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Association the Study of Between Cpeb1 (Rs230846 C>T) Gene Polymorphism and Azoospermia/Severe Oligospermia

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ABSTRACT

BACKGROUND AND OBJECTIVE: : *CPEB1* gene plays a significant role during gametogenesis. Due to remain unclear many causes of male infertility, we aimed to evaluate the association between of *CPEB1* rs2303846 gene polymorphism with the risk of men with idiopathic azoospermia/ severe oligospermia.

METHODS: The present study is a case-control investigation, were performed on 100 peripheral blood samples of men with idiopathic azoospermia/ severe oligozoospermia and 100 blood samples of healthy men, who were referred to department of infertility and sterility of Tabriz Al-Zahra hospital from 2015 to 2017. The PCR-RFLP method was used to determine the frequency of genotypes and then compared the relationship between polymorphism and clinical parameters.

FINDINGS: The genotypes frequency of *CEBP1* gene polymorphism CT+TT/CC did not show a statistically significant difference between groups (P=0.395, OR=1.273; CI=0.730-2.220). In addition, no significant correlation was found between genotypes and FSC, MSC and SMI clinical parameters ($p<0.05$).

CONCLUSION: Findings revealed that *CEBP1* rs2303846 gene polymorphism cannot to be considered as a risk factor for idiopathic azoospermia/ severe oligospermia men.

KEY WORDS: *Azoospermia, Male Infertility, Severe oligospermia*.

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Introduction

About 13-18% of couples have a fertility problem, half of which is related to men. The cause of male infertility in 40% of cases is known and in 60% of cases it cannot be justified in terms of pathology. Therefore, infertility treatment in men is more difficult than women (1). In general, the causes of male infertility are classified into three categories: genetic, non-genetic and infectious (1 and 2). The most important genetic factors include Klein filter syndrome, xx male, Noon's syndrome, Y chromosome microdeletions, mitochondrial DNA mutations, single gene defects, abnormal expression of non-coding RNA, and multifactorial defects (3-3). It is estimated that about 2,000 genes are involved in controlling this process, most of them on autosomal chromosomes and about 30 genes located on the chromosome Y (9). Despite all the above mentioned factors, more than 15-10% of male infertility remains unknown, and so far no cause has been identified, which is known as male idiopathic infertility (10, 11).

Azoospermia and oligospermia, caused by genetic variation, constitute an important part of the causes of male infertility. Azoospermia is one of the most common manifestations of infertility, accounting for 10 to 15 percent of infertility. Studies have shown that genetic factors are responsible for one third of azoospermia cases (12, 13). The relationship between genetic polymorphism and infertility is one of the most important and useful topics (14-17). Recent studies have shown that the presence of a gene polymorphism (rs2303846) CPEB is a predisposing risk factor for azoospermia. The CPEB gene encodes an alpha subunit of glycoprotein hormone, which forms a functional hormone with the beta units of gonadotropins and thyroid stimulating hormone and attaches to the receptors. This gene has a wide distribution in tissues and is expressed in a variety of tissues, including testicles, ovaries, placental cells and fatty tissues (18). The CPEB gene encodes an important protein that is a member of the Cytoplasmic Pyladenylation Element Binding Protein (CPEB) family, a protein product that has a specific sequence playing a role in regulating the translation process in ovulation in vertebrates. This protein has a function in the cytoplasm and nucleus and may play an important role in cell proliferation and tumor formation (19). The CPEB1 gene is located on the chromosome 15q25.2 and has 14 exons, the product of which is a completely protected protein. CPEB proteins cause oocyte growth and follicular development with attachment to several oocytes mRNAs, including Smad1, Smad5, Spindlin, Bub1b, MOS, H1foo, Obox1, Dnmt1o, TiParp, Trim61, and Gdf9. So far, only two studies have reported the role of CPEB1 polymorphism in male infertility (18 , 21). In the first study by Zhang et al., the presence of a polymorphism in the 3'-UTR region of the CPEB1 gene was reported as a risk factor for male infertility in the Chinese population (18). In addition, YadollahyKhaless et al. showed that the prevalence of this polymorphism in the studied groups is significantly different (21). Due to differences in the abundance of alleles in different ethnicities and races, this study was conducted for the first time only on the Azeri breeding specialty in Iran. On the other hand, considering that only the results of small studies cannot be considered to confirm the association of a polymorphism with a particular disorder, the aim of this study was to examine the relationship between CPEB1 polymorphism (rs2303846) and the risk of men with severe azoospermia / oligospermia with unknown causes in patients referred to al-Zahra hospital in Tabriz.

Methods

This case-control study was performed on 100 peripheral blood samples of men with severe azoospermia / oligospermia and 100 peripheral blood samples of healthy men (having a child, normal fertility, lack of family history of infertility, and normal sperm count), which was referred to the infertility department of Al-Zahra Hospital in Tabriz for treatment during the years 2015-2017. Determining the sample size using the Cochran formula, taking into account p = 0.1 (relative to the population with a certain attribute), the standard value of the standard unit in the 95% confidence interval (Z = 1.96) and the tolerable error rate (d=0.04) were estimated 200 samples (100 samples of fertile men and

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100 samples of infertile azosperm/ severe oligospermic male) with unknown causes. Infertile men reported in the urological, infertility and medical genetics evaluations as azoospermic/oligospermic with unknown cause and based on spermogram results, sperm count was less than 5 million/ml of semen and was considered as a cytogenetic abnormality by the G-Banding method and molecular experiments were evaluated for the chromosome Y microdeletion in AZO (Azoospermia Factor) areas, and no specific cases were reported, were selected. In cases where the cause of infertility was mentioned in the patient records, they were excluded from the study. In the healthy group, the fertile men with a child, normal fertility and without family history of infertility with normal spermogram results were selected. After obtaining consent from the subjects and in full compliance with the ethical standards, 2 ml of peripheral blood samples were taken in vials containing EDTA with an abbreviated code without knowledge of the person's identity and genomic DNA was obtained using the Salting-Out method (22). The nucleotide sequence of the primers was designed with Primer 3 software and after BLAST, the specificity of designed primers was evaluated on NCBI website. The gene sequence (Accession number: NM_001079533) was imported in NEB cutter software to select a specific restriction enzyme and finally the specific restriction enzyme RsaI (18) was determined (Table 1). The genomic DNA content and purity were determined using Nano drop device (Thermo Scientific, USA). The PCR-RFLP technique was used to evaluate the polymorphism of CPEB1 gene. In order to amplify the specific area, 1 ul (ng50) DNA, 1 pmole forward and reverse primers, 13 ul of the Master Mix Red 2x (Ampliqon, Denmark) and 9 ul distilled water in a final volume of 25 ul were mixed. Thermocycler (Eppendorf, Germany) was used for PCR reaction. Amplification was repeated with a denaturing step at 95 ºC for 8 minutes, then 32 cycle at 95 ºC for 30 seconds, the annealing temperature 57/7 ºC for 30 seconds and 72 ºC for 60 seconds. Finally, amplification was carried out at a temperature of 72 ° C for 8 minutes. Before enzyme digestion, amplified products were loaded on1% agarose gel and were stained with Safe Stain (Sina-genes, Iran). To determine the frequency of gene polymorphism genotypes of CPEB1, PCR product was digested with restriction enzyme Rsa I (Fermentas, Germany) according to the manufacturer's protocol. To this end, 10 µl of PCR product, 2 µl of Buffer10x, 1 µl of enzyme and 17 µl of distilled water were mixed and incubated for 6 h at 37 C. The fragments resulted from enzymatic digestion were separated with 4% agarose gel and identified by Safe Stain. The results of the evaluation of healthy and patient groups for genotypic frequency were analyzed using SPSS software version 25 and chi-square test. T-test was used for comparing clinical parameters with genotype type and $p < 0.05$ was considered significant.

Table 1. Sequence of primers used for amplification region gene polymorphism (rs2303846) CPEB1 (18).

Polymorphism of *CPEB1 gene*	(5′ → 3′) Primer sequence	Annealing tempreture (°c)	Product size (base pair)	Restriction enzyme
rs2303846	Forward: 5′–TGGCAGGTCAGGCAAGCAGC–3′ Reverse: 5′–GCAGAAACAAAGACAGATTCAGCAAG–3′	57.7	119	Rsa I

Results

The mean age of the healthy subjects was 43±6.22 years (ranging from 23-63 years) and the patient group was 42 ± 5.43 years (ranging from 25- 59 years). There was no significant difference between the two groups. There was expected three types of genotypes consist of (healthy homozygote) CC, (heterozygote) CT and (mutated homozygote) TT (Table 2). After enzymatic digestion, the length of parts for healthy homozygote (CC) 119 bp, for heterozygote (CT) 119, 24 and 95 bp, and for mutant homozygote (TT) 95 and 24 bp were obtained. Genotypic frequency was evaluated for patients and healthy subjects in three codominant, dominant and recessive heritages. The frequency of

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genotypes was not significantly different from the two groups in the hereditary codominant pattern (p=0.395, CI= 0/73-2/22, OR/273) (Table 3).

Table 2. Frequency of polymorphic genotypes of CPEB1 gene (rs230846 C> T) in healthy and patient groups

Group	Healthy N(%)	Healthy N(%)	P-value
healthy homozygote (CC)	22(49)	23(51)	
heterozygote (CT)	44(47)	50(53)	0.546
mutated homozygote (TT)	34(56)	27(44)	

A picture of gel electrophoresis of 119-bp amplified PCR products for the CPEB1 gene was shown in Fig. 1. Additionally, a sample of the gel electrophoresis image from the PCR-RFLP reaction was shown in Fig. 2. In this image, column 1 indicates a person with mutant homozygote genotype (TT), column 2 indicates a person with a healthy homozygote genotype (CC), and a third column indicates a person with a heterozygote genotype (CT). In general, there was no significant difference in genotypic abundance between the evaluated groups in any of the inherited patterns of codominant, dominant and recessive (Table 3). Also, while comparing the frequency of carriers (heterozygotes) with non-carriers, there was no significant difference between the two groups in the hereditary codominant pattern (p=0.886; CI= 0/486-1/834, OR=0.494). Additionally, other clinical parameters including the functional growth sperm concentration (FSC), motile sperm concentration (MSC), movement, morphology and sperm motility index (SMI) there was no significant correlation between genotypes in different groups of patients with different genotypes (Table 4).

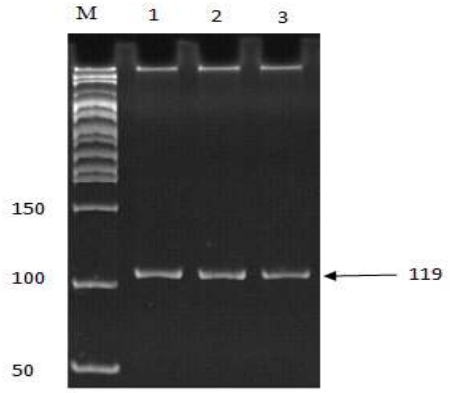

Figure 1. PCR results for polymorphism of CEBP1 gene in 1% agarose gel. M: molecular weight index (50 bp), Columns 1, 2, and 3: amplified product of 119 pairs of CEBP1 gene.

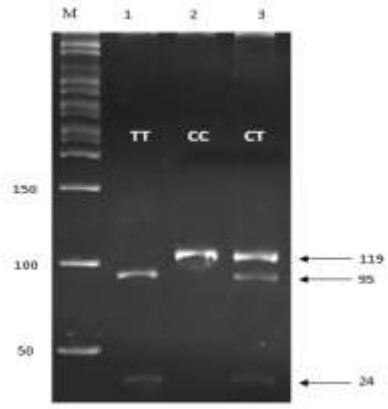

Figure 2. PCR-RFLP results for CEBP1 gene polymorphism in agarose gel 4%. M: 50-bp molecular weight index (50 bp), column 1: mutant homozygote (TT), column 2: healthy homozygote (CC), column 3: heterozygote (CT).

Table 3. Comparison of CPEB1 polymorphism in healthy men and patient groups in different inherited patterns referred to the infertility department of Alzahra hospital in Tabriz during 2015-2017

gene polymorphism of *CPEB1* (rs230846 C>T) pattern		group patient N(%)	group healthy N(%)	OR	CI-95% high	CI-95% low	P-value
dominant	CC	۲۳ (۵۱)	۲۲ (۴۹)	۱/۰۵۹	۱/۰۵۷	۰/۵۴۵	۰/۸۶۶
	CT+TT	۷۷ (۴۹)	۷۸ (۵۱)				
codominant	CT	۵۰ (۵۳)	۴۴ (۴۷)	۱/۲۷۳	۲/۲۲۰	۰/۷۳۰	۰/۳۹۵
	CC+TT	۵۰ (۴۷)	۵۶ (۵۳)				
recessive	TT	۲۷ (۴۴)	۳۴ (۵۶)	۰/۳۹۲	۲/۲۸۲	۱/۳۱۵	۰/۷۱۸

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Table 4. Relationship between clinical parameters and polymorphic genotypes of CPEB1 (rs230846 C> T) in patients

Clinical parameters		Polymorphism of *CPEB1* (rs230846 C>T)			P-value
		CC	CT	TT	
Age (year)	50 ≤	26	34	12	0.634
	50 >	9	12	7	
SMI	50 ≤	14	22	9	0.850
	50 >	18	18	8	
	I	15	20	6	
FSC	II	15	21	9	0.814
	III	5	5	4	
MSC	1-5	14	22	9	0.850
	6-10	18	18	8	

* Statistical analysis with t-test was a significant level of p <0.05; FSC; Functional Sperm Concentration; MSC; Motile Sperm Concentration, SMI; Sperm Motility Index

Discussion

The results of this study, unlike previous evaluations, showed no significant correlation between CPEB1 gene polymorphism and increased risk of men with severe azoospermia / unexplained cause. Studies have shown that the presence of polymorphism in the CPEB1 gene results in changes in miRNA binding positions and increases the risk of complex genetic diseases such as infertility in males (18). This gene is important as a regulator in the translation process in cells (23).

The results of studies by Tay et al. showed that, during the suppression of the CPEB1 gene, the size of the gonads in mice was reduced and thus, the gametes were less produced than healthy mice. Decrease in production of gametes was due to a break in the evolutionary pathway during the pachytene phase of meiosis I. The poly-A tail regulation in the translation process of the mRNA molecules involved in the formation of the proteins involved in the synaptone-complex is via the CPEB1 protein (23). The protein CPEB1 plays a role in the stability of many of the mRNA molecules involved in the gametogenesis process (24,25).

A study by Zhang and colleagues on 449 azoospermic or oligospermic men and 357 healthy men showed that the presence of a polymorphic region in the 3'-UTR region of CPEB1 gene has a significant relationship with the risk of infertility in the Chinese population. In this study, using miRanda and Targetscan tools was showed that the presence of this polymorphism could affect the quality of miR-663 and miR-668 binding (18). However, the results of this study, contrary to the previous report, did not reveal a correlation between this polymorphism and the risk of male infertility. In a study by YadollahyKhaless and colleagues, 70 healthy men and 70 infertile men (azoosperm / oligosperm) referred to the Qom Infertility Center reported a significant difference in the frequency of CC and TT genotypes among the two groups (21). In our research, the most frequent were heterozygote genotypes (CT), but in the study of YadollahyKhaless et al., the frequency of heterozygotes was reported to be zero and genotypic frequency in different races (Kurdish, Lor, Arabs, Gilaki, Fars and Turk) was reported uniform and homogeneous and did not show any significant difference. Such a conclusion must be made with a greater number of people of different ethnicities and races. In the present study, which was performed on 100 men with severe azoospermia/ oligospermia with an Azari Turk race, results were shown to be quite contradictory. In justifying these differences and the importance of evaluating it in male infertility it can be said that the distribution of alleles in different geographical regions is likely to be different between the results of studies on this polymorphism. It may also be probable that differences in race, Ethnicity and genetic background of the various individuals is

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important. In addition, different criteria considered in selecting the patients and healthy groups can lead to differences in the results of the evaluations. Such differences can sometimes be due to the errors caused by sampling and analyzing data.

The characteristics of this study were that in the selection of patient samples, inclusion and exclusion criteria were performed with high accuracy and high sensitivity and frequency of rs2303846 polymorphism in different hereditary patterns was evaluated. In any of the patterns, there was no significant difference in the frequency of genotypes between the two groups. In addition, in this evaluation, the relationship between each of the polymorphic rs2303846 genotypes with clinical parameters such as age, FSC, MSC and SMI was analyzed in infertile men, which there was no significant difference in any of these indices. During bioinformatics assessments, it was shown that as a result of replacing the T allele with C in the rs2303846 polymorphism of the CPEB1 gene, miRNA regulating molecules may be less effective in binding to the region of the polymorphic presence of the mRNA molecules derived from the CPEB1 gene and lead to continued gene expression. So that after playing the role of the CPEB1 gene, its expression must be inhibited, despite the presence of the mutated allele T, this inhibitory function will be stopped and the consequences of it will be negative effects that appear during spermatogenesis (25). In order to confirm the reported results, more studies are needed, and in populations of different races and ethnicities, and the genetic nature of a particular disorder cannot be ascribed to the results of several reports of few studies in particular populations. The study showed that the presence of CPEB1 gene polymorphisms could not justify the potential for severe azoospermia / oligospermia and infertility in men in the population. As a result, it may be argued that the evaluation of this polymorphism cannot be considered as a biomarker in identifying the causes of infertility in men. However, in order to confirm the results of this polymorphism with male infertility, it needs to be reconsidered in populations of different races and geographic regions.

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