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Analysis of *CDK2* mutations in Chinese men with non-obstructive azoospermia who underwent testis biopsy

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KEY MESSAGE
The genotype and allele frequencies of four known single nucleotide polymorphisms in the *CDK2* gene, identified in Chinese men with non-obstructive azoospermia (NOA) who underwent testis biopsy, were similar in patients and controls. Mutations in the coding sequence of the *CDK2* gene may not, therefore, be responsible for idiopathic NOA.

A B S T R A C T

To examine whether mutations of the *CDK2* gene exist in Chinese men with non-obstructive azoospermia (NOA) with different histopathology, we recruited 175 Chinese men with idiopathic NOA who underwent testis biopsy, including hypospermatogenesis, germ cell maturation arrest and Sertoli cell only syndrome. Genomic DNA was extracted from peripheral blood samples. Subsequently, the seven exons of the *CDK2* gene were amplified using polymerase chain reaction with specific primers, respectively. The polymerase chain reaction products were sequenced on an automated sequencer. We identified four known single nucleotide polymorphisms: c.324G>A in exon 1; c.363T>C in exon 2; c.*570G>A; and c.*1160G>C in the 3' UTR of the *CDK2* gene. Comparison of the genotype and allele frequencies showed no significant differences between NOA cases and controls for the four single nucleotide polymorphisms. Furthermore, no significant differences were found between each pathological group and control group, respectively. The results indicate that mutations in the coding sequence of the *CDK2* gene may not be responsible for idiopathic NOA in Chinese men. Future studies in large cohorts of different ethnic populations are warranted to establish whether associations exist between the *CDK2* gene and NOA.

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Introduction

According to the World Health Organization, around one in 10 couples of reproductive age is unable to conceive (Ammar-Khodja et al., 2014). Among these infertile couples, about one-third could be attributed to male factors and an additional one-third to both male and female problems (Bhasin et al., 1994). Several causes of male infertility have previously been proposed, including endocrine disorders, environmental factors, spermatic duct obstruction, anti-sperm antibodies, cryptorchidism, testicular trauma, chromosomal abnormalities and hormone factors (Bonarriba et al., 2013).

Azoospermia, a lack of spermatozoa in the seminal fluid, is a type of male infertility. Incidence of azoospermia is 10–20% (Lee et al., 2011). Types of azoospermia include obstructive azoospermia and non-obstructive azoospermia (NOA) (Xu et al., 2017; Yafi and Zini, 2013). According to testicular biopsy, azoospermia can be categorized into several histopathologies. Hypospermatogenesis is a mild form of azoospermia, with a variable degree of germ-cell loss and spermatozoa in the seminiferous tubule. Germ-cell maturation arrest is a form of infertility, with a cessation at the stage of germ-cell formation. The most severe form of azoospermia is the Sertoli cell only syndrome, which is defined as the complete absence of germ cells in the seminiferous tubule (Alhalabi et al., 2013; Ammar-Khodja et al., 2014; Yatsenko et al., 2015).

Although idiopathic azoospermia remains unexplained (Aboutaleb et al., 2014; Giannouli et al., 2004), nearly 50% of idiopathic infertility cases are thought to have a genetic basis (Bozhedomov, 2016; O'Flynn et al., 2010). Numerous mouse models have linked hundreds of genes with azoospermia, but only a few studies have identified gene mutations in humans with idiopathic azoospermia, such as *TEX11*, *SYCP3*, *PRM1*, *PRM2*, and *NR5A1* (Abbas et al., 2014; Imken et al., 2009; Miyamoto et al., 2003; Niederberger, 2016; Ropke et al., 2013; Yatsenko et al., 2015; Zheng et al., 2012). Other genes that might be involved in idiopathic azoospermia remain to be determined. Furthermore, it has been reported that ethnic diversity exists in related genes that lead to spermatogenic failure, such as *ESR1*, *ESR2*, *eNOS* and *DAZL* (Ge et al., 2014; Krausz et al., 2015). For instance, a missense variant (rs121918346) in the *DAZL* gene was found to be significantly associated with spermatogenic failure in Chinese men, but not in other populations (Becherini et al., 2004; Chen et al., 2016).

The *CDK2* gene, which belongs to the Cyclin-dependent kinases (CDKs), has been reported to be critical for the mammalian cell cycle both at the G1 to S phase transition and throughout the S phase of gamatogenesis (Ashley et al., 2001; Baudat et al., 2000; Malumbres, 2005). Interestingly, *Cdk2*$^{-/-}$ mice did not have severe consequences for embryonic development, but both males and females were infertile (Lopes et al., 2013; Satyanarayana et al., 2008). *Cdk2*$^{-/-}$ male mice showed a reduced testis volume, and an apparent blocking of meiosis took place in prophase I that led to spermatocyte apoptosis and, consequently, the total absence of mature spermatids (Berthet et al., 2003; Sierant et al., 2015). In the present study, we investigated whether perturbations of the *CDK2* gene were present in Chinese idiopathic NOA patients with different histopathology.

Materials and methods

Participants

This study enrolled adult male patients with newly diagnosed NOA who had visited the Center for Reproductive Medicine, Shandong University, from January 2014 to December 2015. All patients were selected on the basis of an andrological examination that included medical history, physical examination, semen analysis, hormone analysis, scrotal ultrasound, karyotype testing, and Y chromosome microdeletion screening. Participants whose infertility was related to known causes or who had any relevant history that could account for their infertility (childhood disease, varicocele, cryptorchidism, environmental exposure, radiation exposure, prescribed drug use, chromosomal abnormalities, obstructive azoospermia, hypogonadotropic hypogonadism, recurrent infections, testis trauma, iatrogenic infertility, karyotype anomalies, or Y-chromosome microdeletions) were excluded in the study. On the basis of World Health Organization recommendations and standards (Shu et al., 2013), testicular biopsies were conducted in patients without available sperm after two or more inspections of semen. The participants comprised 175 Chinese men with NOA. Mean age was 28 ± 4.2 years. All samples were handled in accordance with the National Regulation of Clinical Sampling in China. Informed consent was obtained from all participants. The control group comprised 46 men who are from normal Han Chinese population in Beijing. The genotype and allele frequencies of the control group were obtained from Ensemble database (Http://asia.ensembl.org/index.html). The study was approved by the Institutional Review Board of Reproductive Medicine of Shandong University on 11 October 2014 (reference number 42).

Polymerase-chain-reaction and sequencing analysis

Genomic DNA of 175 patients was obtained from peripheral blood. The seven exons coding for *CDK2* were amplified using polymerase-chain-reaction (PCR) with seven pairs of primers (**Table 1**). Detailed information of PCR conditions is available upon request. The PCR products were first analysed by agarose gel electrophoresis and then sequenced on an automated sequencer (PRISM 310; Applied Biosystems).

Table 1 – The *CDK2* gene-specific primer sequences.

Primer identifier	primer sequences(5'-3')	Product size (bp)
Exon1-F	ACCAATAGAAAGGCCTGGGG	416
Exon1-R	GGAGTCCCGGGTACAGAGG	
Exon2,3-F	TCTTCTCACTTTCCTAGGGG	491
Exon2,3-R	CCCATGATGAGAGGAGCTGA	
Exon4-F	GCAAACCCAGTCTGC	271
Exon4-R	CTCTTGGGAAGCTCAGAGAAA	
Exon5-F	TACCCTATAAACCACCCCGC	205
Exon5-R	GTTCTTGGATGTGGGGAGGA	
Exon6-F	GTCAAGGTGGGTCTTGGTAT	267
Exon6-R	GAAAACAGGTGCCCACTCTC	
Exon7-1-F	CTGCTGCCCATTTAGTCCAC	633
Exon7-1-R	ACTCCTCCCAGTGGTTTTGT	
Exon7-2-F	GGGGCTAAGTTGGTGCTTTT	793
Exon7-2-R	TCTGTCTCCCCACCATTCTTTCA	

Statistical analysis

Data from Sanger sequencing were analysed with the use of Sequencher 4.9 software (Gene Codes Corporation, USA). The Statistical Package for Social Science for Windows (SPSS, version 20.0, IBM Corp., USA) was used for statistical analyses. Chi-squared test or Fisher's exact test was used when appropriate; $P < 0.05$ was considered statistically significant.

Results

The *CDK2* gene was sequenced in 175 patients who had idiopathic NOA, including 48 cases of hypospermatogenesis, 40 cases of maturation arrest, and 87 cases of Sertoli cell only syndrome. Four known single nucleotide polymorphisms (c.324G>A in exon 1, c.363T>C in exon 2, c.*570G>A and c.*1160G>C in 3' UTR) were identified. No novel mutations were found. Comparison of the allele and genotype frequencies between the idiopathic NOA cases and control population showed no statistically significant differences (**Table 2**).

Four single nucleotide polymorphisms (SNP) in the *CDK2* gene were then analysed between each histopathology group and control group, respectively; allele and genotype frequencies of the four SNP showed no significant differences (**Table 3**).

Discussion

The male gametogenesis includes differentiation of spermatogonia, the meiosis process of spermatocyte, and spermatogenesis, which continuously generates mature germ cells in large numbers from a small population of stem cells (Zhao et al., 2012a; Zheng et al., 2012). Mutations in many genes involved in this process could profoundly affect gametogenesis and lead to male infertility (Ashley et al., 2001; Niederberger, 2016; Vogt, 1997; Zhao et al., 2012b).

Meiosis is a critical stage of gametogenesis during which alignment and synapsis of chromosomal pairs occur, allowing for the recombination between maternal and paternal genomes (Adzhubei et al., 2010; Hamer et al., 2008; Hydbring et al., 2016). Therefore, the meiotic genes alternated are not typically tolerated in humans, and lead to variable degree of sterility. Despite many animal models suggesting meiotic arrest in infertility, little is known about meiotic defects in human gametogenesis. *CDK2* is essential for male meiosis, which is present in the prophase I and is critical for synaptonemal complex formation, chromosomal crossover, unpaired double-strand breaks and telomere function (Deans et al., 2006; Viera et al., 2009; Zhao et al., 2012a). Infertility in male *Cdk2*$^{-/-}$ mice is caused by meiotic arrest and azoospermia. Notably, *Cdk2*$^{-/-}$ spermatocytes only reach a pachytene-like stage of prophase I and meiotic defects prevent their passage through the pachytene checkpoint and lead to spermatocyte apoptosis (Deans et al., 2006; Mahadevaiah et al., 2001; Zhao et al., 2012a). These findings indicate that *CDK2* is a potential candidate gene for idiopathic azoospermia in human.

We analysed the *CDK2* gene in 175 Chinese patients with idiopathic NOA. No mutations were detected, and four known SNP were identified, but the genotype and allele frequencies showed no significant differences between idiopathic and control groups, which may be attributed to the small sample size and the limited statistical power. Our findings suggest that mutations in the coding region of the *CDK2* gene are not common in NOA, especially in maturation arrest histopathology, which shows the abnormal spermatogenesis in seminiferous tubule and arrest at the spermatocyte consistent with the *Cdk2*$^{-/-}$ mouse. Recently, a phenotype similar to *Cdk2*$^{-/-}$ mouse was shown in male mice that are homozygous for the *CDK2*^{Y15S} alteration, mimicking human rs3087335, which was reported to have a close association with male infertility and exist at a low frequency of 0.006 (Hu et al., 2014; Singh and Schimenti, 2015; Zhoucun et al., 2006). In the present study, however, we did not identify this missense variation in this study, which was also possibly caused by the small sample size. Furthermore, we analysed the four SNP in different histopathology groups compared with control group, respectively, and found no significant differences. Therefore, it is unlikely that these SNP are related to NOA, but a study powered to detect a smaller difference between men with NOA and control men may show an effect.

As far as we know, the present study is the first to investigate mutations of *CDK2* in a cohort with idiopathic NOA, and no causative

Table 2 – Allele and genotype frequencies of four single nucleotide polymorphisms in Chinese men with non-obstructive azoospermia (*n* = 175).

Location	dbSNP identifier	Sequence variation	Amino acid Variation	Allele	Allele frequency, n (%)[a]		Genotype	Genotype frequency n (%)[a]	
					NOA	Control[b]		NOA	Control[b]
Exon1	rs2069398	c.324G>A	Synonymous	G	348 (99.4)	92 (100)	GG	173 (99)	46 (100)
				A	2 (0.6)	0 (0)	GA	2 (1)	0 (0)
							AA	0 (0)	0 (0)
Exon2	rs568687267	c.363T>C	Synonymous	T	349 (99.7)	92 (100)	TT	174 (99.4)	46 (100)
				C	1 (0.3)	0 (0)	TC	1 (0.6)	0 (0)
							CC	0 (0)	0 (0)
3'UTR	rs2069415	c.*570G>A	–[c]	G	298 (85.1)	89 (85.9)	GG	127 (72.6)	35 (76.1)
				A	52 (14.9)	13 (14.1)	GA	44 (25.1)	9 (19.6)
							AA	4 (2.3)	2 (4.3)
3' UTR	rs1045435	c.*1160G>C	–[c]	G	344 (98.3)	91 (98.9)	GG	169 (96.6)	45 (97.8)
				C	6 (1.7)	1 (1.1)	GC	6 (3.4)	1 (2.2)
							CC	0 (0)	0 (0)

NOA, non-obstructive azoospermia; SNP, single nucleotide polymorphism.

[a] Chi-squared test or Fisher's exact test: $P > 0.05$.

[b] The allele and genotype frequencies of control group were obtained from Ensemble (http://asia.ensembl.org/index.html).

[c] Null.

Table 3 – Allele and genotype frequencies of four single nucleotide polymorphisms in Chinese men with NOA of different histopathology groups (n = 175).

Location	dbSNP ID	Allele	Allele frequency n (%)[a]				Genotype	Genotype frequency, n (%)[a]			
			Hypospermatogenesis n (%)	Germ cell maturation arrest	SCOS	Control[b]		Hypospermatogenesis	Germ cell maturation arrest	SCOS	Control[b]
Exon1	rs2069398	G	95 (99)	80 (100)	173 (99.4)	92 (100))	GG	47 (97.9)	40 (100)	86 (99)	46 (100)
		A	1 (1)	0 (0)	1 (0.6)	0 (0)	GA	1 (2.1)	0 (0)	1 (1)	0 (0)
							AA	0 (0)	0 (0)	0 (0)	0 (0)
Exon2	rs568687267	T	96 (100)	79 (98.8)	174 (100)	92 (100)	TT	48 (100)	39 (97.5)	87 (100)	46 (100)
		C	0 (0)	1 (1.3)	0 (0)	0 (0)	TC	0 (0)	1 (2.5)	0 (0)	0 (0)
							CC	0 (0)	0 (0)	0 (0)	0 (0)
3'UTR	rs2069415	G	87 (90.6)	71 (88.8)	140 (80.5)	79 (85.9)	GG	39 (81.3)	32 (80)	56 (64.4)	35 (76.1)
		A	9 (9.4)	9 (11.3)	34 (19.5)	13 (14.1)	GA	9 (18.8)	7 (17.5)	28 (32.2)	9 (19.6)
							AA	0 (0)	1 (2.5)	3 (3.4)	2 (4.3)
3' UTR	rs1045435	G	94 (97.9)	79 (98.8)	171 (98.3)	91 (98.9)	GG	46 (95.8)	39 (97.5)	84 (96.6)	45 (97.8)
		C	2 (2.1)	1 (1.3)	3 (1.7)	1 (1.1)	GC	2 (4.2)	1 (2.5)	3 (3.4)	1 (2.2)
							CC	0 (0)	0 (0)	0 (0)	0 (0)

NOA, non-obstructive azoospermia; SCOS, Sertoli cell only syndrome; SNP, single nucleotide polymorphism.

[a] Chi-squared test or Fisher's exact test. Hypospermatogenesis versus control, germ cell maturation arrest versus control; SCOS versus control; $P > 0.05$.

[b] The allele and genotype frequencies of control group were obtained from Ensemble (http://asia.ensembl.org/index.html).

variants were found. Our results indicate that mutations in the *CDK2* gene may not be responsible for idiopathic NOA in the Chinese population. Because of ethnic diversity, however, the exact role of *CDK2* in the pathogenesis of idiopathic azoopspermia needs to be further explored in other populations.

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