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Original Article

Cell-free seminalmRNA of DDX4 and TNP1Genes as Potential Biomarkers of the Presence of Sperm in the Testicular Tissue

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Abstract

Introduction: Non-obstructive azoospermia (NOA) is one of the reasons for infertility in men, and different factors including genetic factors are involved in its development. Since taking biopsies of the testicular tissue for assisted reproductive technologies (ARTs) is invasive and time-consuming, and the testicular tissue is heterogeneous, introducing a biomarker for predicting the possibility of the presence of sperm in the testicle can increase the ART efficiency. Accordingly, Cell-free seminal mRNA (CFs-mRNA), which is found in many fluids of the body including the seminal fluid of NOA individuals, can be employed as a biomarker for this purpose.

Materials and methods: This study was conducted on 15 men suffering NOA, candidates for testicular sperm extraction (TESE), along with 15 healthy men as control. The testicular tissue of 10 patients was examined using hematoxylin and eosin staining and then classified according to Johnsen scoring. RNA was extracted from the cell-free plasma of semen samples and cDNA was synthesized. The Expression level of *TNP1* and *DDX4* genes was investigated using real-time polymerase chain reaction (PCR).

Results: The expression of CFS-mRNA of the *DDX4* gene was observed in only one sample of NOA individuals (10%), showing a score of 8. Further, the expression of CFS-mRNA of the *TNP1* gene was observed only in two samples (20%) of NOA patients whose scores were 3 and 8.

Conclusion: Insufficiency or lack of expression of CFS-mRNA of *TNP1* and *DDX4* genes may be helpful in predicting the absence of sperm in the testicular tissue of NOA patients in terms of sperm retrieval for ART. Yet,

further studies with more specific and sensitive techniques are required to achieve a more solid and precise conclusion.

Keywords: Azoospermia, Biomarkers, CFS-mRNA, Sperm retrieval

1. Introduction

Around 10 to 15% of couples suffer infertility, 50% of which is related to men [1, 2]. One of the most important factors of infertility in men is azoospermia, which refers to the absence of sperm in the semen. Azoospermia can occur because of either obstruction of the genital tract known as obstructive azoospermia (OA) or defective spermatogenesis known as non-obstructive azoospermia (NOA)[3,4].

While the known genetic reasons including chromosome defects, mutations, and micro-deletions of chromosome Y, as well as different types of polymorphisms are involved in increasing the susceptibility to NOA [5-8], a major part of the underlying causes and molecular mechanisms have remained unknown so far. In addition, the management and treatment of NOA patients are very challenging; in testicular sperm extraction (TESE), sperm recovery is around 55-70%, and in micro-dissection sperm retrieval (m-TESE) is about 35-55% [9-12]. Since testicle biopsy is an invasive and time-consuming method, and as the testicular tissue in NOA patients can be heterogeneous, i.e. parts of it may have active sperm [13, 14], biomarkers that can predict the chance of sperm retrieval from the testicular tissue could significantly help in enhancing the efficiency of ART.

Cell-free seminal mRNA (CFS-mRNA) is found in many fluids of the body and can reflect the expression profile of genes of relevant tissues. Various studies have proposed these molecules as a reliable biomarker in defects related to male infertility disorders especially NOA. So far, CFs-mRNA of *DDX4*, *ACTB*, *DAZ*, *AKAP4*, *PRM2*, *TGM4* *PRM1*, *SEMG1*, *SMPC* *DDX4*, *ODF2*, *ODF1*, *SP10*, *LDAC*, *BOLL*, *SYP3*, *MAGEA4*, *FGFR3*, and *UTF1* genes has been examined in the semen plasma of men suffering from NOA [15-19]. The expression level of this type of mRNA is greater in the semen fluid than in other fluids (15 µg/ml). Thus, by ejaculation, adequate amounts of mRNA are available for Microarray, RT-PCR, and RNA sequencing techniques. Although secretions of testicles constitute around 10% of the semen fluid, CFs-mRNA present in the seminal fluid can be related to the residual cytoplasm as well as droplets of newly emerging sperms in the testicular tissue [20]. Therefore, this can predict the presence or absence of sufficient sperm in the testicular tissue for reproduction techniques.

TNP1 and *DDX4* genes are known as specific genes for germinal cells, and their mRNA analysis may be helpful in detecting the presence of sperm in the testicular tissue. *DDX4* gene belongs to the RNA family of DEAD-box helicases located on chromosome 5 and is essential in the males'germ cells to initiate meiosis [21]. *TNP1* gene, located on chromosome 2, codes a protein playing a role as an intermediate

between histones and protamines during spermatogenesis [22, 23].

In this study, CFS-mRNA of these two genes was examined to be introduced as possible biomarkers for sperm retrieval from testicles of NOA patients.

2. Materials and Methods

Participants

This study was carried out on 15 NOA men who were candidates for testicular sperm extraction (TESE). These participants referred to Rouyan research center and Qom specialized infertility center in 2015 and 2016 and completed a consent form. In addition, samples were collected from 15 normal men as control. The control samples were chosen based on the normal parameters of sperm and semen according to the guidelines of WHO. These individuals had no previous defects regarding infertility such as cystic fibrosis, varicocele, chemotherapy, Kleinfelter syndrome, AZF microdeletions, etc.

Histological studies

Considering the conditions of laboratory and patients, through biopsy, the testicular tissue of 10 patients was taken for histological studies. Next, the samples were placed inside formalin buffer 4% overnight for fixation and then stabilized in paraffin. Every section was divided into smaller sections of about 5 µm and then dehydrated in alcohols with 75-100% dilutions. Thereafter, they were stained with hematoxylin (2 min) and Eosin (30 s) techniques. Microscopic studies were performed by Olympus microscope (BX53) and camera (Olympus DP73, Tokyo, Japan). To investigate the seminiferous tubes histologically, Johnsen scoring was used [24]. In this type of grading, the mean number of seminiferous tubes examined in the present study was 63-100 tubes. In the system of histological quantitative grading, the maturity level of sperm was graded 1-10 based on the degree of progression of germinal cells in each section of the seminiferous tubes.

Semen Collection

The semen samples were obtained through masturbation and after 3-5 days of avoiding intercourse. After collection, the samples were exposed to 37 °C for 5 minutes to liquefy. Next, the plasma of the cell-free samples was isolated. For this purpose, the samples were centrifuged in two stages. Initially, they were centrifuged at 1600g for 10 min followed by 16000g for 10 min [16]. Thereafter, the supernatant was carefully separated and kept inside liquid nitrogen for the subsequent stage including RNA extraction.

RNA Extraction, cDNA Synthesis, and Real-time PCR

For total RNA isolation from the samples, a Trizol-based protocol (Invitrogen) was used, according to the manufacturer's protocol. The concentration and purity of the extracted RNA were examined by spectrophotometer (NanoDrop, Thermo Fisher) based on OD 260/280, while its quality was checked using Agarose gel. Before Complementary DNA (cDNA) synthesis, to eliminate possible contamination with DNA, Dnase I was used. In the next step, cDNA synthesis was performed, using RevertAaid™ First Strand cDNA Synthesis kit (Fermentas), The expression analysis of *TNP1* and *DDX4* genes was done on an ABI Real-time PCR system (Applied Biosystem,USA), using high-ROX RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). The housekeeping gene, Hypoxanthine-guanine phosphoribosyl transferase (HPRT), was also used as quality control. The primers of the studied genes are mentioned in **Table 2**. The conditions for real-time PCR reaction included: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 10 min. Finally, the results obtained from investigating the expression DDX4 and TNP1 genes were

examined descriptively and analytically for both normal and NOA participants.

3. Results

Gene Expression

CFS-mRNA related to the *DDX4* gene was detected in 11 participants of the control group (73.33%), while it was detectable only in one case of the patient group (6.66%). Further, CFS-RNA of the *TNP1* gene was observed in 13 of the control individuals (86.66%), while it was found in only two of the NOA patients (13.33%) (**Fig. 1**).

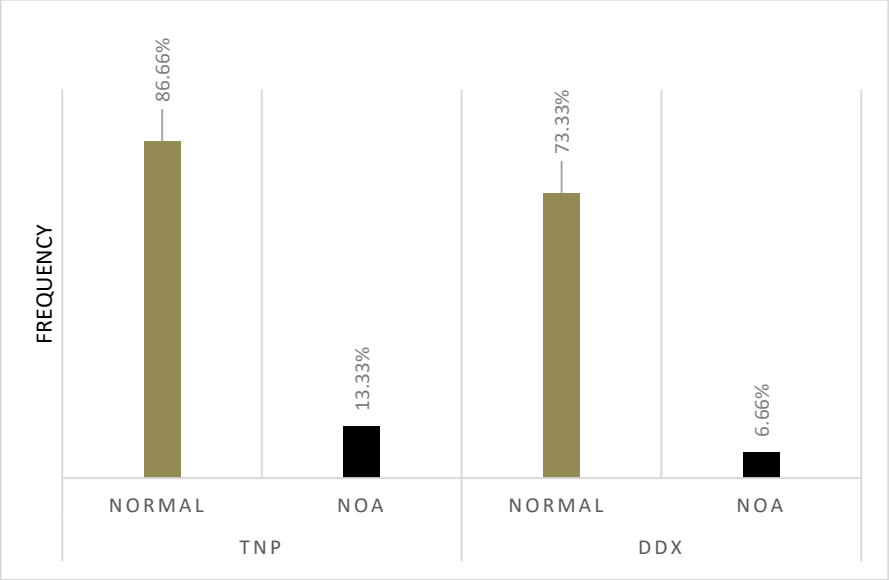

Figure 1. The frequency of CfS-mRNA of the *TNP1* and *DDX4* genes detected in the seminal fluid of normal individuals and NOA patients

Histological Evaluation

Out of the 15 patients with NOA, it was possible to histologically examine the testicular tissue sample in 10 patients. Based on the analysis, most patients (90%) showed grade 1-3, while only one patient (10%) indicated grade 8 (**Table 3**). **Fig. 2** reveals some samples of the sections related to the testicular tissue with various grading in the NOA patients.

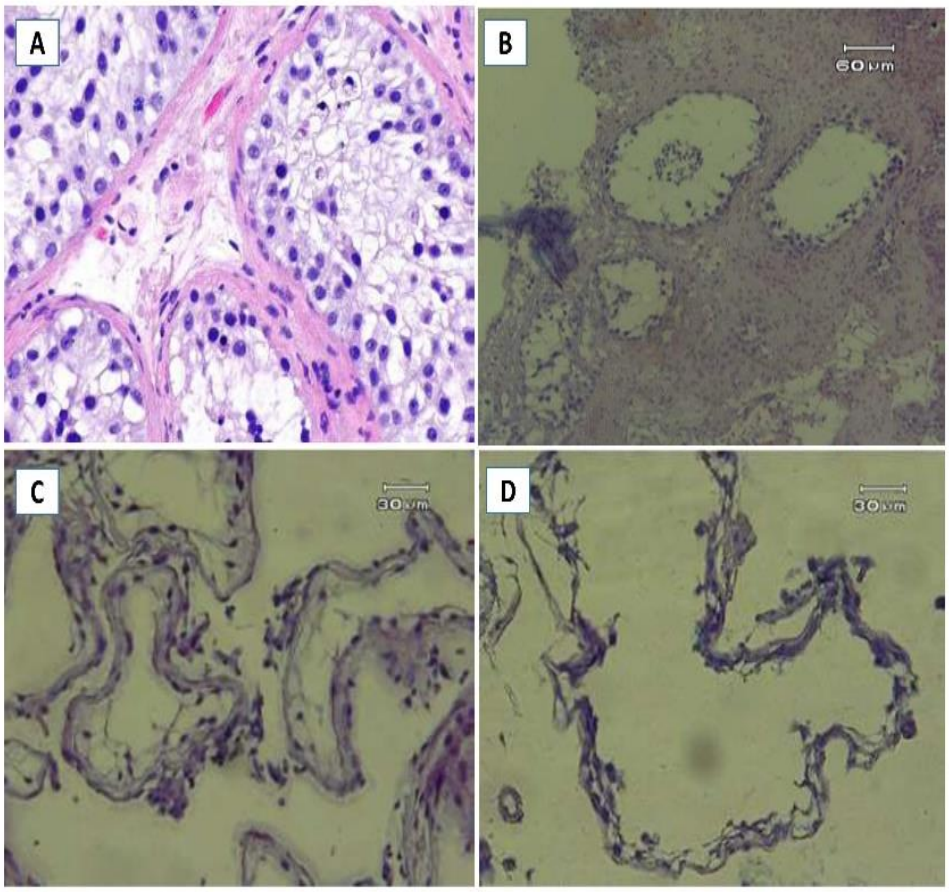

Figure 2 . The sections related to the testicular tissue with various Johnsen score (A): a sample of the tissue with score 8, (B): a sample of tissue with score 3, (C) a sample of tissue with score 2, (D), a sample of tissue with score 1

Comparing the results obtained from real-time PCR and the histopathology of testicular tissue in NOA patients

The results related to CFS-mRNA of *DDX4* gene in the semen fluid based on the histopathology findings of the testicular tissue in NOA patients indicated that only in one sample (10%) expression of the *DDX4* gene was observed, and this sample was in grade 8. In this study, the expression of the CFS-mRNA gene of *TNP1* was observed in only two samples (20%) whose scores were 3 and 8 (**Table 4**).

4. Discussion

Considering the invasiveness and high cost of histopathological investigations, as well as the unreliability of the results obtained from this technique regarding sperm retrieval from the testicular tissue (given the heterogeneity of this tissue), a method is required to be both noninvasive and able to predict the presence or absence of the sperm inside testicular tissue with high sensitivity and specificity (15, 16). In this study, the expression of CFS-mRNA of DDX4 and TNP1 genes was studied to investigate their possible introduction as a biomarker for sperm retrieval from testicular tissue.

DDX4 plays a key role in the males for initiating meiosis and also stimulates the translation of a large group of mRNAs. Moreover, they are involved in assembling ribonucleoprotein granules of germ cells. In the testicle of adults, 585 potential target mRNAs have been identified for DDX4[19]. Another gene examined in this study was TNP1. This gene is highly expressed during spermatogenesis[23]; the proteins coded by it during spermatogenesis replace somatic histones and are then replaced by protamines [22]. TNP1 also decreases the temperature of DNA denaturation, DNA loosening in the nucleosome, as well as stimulating the activity of topoisomerase1. Further, the role of TNP1 has been observed in repairing single-stranded cleavages in vitro and in somatic cells [23].

DDX4 gene was observed in only one (6.66%) of the NOA patients with scores of 8 and 11 (73.33%) of normal participants. As opposed to our study, in a study by Abdallah et al. in 2016, the expression of these genes was observed in around 48% of NOA patients [25]. This reduced or lack of detection of this gene in our study can be due to two reasons. Firstly, the CFS-mRNA of the DDX4 gene does not have high specificity or sensitivity to detect germinal cells in the semen fluid. However, in a study by Yu et al. in 2016, the expression of CFS-mRNA of this gene was seen in all samples with maturation arrest and hypo spermatogenesis. They proposed this gene as a suitable biomarker for detecting sperm in germinal cells [26]. Also, the study by Li et al. showed that expression of this gene in the plasma of semen fluid can be a good criterion to classify different types of both obstructive and nondestructive azoospermia [16]. Secondly, during the preparation of patient samples as well as within the time interval between sample collection and conducting real-time PCR reaction, the transcripts of the DDX4 gene in the semen fluid have been degraded to a large extent. A study explored the stability of CFS-mRNA of DDX4 gene and the results revealed high degradability of the transcripts of this gene [16]. Thus, using this gene as a biomarker for reproductive cells may be error-prone, since cfs-mRNA may be degraded considering the laboratory conditions, whereby the measurement time may be delayed.

The expression of TNP1 gene in our study was detected in 13 (86.66%) of normal participants and only 2 (13.33%) of the NOA patients. Based on the histological examinations, the two NOA patients expressing this gene had scores of 3 and 8. In score 8, the testicular tissue of the person contained mature germinal cells; since the TNP1 gene is expressed in the final stages of sperm maturity, at score of 3. When there are only spermatogonial cells in the testicle, expression of this gene was not expected.

Thus, this may indicate heterogeneous testicular tissue, i.e. possibly spermatogenesis may occur in other areas of the same testicle or in the other testicle. Given that, mature germinal cells may exist and have caused the expression of this gene. Accordingly, CFS-mRNAs of this gene may be a suitable biomarker for identifying people whose testicular tissue contains reproductive cells in the final stages. Also, in a study in 2019 by Hashemi et al. on the relative expression of CFS-mRNA of this gene on Iranian samples, it was found that expression of this gene was significantly lower in NOA patients and more specifically in negative sperm retrieval (SR-) individuals compared to the control group. They proposed this gene as a biomarker that can predict the extent of sperm retrieval success from testicular tissue with high specificity and sensitivity [27].

The limitations of this study included small sample size, thus necessitating further and more accurate examinations with larger sample size. Additionally, investigation of the expression of these genes in the testicular tissue alongside CFS-mRNA of the semen fluid, examination of the level of relative expression, and more functional studies can lead to more robust results. Apart from that, in some cases failure to observe the expression of a gene in semen does not mean that the desired gene is not expressed. This is because some studies have indicated rapid RNA degradation from 3-UTR, which may lead to a reduced number of full RNA transcripts in the samples. Therefore, the efficiency of replication in routine methods such as real-time may decrease, even resulting in failure to detect the expression of that gene [15]. Hence, for higher efficiency, more specific and sensitive methods such as RNA-seq are recommended.

Overall, investigation of the expression of CFS-mRNA of different genes associated with differentiation of sperm and germ cells in the semen fluid can lead to the development of an expression profile offering excessive information for detecting biomarkers to predict the extent of success of sperm retrieval from the testicular tissue for ART. Indeed, it can be considered a noninvasive and inexpensive method for treating NOA patients.

5. Conclusion

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Ethical approval

This study was drawn from a research project (No.IR.IAU.TMU.REC.139 4.59) approved by

Research Ethics Committee of Tehran Islamic Azad University of Medical Sciences

Conflict of interest

There is no conflict of interests.

Competing interest

The authors declare that they have no competing interests.

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Tables :

Score	Histological Criteria
10	Full spermatogenesis
9	Slightly impaired spermatogenesis, many late spermatids,disorganized epithelium
8	Less than five spermatozoa per tubule, few late spermatids
7	No spermatozoa, no late spermatids, many early spermatids
6	No spermatozoa, no late spermatids, few early spermatids
5	No spermatozoa or spermatids, many spermatocytes
4	No spermatozoa or spermatids, few spermatocytes
3	Spermatogonia only
2	No germinal cells, Sertoli cells only
1	No seminiferous epithelium

Table 1. Johnsen score and its criteria

Gene	Primers
TNP	Forward primer: 5' CCTCATTTTGGCAGAACTTACC 3'
	Reverse primer: 5' CGGTAATTGCGATTGGCGTC 3'
DDX4	Forward primer: 5'ATCAGAAATGGATGATGGACCTTCT 3'
	Reverse primer: 5'CTTATTACACTCACCAGCATCTCTGTT 3'
HPRT	Forward primer: 5' TGGACTAATTATGGACAGGACTGAAC 3'
	Reverse primer: 5' GCACACAGAGGGCTACAATGTG 3'

Table 2. Primers used in this study

Number of cases (Total=10)	Johnsen Scoring
1	8
4	3
3	2
2	1

Table 3. The Johnsen score of the collected samples

Johnsen score	**DDX4**		**TNP1**	
	-	+	-	+
Score 1	2	0	2	0
Score 2	3	0	3	0
Score 3	4	0	3	1
Score 8	0	1	0	1
Total	10		10	

Table 4. The frequency of expression of CFS-mRNA of TNP1 and DDX4 genes in the samples considering the histopathology of testicular tissue in NOA patients

+: gene expression was detected; -: No gene expression was detected