Copy number variations in spermatogenic failure patients with chromosomal abnormalities and unexplained azoospermia

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ABSTRACT. Male infertility is mostly caused by spermatogenic failure. Currently, routine genetic analyses of unexplained azoospermia or oligozoospermia are limited to the investigation of Y chromosomal microdeletions and chromosome karyotype analyses. The aim of this study was to find spermatogenic failure genes in patients with chromosomal abnormalities and unexplained azoospermia caused by copy number variations in order to provide a theoretical basis for further research. Spermatogenic failure patients consisting of 13 males with chromosomal abnormalities and 20 with unexplained azoospermia were enrolled. The subjects underwent high-throughput genome-wide sequencing to find copy number variants (CNVs), and the results were analyzed using the Database of Genomic Variants, Online Mendelian Inheritance in Man database, and PubMed. The results showed that 16 CNVs were detected in 11 patients with chromosome abnormalities, and 26 CNVs were found in 16 males with azoospermia. Our data showed CNV-involved loci including: three times on 11p11.12 and 14q11.2 and twice on 6p21.32, 13q11, 15q11.11, 16p12.2,

and 21q22.3. Some CNVs may involve changes in genetic structure and function or gene mutations, which may affect gene expression in testicular tissues and lead to spermatogenic failure. The involved genes include *EDDM3A*, *EDDM3B*, *HLA-DRB1*, *HLA-DQA1*, *POTE B*, *GOLGA8C*, *DNMT3L*, *ALF*, *NPHP1*, *NRG1*, *RID2*, *ADAMTS20*, *TWF1*, *COX10*, *MAK*, and *DNEL1*. By applying high throughput genome-wide sequencing to determine CNVs, we provide a number of candidate genes possibly contributing to spermatogenic failure.

Key words: Copy number variations; Spermatogenic failure; Chromosomal abnormalities; Y-chromosomal microdeletions; Azoospermia; Oligozoospermia

INTRODUCTION

Male infertility, which refers to a male's inability to cause pregnancy in a fertile female, is mostly caused by spermatogenic failure. Problems during spermatogenesis contribute to a lower or absent production of spermatozoa, are determined by routine semen analysis, and described using terms such as 'azoospermia', 'oligozoospermia', 'teratozoospermia', or 'asthenozoospermia', or a combination of the above ('oligoasthenozoospermia', 'oligoteratozoospermia', or 'oligoasthenoteratozoospermia') (Massart et al., 2012).

Currently, clinical diagnosis of unexplained azoospermia or oligozoospermia involving a routine genetic analysis is limited to the investigation of Y chromosomal microdeletions and chromosome karyotype analyses. The incidence of Y chromosomal microdeletions and chromosome abnormalities is ~7.4 and ~5% in male infertility patients, and the prevalence is increased to ~9.7 and ~13% in azoospermic males (Van Assche et al., 1996, Ferlin et al., 2007; McLachlan and O'Bryan, 2010).

Recently, gene mutations and polymorphisms, unbalanced translocations, and protamine defects relevant to spermatogenesis have been elucidated (Carrell et al., 2006). The current study includes two groups of infertile patients without Y chromosomal microdeletions, with one group only having chromosome abnormalities. Martin et al. (2008) suggested a possible role of additional copy number variants (CNVs) in chromosome inversions and translocation events. Therefore, we tried to determine whether there is any other cause of spermatogenic failure other than abnormal karyotype. The other group of patients has unexplained azoospermia, and we also attempt to explain the possible pathogenesis of problems during spermatogenesis.

CNV is defined as a DNA segment longer than 1 kb with a variable copy number compared with a reference genome (Feuk et al., 2006; Freeman et al., 2006). The research methods for CNVs include fluorescent *in situ* hybridization (FISH), comparative genomic hybridization (CGH), multiplex amplifiable probe hybridization (MAPH), multiplex ligation-dependent probe amplification (MLPA), etc. CNVs have recently been shown to be an important source of genetic diversity, exhibiting remarkable differences between individuals and playing a role in complex diseases such as mental retardation, schizophrenia, and cancer (Redon et al., 2006; Lee et al., 2007; Fanciulli et al., 2010).

Recently, CNVs have also been applied to the analysis of male infertility by CGH (Lee et al., 2007; Tuttelmann et al., 2011). However, to the best of our knowledge, little information is available regarding CNVs in males with chromosomal abnormalities and unexplained azoospermia using high throughout genome-wide sequencing. Hence, we will focus on those cases in order to determine the pathogenesis of spermatogenic failure.

MATERIAL AND METHODS

Patients

Spermatogenic failure patients with chromosomal abnormalities (N = 13) and unexplained azoospermia (N = 20) were enrolled. The mean ages were 29.0 years (23-37 years) and 27.6 years (22-39 years). All men with known clinical problems (e.g., undescended testes, varicocele, retrograde ejaculation, infections, and obstructive azoospermia), abnormal examination results (reproductive hormone levels, serum inhibin-B, seminal plasma fructose, and α-glycosidase levels), and genetic causes of spermatogenic failure (chromosome karyotype anomalies, Y-chromosomal mircodeletions) were excluded. All participants gave written informed consent for evaluation of their clinical data and genetic analyses of their donated DNA samples according to a protocol approved by the Chinese Association of Humanitarianism and Ethics.

Semen analysis

Semen samples were obtained after a 3-7 day period of ejaculatory abstinence, and semen analyses were performed three times within an interval of three months according to the World Health Organization guidelines (5th) (World Health Organization, 2010). Absence of spermatozoa in the semen ejaculate, if detected three times, was considered azoospermia (A). A sperm concentration of <15 x 10^6/mL was considered oligozoospermia (O). A sperm concentration of <15 x 10^6/mL with progressive motility <32% or total (progressive + nonprogressive) motility <40% was considered oligoasthenozoospermia (OA). Oligoteratozoospermia (OT) was defined as a sperm concentration of <15 x 10^6/mL with a percentage of morphologically normal sperm of <4%. Oligoasthenoteratozoospermia (OAT) was defined as a sperm concentration of <15 x 10^6/mL progressive motility <32% or total (progressive + nonprogressive) motility <40%, and a percentage of morphologically normal sperm of <4%.

High throughput genome-wide sequencing and data analysis

Peripheral blood (1 mL) containing EDTA (2.25 mg/mL ethylenediamine tetra-acetic acid) was sent to Berry Genomics, Beijing, China for processing and high throughput sequencing to determine the CNVs. The results were analyzed using the Database of Genomic Variants (DGV) (Genomic Variants in Human Genome Build GRCh37: Feb. 2009, hg19) (http://dgv.tcag.ca/dgv/app/home), OMIM (Online Mendelian Inheritance in Man) (http://omim.org/), and PubMed (Key words were infertility, spermiogenesis, azoospermia, oligozooospermia, and testis).

RESULTS

In the present study, 16 CNVs were detected in 11 patients with chromosome abnormalities, and 26 CNVs were found in 16 males with azoospermia. There were no obvious abnormalities in the remaining two chromosomal abnormality patients (Nos. 8136 and 8548) (Table 1) and the other four unexplained azoospermic males (Nos. 7742, 7958, 8063, and 8077) (Table 2). CNV sizes ranged from 100 kb to 2.38 Mb and 40 kb to 2.2 Mb in patients with chromosome abnormalities and azoospermia, respectively. For chromosome abnormality carriers, as shown in Table 1, out of 13 patients, five cases were diagnosed with azoospermia (A), three with oligoasthenozoospermia

(OA), two with oligozoospermia (O), two with oligoasthenoteratozoospermia (OAT), and one with oligoteratozoospermia (OT).

Our data showed CNV-involved loci three times on 11p11.12 and 14q11.2, twice on 6p21.32, 13q11, 15q11.1-11.2, 16p12.2, 21q22.3, and once on 1p33, 1p36.21, 2p16.3, 2q13, 2q21.1, 2q36.1, 3p14.1, 4q13.2, 5q11.1, 5q23.1, 5q35.3, 6q22.31, 7p21.3, 7q31.1, 8p12, 8p22, 9p21.1, 10p11.1, 10q11.22, 10q21.1, 12q12, 12q14.1, 14q24.3, 15q25.1, 17p12, and 22q11.21 (Figure 1).

Table 1. Detailed analysis of 13 patients with chromosomal abnormalities.

Case No.	Karyotypes by G-banding	Semen analysis	Sequencing results	Type	Size (bp)	Regions on chromosomes
7050	46,XY,t(1;2)(q21;p23)	OA	chr16:(21960001-22440000)x1	Loss	480K	16p12.2
8004	46,XY,t(2;8)(q31;q22)	OAT	chr2:(110860001-111000000)x1	Loss	140K	2q13
5107	45,XY,-13,-19,+der(19)t(13;19)(q12;p13)	A	chr6:(121500001-121600000)x1	Loss	100K	6q22.31
5118	46,XY,t(X;2)(p22;p11)	A	chr2:(52100001-52280000)x1	Gain	180K	2p16.3
			chr6:(32460001-32580000)x3	Gain	120K	6p21.32
6632	45,X,der(Y;22)(q10;q10)	OA	chr9:(29520001-29960000)x3	Gain	440K	9p21.1
7504	46,XY,21pstk-	OT	chr22:(21100001-21460000)x1	Loss	360K	22q11.21
8557	46,XY,21p-	A	chr21:(46740001-48100000)x1	Loss	1.36M	21q22.3
8033	46,XY,16qh+	OAT	chr14:(73940001-74140000)x1	Loss	200K	14q24.3
			chr15:(79000001-79100000)x1	Loss	100K	15q25.1
7523	46,XY,inv(1)(p32q42)	A	chr17:(13760001-14200000)x3	Gain	440K	17p12
4894	46,XY,inv(1)(p36q25), inv(9)(p11q12)	A	chr2:(132340001-133020000)x1	Loss	680K	2q21.1
			chr11:(48820001-49400000)x3	Gain	580K	11p11.12
			chr13:(19440001-19640000)x3	Gain	200K	13q11
3849	47,XY,+mar	O	chr10:(38480001-39160000)x3	Gain	680K	10p11.1
			chr15:(20180001-22560000)x3	Gain	2.38M	15q11.1-q11.2
8136	46,XY,t(1;3)(p22;q29)	OA	No obvious abnormalities			
8548	46,XY,13p+	O	No obvious abnormalities			

A = azoospermia; O = oligozoospermia; OA = oligoasthenospermia; OT = oligoteratozoospermia; OAT = oligoastenoteratozoospermia.

Table 2. Detailed analysis of 20 patients with unexplained azoospermia.

Case No.	Sequencing results	Type	Size(bp)	Regions on chromosomes
7516	chr8:(31500001-32040000)x3	Gain	540K	8p12
	chr12:(38580001-38740000)x1	Loss	160K	12q12
7524	chr1: (15300001-15480000)x3	Gain	180K	1p36.21
7647	chr5: (49440001-49480000)x1	Loss	80K	5q11.1
7688	chr7: (12860001-13100000)x1	Loss	240K	7p21.3
	chr11: (49240001-49980000)x1	Loss	740K	11p11.12
7937	chr2: (222200001-223020000)x3	Gain	820K	2q36.1
7998	chr4: (70180001-70440000)x3	Gain	260K	4q13.2
8064	chr7: (112180001-112360000)x1	Loss	180K	7q31.1
8080	chr13: (19440001-19500000)x1	Loss	60K	13q11
	chr16: (22400001-22720000)x1	Loss	320K	16p12.2
8196	chr5: (117380001-117660000)x1	Loss	280K	5q23.1
	chr12: (60680001-60940000)x1	Loss	260K	12q14.1
8235	chr10: (46980001-47700000)x3	Gain	720K	10q11.22
	chr10: (59560001-59760000)x1	Loss	200K	10q21.1
	chr21: (44700001-45240000)x3	Gain	540K	21q22.3
7467	chr1: (47580001-47680000)x1	Loss	100K	1p33
	chr8: (13720001-14040000)x3	Gain	320K	8p22
7468	chr5: (180400001-180440000)x1	Loss	40K	5q35.3
	chr14: (19460001-20420000)x3	Gain	960K	14q11.2
7477	chr14: (19460001-20280000)x3	Gain	820K	14q11.2
7333	chr3: (65780001-66340000)x3	Gain	560K	3p14.1
	chr14: (19460001-20440000)x3	Gain	980K	14q11.2
6035	chr6: (32440001-32700000)x3	Gain	260K	6p21.32
	chr11: (48800001-49380000)x3	Gain	580K	11p11.12
5190	chr15: (20180001-22380000)x3	Gain	2.2M	15q11.1
7742	No obvious abnormalities			
7958	No obvious abnormalities			
8063	No obvious abnormalities			
8077	No obvious abnormalities			

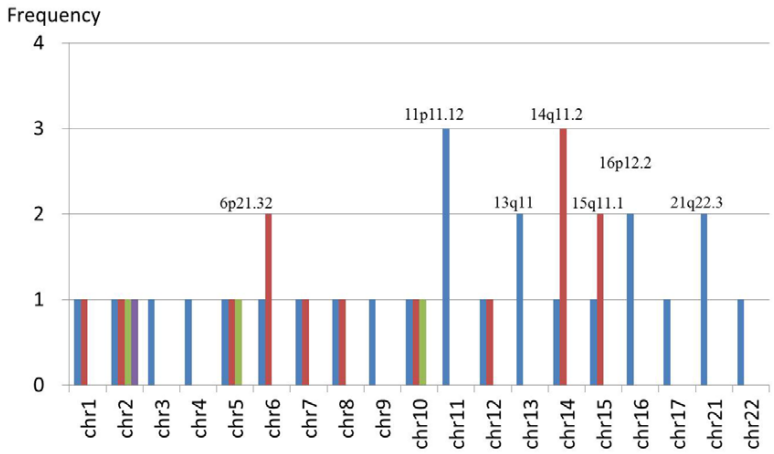

Figure 1. Frequency of CNVs on different regions of chromosomes. CNVs occurred on 11p11.12 and 14q11.2 three times; on 6p21.32, 13q11, 15q11.1, 16p12.2, and 21q22.3 twice; and on 1p33, 1p36.21, 2p16.3, 2q13, 2q21.1, 2q36.1, 3p14.1, 4q13.2, 5q11.1, 5q23.1, 5q35.3, 6q22.31, 7p21.3, 7q31.1, 8p12, 8p22, 9p21.1, 10p11.1, 10q11.22, 10q21.1, 12q12, 12q14.1, 14q24.3, 15q25.1, 17p12, and 22q11.21 only once.

DISCUSSION

In this present study, 42 CNVs were detected in 33 cases of infertile males by high throughput genome-wide sequencing. Among them, there were 16 CNVs in 13 males with chromosomal abnormalities and 26 CNVs in 20 unexplained azoospermic patients. Therefore, it is important to differentiate between CNVs that are likely to contribute to an affected individual's spermatogenic failure and CNVs that may be polymorphism variations. Then, the loci and loci-associated genes of CNVs were examined using DGV, OMIM database, and PubMed with the following key words: infertility, azoospermia, oligozoospermia, spermiogenesis, and testis. The loci related to the spermatogenic failure gene will be analyzed emphatically.

CNVs on the regions of 11p11.12, 13q11, 16p12.2, 1p33, 1p36.21, 2q21.1, 2q36.1, 3p14.1, 4q13.2, 5q11.1, 5q23.1, 5q35.3, 7p21.3, 7q31.1, 8p22, 9p21.1, 10p11.1, 10q11.22, 10q21.1, 12q14.1, 14q24.3, 15q25.1, and 22 q11.21 on spermatogenic failure-related genes were not discovered by the time of this literature reported. However, CNVs associated with the genes of spermatogenesis impairment on 14q11.2, 6p21.32, 15q11.2, 21q22.3, 2p16.3, 2q13, 6q22.31, 8p12, 12q12, and 17p12 are shown in Table 3.

The CNVs in three patients, Nos. 7468, 7477, and 7333, on 14q11.2 involved *EDDM3A* and *EDDM3B*. Damyanova et al. (2013) reported that the loss of chr.14q11.2 can affect the proteins that are synthesized and secreted by epididymal epithelial cells that have been found to be up-regulated in the epididimis of non-obstructive azoospermia (NOA) men. In spite of the gains exhibited in our three patients, the CNVs may also affect the function of the genes and lead to azoospermia.

Patient Nos. 6035 and 5118 exhibited CNVs on 6p21.32. Hu et al. (2014) investigated 3608 NOA cases and 5909 controls to identify additional risk loci, and found that 6p21.32 as one of the strong evidence of the NOA susceptibility loci. The CNVs on 6p21.32 involved OMIM genes, *HLA-DRB1* and *HLA-DQA1*, which belong to human leukocyte antigen (*HLA*) class II genes. van

der Ven et al. (2000) reported that patients with male factor infertility differ in their *HLA* class II allele constitution from males with normozoospermia. It was suggested that genes identical to or located in close vicinity to *HLA* class II genes may influence spermatogenesis and male gamete function (van der Ven et al., 2000). Other studies have also reported that specific alleles and single nucleotide polymorphisms (SNPs) in the *HLA* region were associated with NOA. It is likely that variations in this *HLA* class II region might mediate the response to testicular microenvironmental antigens and cause testicular azoospermia through autoimmune inflammatory responses (Kurpisz et al., 2011; Zhao et al., 2012; Jinam et al., 2013). This may be an explanation for the azoospermia observed in two of the patients.

Table 3. Region, type, size, polymorphism status, and genes related to spermatogenesis or infertility for CNVs of all 27 patients.

Case No.	Phenotype	Region	Type	Size (bp)	Polymorphisms from DGV*	Gene symbols
7688 6035 4894	3xA	11p11.12	1xloss/2xgain	740K/580K/580K	Y	
7468 7477 7333	3xA	14q11.2	3xgain	960K/820K/980K	Y	*EDDM3A*, *EDDM3B*[14]
6035 5118	2xA	6p21.32	2xgain	260Kb/120K		*HLA-DRB1*, *HLA-DQA1*[16-19]
4894 8080	2xA	13q11	1xgain/1xloss	200K/60K		
5190 3849	1xA/1xO	15q11.1-11.2	2xgain	2.2M/2.38M	Y	*POTE B*[20-21], *GOLGA8C*[22]
8080 7050	1xA/1xOA	16p12.2	2xloss	320K/480K	Y	
8235 8557	2xA	21q22.3	1xgain/1xloss	540K/1.36M		*DNMT3L*[23]
7467	A	1p33	Loss	100K		
7524	A	1p36.21	Gain	180K		
5118	A	2p16.3	Gain	180K		*ALF*[24]
8004	OAT	2q13	Loss	140K		*NPHP1*[25-26]
4894	A	2q21.1	Loss	680K		
7937	A	2q36.1	Gain	820K		
7333	A	3p14.1	Gain	560K	Y	
7998	A	4q13.2	Gain	260K		
7647	A	5q11.1	Loss	80K		
8196	A	5q23.1	Loss	280K		
7468	A	5q35.3	Loss	40K	Y	
5107	A	6q22.31	Loss	100K		
7688	A	7p21.3	Loss	240K		
8064	A	7q31.1	Loss	180K	Y	
7516	A	8p12	Gain	540K		*NRG1*[27-28]
7467	A	8p22	Gain	320K		
6632	OA	9p21.1	Gain	440K	Y	
3849	A	10p11.1	Gain	680K		
8235	A	10q11.22	Gain	720K	Y	
8235	A	10q21.1	Loss	200K	Y	
7516	A	12q12	Loss	160K	Y	*RID2*, *ADAMTS20*, *TWF1*[29]
8196	A	12q14.1	Loss	260K	Y	
8033	OAT	14q24.3	Loss	200K		
8033	OAT	15q25.1	Loss	100K		
7523	A	17p12	Gain	440K		*COX10*[30-31], *MAK*[32], *DNEL1*[33]
7504	OT	22q11.21	Loss	360K	Y	

A = azoospermia; O = oligozoospermia; OA = oligoasthenospermia; OT = oligoteratozoospermia; OAT = oligoastenoteratozoospermia. *In the DGV database, there were noquery results for diseases of CNVs (polymorphisms) showing whole red or blue lines. This suggests that the CNVs (including gains and losses) are also found in the normal population and are likely not pathogenic.

Guediche et al. (2012) detected small supernumerary marker chromosomes in patients using array CGH to evaluate the size and the precise genomic constitution. *POTE B* (*POTE* ankyrin domain family (member B)), located on 15q11.2, is expressed in human testis, particularly in a specific cell type in primary spermatocytes (Ise et al., 2008). It is possible that *POTE* plays a role in human sperm maturation and its involvement in diverse regulatory pathways serves an important

signaling function in the reproductive system (Guediche et al., 2012). It has also been shown that *GOLGA8C* replication on the 15q11.2 region is associated with male infertility (Tu et al., 2013). The CNVs of patient Nos. 5190 and 3849, both with gains on 15q11.2, may result in an increase in replication leading to spermatogenic failure.

Huang et al. (2012) reported that the gene for DNA methyltransferase 3-like protein (*DNMT3L*) on 22q22.3 is essential for normal spermatogenesis and may be involved in spermatogenetic impairment and male infertility. They investigated allele, genotype, and haplotype frequencies of three SNP loci of *DNMT3L* to explore the possible association between *DNMT3L* and male infertility in 233 azoospermic males and 249 fertile controls. The results indicated that SNP rs2070565, as well as haplotypes AAA and GAA, may be associated with male infertility and that *DNMT3L* may contribute to azoospermia susceptibility in humans (Huang et al., 2012). In this study, two cases, Nos. 8235 and 8557, showed that the gain and loss of CNVs may lead to structural or functional changes of *DNMT3L*, resulting in azoospermia or oligozoospermia.

ALF on 2p16.3 is highly expressed in adult testis and is located in the spermatid nuclei and in the annulus of spermatozoa. Huang et al. (2006) identified *ALF* as a human spermatogenesis-related gene, and reported that the abnormal expression of *ALF* might be a partial cause of human infertility. The 180 k gain in CNVs observed in patient No. 5118 may affect normal expression of *ALF*, leading to azoospermia.

The CNVs involved in *NPHP1* occurred on 2q13. It has previously been shown that defects in *NPHP1* can lead to juvenile nephronophthisis type I, the most common genetic disorder causing end-stage renal failure in children and young adults (Hildebrandt et al., 1997). However, homozygous *NPHP1* mutant mice were viable with renal manifestations of nephronophthisis and also appeared normal except for OT in infertile males. These novel findings indicate that nephrocystin is critical for the differentiation of early elongating spermatids into spermatozoa.

The azoospermic patient No. 7516 had a CNV gain of 540 k on 8p12. Oral et al. (2008) identified *NRG1* (neuregulin 1) as a novel FSH-upregulated clone homologous to mouse. *NRG1* is known to control cell proliferation, differentiation, and survival in various tissues. Zhang et al. (2011) reported that *NRG1* promotes spermatogonial proliferation and initiates meiosis. It is assumed that the CNV on 8p12 involving *NRG1* does not display normal function during spermiogenesis.

On the 12q12 locus, there are three genes expressed in the testis: AT-Rich Interaction Domain-Containing Protein 2 (*ARID2*), A Disintegrin-Like and Metalloproteinase with Thrombospondin Type 1 Motif 20 (*ADAMTS20*), and Twinfilin Drosophila Homolog of 1 (*TWF1*) (Ghorbel et al., 2013). The CNVs of patient No. 7516 may lead to certain changes in genetic structure or function, which may affect gene expression in testicular tissues and lead to spermatogenesis impairment.

Three spermatogenic failure-related genes on 17p12 of patient No. 7523 were *COX10*, *MAK*, and *DNEL1*. *COX10* is expressed in multiple tissues with the highest expression occurring in the heart, skeletal muscle, and testis (Murakami et al., 1997). *In situ* hybridization confirmed that the mRNA expression of *COX10* was stronger in the spermatogenic cells of normal fertile testes than azoospermic testes. It was suggested that *COX10* may play a specific role in the development and progression of azoospermia (Yang et al., 2009). Expression of *MAK* was highly tissue-specific, and its transcripts were detected almost exclusively in testicular cells at the beginning and end of meiosis but hardly detectable in ovarian cells, including oocytes, after the dictyotene stage. These results suggest that *MAK* plays an important role in spermatogenesis (Matsushime et al., 1990). *DNEL1* is also expressed specifically in the testis, as determined by northern blot analysis of mRNA from 16 different tissues. The close similarity of *DNEL1* to the C-terminal part of the axonemal beta-

heavy chain may suggest an origin from a common progenitor gene, and the testis-specific pattern of expression indicates a possible role in sperm development and motility (Milisav et al., 1996). Further study is needed to determine if changes in expression of the above-mentioned genes result in azoospermia.

None of the described CNVs associated with spermatogenic failure in patients with chromosomal abnormalities and unexplained azoospermia were identified through high throughput genome-wide sequencing. In our present research, we provide evidence that CNVs contribute to the complex origin of male infertility and present a number of candidate genes that are potential risk factors for spermatogenic failure including *EDDM3A* and *EDDM3B* on 14q11.2, *HLA-DRB1* and *HLA-DQA1* on 6p21.32, *POTE B* and *GOLGA8C* on 15q11.2, *DNMT3L* on 21q22.3, *ALF* on 2p16.3, *NPHP1* on 2q13, *NRG1* on 8p12, *RID2*, *ADAMTS20* and *TWF1* on 12q12, *COX10*, *MAK*, and *DNEL1* on 17p12. However, further studies with larger sample sizes and different ethnic populations are necessary to confirm these findings, and further functional analyses are needed to elucidate the roles of these genes in pathological male infertility.

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