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International Journal of Recent Scientific Research
Vol. 5, Issue, 6, pp. 1222-1225, June, 2014

International Journal of Recent Scientific Research

ISSN: 0976-3031

RESEARCH ARTICLE

STUDY OF ASSOCIATION BETWEEN 4 DIFFERENT SNPS LOCATED ON *CHDH* GENE WITH AZOOSPERMIA MALE INFERTILITY IN IRANIAN POPULATION

Narjes Bakhtari, Masoud Sheidai, Hamid Gourabi, Zahra Noormohammadi, Esmat Ghalkhani, and Ali Mohammad Malekasgar

[1,2,4] Biology Department, Shahid Beheshti University, Tehran, Iran
[3]Department of Biology, School of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
[5]Department of Medical Science, Genetic Unit, Qom University of Medical Sciences, Iran

ARTICLE INFO

Article History:

Received 11th, May, 2014
Received in revised form 16th, May, 2014
Accepted 14th, June, 2014
Published online 28th, June, 2014

Key words:

Infertility. Azoospermia .Choline Dehydrogenase. Genetic diversity
.

ABSTRACT

Purpose A case-control population study was performed to investigatie the association of the SNPs (rs*: 12676*), (*rs: 9001*), (*rs: 200569248*) and (*rs: 78371839*) located on *CHDH* gene with azoospermiamale infertility in Iranian population.

Methods Two separated population of Center and North of Iran (In Royan Institute) were collected. DNA was extracted from each samples through salting-out method. A 345bp segment of the gene was amplified using PCR. All samples were genotyped using direct sequencing. Chi-squared test and Odds Ratio were recruited to check the association of the SNPs with azoospermia, and to investigate the genetic difference in populations AMOVA was performed.

Result There was no significant genetic difference between our populations, which meant they were almost genetically similar, so we merged data from both populations in one single group. We also find a positive association between SNPs (*rs: 12676*) and (*rs: 9001*) and azoospermia infertility but no association was found in SNPs (*rs: 200569248*) and (*rs: 78371839*) with azoospermia male infertility.

Conclusion Positive association probably reveals an important role of this gene in male infertility. Further studies are needed to support the obtained result. It is important to know if such an association plays a key role in male infertility so probably with a Betaine rich diet treatment we can overcome to the *CHDH* enzyme defects.

INTRODUCTION

Infertility is a condition in which a couple cannot conceive after 12 months of attempt [1, 2].About 50% of infertility problems are associated to the male factor [3]. Genetic factors leading to infertility are: chromosomal abnormalities, monogenetic and multifactorial defects as well as genetic endocrine failure [4]. Another major cause of infertility is the impact of destructive environmental factors [5-7].

Methyl folate, methionine and choline are the main source of methyl groups in our diet. [8, 9]. Metabolisms of these micro-nutritions play an essential role in the formation of the S-adenosyl-methionine (SAM), as the general donor of the methyl group. [10]. Key enzymes in methionine metabolism pathway are: phosphatidyl choline N-methyl transferase (PEMT), choline dehydrogenase (*CHDH*), betaine-homocysteine methyl transferase (BHMT) [11].

CHDH enzyme catalyses the oxidation of choline to betaine using an intermediate molecule called betaine aldehyde [10]. Histological studies revealed that *CHDH* gene deletion caused a high reducing of betaine. It was shown that *CHDH* is expressed highly in testis, liver and kidney [11]. Dietary sources of betaine include wheat, shellfish, spinach and sugar beets [12, 13]. In addition, betaine can be made de novo via the oxidation of choline in a series of reactions catalyzed by *CHDH* and betaine aldehyde dehydrogenase (BADH, EC 1.2.1.8) [14-19].

Sperm Mitochondria is located in the mid piece. Lack of the *CHDH* enzyme, which is located generally in the inner membrane of mitochondria, can lead to the abnormal shape of it according to the electron microscope studies. In fact, it causes reducing in ATP concentration in mitochondria of the testis [11].

In the present study, molecular diversity of *rs: 12676* (G674T), a functional non-synonymous SNP results in the replacement of arginine, a polar, hydrophilic amino acid at position 78 with leucine, The *CHDH* minor T allele is associated with increased susceptibility to developing clinical symptoms of dietary choline deficiency (steatosis and muscle cell damage) [20] as well as increased risk of breast cancer [21], also SNP, *rs: 9001* (A560C) a non-synonymous SNP leads to substitution of glutamate to alanine, a change from medium-size and acidic (E) to small size and hydrophobic (A) at position 40, *rs:78371839* (C661T) a non-synonymous SNP results in changing of proline to serine and finally *rs:200569248*(C673T) which leads to change.

* Corresponding author: **Narjes Bakhtari**
Department of Biology Department, Shahid Beheshti University, Tehran, Iran

Phenylalanine a non-polar amino acid to leucine a polar amino acid which all may result changing in *CHDH* structure and function [22].

In this study, we present data that show a positive association between SNPs (*rs: 12676*) and (*rs: 9001*) and azoospermia male infertility in our population. We also tried to investigate the relationship between the patients' geographical population and their molecular diversity by using multivariate statistical methods.

The main network that *CHDH* takes part in, is also displayed [22], with finding other enzymes, which act along with *CHDH*, maybe we can have other cases to investigate their functional association with male infertility or other disease.

MATERIALS AND METHODS

Ethics statement

The study design and all procedures used in this study were approved by the Shahid Beheshti University of Iran. Subjects were at least 25 years of age and were recruited from the Center and North regions of Iran. Informed written consent was obtained from all subjects at the initial clinic visit.

Design of study

Subjects were azoospermia men and they were all undergone through the semen analysis and karyotyping by Royan Institute of Iran. We chose the subjects with normal karyotype. They finally were screened to determine the frequency of studied SNPs in the target populations and allowed for enrichment of the homozygous variant genotypes.

Sample collection and DNA isolation

In order to screen subjects for the *rs12676*, blood samples were collected into EDTA Tubes. All tubes were stored on ice if not processed immediately; DNA of all blood samples was extracted by Salting-Out method. DNA quality and concentration was determined using a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

SNPs genotyping

SNPs genotype was determined by direct sequencing. A 345 bp fragment of the *CHDH* gene containing four mentioned SNPs was PCR amplified using Taq DNA polymerase (Bioron, Germany) .The primers used for amplification were: *CHDH* forward 5'-ATTCCCCTCCGTGGATCAG-3' and *CHDH* reverse 5'-TGTCGTCGCACAGGTTGG-3', designed using Oligo5.0 software. Each 20 µL reaction contained 400 ng of genomic DNA, with primers at a final concentration of 14.72 ng/µl, and 1 µl of 5u/µl of Taq DNA Polymerase, 2 µl of 10X buffer, 0.4 µl of 100mM $MgCl_2$ as well as 0.5 µl of 40mM dNTP-Mix.

The PCR conditions were: an initial denaturing step at 98℃ for 6 minutes followed by 40 cycles of denaturing at 98℃ for 30 seconds. Annealing was done at 66℃ for 30 seconds, while primary extension was done at 72℃ for 45 seconds. The final extension was performed at 72℃ for 5 minutes. PCR cycling was performed by using the Applied Biosystems2720 Thermal Cycler. The resulting DNA concentration was determined by using Nanodrop. *CHDH* fragments were purified and then sequenced in Macrogen Company in Korea, using the *CHDH* forward primer. *SNPs* genotype was screened by examining sequencing chromatograms through Sequence Scanner software (version 1.0, Applied Biosystems, Carlsbad, CA).

Statistical analyses

Chi-squared test using SPSS Version 19.0 (2010) software was recruited to investigate the association of each SNP with azoospermia male infertility. Then Odds Ratio, with 95% confidence intervals (CIs) was carried out via SPSS Version 19.0 software. *P*-values = 0.05 was taken as statistically significant.

In order to find out the genetic differences between the studied population, we used AMOVA (Analysis of Molecular Variance) after 1000 permutations by GenAlex 6.4, [23].

RESULT

In this study, 50 cases and 20 fertile controls were genotyped. The average ages of controls and azoospermia infertile males were at least 25 years. We considered ethnical features and collected the samples from two geographical regions of Iran (North and South provinces). Distributions of Genotype frequencies for each SNP are listed in tables1to 4 respectively.

Table 1 Genotype frequencies of SNP (*rs12676*) in the *CHDH* gene

Genotype	Azoospermia N=50	Controls N= 20	Total 70N=
GG	39(78%)	18(90%)	57(81.43%)
GT	8(16%)	2(10%)	10(14.29%)
TT	3(6%)	0 (0%)	3(4.28%)

Table 2 Genotype frequencies of SNP (*rs: 9001*) in the *CHDH* gene

Genotype	Azoospermia N=50	Controls N= 20	Total 70N=
AA	40(80%)	18(90%)	58 (82.86%)
AC	7(14%)	2(10%)	9 (12.86%)
CC	3(6%)	0 (0%)	3(4.28%)

Table 3 Genotype frequencies of SNP (*rs:78371839*)in the *CHDH* gene

Genotype	Azoospermia N=50	Controls N= 20	Total 70N=
CC	45(90%)	19(95%)	64(91.43%)
CT	4(8%)	1(5%)	5(7.14%)
TT	1(2%)	0 (0%)	1(1.43%)

Table 4 Genotype frequencies of SNP (*rs:200569248*) in the *CHDH* gene

Genotype	Azoospermia N=50	Controls N= 20	Total 70N=
CC	44(88%)	19(95%)	63(90%)
CT	4(8%)	1(5%)	5(7.14%)
TT	2(4%)	0 (0%)	2(2.86%)

Paradigms of sequencing for each SNP are shown in (Figs.1, 2, 3).

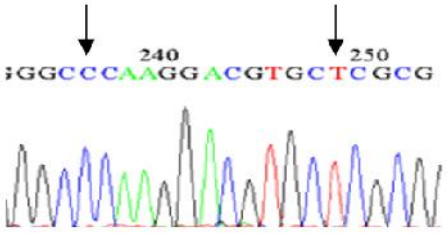

Fig 1 rs: 12676(T) mutated rs: 200569248(C) wild type

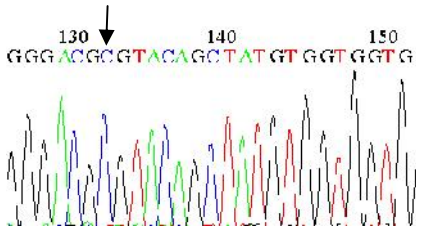

Fig 2 mutated sample of rs: 9001(C)

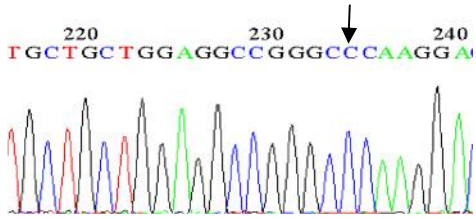

Fig 3 rs:78371839 (C) wild type.

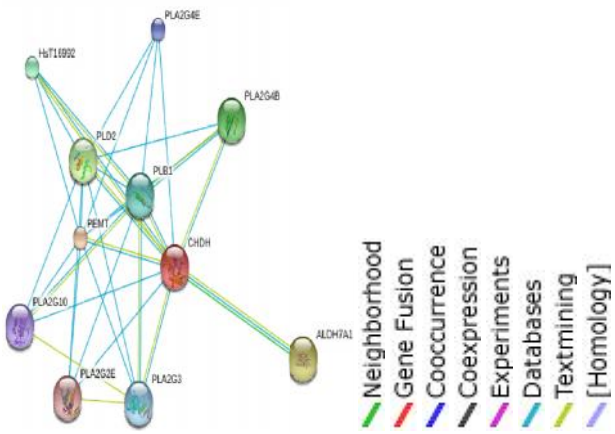

Fig 4 CHDH (596 aa), *Homo sapiens*, network [22]

Table 5 Predicted Functional Partners of CHDH protein [22]

Partner	Descriptions	Score
PEMT	Phosphatidylethanolamine N-methyltransferase; Catalyzes three sequential methylation of phosphatidylethanolamine (PE) by AdoMet, thereby producing phosphatidylcholine (PC). (236 aa)	0.916
ALDH7A1	Aldehyde dehydrogenase 7 family, member A1Multifunctional enzyme mediating important protective effects. Metabolizes betaine aldehyde to betaine, an important cellular osmolyte and methyl donor. (539 aa)	0.909
PLD2	Phospholipase D2; May have a role in signal-induced cytoskeletal regulation and/or endocytosis (933 aa)	0.826
PLA2G4B	Phospholipase A2, group IVB (cytosolic) (781 aa)	0.804
HsT16992	JMJD7-PLA2G4B readthrough; Calcium-dependent phospholipase A2 that is a selectively hydrolyse. (1012 aa)	0.803
PLB1	Phospholipase B1; Membrane-associated phospholipase. Exhibits a calcium- independent broad substrate specificity including phospholipase A2/lysophospholipase activity. (1458 aa)	0.803
PLA2G3	Phospholipase A2, group III; PA2 catalyzes the calcium-dependent hydrolysis of the 2- acyl groups in 3-sn-phosphoglycerides. Shows an 11-fold preference for phosphatidylglycerol over phosphatidylcholine (PC). (509 aa)	0.801
PLA2G4E	Phospholipase A2, group IVE; Calcium-dependent phospholipase A2 that selectively hydrolyzes glyglycerophospholipids in the sn-2 position. (839 aa)	0.800
PLA2G10	Phospholipase A2, group X; PA2 catalyzes the calcium-dependent hydrolysis of the 2- acyl groups in 3-sn-phosphoglycerides.(165 aa)	0.800
PLA2G2E	Phospholipase A2, group IIE; PA2 catalyzes the calcium-dependent hydrolysis of the 2- acyl groups in 3-sn-phosphoglycerides. (142 aa)	0.800

In order to check genetic diversity, we performed AMOVA test in both populations. This analysis produced Fst = -0.117 (p = 0.43) for the samples studied from Central Iran and Fst =-0.143 (p = 0.71) for the samples studied from North of Iran.

Both values are not significant and indicate minor genetic difference between these samples, so we decided to merge the data from both pop. To one single group as Iranian Population.

Chi-squared test showed significant difference between cases and controls in (*rs: 12676*) with (χ^2 = 5.357, df = 1, P = 0.021). We also found positive association in (*rs: 9001*) with azoospermia in our study with (χ^2 = 3.922, df = 1, P = 0.048) .

No positive association was found neither in (*rs: 78371839*) with (χ^2 = 1.802, df = 1, P = 0.179) nor in (*rs: 200569248*) with (χ^2 = 3.150, df = 1, P = 0.076) in our study.

Odd Ratio was also calculated for the SNPs with positive association with following results: 2.538in *rs: 12676*, while it was 2.250 in *rs: 9001*.

At the end, we displayed the network which *CHDH* enzyme takes part in fig.4, from data presented in SWISS-Prot database [22]. Extra information about *CHDH* enzyme is located briefly in table 5.

It shows partners of *CHDH* in this network, considering this, we can suggest they are probably other good cases to be studied on such matter, but it definitely needs more studies to find out their exact role in this subject.

DISCUSSION

It has been reported *CHDH* gene knockout in male mice causes to male infertility because of sperm motility disruption. Further studies in humans were relatively the same. SNP *rs12676* is associated with sperm motility pattern, abnormal changes in sub-structure of sperm mitochondria and a remarkable reducing in sperm ATP concentration [24]. Homozygote and heterozygote individuals for this SNP have less *CHDH* enzyme concentration in their sperm than normal genotypes. It has been shown that the TT and GT genotypes are related to very low and low sperm motility respectively, but the GG plays a conversed role [24]. (G674T) *rs12676* is a non-synonymous polymorphism located in nucleotide 674 of exon 3 in *CHDH*. Substitution of T allele with G causes the change of Arginine, a hydrophilic amino acid, to Leucine, a hydrophobic amino acid, at position 78. In this study, we wanted to investigate the association between this SNP within azoospermia male infertility in Iranian population.

We also found there is another functional SNP (*rs: 9001*) located on *CHDH*, which has been located on nucleotide 560 and leads to substitution of glutamate to alanine, a change from medium-size and acidic (E) to small size and hydrophobic (A) at position 40, which seems to have an important role in *CHDH* activity (22) so we also tried to check the association between this SNP with azoospermia in our population.

After knowing that there are another two non-synonymous SNPs *rs: 78371839* (C661T) SNP which results in changing

proline to serine and *rs:200569248*(C673T) which leads to change Phenylalanine a non-polar aminoacid to leusine a polar amino acid, next to these two SNPs we planned to amplify a 345bp fragment of exon3 of *CHDH* genecontaining these four SNPs to investigate the association between all mention SNPs and azoospermia at the same time .

Both populations were genetically homogenous according to AMOVA, so we merged their results. Considering that azoospermia is a condition which caused by a lot of factors in which sperm production failed in patients, so positive association probably reveals an important role of this gene in male infertility .

With respect to the knowledge that most of the association studies vary among different populations, the following results are related to Iranian population .

Further studies are needed to support the obtained result. It is important to know if such an association plays a key role in male infertility so probably with a Betaine rich diet treatment we can overcome to the *CHDH* enzyme defects.

Acknowledgment

We would like to thank Shahid Beheshti University to support us handling of this study. We appreciate Dr. Mohseni and Mrs. Mokhtari as well as the staff on Royan Institute of Tehran who helped us through collecting the samples. We also thank the subjects for participating in our study.

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