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The Association between rs763763348 and rs190628533 SNPs in CLCA4 Gene and Azoospermia

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ABSTRACT

Background and Objective: Spermatogenesis is a complex phenomenon that is influenced by various genes. One of these genes that is likely to be effective in causing azoospermia is CLCA4. This study was conducted to investigate the association between rs763763348 and rs190628533 single nucleotide polymorphisms in CLCA4 gene and azoospermia in Iranian men.

Methods: In this cross-sectional study, blood samples were collected from 100 men suffering from non-obstructive azoospermia referred to Jihad Daneshgahi Infertility Treatment Center in Qom, as well as 100 fertile men who had at least one child and had healthy sperm test. The DNA of the samples was extracted by salting out method. Then, Tetra-primer ARMS PCR technique was used to check single nucleotide polymorphisms and the relationship between single nucleotide polymorphisms (SNPs) and male infertility was investigated.

Findings: The mean age of healthy people was 33.12±2.789 years and the mean age of patients was 32.54±2.571 years. The mean level of FSH hormone in healthy and sick subjects was 6.86±1.214 and 15.05±2.078, respectively ($p<0.0001$). The mean level of LH hormone in healthy people (4.12±1.04) and in sick people (11.44±1.54) showed a significant relationship ($p<0.0001$). In all studied subjects, the genotype of 100% of the subjects regarding rs190628533 was CC and the genotype of 100% of subjects regarding rs763334876 was GG. There was no significant difference between the healthy and sick groups in the evaluation of these two SNPs.

Conclusion: According to the results of this study, single nucleotide polymorphisms of rs190628533 and rs763334876 are not the cause of infertility due to azoospermia among Iranian men.

Keywords: *Azoospermia, Single Nucleotide Polymorphism, CLCA4 Gene, Male Infertility*.

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Introduction

Male infertility is a complex clinical condition that may occur due to various causes such as anatomical problems, infections, trauma, endocrine disorders, immune system problems, and genetic defects. So far, there have been many studies on the role of genetics in male infertility, and it is estimated that genetic factors cause 15-30% of male infertility (1). Infertility refers to the inability of couples to have children after at least one year of intercourse without using contraceptives (2). According to the report of the World Health Organization, infertility has affected about 80 million couples around the world (3) and 15% of the population of Iran is suffering from infertility (1), of which 50% are related to male causes (4). The main cause of infertility in men is a defect in spermatogenesis, which is divided into three general categories: azoospermia, oligospermia, and asthenospermia. In addition to lifestyle factors (5), genetic mutations and chromosomal abnormalities can be related to male infertility. Spermatogenesis or the process of producing male sex cells takes place as a result of mitosis and meiosis in the testes (6).

It seems that the disorder and mutation of any of the genes effective in spermatogenesis can cause male infertility. One of the main problems in infertile men is the decrease in normal sperm count or sperm motility. Currently, despite the importance of sperm motility in the reproductive process, there is limited information about the molecular mechanisms related to the structure and motility of sperm (1, 7). Several studies have shown that molecular methods can be used as a useful tool in the evaluation of spermatogenesis disorders in men with non-obstructive azoospermia. In addition, the effect of many autosomal genes on male infertility has been investigated (7-9). Arabi et al. in their study introduced the expression of SYCP3 (Synaptonemal Complex Protection 3) gene as a potential molecular marker for spermatogenesis; that's because the expression of this gene in the testis has a specific relationship with the development stages of spermatogenesis (10). It was also found that the development of GT repeats in the Heme-Oxygenase gene promoter is effective on the infertility of men with azoospermia and oligospermia (11).

According to the studies, the frequency of disomy for all chromosomes in infertile patients was higher than that of control subjects, and the amount of DNA damage in the sperms of infertile subjects was significantly higher than that of normal subjects (12). In addition, considering the effect of gamma rays on the genomic instability of azoospermia factor (AZF) regions in men with azoospermia and oligospermia, it was found that the frequency of genomic instability in the samples of infertile men was significantly higher than that of fertile men (13). Based on the experiments, Yatsenko et al. concluded that the main cause of meiotic arrest and azoospermia in infertile men is homozygous TEX11 (Testis Expressed 11) mutations, and almost half of infertility cases in men are related to genetic defects (14). In 2018, for the first time, a study was conducted about the TUSC1 (Tumor Suppressor Candidate 1) gene and its single nucleotide polymorphisms on male infertility in the Hutterite population in America. In this study, scientists found that rs12348 in the TUSC1 gene is associated with azoospermia and oligospermia (5).

CLCA4 gene is located in chromosomal region 1p22.3. It has 15 exons and its size is 33681 bp, and three transcripts are made from it. This gene acts as a tumor suppressor and is involved in cancers through the PI3K/AKT signaling pathway (15-18). This gene is a regulator of the chloride channel and is activated by calcium, and as a result, it is related to the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene (17). It should be noted that the CFTR gene located on chromosome 7 is very common among

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European populations and is known as the main cause of non-obstructive azoospermia (19). According to the studies conducted and the relationship between the CLCA4 gene and other genes and different physiological functions, two SNPs related to this gene were selected to be investigated in healthy individuals and patients with azoospermia, and if this change shows a significant difference, the association between azoospermia and the two SNPs will be determined. The reason for choosing two SNPs is due to an article by Wang et al. (20) and their relationship with azoospermia, as well as not being investigated in the Iranian population. Another goal is to determine the difference in the frequency of CC, CT, and TT genotypes and C and T alleles in the rs190628533 two SNP in two healthy and azoospermia groups, and to determine the frequency of GG, GA, and AA genotypes and G and A alleles in rs763334876 in both healthy and azoospermia groups.

Methods

This cross-sectional study was approved by the ethics committee of Islamic Azad University, Qom branch with code IR.IAU.QOM.REC.1399.026 and written consent was obtained from the participants. The study was conducted on 100 men with non-obstructive azoospermia and 100 healthy men as control group from among those who referred to the Jihad Daneshgahi Infertility Treatment Center in Qom in 2018-2019. The healthy group included people who became father without using assisted reproductive methods and with at least one healthy child. Patients are people who have been approved by a urologist. They did not have anatomical abnormalities of the reproductive system, testicular neoplasms, numerical and structural chromosomal abnormalities, or Y chromosome microdeletions (YCMDs).

After the diagnosis of non-obstructive azoospermia by a urologist at the Jihad Daneshgahi Infertility Treatment Center in Qom, a questionnaire containing demographic information, sex hormone levels, body mass index, kin relationships between parents, and history of smoking in relatives was prepared. In order to determine the genotype of the studied subjects, 3 cc of blood was collected from them and placed in tubes containing EDTA. The collected samples were transferred to the laboratory and stored at -20 °C. DNA extraction from blood was done using salting out method. Blood samples were lysed with EDTA and routine steps were performed which included the addition of SDS and protease, 6 M salt and chloroform, as well as DNA ethanol precipitation, and finally, DNA was dissolved in 50 λ deionized distilled water and stored at -20 °C. For quantitative and qualitative evaluation of the extracted DNA, nanodrop and electrophoresis devices were used, respectively. Finally, in the DNA samples, the purest DNA samples were selected by measuring the absorbance ratio of 260/280 nm and also the absorbance ratio of 260/230 nm and matched with the qualitative electrophoresis bonds.

In this research, the Tetra-Primer ARMS PCR method was used to determine the genotype of SNPs. This method requires two outer primers and two allele-specific inner primers to determine the single nucleotide polymorphism genotype. Considering that the above-mentioned method uses 4 primers in one reaction, both allele-specific markers can be examined simultaneously. For this reason, the desired FASTA sequence was first extracted from the NCBI website, and primers were designed based on PRIMER1 database: primer design for tetra-primer ARMS-PCR (Table 1).

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Table 1. The sequence of the four primers used for rs190628533 and rs763334876 SNPs

SNPs	Number of primer	Type of primer	Sequence of the primers
rs190628533	1	Inner forward primer (T allele)	CAGAATGTGGAGAGAAAGGCGAAGAT
	2	Inner reverse primer (C allele)	GTAGAAGGTCAGGGGTGAAGTGACTG
	3	Outer forward primer (3'-5')	TTTTCTGATATTAACCATTTTTGCCACAAA
	4	Outer reverse primer (3'-5')	GAGCCAGGATTTGATTCTAGGGACTTT
rs763334876	1	Inner forward primer (G allele)	TTTTTCTTGTCTTTTTAATCTAGGGATCAG
	2	Inner reverse primer (A allele)	CTTGATTCATTCGATTTAGGCGGGCT
	3	Outer forward primer (3'-5')	TATACGTGTCCCATAATACACACCACCA
	4	Outer reverse primer (3'-5')	TAGGTAATCCTGCCATGAGTGTGTTTCT

To perform PCR, the level of mixed materials and PCR steps for two SNPs was as follows:

- In the amplification of rs190628533 SNP, to identify nucleotide T, 10 µL Taq DNA Polymerase Master Mix RED, primers 1, 3, and 4 in amounts of 1, 1, and 2 µL, respectively, 3 µL DNA, and 3 µL distilled water were used.
- In order to identify C nucleotide, exactly the same materials and amounts were used; but primer number 2 was used instead of primer number 1.
- In the amplification of rs763334876 SNP, in order to identify the nucleotide G, 10 µL Taq DNA Polymerase Master Mix RED, primers 1, 3 and 4 in amounts of 0.75, 0.75 and 1.5 µL, respectively, 3 µL DNA, and 4 µL distilled water were used.
- In order to identify nucleotide A, exactly the same materials and quantities were used; however, primer number 2 was used instead of primer number 1, and the amounts of primers 2, 3 and 4 were 0.75, 1.5 and 0.75 µL, respectively. After preparing the mixture, all the vials were spun for a few seconds.

PCR program for rs190628533 SNP was performed in 3 minutes at 95 °C, (30 seconds at 93 °C, 40 seconds at 57 °C, 40 seconds at 72 °C) in 35 cycles, 5 minutes at 72 °C and for rs763334876 SNP, the steps were the same as the previous SNP, but initial denaturation was increased for 5 minutes, the PCR cycle was reduced to 33 cycles, and the bonding step was performed at a temperature of 59 °C for 45 seconds. After PCR, to conduct electrophoresis and observe the amplified DNA, the DNA bonds were observed using a transilluminator.

SPSS software was used to perform statistical tests. The Kolmogorov-Smirnov test was used to determine whether or not a quality was normal. If the quality was normal, the independent t test and if it was not normal, chi-square and Mann-Whitney U tests were used for analysis, and $p<0.05$ was considered significant.

Results

The mean age of the examined samples in this study in healthy people was 33.12±2.789 years and in the age range of 27 to 36 years. Furthermore, the mean age of the patients was 32.54±2.571 years and in the age range of 29 to 36 years.

The mean level of FSH hormone in healthy people was 6.89±1.214 and in sick people was 15.05±2.078, and the evaluations showed that the mean difference observed in the level of FSH hormone between healthy

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people and sick people was significant (p<0.0001). The mean level of LH hormone was 4.12±1.04 in healthy people and 11.44±1.54 in sick people, and the observed mean difference was significant (p<0.0001) and in the group of infertile men, the level of this hormone was significantly higher. The observed mean difference in testosterone levels in healthy subjects (4.23±1.144) and patients (4.91±1.029) showed no significant relationship (p=0.752). The aforementioned hormones were measured based on mIU/ml.

In the evaluation of the rs190628533 SNP, based on the bonds seen in the electrophoresis, all healthy and sick people had CC genotype and no other genotypes were observed (Figure 1 A), and in the evaluation of the rs763334876 SNP, all the people had the GG genotype (Figure 1 B).

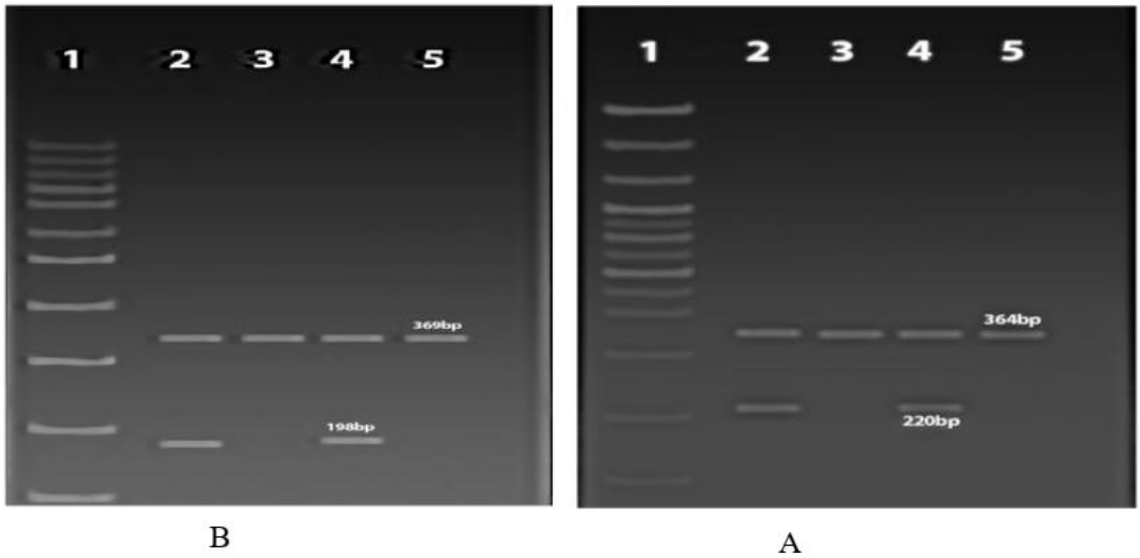

Figure 1. A) 1: 100bp marker, 2 and 3: GG homozygous person, 4 and 5: GG homozygous person and B) 1: 100bp marker, 2 and 3: CC homozygous person, 4 and 5: CC homozygous person.

Discussion

According to the results obtained in this study, only CC genotype was observed in rs190628533 SNP. Moreover, only the GG genotype was observed in the rs763334876 SNP and both cases, on the NCBI website and the section related to the Allele Frequency Aggregator (ALFA), were consistent and identical with the populations studied in other countries and are the same (21) (Figure 2).

CLCA4 gene is located in chromosomal region 1q21.3 and is involved in the Wnt/B-Catenin signaling pathway as an activator. This gene is a regulator of the chloride channel that is activated by calcium and was expected to be effective in causing azoospermia and sperm disorder due to its connection with the CFTR gene (17).

In this study, the frequency of C allele was 100% in both groups of healthy and sick people in the rs190628533 SNP, and T allele was not observed in the population of healthy and sick people. In addition, in the other SNP (rs763334876), 100% frequency in both healthy and sick people was related to the G allele, and the A allele was not observed in the studied population at all. Therefore, 100% genotypic frequency in all healthy and sick people in rs190628533 and rs763334876 SNPs was related to CC and GG, respectively, and the expected genotypes, i.e., TT and TC genotypes in rs190628533 SNP, and AA and GA genotypes in

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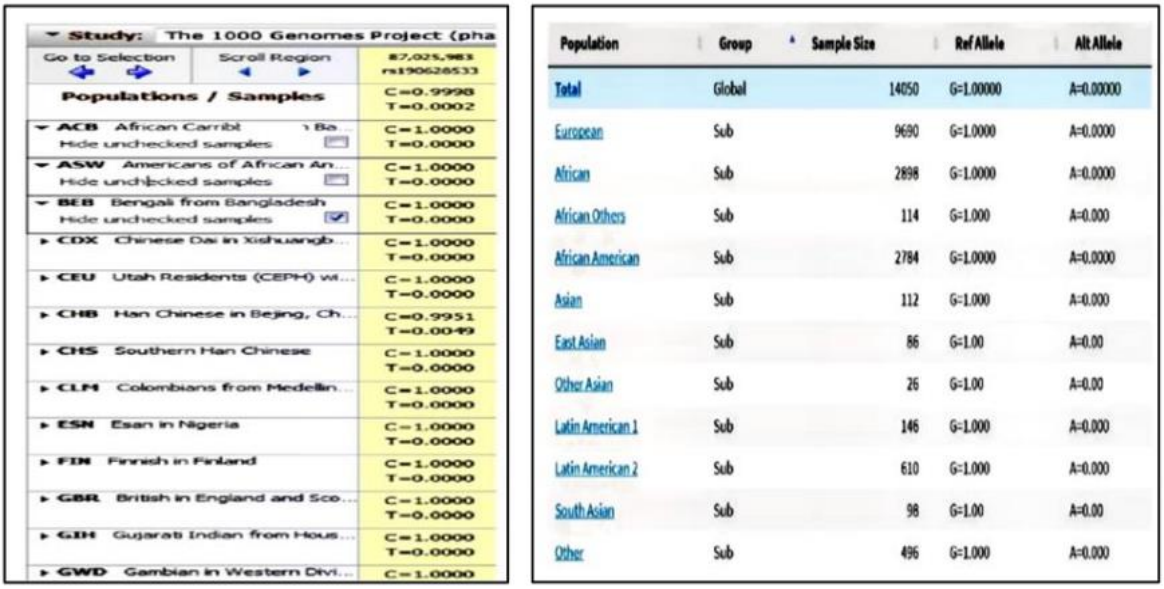
rs763334876 SNP, were not observed in the studied populations. Based on comparative evaluations, the frequency of genotypes studied in this research is consistent with the populations studied in other countries (20-22).



B A

Figure 2. A) frequency of rs190628533 alleles and B) frequency of rs763334876 alleles in studied populations in the world (taken from NCBI website)

Overall, new technologies that analyze the influence of genetics from a global perspective may lead to further advances in understanding the cause of male infertility through the identification of specific infertility phenotypes (1). In this regard, examining each specific gene and SNP can be very helpful. In previous studies, the roles of the CLCA4 gene have been investigated, as Frühmesser et al. showed that CLCA4 plays an important role in human spermatogenesis and may act through CFTR gene expression. Hence, CLCA4 dysfunction may play a role in male infertility. CFTR protein is made of a polypeptide sequence with a length of 1480 amino acids, which finally forms a transmembrane protein. Mutation in the CFTR gene is the cause of cystic fibrosis. There is a very common mutation (5T allele) in this gene, which does not cause cystic fibrosis, but along with other genetic causes, it can be the cause of congenital bilateral absence of the vas deferens (CBAVD) and consequently infertility (23). In another study, the loss of CLCA4 and epithelial-mesenchymal transition in breast cancer cells eventually lead to breast cancer (16). Furthermore, Chen et al. determined that this gene inhibits epithelial-mesenchymal transition through PI3K/AKT signaling, preventing cell proliferation and metastasis in hepatocellular carcinoma (17). Types of CLCA4 gene variants in the development of cystic fibrosis have also been identified by Yu et al. (18). All the above studies confirm the possible effect of CLCA4 gene on infertility. According to the evaluations conducted in the present study in the mentioned gene, there is no correlation between these two selected SNPs (rs190628533 and rs190628533) and azoospermia in men referred to the Jihad Daneshgahi Infertility Treatment Center in Qom. In other studies, lack of association between gene polymorphisms and male infertility has been shown. In a study by Wang et al., 6 polymorphisms c.390C>T (rs190628533),

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c.1474A>G (rs2231599), c.2105C>G (rs757773924), c.2371A>T (rs759981524), c.956G>A (rs763334876) and c.895T>C (rs79822589) were investigated in North China, and it was found that there is no significant difference between them and azoospermia (20).

Similar to the results of the present study, Siasi et al. investigated the two SNPs rs551373 and rs683155 in the DDX25 gene in patients with azoospermia. This research also showed that the two polymorphisms mentioned in the studied population cannot be considered as genetic factors in infertility among Iranian men (11). In the northeastern region of Iran, the effect of other genes such as methionine synthase (MTR) on male infertility was investigated and it was found that there is no significant difference in the frequency of the relevant alleles between sick and healthy people (22).

Although a lot of work still needs to be done to fully determine the involvement of genes in the production of infertility phenotypes, the findings of this research show that in the studied population, there is no significant difference between the two SNPs rs190628533 and rs763334876 in the CLCA4 gene and male infertility. However, differences may be shown in the studies of other SNPs of this gene or in a larger society or other ethnicities. In general, more comprehensive investigations are suggested in this area.

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