



Upregulation of the *RNF8* gene can predict the presence of sperm in azoospermic individuals

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Objective: In this study, specimens from testicular biopsies of men with nonobstructive azoospermia (NOA) were used to investigate whether *RNF8* gene could serve as a biomarker to predict the presence of sperm in these patients.

Methods: Testicular biopsy specimens from 47 patients were classified according to the presence of sperm (positive vs. negative groups) and investigated for the expression of *RNF8*. The level of *RNF8* gene expression in the testes was compared between these groups using reverse-transcription polymerase chain reaction.

Results: The expression level of *RNF8* was significantly higher in testicular samples from the positive group than in those from the negative group. Moreover, the area under the curve of *RNF8* expression for the entire study population was 0.84, showing the discriminatory power of *RNF8* expression in differentiating between the positive and negative groups of men with NOA. A receiver operating characteristic curve analysis showed that *RNF8* expression had a sensitivity of 81% and a specificity of 84%, with a cutoff level of 1.76.

Conclusion: This study points out a significant association between the expression of *RNF8* and the presence of sperm in NOA patients, which suggests that quantified *RNF8* expression in testicular biopsy samples may be a valuable biomarker for predicting the presence of spermatozoa in biopsy samples.

Keywords: Azoospermia; Biomarker; *RNF8*; Spermatogenesis

Introduction

Infertility, which is defined as a disability to conceive a child after regular unprotected intercourse over the course of 1 year, affects

13%–18% of couples globally [1]. Male factors account for infertility in roughly half of all cases [2], and azoospermia is the most common male factor leading to infertility [3]. Azoospermia can be concisely described as the absence of sperm in the ejaculate, and it is classified into obstructive azoospermia (OA), which derives from a blockage in the genital tract, and nonobstructive azoospermia (NOA), which results from a failure of spermatogenesis [4]. In some NOA samples, germ cells are absent and the men are diagnosed as having Sertoli cell-only syndrome [5]. Men whose germ cells have no detectable mature spermatids are diagnosed with maturation arrest [6]. In other samples, spermatogenesis can sporadically progress to mature spermatids in some tubules, a process known as hypospermatogenesis [7].

If sperm can be retrieved by microdissection testicular sperm extraction (mTESE), men with NOA may be able to fertilize their partners by

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intracytoplasmic sperm injection (ICSI) [8]. The rate of sperm retrieval in testicular operations is about 50% [9], and to date, the only generally accepted reliable predictor of successful mTESE is testicular histology [10]. Failure to retrieve sperm may have devastating consequence for couples who may not want to undergo sperm or embryo donation. Subsequent surgery after the first mTESE procedure with the hope of finding possible spermatogenic loci may be the best option for many of these couples. Since some researchers believe that spermatozoa retrieved from NOA patients are not appropriate for freezing [11], and a second or repeated mTESE procedure may therefore be considered in these patients for future ICSI, it seems necessary to find additional criteria based on a noninvasive approach in order to predict the success of sperm retrieval in patients with NOA.

RNF8 is an E3 ubiquitin ligase located on chromosome 6p21.3 that spans 8 exons. It plays important roles in many biological processes, including DNA repair [12,13] and chromatin remodeling [14]. Ubiquitination of histones in the testes by *RNF8* leads to nucleosomal replacement by protamines, which is essential for the condensation of chromatin and spermatogenesis [15]. A lack of *RNF8* in mice leads to infertility due to defective spermiogenesis [16], and *RNF8*-knockout mice are unable to generate mature sperm [17]. Additionally, through *RNF8*-dependent mechanisms, sex-linked genes (including the *Ssty* gene family) escape post-meiotic silencing and are activated in round spermatids and throughout the course of spermiogenesis [18,19]. Downregulation of the *Ssty* gene family was found to be correlated with a reduced amount of sperm in the testes of mice [20]. These studies indicate that *RNF8* plays an essential role in modulating mechanisms related to spermatogenesis and that its absence could result in infertility.

Methods

1. Patients

Forty-seven men with OA and NOA who underwent mTESE to obtain spermatozoa for ICSI were enrolled in this study. The patients were chosen based on the availability of well-preserved testicular biopsy samples for research. The Institutional Ethics Committee of Shahid Sadoughi Medical University approved this study (IRB No. 1396.219) and written informed consent was obtained from all patients before the collection of tissue samples. The preoperative examination included history, measurement of testis size, semen analysis, and quantification of serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels. Moreover, no patients were on hormonal therapy and all had primary infertility. None of the participants had a history of TESE or cryptorchidism. Patients with cystic fibrosis or chromosomal abnormalities such as Klinefelter syndrome and Y chromosome microdeletion were excluded from the study.

2. Tissue acquisition and histological analysis

Spermatozoa were obtained by testicular biopsy. The pieces of testicular tissue weighing approximately 20 mg that were used for RNA isolation were placed into a cryovial and instantly snap-frozen in liquid nitrogen. Depending on whether sperm were recovered, the azoospermic patients were divided into OA and NOA groups. Sperm retrieval was considered to be positive if, in the laboratory, at least one spermatozoon was found that was morphologically suitable for ICSI. Additionally, another small piece of testicular tissue was paraffin-embedded and fixed using Bouin solution for histopathological evaluation. After staining with hematoxylin and eosin, biopsy samples were evaluated as described elsewhere [21].

3. RNA extraction and cDNA synthesis

Total RNA was extracted from the tissue samples using RNeasy Plus Universal (Qiagen, Hilden, Germany) based on the manufacturer's protocol and kept at -80°C . The concentration and purity of the isolated RNA were determined by the A260/A280 ratio using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and confirmed by agarose gel electrophoresis. Template cDNA was synthesized from 1 μg of whole extracted RNA with a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania) using oligo-dT and random hexamer primers simultaneously for each reaction in an Eppendorf Mastercycler Gradient device (Hamburg, Germany).

4. Quantitative reverse-transcription polymerase chain reaction and primer design

For reverse-transcription polymerase chain reaction (RT-PCR), the initial denaturation step took 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds. The melting curve was derived by increasing the temperature from 72°C to 95°C to guarantee that no genomic DNA or primer dimers were amplified in the reactions. Quantitative PCR (qPCR) was run in triplicates on 48-well plates (Applied Biosystems, Step One Plus, Foster City, CA, USA) and the average CT value was used for further analysis. All RT-PCR runs contained non-template (cDNA) controls in order to avoid potential contamination. Relative gene expression analysis was performed using the comparative CT method ($2^{-\Delta\Delta\text{CT}}$). The $2^{-\Delta\Delta\text{CT}}$ parameter represents the expression of a gene of interest (here, *RNF8*) as a fold multiple with respect to the housekeeping *ACTB* gene. To design the primers, the following criteria were applied consecutively: mutual exons were retrieved between all transcripts from Ensembl, primers were chosen for exon-exon junction sites so that 6–10 nucleotides at the 3' end of the primer sequence would hybridize to 1 exon and 10–16 nucleotides at the 5' end would bind to the adjacent exon, the dynamic and structural fea-

tures of primers were evaluated using OligoAnalyzer, the primers' specificity was verified by NCBI BLAST, and Ensembl was used to ensure that no polymorphism existed (with a minor allele frequency lower than 0.01) at the last base of the 3' end of the primer sequence. The features of the primers are presented in Table 1. Lowercase base pairs are located at the 3' end of the primer sequence.

Table 1. Real-time RT-PCR primers used in this study

Gene	Primer sequencing (5'→3')	Product size (bp)	Annealing temperature (°C)
<i>RNF8</i>	F: GATGGGTGCGAGgtgactg	153	59
	R: CCACATTAGACTcttgtgtcc		
<i>ACTB</i>	F: CCTTCCTGGgcatggag	204	57
	R: CGGAGCAATGATcttgatctt		

RT-PCR, reverse-transcription polymerase chain reaction; F, forward; R, reverse.

Table 2. Clinical characteristics of the patients

Variable	OA	NOA+	NOA-	p-value
Number	7	19	21	-
Age (yr)	29.0±4.6	30.2±1.7	33.1±3.5	-
Testicular volume (mL)	14.1±1.2	10.9±4.6	9.7±3.1	<0.001
LH (mIU/mL)	5.1±0.4	8.9±0.5	13.4±1.2	<0.001
FSH (mIU/mL)	8.9±0.4	9.2±2.4	15.9±1.1	<0.001
Testosterone (ng/mL)	3.6±0.1	4.1±0.9	5.0±1.3	NS
Transcript expression	1.33±0.17	1.61±0.23	1.9±0.42	<0.001

Values are presented as mean ± standard deviation.

OA, obstructive azoospermia; NOA, nonobstructive azoospermia; +, presence of sperm; -, absence of sperm; LH, luteinizing hormone; FSH, follicle-stimulating hormone; NS, not significant.

5. Statistical analysis

Serum LH and FSH levels, testosterone levels, average testicular volume, age, and mean *RNF8* expression were examined with respect to the mTESE results using the independent *t*-test, with *p*-values <0.05 considered to indicate statistical significance. Comparisons among the OA, NOA-, and NOA+ groups were made by utilizing one-way analysis of variance followed by the Dunnett multiple comparison post-test. A receiver operating characteristic (ROC) curve was constructed to predict the presence of retrievable sperm based on *RNF8* expression. All analyses were performed in IBM SPSS ver. 23.0 (IBM Corp., Armonk, NY, USA).

Results

As shown in Table 2, sperm retrieval was successful in 19 of the 40

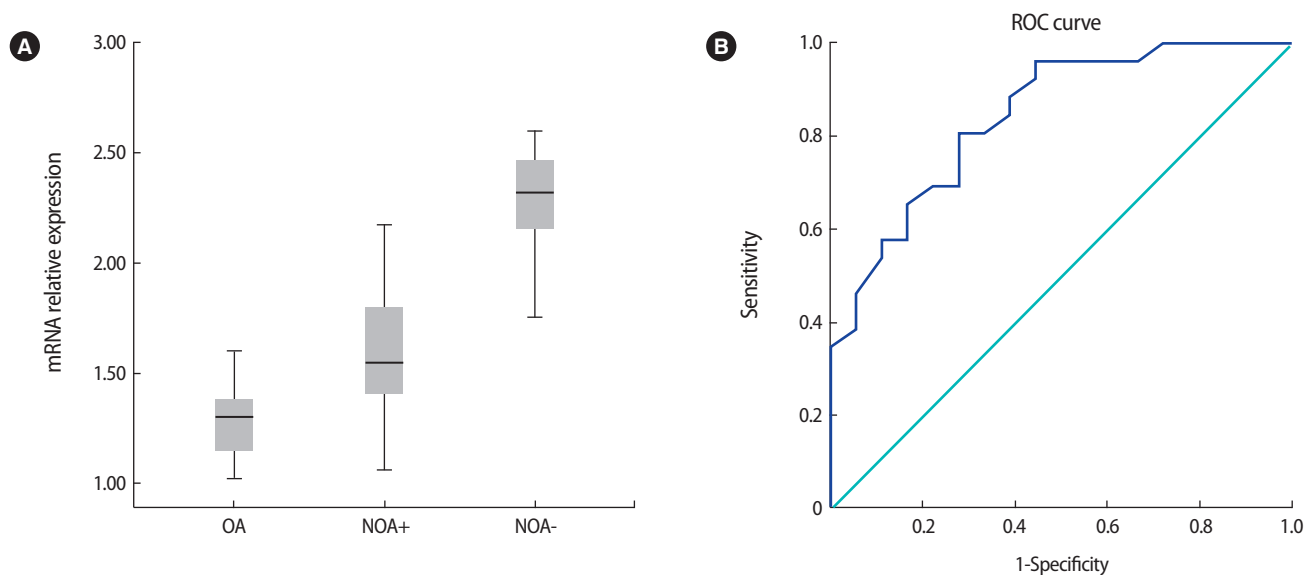


Figure 1. (A) Differential expression of the *RNF8* gene in the obstructive azoospermia (OA), nonobstructive azoospermia (NOA)+ (presence of sperm), and NOA- (absence of sperm) groups. *ACTB* was used as a housekeeping gene. Each reaction was performed in triplicate. Expression of *RNF8* was upregulated in the NOA- group (2.27 ± 0.18) compared with the NOA+ (1.6 ± 0.23) and OA (1.3 ± 0.17) groups. The difference was significant ($p = 0.001$). (B) Receiver operating characteristic (ROC) curve of *RNF8* expression in predicting sperm recovery outcomes.

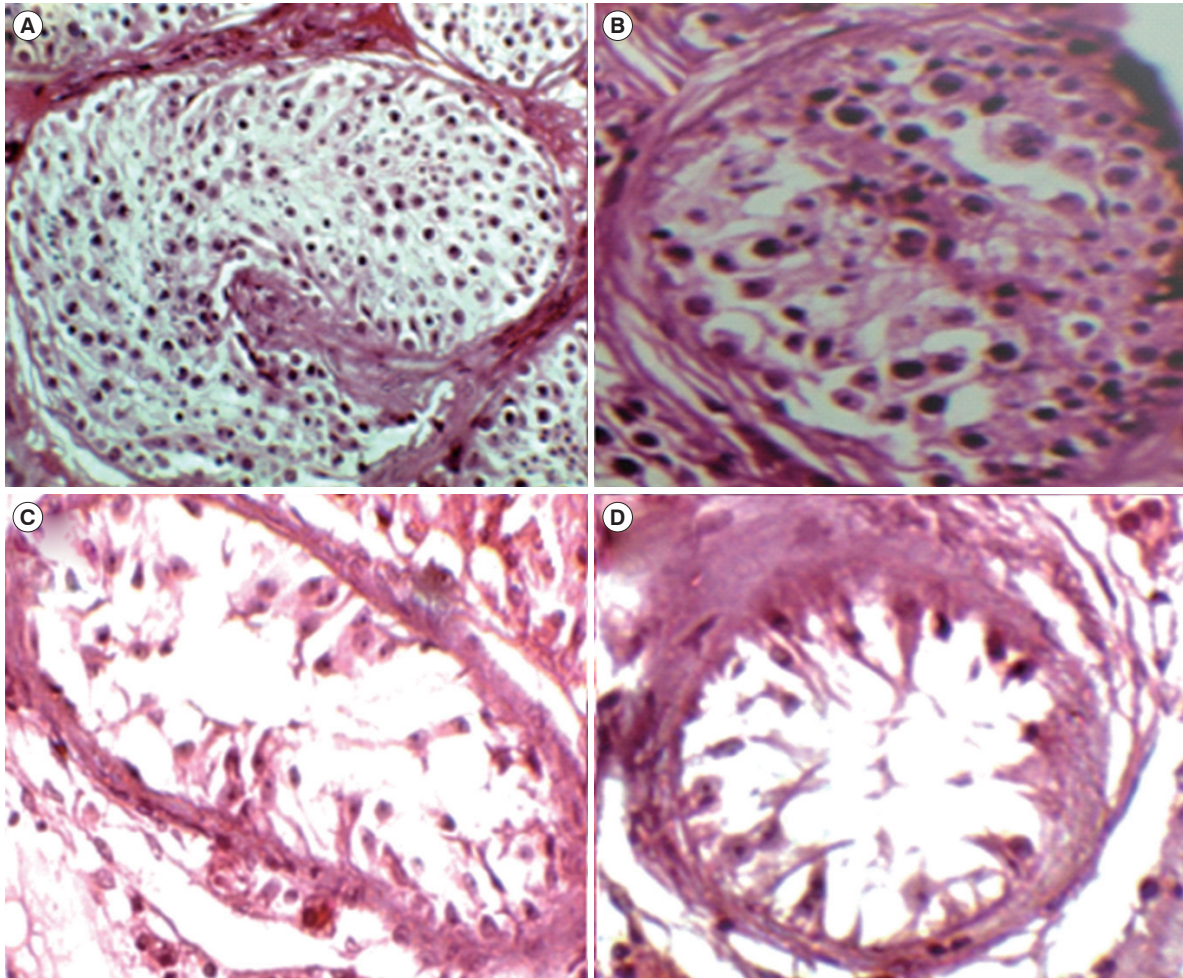


Figure 2. H&E staining of histological samples from men with azoospermia. Specimens were categorized into the following four groups: (A) obstructive azoospermia, (B) hypospermatogenesis, (C) maturation arrest, and (D) Sertoli cell-only.

men with NOA. There were no significant differences in mean age and testosterone levels between the OA, NOA+, and NOA– groups. The NOA– patients showed the highest mean FSH and LH levels. In addition, testicular volume had a lower mean value in the NOA– group than in the NOA+ and OA groups (Table 2). The level of *RNF8* mRNA expression in testicular biopsy samples was assessed using qPCR, and as illustrated in Figure 1A, it was significantly higher in the NOA– group than in the NOA+ and OA groups ($p < 0.001$). As shown in Figure 2, histopathological analyses revealed that 12, 15, and 13 individuals had Sertoli cell-only syndrome, maturation arrest, and hypospermatogenesis, respectively.

The diagnostic power of *RNF8* expression was evaluated based on the area under the curve (AUC), and its specificity and sensitivity were determined based on the ROC curve. To discriminate azoospermic patients with and without successful sperm recovery, the optimal cutoff point was determined to be 1.76, which yielded an AUC of 0.84 (95% confidence interval, 0.764–0.974; $p < 0.001$) with sensitivity

and specificity values of 0.80 and 0.83, respectively (Figure 1B).

Discussion

Due to the heterogeneous nature of the testis, researchers have struggled to find a definitive marker capable of predicting the presence of spermatozoa in the testis [16]. Many noninvasive assessments have been used to predict the likelihood of sperm retrieval in NOA patients, including histological analyses, testicular volume measurements, assessments of hormone levels, and semen analysis; however, these techniques have not shown sufficient predictive power to produce reliable results [22,23]. Only patients who have been diagnosed with complete loss of the *AZFa* or *AZFb* regions are definitively considered to have no spermatozoa in their testicular biopsy samples, meaning that it is recommended to avoid mTESE in those patients [18]. More recently, it has been suggested that alterations of molecular markers associated with spermatogenesis may predict the pres-

ence of mature spermatozoa in men with NOA [24,25].

High levels of proliferative activity during spermatogenesis make germ cells more susceptible to DNA-damaging factors than other cells. More severe DNA damage caused by various endogenous and exogenous agents can result in germ cell loss and subsequent adverse outcomes, such as impaired spermatogenesis and male infertility [26]. In response to DNA damage, the phosphorylation of MDC1 causes *RNF8* to be recruited to the locations of DNA double-strand breaks (DSBs), where *RNF8* couples with Ubc13 catalyzes the construction of lysine 63-linked polyubiquitin chains on histones. The ubiquitination of histones at DSBs provides a fundamental chromatin structure for the subsequent function of proteins vital for DNA damage repair, such as 53BP1 and BRCA1 [27,28].

Some molecular markers have previously been proposed; for example, *ESX1* was identified in 95.4% of studied cases and showed a specificity of 74% and sensitivity of 80% for predicting residual spermatogenesis [29]. In a study by Ando et al. [22], the level of *VASA* transcription was evaluated using RT-qPCR in 52 samples from men with NOA, and it was demonstrated to be a predictive factor for sperm recovery, with 86.2% specificity and 87.0% sensitivity. In another study, *HSFY* was proposed as a potential diagnostic marker for sperm recovery in NOA patients, with a specificity of 92.6% and a sensitivity of 66.7% [23]. Despite the identification of these biomarkers, the quest to identify more accurate diagnostic markers is still continuing. Recently, histone demethylase *JMJD1A*, with a cutoff level of 0.74 and a specificity of 89.29% and a sensitivity of 90.91%, demonstrated a more reliable predictive value for sperm recovery than the other previously suggested markers [30].

In the present study, the expression of *RNF8* was evaluated in 40 NOA patients to assess the ability of the level of *RNF8* expression to serve as a biomarker for the presence of mature spermatids. The expression of *RNF8* at the transcript level was significantly higher in the NOA– group than in the NOA+ group. Moreover, the AUC of *RNF8* expression for the entire study population was 0.84, showing the discriminatory power of *RNF8* expression for differentiating between the NOA– group and the NOA+ group. In the ROC curve analysis, *RNF8* expression showed a sensitivity of 81% and a specificity of 84%, with a cutoff level of 1.76.

This study is the first to report the upregulation of *RNF8* expression using RT-qPCR. However, a microarray study by Spiess et al. [31] showed significantly higher expression of *RNF8* in individuals with Sertoli cell-only syndrome histology than in men with normal spermatogenesis. Moreover, a haplotype analysis of rs104669 and rs195432 of *RNF8*, which were in strong linkage disequilibrium, revealed that the AC haplotype was correlated with a reduced risk of NOA and smaller testis volume in a Chinese population [32]. Considering the elevated level of *RNF8* expression in the patients with im-

paired spermatogenesis in this study, this alteration likely reflects changes in somatic cells, which may imply that *RNF8* has potential for interventional therapies [33].

Although our study suggests that *RNF8* has potential as a novel biomarker for sperm recovery, some limitations are worth mentioning. First, sperm recovery based on mTESE is not sufficiently precise, because spermatogenesis is well known to exhibit heterogeneity in the testes of men with NOA, such that loci of spermatogenesis can coexist within Sertoli-only seminiferous tubules. Second, seminal plasma from azoospermic patients may be a better target for biomarker investigation, as it reflects more complex patterns of whole-testis physiology than mTESE samples, which only enable the analysis of a limited region of tissue. With these considerations in mind, it is recommended to utilize samples with high homogeneity and to promote the investigation of gene expression patterns as biomarkers in seminal plasma by focusing on finding biomarkers for sperm retrieval in NOA patients before performing mTESE.

This study presents a significant association between the expression of *RNF8* and the presence of sperm in NOA patients, and therefore suggests that quantified *RNF8* expression in testicular biopsy samples may be a valuable biomarker for predicting the presence of spermatozoa in biopsy samples.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Author contributions

Conceptualization: NG, MN, EB. Data curation: MN, EB, SMAZ. Formal analysis: SMAZ, MN, MT. Methodology: NG, NN, MS, MN, MD. Project administration: MN, NN, MS, MD. Writing - original draft: MN, MT. Writing - review & editing: NG, EB, MN.

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