

Determination of CAPN11 and UBQLN3 gene expression in men with Non-Obstructive Azoospermia

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ABSTRACT

Genetic disorders play an important role in male infertility, especially in Non-Obstructive Azoospermia (NOA). Many studies have been performed on the expression profiles of gonadal genes in order to accurately identify the genes involved in impaired spermatogenesis. In the current study, testicular biopsy, hormonal assay, and Real-Time PCR were done to check hormonal and gene expression levels of the CAPN11 and UBQLN3 genes in the testicular biopsy samples of 30 NOA and 30 Obstructive Azoospermia (OA) patients. The Real-time PCR showed that the expression of these genes decreased in azoospermia patients. The hormonal evaluation showed that FSH and LH significantly increased in NOA men and there was no difference in testosterone level between the two groups. Due to the obtained results of this study, CAPN11 and UBQLN3 can introduce as two biomarkers of NOA.

Keywords: CAPN11, gene expression, infertility, UBQLN3, unnatural azoospermia

INTRODUCTION

Infertility refers to cases in which couples fail to conceive after one year of unprotected sexual intercourse. Infertility can cause serious emotional problems for

families, affecting one in seven people. About 15 % of couples worldwide can be impacted by infertility, and half of the cases are related to men [1]. One of the types of male infertility, which has been studied today, is azoospermia [2].

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Azoospermia in medicine is referred to a male whose semen has no sperm or in other words sperm count is zero. In this case, nothing can be detected in the appearance of semen because only one percent of semen is sperm [3]. Up to 20 % of male infertility cases and about 1 % of the male population are affected by azoospermia [4]. Azoospermia is generally divided into two types: obstructive, which can be congenital or acquired and non-obstructive, which can be congenital, acquired, or genetic [4,5]. Spermatogenesis is normal in Obstructive Azoospermia (OA) but a barrier or mechanical disturbance prevents sperm from reaching the ejaculatory duct, while spermatogenesis in Non-Obstructive Azoospermia (NOA) is stopped or severely impaired [5].

Azoospermia with a genetic background classified as an idiopathic case and accounts for a significant proportion of male infertility [6]. The knowledge on genetic causes of azoospermia is scarce, because there are limitations in evaluating gene function in *in vivo* models and accessing testicular tissues. Despite all the recent advances, the molecular etiology of azoospermia (which is complex) has not yet been satisfactorily identified and more research is needed. The routine tests to

Key genes in non-obstructive azoospermia diagnose infertility include hormone evaluation, semen analysis, and cytogenetic test, which can be supplemented by Y-micro deletion and CFTR gene mutation analysis [7]. Besides, the identification of pivotal spermatogenesis genes seems to be helpful in finding potential molecular biomarkers.

In recent years, many studies have been performed on gonad gene expression profiles to precisely identify genes involved in impaired spermatogenesis. The general analysis of transcriptome through microarray has identified the impaired genes [8–10]. Hitherto studies have been limited to elucidating the molecular background of NOA development. In 2003, Malcher *et al.* reported several genes that were down-regulated in infertile men compared to normal men by using microarray and Real-time PCR analyses. These genes included SPATA3, GGN, CAPN11, UBQLN3, SPACA4, AKAP4, and FAM71F1 [7].

The Ubiquitin-Proteasome Pathway (UPP) plays a major role in spermatogenesis [11]. Ubiquilins are proteins that have an amino-terminal UBL domain and a carboxy-terminal Ubiquitin-Associated (UBA) domain in their structure. So far, seven

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ubiquitins have been identified in vertebrates including UBQLN1, UBQLN2, UBQLN3, UBQLN4, UBQLN5, UBQLN6, and UBQLNL. Among the ubiquitin proteins, UBQLN3, UBQLN5, and UBQLN-like (UBQLNL) are testis-specific and have the highest level in the adult testes [12]. The UBQLN3 gene is located on 11p15.4 and is functionally linked to the ubiquitin-proteasome system [13]. The main expression of UBQLN3 is related to the elongation phase of spermatids and starts at postnatal day 28 [14]. The UBQLN3 gene sequence is similar to XDRP1 in *Xenopus*, which is involved in cell cycle regulation by cyclins A1 and A2, so low expression of UBQLN3 in infertile men may be related to spermatogenic failure [15]. Another gene reported by Malcher et al. is CAPN11 that located on 6p12. CAPN11 is a member of the Calpains' superfamily consisting of eight intracellular calcium-dependent cysteine proteases. The Calpains family is ubiquitously expressed in mammals and has also been shown to be involved in spermatogenesis [16,17].

To date, studies have been conducted on differential gene expression in control and infertility groups. FAM71F1, UBQLN3, GGN, CAPN11, SPATA3 and SPACA4

Key genes in non-obstructive azoospermia can be mentioned among the genes that are down regulated in testicular tissues of infertile men with NOA. Therefore, in the present study, the expression level of UBQLN3 and CAPN11 genes in mRNA levels was assessed using the quantitative Real-time PCR (RT-PCR) technique in testicular biopsy samples of NOA groups, which underwent Testicular Sperm Extraction (TESE) surgery.

MATERIALS AND METHODS

Testicular Biopsies

Testicular biopsy specimens were collected from 30 infertile men, 28 to 45 years old, who underwent Testicular Sperm Extraction (TESE) surgery. After pathological evaluation, specimens were divided into two groups NOA and OA (control group). This study was approved by the Ethics Committee of Tehran Azad University for research on human subjects (approval reference no: IR. IAU. PS. REC. 1398.043) and informed written consent was obtained from all patients. Clinical examination of infertile patients was performed according to the guidelines of World Health Organization (WHO) [18] and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

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During a standard infertility workup including histopathological evaluation of the testis, testicular biopsy specimens were dissected into three small pieces (1–2 mm³ each one) under a dissecting microscope (Zeiss, Germany) and collected in RNA later (Ambion Life Technologies)

Hormonal assay

After venous collection of blood samples and serum prepared, levels of LH, FSH, and testosterone were assessed. The concentrations were determined in each of the two NOA and control groups. According to the manufacturer's instructions, the concentrations were determined using the ELISA technique in Cobas e411 (Roche, Germany).

The extraction of RNA and complementary DNA (cDNA) synthesis

RNA extraction and cDNA synthesis from testicular biopsy samples were carried out by GeneALL kits (Dongnam-ro, songpa-gu, Seoul, Korea) according to the manufacturer's instruction and kept at –80 °C until use. The Real-Time PCR technique was employed to evaluate the expression levels of UBQLN3 and CAPN11 genes in testicular biopsy samples. The sequences and qualities of the UBQLN3, CAPN1, and GAPDH (as internal control) primers are

Key genes in non-obstructive azoospermia presented in Table 1 and steps are summarized in Table 2. GAPDH was used to normalize the expression of UBQLN3 and CAPN11 genes. Finally, the 2– $\Delta\Delta C_t$ (cycle threshold) method was used to calculate fold changes and determine the relative expression profiles of UBQLN3 and CAPN11 transcripts.

Statistical analysis

T-test and U Mann Whitney test were conducted to statistically significance analysis of Real-Time PCR findings of both CAPN11 and UBQLN3 genes in testicular biopsy samples. On the other hand, T-test and U Mann Whitney test were also used for analyzing hormones data of the participants. The statistical calculations were performed using SPSS (IBM SPSS Statistics version 16; IBM Corporation, NY, USA). In all tests, $p < 0.05$ was considered statistically significant.

RESULTS

Pathological analysis

The results of the pathology test are shown in Figure 1. As shown in Figure 1a, the density of the background material is increased and destruction is occurred in spermatogenic tubes, spermatogonia, and

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primary spermatocytes, leaving only Sertoli cells visible.

Figure 1b shows a decrease in cell cohesion in primary and secondary spermatogonia and secondary spermatocytes, as well as a

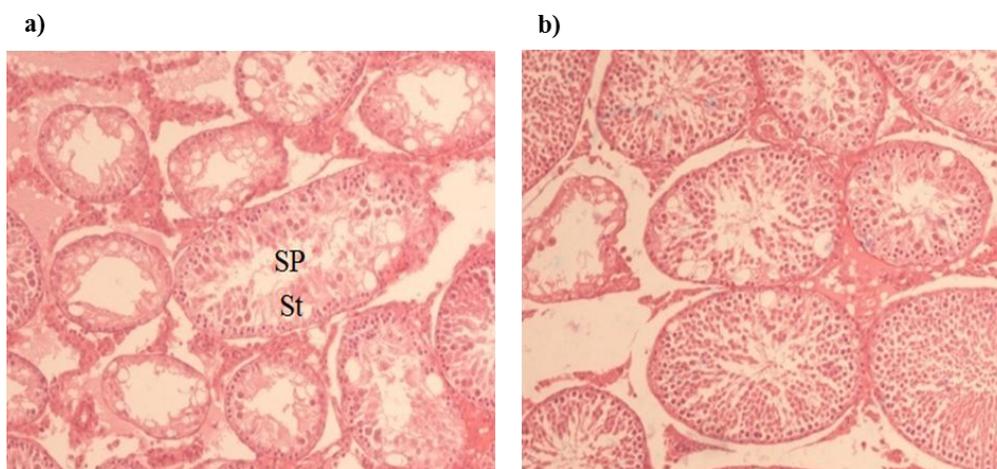
Key genes in non-obstructive azoospermia
reduction in the number of all cells in the seminiferous tubes is quite evident in this Figure.

Table 1. Real-Time PCR primer sequences

Gene	Primer 5'-3'	Tm (°C)	GC%	Self-complementary
UBQLN3	F-GCAATGTTGTTCCAGAGTCAA	57.01	42.86	4.00
	R-CAAGATAGGCATAAGCAGAGC	56.55	47.62	4.00
CAPN11	F-ATGGAGTGGTTTGCCTTATC	55.39	45.00	2.00
	R-CCACCATTCCCGTATCAGTA	56.42	50.00	2.00
GAPDH	F-TGGCTACAGCAACAGGGTG	59.93	57.89	4.00
	R-CTCTTGTGCTCTTGCTGGG	58.45	57.89	2.00

Table 2. Real-Time PCR steps

Gene	1 cycle		40 cycle		1 cycle
	Initial Denaturation	Denaturation	Annealing	Extension	Melting Curve Analysis
UBQLN3	95 °C 10 m	93 °C 30 s	58 °C 30 s	72 °C 25 s	55–95 °C 10 s
CAPN11	95 °C 12 m	93 °C 20 s	56 °C 30 s	72 °C 20 s	55–95 °C 10 s
GAPDH	95 °C 10 m	93 °C 30 s	57 °C 30 s	72 °C 25 s	55–95 °C 10 s

**Figure 1.** Testis tissue of a) Obstructive Azoospermia and b) Non-Obstructive Azoospermia males.

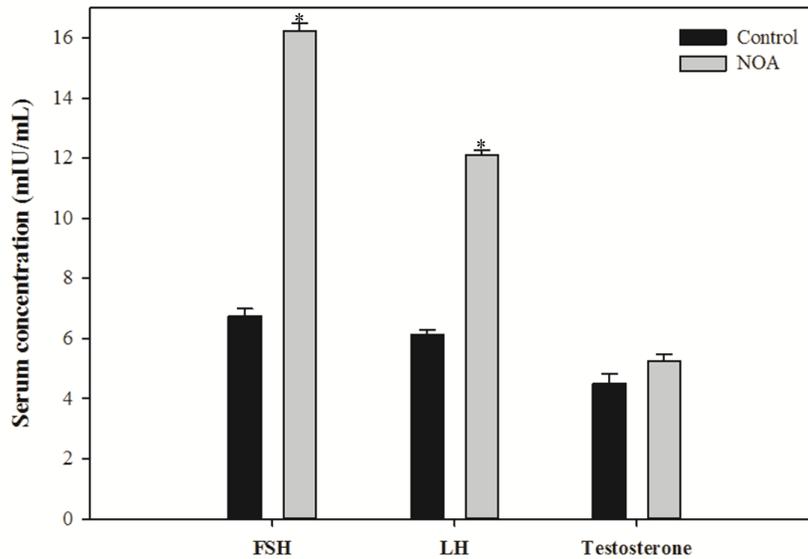


Figure 2. Demographic data analysis.

Demographic analysis

The results of demographic data showed that there is no significant difference between NOA and control subjects in terms of age ($p>0.05$). The mean age of control and NOA were 30.27 and 29.87, respectively. As shown in Fig. 2, the mean difference of FSH and LH hormones between NOA and control was statistically significant and no significant difference was observed in testosterone levels. The mean values of FSH in control and NOA were 6.71 and 16.18, respectively. Mean FSH in the NOA group increased

significantly ($p<0.0001$). The mean LH level in the control group was 6.1 and in the NOA group was 12.02, so the LH level in the NOA group also increased significantly ($p<0.0001$). The mean testosterone level in the control group was 4.53 and in the NOA group was 5.2 and there were no significant differences between the two groups ($p=0.06$).

Expression analysis of the UBQLN3 and CAPN11 genes

The expression levels of UBQLN3 and CAPN11 mRNAs in control and NOA groups were analyzed using qRT-PCR. As

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mentioned before, the GAPDH gene was used as a control gene. The results showed that the expression level in both genes was significantly reduced. The mean expressions of UBQLN3 gene in control and NOA groups were 1 and 0.431, respectively, and similarly, CAPN11 gene expression in the control group was 1 and in the NOA group was 0.368 (Figure 3).

DISCUSSION

Genetic disorders seem to play an important role in male infertility, especially in cases of Non-Obstructive Azoospermia. Spermatogenesis as a complex process of cell differentiation and the development and maturation of germ cells is regulated by 2300 genes. Impaired expression of each gene involved in spermatogenesis is considered as one of the possible causes of non-congenital azoospermia and male infertility [19]. Meiosis in humans is controlled by many genes, some of which are expressed throughout meiosis and some in certain stages of spermatogenesis [20,21]. The result of these genes is the pairing of homologous chromosomes and the reduction of the number of chromosomes from diploid to haploid. Proper spermatogenesis requires the mating of the homologous, synapse, and recombinant

Key genes in non-obstructive azoospermia chromosomes, followed by the separation of chromosomes. In recent years, the study of effective genes in the process of male infertility has been considered due to their important role in treatment planning on the one hand and the possibility of studying genetic problems in children through methods such as genetic diagnosis before implantation [22]. On the other hand, if a gene is expressed at a certain stage of spermatogenesis, it is possible to predict how spermatogenesis progresses through molecular methods and its adaptation to histopathological findings [23,24]. Therefore, identifying specific genes involved in the human spermatogenesis process in order to find potential biomarkers seems necessary. This will facilitate diagnostic testing of male infertility using gene expression profiling in gonads to accurately assess the history of defective spermatogenesis and potentially predict it. Therefore, considering that some candidate genes for Azoospermia, such as UBQLN3 and CAPN11, have not been fully investigated, especially in human samples, the expression level of the mentioned genes in men with Non-Obstructive Azoospermia was investigated in this study. In addition, hormonal changes of

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FSH, LH and testosterone and their relationship with the expression of UBQLN3 and CAPN11 genes were investigated.

The UBQLN3 gene is located on chromosome 11P15.4 and is an ubiquitin-like protein. Ubiquitins contain a second quasi-ubiquitin (N-terminal) and a latter with an ubiquitin (C-terminal) at the end of carboxyl. This gene is specifically expressed in the testicles. CAPN11 is located on the 6P12 chromosome. Kalpins are a family of cysteine-dependent proteases calcium is intracellular. The presence of this gene will regulate spermatogenesis and sperm penetration into the zona pellucida [25].

To date, several studies in which differential expression of gene expression has been performed in control and infertility groups have led to the identification of genes that were never previously associated with infertility [6]. The genes FAM71F1, UBQLN3, GGN, CAPN11, SPATA3, and SPACA4 are different in men with Azoospermia. However, these genes have not yet been fully studied, although a number of them have been studied in rodent models [26,27].

Key genes in non-obstructive azoospermia

In 2013, Mulcher *et al.* reported several of susceptible biomarkers in azoospermia using the microarray method. A comparative analysis of gene expression profiles between infertile individuals and the control group led to the selection of 4946 distinct genes in terms of expression. Of these, the seven genes most commonly shown in infertile individuals were CAPN11 and UBQLN3. This significant reduction in expression was confirmed by two quantitative polymerase chain reaction methods and Western blotting [28,29].

In 2014, Corpiz *et al.* studied 27 testicular biopsies of 27 men with NOA with varying degrees of cessation in spermatogenesis. The results of this study also showed that out of 49436 genes with a different expression, 14 of them significantly decreased or increased expression in Azoospermic individuals, among which CAPN11 and UBQLN3 among Azoospermic individuals decreased expression. In this study, this decrease in expression was observed and reported [30].

A 2012 study by Kim *et al.* on the effect of heat on sex cells reported some genes that reduce or increase expression due to heat.

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One of those genes is CAPN11, which has been shown to reduce expression, and, as previously reported in this study, decreased gene expression could be involved in infertility [31].

A 2017 study by Vahid *et al.* in Pakistan examined and introduced in depth the reproductive biomarkers of the male sex. In this study, the reduced expression of UBQLN3, along with many other genes, was introduced as a familiar name biomarker. The concentration of some proteins in the plasma seminal as well as the increase in expression of some other

Key genes in non-obstructive azoospermia

genes, along with the decrease in UBQLN3 expression, has been introduced as a molecular diagnostic method. In our study, it was also observed that in non-obstructive Azoospermia, this gene shows a decrease in expression [32].

The selection and evaluation of such genes could be useful as a molecular diagnostic tool in determining the degree and level of spermatogenic defects in men with idiopathic nonspecific Azoospermia.

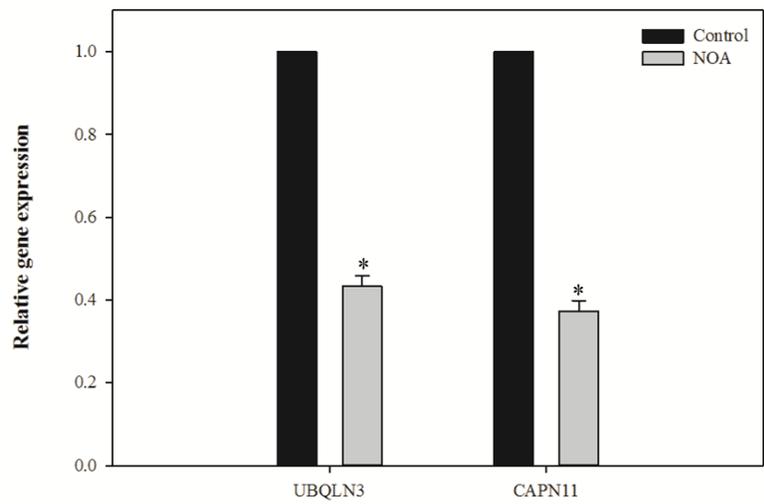


Figure 3. Expression analysis of the UBQLN3 and CAPN11 genes.

CONCLUSION

The selection and evaluation of such genes could be useful as a molecular diagnostic tool in determining the degree and level of spermatogenic defects in men with idiopathic nonspecific Azoospermia.

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