MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 2043-2048
DOI: 10.12659/MSM.915393

Received: 2019.01.27
Accepted: 2019.03.04
Published: 2019.03.19

Chloride Channel Accessory 4 (*CLCA4*) Gene Polymorphisms and Non-Obstructive Azoospermia: A Case-Control Study

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Source of support: This study was supported by the Science and Technology Funds of Education Department of Jilin Province, Peoples' Republic of China (JJKH20170846KJ)

Background: Genetic mechanisms are associated with male infertility, but the association with non-obstructive azoospermia (NOA) remains unclear. Mutations in the chloride channel accessory 4 (*CLCA4*) gene have been shown to have a role in male infertility. The aim of this study was to investigate the associations between single nucleotide polymorphisms (SNPs) of the *CLCA4* gene and NOA in a Chinese Han population of Northeast China using combined targeted gene capture next-generation sequencing and bioinformatics analysis.

Material/Methods: The study group included 100 men with NOA, and there were 100 normal controls. Targeted gene capture next-generation sequencing was performed combined with bioinformatics analysis. Ten *CLCA4* SNPs were screened in the cases of NOA and control subjects. The associations between SNPs and NOA were analyzed.

Results: Six SNPs, c.390C>T (rs190628533), c.1474A>G (rs2231599), c.2105C>G (rs757773924), c.2371A>T) (rs759981524), c.956G>A (rs763334876), and c.895T>C (rs79822589) were identified in the study group of cases in NOA but not in control subjects. All *CLCA4* SNPs were in Hardy-Weinberg equilibrium. The allele and genotype frequencies of the six SNPs were not significantly different between the study group and the controls. Haplotype analysis showed the existence of two haplotypes, CTAGACTACG and CTCGACTACG, which showed statistical significance of 0.074, and 0.088 between cases of NOA and the controls, respectively.

Conclusions: There were no significant associations between *CLCA4* SNPs and NOA in men in a Chinese Han population of Northeast China.

MeSH Keywords: Azoospermia • Infertility, Male • Polymorphism, Single Nucleotide

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915393

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Background

Genetic mechanisms are associated with male infertility, but the association with non-obstructive azoospermia (NOA) remains unclear [1]. Azoospermia is the most severe form of male infertility, in which genetic abnormalities have been identified in between 15–20% of cases [1]. Compared with obstructive azoospermia, patients with non-obstructive azoospermia (NOA) are less likely to be fertile. The genetic mechanism of NOA remains unclear, and has recently become a focus for research in reproductive medicine [2]. Currently, the genetic causes of azoospermia are known to include chromosomal abnormalities, azoospermia factor (AZF) microdeletion of the Y chromosome, copy number variations (CNVs), and monogenic mitochondrial and epigenetic abnormalities [3]. Recently, associations between gene polymorphisms and male infertility have been identified [4–7]. The relationships between additional genes and abnormal spermatogenesis require further investigation.

The chloride channel accessory 4 (*CLCA4*) gene (MIM: 616857) maps to a cluster of *CLCA* genes on chromosome 1p22.3 [8]. CLCA4 belongs to the calcium-dependent chloride channel family and modulates the basic defect in cystic fibrosis [9]. Kolbe et al. [10] also reported that the basic defect of cystic fibrosis was associated with markers that included the *CLCA4* gene promotor. Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene are known to cause cystic fibrosis and are also associated with male infertility [11]. Using DNA arrays to identify novel CNVs, Frühmesser et al. [12] found that *CLCA4* plays an important role in human spermatogenesis, and may act by mediating *CFTR* gene expression. Therefore, disrupted *CLCA4* function may play a role in male infertility. However, these results require further confirmation.

Therefore, the aim of this study was to investigate the associations between single nucleotide polymorphisms (SNPs) of the *CLCA4* gene and NOA in a Chinese Han population of men in Northeast China using combined targeted gene capture next-generation sequencing and bioinformatics analysis.

Material and Methods

Subjects

A case-control study included 100 subjects from the Center for Reproductive Medicine of the First Hospital, Jilin University, China, enrolled between January 2015 and December 2016. Patients were diagnosed with NOA by semen analysis and testicular fine needle aspiration cytology. One hundred control subjects were randomly selected from sperm donors at the Human Sperm Bank of Jilin Province between January 2015 and December 2016. All patients and controls were of Han ethnicity and were from Northeast China. Karyotype analysis and Y chromosome AZF microdeletion detection were performed for all cases, and those with chromosomal abnormalities or AZF microdeletions were excluded. Written informed consent was provided by each participant. The present study was approved by the Ethics Committee of the First Hospital of Jilin University (no. 2016-419, dated 10th Dec. 2016). All participants signed informed consent.

DNA extraction

Peripheral venous blood was collected from each participant into anticoagulant tubes containing ethylenediaminetetraacetic acid using standard protocols. Total DNA was extracted from blood using a blood genomic DNA extraction kit (TIANGEN Biotech Co., Ltd, Beijing) according to the manufacturer's instructions and then kept at –20°C until use.

Targeted gene capture sequencing

Targeted gene capture sequencing was performed by MyGenostics (Beijing, China). Genomic DNA samples were fragmented and prepared for standard Illumina library construction. Biotinylated capture probes were designed for the exons of *CLCA4* gene and then sequenced using Illumina HiSeq2000 Next-Generation Sequencing platform and bioinformatical analyses (MyGenostics, Beijing, China). Data analysis was performed according to MyGenostics protocols.

Statistical analysis

Clinical data were assessed using the t-test with SPSS software version 17.0 (IBM Corporation, Armonk, NY, USA). Assessment of the Hardy-Weinberg equilibrium (HWE) was determined using SHE software (*http://analysis.bio-x.cn*) for each single nucleotide polymorphism (SNP) of the CLCA4 gene in the cases and controls separately. The allele frequencies of *CLCA4* polymorphisms were compared using the chi-squared (χ^2) test and Fisher's exact test (two-sided). SNP genotype frequencies and dominant/recessive model analysis of cases and controls were calculated using logistic regression analysis. Haplotype analysis was conducted using SHE software (*http://analysis.bio-x.cn/SHEsisMain.htm*). Linkage disequilibrium analysis was performed using Haploview bioinformatics software (*http://www.broadinstitute.org/haploview/*). P-values <0.05 were considered as statistically significant.

Results

Subject characteristics

The clinical characteristics of the subjects are shown in Table 1. Clinical information includes age, body mass index (BMI), semen

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Table 1. Clinical characteristics of the study population.

Variables	Cases group (100)	Control group (100)	*P* value
Age(years)	29.14±4.40	25.10±5.68	<0.001*
BMI(kg/m^2)	25.51±4.17	22.79±3.70	<0.001*
Semen volume	2.84±1.37	3.66±1.26	<0.001*
Semen pH	7.48±0.32	7.50±0.03	0.537
Sperm concentration (10^6/mL)	0±0	62.95±6.49	NA

NA – not available; * *P*<0.05 has statistical significance.

Table 2. Distribution of *CLCA4* SNPs.

Number	SNV	Site	dbSNP code	n		OR (95%)	*P* value
				Case group	Control group		
1	c.2151T>A	1p22.3-87045065	rs185369520	0	2	–	0.999
2	c.390C>T	1p22.3-87025983	rs190628533	1	0	–	1
3	c.907A>C	1p22.3-87031656	rs2231592	20	32	0.583 (0.321–1.060)	0.077
4	c.1474A>G	1p22.3-87040229	rs2231599	2	0	–	0.999
5	c.2428G>C	1p22.3-87045696	rs2231604	0	2	–	0.999
6	c.2398C>A	1p22.3-87045666	rs539177280	1	3	0.330 (0.034–3.200)	0.339
7	c.2105C>G	1p22.3-87043738	rs757773924	1	0	–	1
8	c.2371A>T	1p22.3-87045639	rs759981524	2	0	–	0.999
9	c.956G>A	1p22.3-87033108	rs763334876	1	0	–	1
10	c.895T>C	1p22.3-87031644	rs79822589	1	0	–	1

volume, pH value, and sperm concentration. The study group was statistically older than the control group because for the control group we enrolled fertile men who were donating to the human sperm bank (P<0.001). BMI and semen volume were also significantly different between the two groups (P<0.001).

Genotype and frequency distribution

The genotype distribution of the *CLCA4* single nucleotide polymorphisms (SNPs) are shown in Table 2. In the study group, c.390C>T was present in one case, c.907A>C was present in 20 cases, c.1474A>G was present in two cases, c.2398C>A was present in one case, c.2105C>G was present in one case, c. 2371A>T was present in two cases, c.956G>A was present in one case, and c.895T>C was present in one case. In the control group, c.2151T>A was present in two cases, c.907A>C was present in 32 cases, c.2428G>C was present in two cases, and c.2398C>A was present in three cases. There was no significant difference in genotype distribution between the two groups (P>.05). However, six sites, c.390C>T (rs190628533), c.1474A>G (rs2231599), c.2105C>G (rs757773924), c.2371A>T (rs759981524), c.956G>A (rs763334876), and c.895T>C (rs79822589) appeared only in the study group, and their role in azoospermia requires further study.

Correlation analysis between SNPs and non-obstructive azoospermia (NOA)

This study classified the minimum genotype frequencies of the ten polymorphic *loci* and a Hardy-Weinberg equilibrium test was performed on the genotype distribution of each polymorphic locus. The results are shown in Table 3. There was no statistical difference between the two groups at any site (P>0.05). These results indicated that the *CLCA4* SNPs were not associated with the occurrence of NOA.

Genotype and the correlation with the dominant/recessive model and NOA

In the genotype analysis of the ten SNPs, the genotypes for each group were classified into dominant and recessive genotypes, and binary logistic regression analysis methods were applied. Neither the dominant nor the recessive model were associated with the occurrence of NOA. The results are shown

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Table 3. Correlation analysis between *CLCA4* and NOA.

SNV	dbSNP code	Case group (n=100)			Control group (n=100)			*P* value	P_{HWE}	
		het	hom	MAF%	het	hom	MAF%		Case group	Control group
c.2151T>A	rs185369520	0	0	0.00	2	0	1.00	0.156	1	0.919
c.390C>T	rs190628533	1	0	0.50	0	0	0.00	0.316	0.959	1
c.907A>C	rs2231592	18	1	10.00	28	2	16.00	0.074	1	0.677
c.1474A>G	rs2231599	2	0	1.00	0	0	0.00	0.156	0.919	1
c.2428G>C	rs2231604	0	0	0.00	2	0	1.00	0.156	1	0.919
c.2398C>A	rs539177280	1	0	0.50	3	0	1.50	0.315	0.959	0.878
c.2105C>G	rs757773924	1	0	0.50	0	0	0.00	0.316	0.959	1
c.2371A>T	rs759981524	2	0	1.00	0	0	0.00	0.156	0.919	1
c.956G>A	rs763334876	1	0	0.50	0	0	0.00	0.316	0.959	1
c.895T>C	rs79822589	1	0	0.50	0	0	0.00	0.316	0.959	1

Table 4. Genotypes and dominant/recessive model analysis of NOA-associated SNPs.

SNV	dbSNP code	Model	Genotypes	n		OR (95%)	*P* value
				Case group	Control group		
c.2151T>A	rs185369520	Dominant	AT/AA+TT	0/100	2/98	–	0.999
		Recessive	AA/AT+TT	–	–	NA	NA
c.390C>T	rs190628533	Dominant	TC/TT+CC	1/99	0/100	–	1
		Recessive	TT/TC+CC	–	–	NA	NA
c.907A>C	rs2231592	Dominant	CA/CC+AA	19/81	30/70	0.547 (0.284–1.056)	0.072
		Recessive	CC/CA+AA	1/99	2/98	0.495 (0.044–5.548)	0.568
c.1474A>G	rs2231599	Dominant	GA/GG+AA	2/98	0/100	–	0.999
		Recessive	GG/GA+AA	–	–	NA	NA
c.2428G>C	rs2231604	Dominant	CG/CC+GG	0/100	2/98	–	0.999
		Recessive	CC/CG+GG	–	–	NA	NA
c.2398C>A	rs539177280	Dominant	AC/AA+CC	1/99	3/97	0.327 (0.033–3.194)	0.336
		Recessive	AA/AC+CC	–	–	NA	NA
c.2105C>G	rs757773924	Dominant	GC/GG+CC	1/99	0/100	–	1
		Recessive	GG/GC+CC	–	–	NA	NA
c.2371A>T	rs759981524	Dominant	TA/TT+AA	2/98	0/100	–	0.999
		Recessive	TT/TA+AA	–	–	NA	NA
c.956G>A	rs763334876	Dominant	AG/AA+GG	1/99	0/100	–	1
		Recessive	AA/GA+GG	–	–	NA	NA
c.895T>C	rs79822589	Dominant	CT/CC+TT	1/99	0/100	–	1
		Recessive	CC/CT+TT	–	–	NA	NA

* Binary logistic regression analysis; $P<0.05$ has statistical significance.

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Table 5. Haplotype analysis of NOA-associated SNPs.

Haplotype	Frequency (%)		*P* value
	Case group	Control group	
BLOCK 1			
CTAGACTACG	0.900	0.840	0.074
CTCGACTACG	0.090	0.145	0.088

P<0.05 has statistical significance.

in Table 4, and there was no statistical difference between the two groups (P>0.05). These results indicate that there is no significant correlation between the dominant or recessive models of the genotypes of the ten SNPs and the occurrence of NOA.

Candidate SNP haplotype analysis

The ten candidate SNPs were distributed on chromosome 1. Haplotype analysis was performed using Haploview bioinformatics software and the results are shown in Table 5. The *CLCA4* SNPs, rs185369520, rs190628533, rs2231592, rs2231599, rs2231604, rs539177280, rs757773924, rs759981524, rs763334876 and rs79822589 form a haploid block; haplotypes were CTAGACTACG and CTCGACTACG. Haplotype frequency results are shown in Figure 1. There was no significant difference between the two groups for the two *CLCA4* haplotypes (P>0.005). This finding showed that haplotypes, CTAGACTACG and CTCGACTACG were not associated with NOA.

Discussion

Male infertility is a complex multifactorial condition, in which azoospermia is the most severe cause. Genetic factors are major contributors to non-obstructive azoospermia (NOA) [13]. Some studies have suggested that up to 2,300 genes are involved in spermatogenesis, and change in any one of these genes may lead to male infertility. These genes can provide targets for diagnostic tests of male factor infertility [14]. Next-generation sequencing technology has been applied to many genomic features of physiology and disease, including male infertility [15]. Targeted gene sequencing in cohorts of infertile men has identified several gene polymorphisms that are associated with male infertility [15]. This study explored the relationship between *CLCA4* polymorphisms and NOA in a Northeastern Han Chinese population.

In this study, ten *CLCA4* SNPs were examined in 100 infertile men and 100 fertile controls, and c.907A>C (rs2231592) and c.2398C>A (rs539177280) were detected in both the case and control groups. These two SNPs showed no significant association between infertile men and controls in the study cohort. Also, c.2151T>A (rs185369520) and c.2428G>C (rs2231604) were only detected in the control group. The other six SNPs were only detected in the study group. Further statistical analysis indicated that the SNPs are not associated with NOA occurrence. There was no significant correlation between dominant or recessive models of the SNPs and the occurrence of NOA. Using Haploview bioinformatics software to analyze the SNPs, haplotype was not associated with NOA. Together these results indicated that the candidates SNPs (under dominant and recessive models and haplotypes) are not related to the

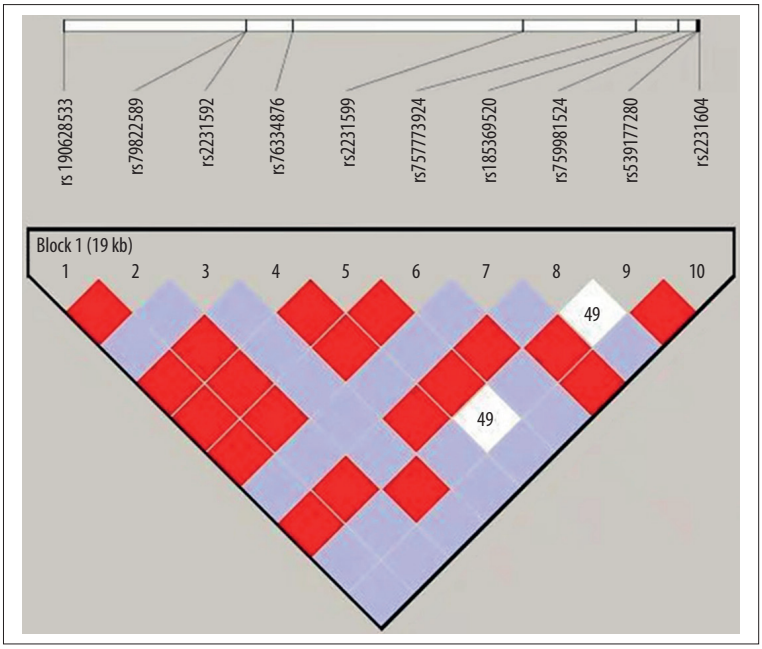

Figure 1. Haplotype analysis of non-obstructive azoospermia (NOA)-associated single nucleotide polymorphisms (SNPs) on chromosome 1.

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occurrence of NOA. This result is inconsistent with previous findings [12]. This discrepancy might be related to sample size, detection or analysis methods used, or, more likely, to the ethnicity of the study populations. Further study is required to understand the full significance of *CLCA4* in infertile men.

The major limitation of this study was the relatively small number of men included in the study with NOA. Also, this study only included a Han ethnic population from Northeast China. However, linkage disequilibrium structures associated with the tested markers are known to be different between ethnic groups [16].

Conclusions

This study showed no significant differences in genotype and allele frequencies or haplotype for *CLCA4* gene single nucleotide polymorphisms (SNPs) between the men with non-obstructive azoospermia (NOA) and healthy controls in a Chinese Han population from Northeast China. These findings indicate that the *CLCA4* gene is not involved in genetic susceptibility to infertility in Chinese men.

Conflict of interest

None.

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