essential role in

regulating genes during human spermatogenesis, which mainly takes place in the fifth position of cytosine bases and followed by guanine (CpG). Male germ cells acquire DNA methylation beginning at mitotic and meiotic germ cells, and it is completed at the stage of the pachytene phase of meiosis [51, 52]. Abnormal DNA methylation in sperm may contribute to male infertility and pass onto offspring, who may become more susceptible of developing illnesses later on in life [53]. Several studies have reported that there is a significant difference on DNA methylation levels between normal and infertile men, which also leads to lower sperm count, reduced semen volume and lower sperm progressive motility [54]. Studies have also shown that DNA methylation can be induced by environmental factors, including exposure to endocrine disrupting chemicals [e.g., perfluorooctanesulfonate (PFOS), phthalates) and heavy metals (e.g., cadmium, mercury, lead), nutritional status, air pollution, smoking, and unhealthy lifestyle, since these are contributing factors to gene-specific and global DNA methylation [55, 56]. For example, cadmium, an environment toxicant that exists as $CdCl_2$ in the environment has been shown to reduce fetal growth by hypomethylation of the *PCDHAC1* promoter region, which leads to a positive expression of *PCDHAC1* [57]. Air pollutants, containing massive different environmental exposures, was also found to alter DNA methylation levels of the genes encoding the mitogen-activated protein kinase (MAPK) regulatory network and other blood-based proteins [58, 59]. Male infertility is also influenced by smoking via epigenetic pathways [60]. Smoking was also shown to have a strong influence on DNA methylation, which alters the CpG methylation patterns in the regions of MAPK8IP and TKR genes, leading to reduced sperm count, motility, and defects in sperm morphology [61]. In some azoospermia sperm samples, there was a considerable increase in the levels of KCNQ1OT1 (KCNQ1 Opposite Strand/Antisense Transcript 1, is a long non-coding RNA gene found in the KCNQ1 locus), compared to the normal group [62]. Also, global methylation level of sperm DNA did not affect the pregnancy rate in IVF, but it affected embryo development when global DNA methylation level was below a threshold value [63]. Furthermore, smokers displayed hypomethylation of reproductive related genes, including Nme2, Trim27, ICR, H19, SNRPN, Sort and Pebp1, which negatively impeded spermatogenesis and sperm motility [64–67].

Also, distinctive DNA methylation modifications are noted in promoters and repeat elements during spermatogenesis [68]. As a crucial transcription factor for mitochondrial biogenesis, nuclear respiratory factor (NRF)-1 cooperates with DNA methylation to directly regulate the expression of various germ cell-specific genes, including *Asz1* [69]. The hypermethylation at the promoter of SOX30 contributes to its silencing of expression in NOA, and the decreasing level of SOX30 is related to the severity of NOA disease. Furthermore, the absence of *Sox30* in mice led to male infertility with a complete lack of spermatozoa, which impaired testis development and spermatogenesis. However, *Sox30* does not influence ovary development and female fertility [6]. Hypermethylation of the MAEL promoter increased the expression of the transposable element LINE-1, leading to a decrease in the appearance of MAEL, and the methylation of the MALE promoter in infertility men correlates

with the severity of spermatogenic failure [70]. On the other hand, aberrant methylation of the GTF2A1L promoter did not affect fertilization rates, but its expression was reduced in NOA patients.

A recent case-control study in NOA and OA patients by investigating the differences and conservations in DNA methylation based on genome-wide DNA methylation and bulk RNA-Seq between these groups for transcriptome profiling. These results have shown that the genome modification of testicular cells from NOA patients is disordered, and the reproductive related gene expression is considerably different [71]. Four functional regions (CGI, gene body, promoter, and TEs) were identified and it was noted that the NOA patient's entropy values in these regions were considerably lower *versus* the OA group. Meanwhile, the methylation level of the OA patients was lower, and the gene expression level was higher than the NOA patients. Likewise, the methylation level of Dazl gene, an RNA binding protein deleted in azoospermia [72], in OA patients was lower than that of the NOA patients. A series of reproductive genes, including testis and ovary-specific PAZ domain gene1 (*Topaz1*), the nuclear receptor *NR5A1*, and the vertebrate-conserved RNA-binding protein gene *DND1*, all displayed lower NDA methylation level and higher gene expression level in OA patients compared to NOA, which are related to the development of spermatogonia that may contribute to male infertility. Transposons are often silenced by DNA methylation, and some functional transposons exhibited higher enrichment scores in NOA patients, including ALU, ERV1, HAT, and MIR. These findings are summarized in Table 3.

Chromosomal Aberrations and Y chromosome (Yq) Microdeletions

Genetic disorders are one of the primary causes of azoospermia, including chromosomal abnormalities, monogenic disorder, multifactorial genetic diseases, and epigenetic disorders, which also constitute the genetic basis of reproductive failure [73]. The prevalence of chromosomal aberrations in azoospermic patients was between 15% and 25% [28, 74–76]. Klinefelter syndrome and its variants (47, XXY and mosaics 46, XY/47, XXY) is the most frequent chromosomal anomaly in NOA, whereas oligozoospermia is more prevalent in men with autosomal structural defects [77]. Klinefelter syndrome (KS), identified over 70 years ago, also remains one of the prevalent causes of infertility, which is typified by small testes, hypogonadotropic hypogonadism, and cognitive impairment. As a syndromic disease, KS is associated with cardiovascular abnormalities, autoimmune diseases, metabolic disorders and cognitive or psychiatric health problems, which may also increase the risk of death [78, 79]. The average prevalence of KS is 152 per 100,000 newborn males, based on several lager cytogenetic chromosome surveys in countries around the world as noted in 2017 [80]. However, KS is often insufficiently diagnosed, and treatment is limited mostly to testosterone therapy, which overcomes some but not

Table 3 Summary of the study involved in DNA methylation and mRNA expression

Gene	Full name	Gene in chromosome
Hypermethylation and low mRNA expression		
ANKRD60	Ankyrin repeat domain 60	20
TMPRSS11E	Transmembrane serine protease 11E	4
PADI3	Peptidyl arginine deiminase 3	1
GPR149	G protein-coupled receptor 149	3
C8B	Complement C8 beta chain	1
SLC45A2	Solute carrier family 45 member 2	5
GJA8	Gap junction protein alpha 8	1
OR5AC2	Olfactory receptor family 5 subfamily AC member 2	3
CELA1	Chymotrypsin like elastase 1	12
CCDC144A	Coiled-coil domain containing 144A	17
C10orf142	Long intergenic non-protein coding RNA 2881	10
GPR25	G protein-coupled receptor 25	1
TEX13B	Testis expressed 13B	X
HIST3H3	Histone cluster 3	1
TAF1L	TATA-box binding protein associated factor 1 like	9
RGPD1	RANBP2 like and GRIP domain containing 1	2
NME8	NME/NM23 family member 8	7
FRG2C	FSHD region gene 2 family member C	3
ELOA2	Elongin A2	18
NDUFA13	NADH:ubiquinone oxidoreductase subunit A13	19
HIST1H2AA	Histone cluster 1	6
TGIF2LY	TGFB induced factor homeobox 2 like Y-linked	Y
ZNF723	Zinc finger protein 723	19
CSNK1A1L	Casein kinase 1 alpha 1 like	13
AL162231	Galactose-1-phosphate uridylyltransferase	9
ABRA	Actin binding Rho activating protein	8
CMTM1	CKLF like MARVEL transmembrane domain containing 1	16
RGPD3	RANBP2 like and GRIP domain containing 3	2
C10orf82	Chromosome 10 open reading frame 82	10
UBXN10	UBX domain protein 10	1
Hypomethylation and high mRNA expression		
ID3	Inhibitor of DNA binding 3	1
S100A1	S100 calcium binding protein A	1
GJC2	Gap junction protein gamma 2	1
TNFRSF14	TNF receptor superfamily member 14	1
MGP	Matrix Gla protein	12
C1QC	Complement C1q C chain	1
ANGPTL1	Angiopoietin like 1	1
ISLR	Immunoglobulin superfamily containing leucine rich repeat	15
DCN	Decorin	12
B3GALT2	Beta-1,3-galactosyltransferase 2	1

all comorbidities [77]. Y chromosome harbors a large number of genes that are necessary for testis development and function. The azoospermia factor (AZF) deletions impaired spermatogenesis, which is also a major molecular cause of male infertility [81]. Meanwhile, Y chromosome (Yq) microdeletions constitute a significant cause of male infertility. European infertile men are less susceptible to Yq microdeletions compared to East Asian and Americans infertile men. Y chromosome is composed of a short arm (Yp), a long arm (Yq), and two pseudo autosomal regions (PARs), which are separated by a centromere [82]. Studies have demonstrated that the deletion of human PARs in men reduced recombination in PARs, leading to sterility [83, 84]. This thus increases the frequency of sex chromosome aneuploidy in sperm, contributing to X-chromosome monosomy (Turner syndrome) or XXY (Klinefelter syndrome) in the offspring [85, 86]. X-chromosome monosomy (Turner syndrome), accounted for approximately 2% of all conceptions, is due to a partial or total loss of the second sexual chromosome, leading to an abnormal development phenotype, including typical dysmorphic stigmata, sexual infantilism, short stature, and partial organs and metabolic abnormalities [87]. However, the TS phenotype may be associated with a genomic imbalance from the absence of genes linked to the second sex chromosome and altered regulation of gene expression that triggered by epigenetic factors. Thus, both copy number variations and epigenetic changes are crucial contributing factors in the TS phenotype [87]. Epigenetic alterations in pericentromeric heterochromatin may also contribute to reconstructing of chromatin conformation, leading to chromosomes that have defects in their ability to align, attach to mitotic spindle fibers, and segregate during mitosis [88].

Concluding Remarks and Future Perspectives

DNA methylation as an epigenetic marker which plays an important role in male spermatogenesis. In this chapter, we discuss the importance of DNA methylation and gene expression that contribute to NOA and OA. We also summarized the several important reproductive genes in NOA and OA that show different DNA methylation and expression level. Also, we discuss findings based on the use of advanced technology to detect genetic mutations in NOA vs. OA that lead to male infertility. More studies are needed by increasing the sample sizes to integrate multiple epigenomic and RNA-seq analysis, which will help in the identification of epigenetic markers and genes pertinent to the regulation of fertility and infertility. Future investigations using single cell (sc) RNA-seq, scATAC-seq and epigenomics will be important to define the etiology and pathogenesis in NOA and OA [89, 90].

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