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Sperm Biology

# MicroRNA-27a-mediated repression of cysteine-rich secretory protein 2 translation in asthenoteratozoospermic patients

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Cysteine-rich secretory protein 2 (CRISP2) is an important protein in spermatozoa that plays roles in modulating sperm flagellar motility, the acrosome reaction, and gamete fusion. Spermatozoa lacking CRISP2 exhibit low sperm motility and abnormal morphology. However, the molecular mechanisms underlying the reduction of CRISP2 in asthenoteratozoospermia (ATZ) remain unknown. In this study, low expression of CRISP2 protein rather than its mRNA was observed in the ejaculated spermatozoa from ATZ patients as compared with normozoospermic males. Subsequently, bioinformatic prediction, luciferase reporter assays, and microRNA-27a (miR-27a) transfection experiments revealed that miR-27a specifically targets *CRISP2* by binding to its 3' untranslated region (3'-UTR), suppressing *CRISP2* expression posttranscriptionally. Further evidence was provided by the clinical observation of high miR-27a expression in ejaculated spermatozoa from ATZ patients and a negative correlation between miR-27a expression and *CRISP2* protein expression. Finally, a retrospective follow-up study supported that both high miR-27a expression and low *CRISP2* protein expression were associated with low progressive sperm motility, abnormal morphology, and infertility. This study demonstrates a novel mechanism responsible for reduced *CRISP2* expression in ATZ, which may offer a potential therapeutic target for treating male infertility, or for male contraception.

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**Keywords:** asthenoteratozoospermia; cysteine-rich secretory protein 2; male infertility; microRNA-27a

## INTRODUCTION

Infertility is the inability of a sexually active, nonconceiving couple to achieve pregnancy in 1 year<sup>1</sup> and it affects approximately 15% of couples trying to conceive in Western countries.<sup>2</sup> Approximately 50% of infertility cases are attributed to male factors, and no causal factor is found in 40%–60% of these cases, which are referred to as idiopathic male infertility.<sup>3</sup> These men present with a decreased number of spermatozoa (oligozoospermia), decreased motility (asthenozoospermia, AZ), and diverse abnormal sperm morphologies (teratozoospermia). Together, these symptoms are described as the oligo-astheno-teratozoospermia (OAT) syndrome. Commonly observed in the clinic, asthenoteratozoospermia (ATZ) is defined as semen containing both <32% progressively motile (PR) spermatozoa and <4% morphologically normal spermatozoa, according to the World Health Organization (WHO) guidelines (5<sup>th</sup> ed.).<sup>4</sup> Abnormal sperm morphology and reduced sperm motility can be the result of urogenital infections, urogenital abnormalities, endocrine disturbances, varicocele, or other causes,<sup>3,5</sup> but the exact causal factors underlying ATZ remain largely unknown. As an important factor in OAT, ATZ plays a role in male infertility.<sup>3</sup>

A number of genes involved in sperm motility have been discovered.<sup>6,7</sup> Gene expression profiling has demonstrated that cysteine-rich secretory protein 2 (*CRISP2*) is closely associated with spermatogenesis and infertility.<sup>8,9</sup> Reduced expression of *CRISP2* in the spermatozoa of patients with asthenozoospermia has been previously observed, and it is correlated with low sperm motility and infertility.<sup>10</sup> However, the expression level of *CRISP2* in the spermatozoa of patients with ATZ remains unclear. As a member of the cysteine-rich secretory proteins (CRISPs)/antigen 5 (Ag5)/pathogenesis-related 1 (Pr-1) (CAP) super-family of proteins,<sup>11,12</sup> *CRISP2* is a component in the sperm acrosome and the outer dense fibers of the sperm tail.<sup>13</sup> Functionally, it could modulate sperm flagellar motility,<sup>14</sup> be released from the acrosome during the acrosome reaction,<sup>15</sup> or be involved in sperm-egg fusion.<sup>16,17</sup> Its absence may cause sperm abnormality as well as disruption of sperm motility. Recent research suggests that reduced *CRISP2* expression in ejaculated spermatozoa is correlated with reduced pregnancy rates in cattle.<sup>9</sup>

In this work, we determined the expression of *CRISP2* mRNA and protein in samples of ejaculated spermatozoa from patients with ATZ and in normozoospermic volunteers and analyzed the regulatory

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effects of miR-27a on the reduction of CRISP2 expression in ATZ. Furthermore, we investigated the roles of CRISP2 and miR-27a in male infertility by follow-up study of patient reproductive histories.

## MATERIALS AND METHODS

### Human semen sample collection and preparation

Our study was approved by the Bioethics Committees of Nanfang Hospital and the Third Affiliated Hospital of Southern Medical University. Written confirmation of informed consent was also obtained from all participants. Twenty asthenoteratozoospermia (ATZ) semen samples and twenty normozoospermia semen samples were collected from patients with ATZ or from normozoospermic volunteers, respectively, in Nanfang Hospital from January 2014 to August 2014. ATZ is defined as <32% PR spermatozoa and <4% morphologically normal spermatozoa according to the World Health Organization (WHO) guidelines (5<sup>th</sup> ed.).<sup>4</sup> The semen samples were obtained by masturbation after 3 days of sexual abstinence, and then allowed to liquefy at 37°C for 30 min. We excluded samples with abnormal semen liquefaction, pH, seminal fructose, acid phosphatase,  $\alpha$ -glucosidase, and other markers of semen quality. Other factors leading to sample exclusion were abnormal hormones (such as testosterone, follicle-stimulating hormone (FSH), estradiol), varicocele, anti-sperm antibody (+), leukocytospermia, reproduction tract infections, and a history of cryptorchidism, orchitis, or epididymitis.

All liquefied semen samples were analyzed by Sperm Class Analyzer (SCA, Microptic, Barcelona, Spain), and stained with Diff-Quik (Dade Behring, Newark, NJ, USA) for evaluating sperm morphology. Basic semen characteristics were then noted (Table 1). Each sample was loaded onto a 50% discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) and centrifuged at 2000 g for 15 min at room temperature.<sup>18,19</sup> The sperm pellet was washed twice with phosphate-buffered saline (PBS) and stored at -80°C until further use.

### RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from sperm pellets with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using a PrimerScript™ RT Kit (TaKaRa, Dalian, China) for detection of mRNA and using a SYBR® PrimeScript™ miRNA RT-PCR Kit (TaKaRa, Dalian, China) for detection of miRNA.

Quantitative real-time PCR for mRNA detection was performed on a Mx3005P thermal cycler (Stratagene, Santa Clara, CA, USA) using a SYBR® Premix Ex Taq™ Real-Time PCR Kit (TaKaRa, Dalian, China). In a final volume of 20  $\mu$ l, the amplification reactions were thermally cycled as follows: denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s. Human  $\beta$ -actin (ACTB) was selected as a normalization standard.

**Table 1: Sperm parameters of the ATZ patients and Norm controls**

Characteristic	Norm (n=20)	ATZ (n=20)
Age (year)	29.60±1.05	33.30±1.46
Semen volume (ml)	3.02±0.24	3.64±0.34
Semen pH	7.55±0.03	7.52±0.03
Sperm concentration (10 <sup>6</sup> ml <sup>-1</sup> )	68.78±12.56	64.90±19.75
Sperm progressive motility (PR, %)	62.29±2.44*	10.09±1.83
Sperm morphology (normal forms, %)	20.26±1.68*	2.86±0.23

\**P*<0.05, Student's *t*-test, Norm versus ATZ. Data are presented as mean±s.d.

Norm: normozoospermic control group; ATZ: asthenoteratozoospermic group; s.d.: standard deviation; PR: progressively motile

The qRT-PCR for miRNA was carried out on the Mx3005P thermal cycler using the SYBR® PrimeScript™ miRNA RT-PCR Kit. Amplification reactions were performed in a final volume of 20  $\mu$ l by following these thermal cycling conditions: 95°C for 1 min; 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s. Sample intensities were normalized to RNU6B snRNA abundance. Data were analyzed using Applied Mx3005P Software and using the relative cycles to threshold method. The primers used for detecting the expression of CRISP2 and miRNAs are listed in **Supplementary Table 1**.

### Prediction of miRNAs targeting CRISP2

miRWalk, an online database of predicted and validated microRNA targets (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk>), was used to predict miRNAs targeting CRISP2.

### Western blotting

Of 40 semen samples, 12 of 20 ATZ samples and 12 of 20 normozoospermia samples were randomly selected for protein extraction. Total protein was extracted from the sperm pellets with a Total Protein Kit (BestBio, Shanghai, China) according to the manufacturer's instructions. Thirty micrograms of total protein was electrophoresed on 12% (*w/v*) SDS-PAGE gels and transferred to PVDF membranes (BioTrace, Pall, Mexico). The membranes were blocked with 5% (*w/v*) skimmed milk, and then incubated with antibodies against CRISP2 (1:500, 19066-1-AP, Proteintech, Chicago, IL, USA) or ACTB (1:1000, 60008-1-Ig, Proteintech, Chicago, IL, USA) at 4°C overnight. Anti-mouse or anti-rabbit IgG HRP-conjugated antibodies were added, and signals were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). The gray value of each protein band was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized to that of ACTB.

### Dual luciferase reporter assay

A 414 bp region of the CRISP2 3'-UTR was amplified from human genomic DNA and cloned into the pEZX-MT05 vector containing *Gussia* luciferase (GLuc) and secreted alkaline phosphatase (SeAP) dual-luciferase reporter genes (GeneCopoeia, Guangzhou, China). Human embryonic kidney 293T cells (HEK 293T cells) were plated in 24-well plates and cultured to 80% confluence before transfection. Vectors containing either the CRISP2 3'-UTR or a negative control, and a miR-27a mimic or a negative control, were transfected. Luciferase activity at 72 h after transfection was detected using a Secrete-Pair™ Dual Luminescence Assay Kit (GeneCopoeia, Guangzhou, China) according to the manufacturer's instructions. The Gluc activity was normalized to SeAP activity. The experiments were carried out in duplicate and repeated at least three times.

### Follow-up study of reproductive history

A follow-up study of reproductive history was carried out to determine the fertility status of each of the 40 study subjects. Study subjects were excluded if they had not been married, or had a partner diagnosed with infertility. Male infertility is defined as the inability to induce a pregnancy with unprotected intercourse with a presumably fertile female partner during a minimum period of 1 year.<sup>3</sup>

### Statistical analysis

Data were presented as mean ± standard deviation (s.d.) or standard error of the mean (s.e.m.) and analyzed using GraphPad Prism 5 Software version 5.00 (GraphPad Software Inc., San Diego, CA, USA). Direct comparisons between paired datasets were performed using Student's *t*-test. One-way ANOVA and Bonferroni test were carried out to determine significant differences in luciferase activity.

Spearman's correlation was used to measure the association between semen characteristics and miR-27a expression. Fisher's exact test was performed to determine significant differences of infertility rate between different groups.  $P < 0.05$  was considered statistically significant.

## RESULTS

### The expression levels of CRISP2 protein and its mRNA in the ejaculated spermatozoa of patients with asthenoteratozoospermia (ATZ)

Initially, CRISP2 mRNA and protein was detected by qRT-PCR and Western blot, respectively, in the spermatozoa of patients with ATZ. No difference in sperm CRISP2 mRNA levels was found between ATZ and normozoospermic participants ( $P = 0.8580$ , **Figure 1a**), but an obvious reduction in protein levels was observed in the spermatozoa of ATZ compared with those of normozoospermic controls (**Figure 1b**).

### Binding of miR-27a to the 3'-UTR of CRISP2 mRNA and repression of its translation

From the comprehensive data miRWalk, we predicted that six candidate miRNAs (miR-27a, miR-27b, miR-502-3p, miR-510, miR-640, and miR-767-5p) could target CRISP2 (**Figure 2a**). Of these candidates, miR-27a was the only micro-RNA with increased expression in ATZ spermatozoa relative to normozoospermic controls as determined by qRT-PCR ( $P < 0.05$ , **Figure 2c**). A diagram of the CRISP2 3'-UTR with the predicted miR-27a binding-site is shown in **Figure 2b**. To confirm regulation of CRISP2 translation by miR-27a, we performed luciferase activity assays. Co-transfection of 293T cells with a miR-27a mimic and a luciferase expression vector containing the CRISP2 3'-UTR resulted in decreased luciferase activity compared with controls ( $P < 0.05$ , **Figure 3c**).

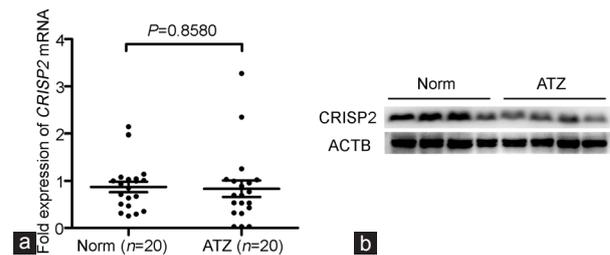
### Correlation of miR-27a and CRISP2 protein expression with normal sperm morphology and progressive motility

We compared miR-27a and CRISP2 protein expression levels with clinical semen parameters. As shown in **Figure 4**, the expression level of miR-27a was negatively correlated with progressive motility ( $P < 0.05$ ,  $r = -0.4006$ , **Figure 4a**) and normal sperm morphology ( $P < 0.05$ ,  $r = -0.3775$ , **Figure 4b**). Other semen characteristics such as age, sperm concentration or semen volume were not correlated with the expression level of miR-27a ( $P > 0.05$ , **Supplementary Tables 2 and 3**). Furthermore, the expression level of CRISP2 protein was positively correlated with progressive motility ( $P < 0.05$ ,  $r = 0.5830$ , **Figure 4c**) and normal sperm morphology ( $P < 0.05$ ,  $r = 0.5588$ , **Figure 4d**).

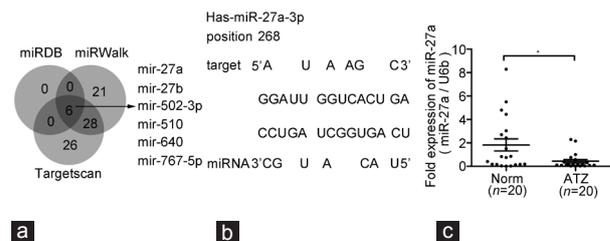
Notably, the expression level of miR-27a was negatively correlated with CRISP2 protein expression (**Figure 3a**). However, the expression levels of miR-27a and CRISP2 mRNA were not correlated ( $P = 0.0971$ , **Figure 3b**).

### Association of miR-27a and CRISP2 protein expression with infertility

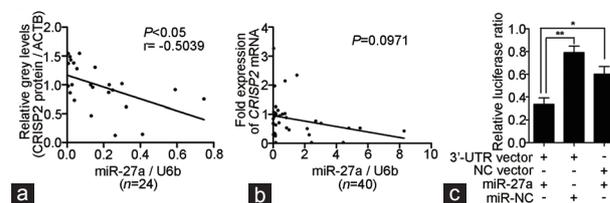
A follow-up study of reproductive history was carried out to evaluate our clinical correlations. Eight study subjects were excluded for individual reasons (six lost contact or refused to cooperate, one was unmarried, and one had a partner diagnosed with infertility), resulting in 17 normozoospermic volunteers and 15 ATZ patients. All of the subjects were divided into high- and low-expression groups according to expression levels of miR-27a or CRISP2 protein in their spermatozoa. The infertility rate was then calculated for each group and the statistical significance of the infertility rate between groups was determined by Fisher exact test (**Supplementary Table 4a–4c**). The infertility rate in the ATZ group was higher than that in the normozoospermic



**Figure 1:** The expression of CRISP2 in the ejaculated spermatozoa between asthenoteratozoospermic patients and normozoospermic volunteers. **(a)** The expression level of CRISP2 mRNA is detected by qRT-PCR in the ejaculated spermatozoa between ATZ patients and normozoospermic volunteers (Student's *t*-test, data are shown as mean  $\pm$  s.e.m.). **(b)** The CRISP2 protein expression is detected by Western blot analysis in 24 (12 Norm vs 12 ATZ) randomly selected ejaculated spermatozoa. CRISP2: cysteine-rich secretory protein 2; Norm: normozoospermic control group; ATZ: asthenoteratozoospermic patients group; s.e.m.: standard error of the mean.



**Figure 2:** High expression level of miR-27a is predicted to target CRISP2. **(a and b)** Prediction of six candidate microRNAs potentially binding to the CRISP2 3'-UTR and a potential binding-site for miR-27a. **(c)** High expression of miR-27a is observed in the ejaculated spermatozoa of patients with asthenoteratozoospermia by qRT-PCR (Student's *t*-test, data are shown as mean  $\pm$  s.e.m.;  $*P < 0.05$ ). CRISP2: cysteine-rich secretory protein 2; Norm: normozoospermic control group; ATZ: asthenoteratozoospermic patients group; s.e.m.: standard error of the mean.

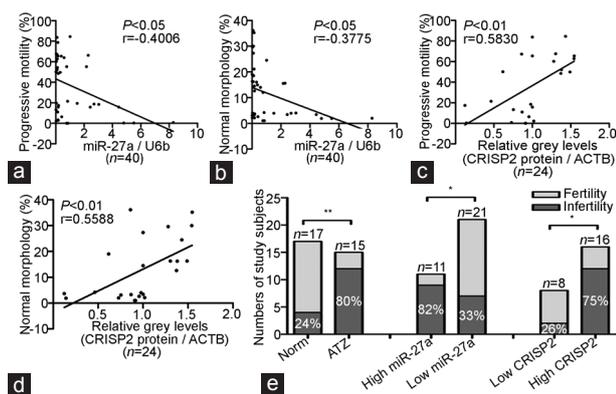


**Figure 3:** miR-27a may bind to the 3'-UTR of CRISP2 mRNA and repress translation of CRISP2. **(a and b)** miR-27a levels are negatively correlated with CRISP2 protein abundance, but not CRISP2 mRNA abundance. **(c)** Results of luciferase reporter assay in HEK 293T cells with co-transfection of 3'-UTR vector or NC vector. The bar graph shows the mean  $\pm$  s.d. in the three independent experiments. One-way ANOVA and Bonferroni test are carried out to determine significant differences in luciferase activity ( $*P < 0.05$ ,  $**P < 0.01$ ). CRISP2: cysteine-rich secretory protein 2; NC: negative control; s.d.: standard deviation.

group ( $P < 0.05$ , **Figure 4e**). A higher infertility rate appeared in the group with relatively high miR-27a expression ( $P < 0.05$ ) and in the group with relatively low CRISP2 protein expression ( $P < 0.05$ ).

## DISCUSSION

CRISP2 is an important sperm protein that modulates sperm flagellar motility<sup>14</sup> and is involved in sperm-egg fusion.<sup>17</sup> It is less abundant in the spermatozoa of patients with ATZ. For further exploration of the mechanism underlying the differential expression of CRISP2 in ATZ,



**Figure 4:** Relatively high miR-27a expression or low CRISP2 protein expression is associated with abnormal sperm morphology, reduced sperm motility and infertility. (a and b) miR-27a expression is negatively correlated with progressive sperm motility and normal morphology, while (c and d) CRISP2 protein abundance is positively correlated. (e) The infertility rates are displayed in the indicated groups. Fisher's exact test was used to assess the statistical significance of infertility rate differences between different groups (\* $P < 0.05$ , \*\* $P < 0.01$ ). CRISP2: cysteine-rich secretory protein 2; Norm: normozoospermic control group; ATZ: asthenoteratozoospermic patients group.

we analyzed the regulatory link between microRNA and CRISP2 in ATZ in this work.

MicroRNAs are widely expressed in the testis, epididymis, ejaculated spermatozoa,<sup>20</sup> and seminal plasma,<sup>21</sup> suggesting important roles of microRNAs in testicular development, spermatogenesis, and even embryogenesis.<sup>22–25</sup> Some studies have reported that miRNA-mediated gene regulation is involved in the maintenance of the undifferentiated state or the induction of differentiation of spermatogonia.<sup>26–28</sup> Some altered sperm miRNAs have been identified by microarray-based approaches in the ejaculated spermatozoa of patients with different spermatogenic impairments.<sup>29–31</sup> The expression of miR-15a and its target gene *HSPA1B* have been recently reported in ejaculated spermatozoa from patients with varicocele.<sup>32</sup> Although it is widely accepted that spermatozoa are translationally silent, some studies<sup>33–35</sup> have shown that protein translation does take place in mammalian spermatozoa before fertilization. Consistent with this, in our previous work, we found that miR-27b could target the 3'-UTR of *CRISP2* and downregulate its expression in asthenozoospermia.<sup>36</sup> In this work, we observed that miR-27a is another specific regulator of *CRISP2* and it could mediate translational repression of *CRISP2* in the spermatozoa of patients with ATZ.

miR-27a, generally considered an oncogenic microRNA, promotes cell proliferation and metastasis in many types of cancer,<sup>37–39</sup> but its function in spermatozoa is still largely unknown. In the spermatozoa of patients with asthenozoospermia, miR-27a is highly expressed,<sup>30</sup> suggesting a correlation between miR-27a and sperm motility. Furthermore, miR-27a is differentially expressed between the ejaculated spermatozoa of bulls with moderate and high nonreturn rates,<sup>40</sup> suggesting that high miR-27a expression in bulls is associated with moderate fertility. Consistently, in our work, we demonstrated that the study subjects with relatively low CRISP2 protein or with high miR-27a expression in their spermatozoa tended to have low progressive sperm motility and normal morphology. A further follow-up study of reproductive history revealed that low CRISP2 protein or high miR-27a expression was correlated with male infertility. All of these data suggest that miR-27a represses CRISP2 protein expression in a posttranscriptional regulation, clinically involving in ATZ and male infertility probably through influencing sperm motility and morphology.

ATZ and AZ are mainly characterized by abnormal sperm morphology or decreased sperm motility or both. CRISP2 is a sperm protein that influences sperm motility as well as sperm morphology. Interestingly, our previous study<sup>36</sup> and this study identified different upstream regulators of *CRISP2* in AZ and ATZ; miR-27b was highly expressed in AZ, mainly inhibiting sperm motility by suppressing *CRISP2*, whereas miR-27a was highly expressed in ATZ, mainly causing sperm malformation by repressing the same target gene. This implies the heterogeneity or specificity of miRNAs in various biological conditions. In addition, microRNAs are involved in diverse functions through targeting multiple target genes. Although both miR-27a and miR-27b regulate *CRISP2*, the differences in disease etiology associated with increased expression of either suggest that each may have a set of unique regulatory targets that are deserving of further investigation.

## CONCLUSION

We have demonstrated that CRISP2 protein is less abundant in the spermatozoa of patients with ATZ. Furthermore, we demonstrated that the observed decrease in CRISP2 protein levels is likely influenced by translational repression of *CRISP2* mRNA by miR-27a. Further clinical correlation analysis and follow-up studies of the reproductive history of study patients suggested that elevated miR-27a levels are associated with ATZ and male infertility, probably through influencing sperm motility and morphology. This study provides an insight into part of the mechanism leading to reduced CRISP2 expression in ATZ, offering a potential therapeutic target for male infertility, or for male contraception.

## AUTHOR CONTRIBUTIONS

CDL and XL designed the study. XML and ZJC carried out the experiments of cytobiology. WBG conducted the follow-up study. HX and TQ participated in acquisition of data and collection of clinical samples. JHZ and QZZ analyzed the experimental data and drafted the manuscript. JKY, JB, and MX participated in the revising of the manuscript. All authors read and approved the final manuscript.

## COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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**Supplementary Table 1: Primers used for detecting the expression of CRISP2 and miRs**

Primer	Sequence(5'-3')
CRISP2 primer	F: GGAAAGCAGTCTCTCCACCTG R: CGGTCCTCTGGATCACTATGTTG
ACTB primer	F: CATGTACGTTGCTATCCAGGC R: CTCCTTAATGTCACGCACGAT
miR-27a	TTCACAGTGGCTAAGTTCCGC
miR-27b	TTCACAGTGGCTAAGTTCTGC
miR-502-3p	ATCCTTGCTATCTGGGTGCTA
miR-510	TACTCAGGAGAGTGGCAATCAC
miR-640	ATGATCCAGGAACCTGCCTCT
miR-767-5p	TGCACCATGGTTGTCTGAGCATG
U6B	CGCAAGGATGACACGCAAATTCG

CRISP2: cysteine-rich secretory protein 2; miRs: microRNAs; ACTB: human  $\beta$ -actin

**Supplementary Table 2: Correlations between miR-27a expression and clinical features**

miR-27a	Age	Sperm concentration	Semen volume	Sperm progressive motility	Normal sperm morphology
<i>P</i>	0.0673	0.2409	0.1885	0.0104*	0.0163*
<i>r</i>	0.2922	-0.1897	0.2123	-0.4006	-0.3775

*n*=40; \**P*<0.05, Spearman rank correlation. miR-27a: microRNA-27a

**Supplementary Table 3: Correlations between CRISP2 protein expression and clinical features**

CRISP2	Age	Sperm concentration	Semen volume	Sperm progressive motility	Normal sperm morphology
<i>P</i>	0.4833	0.5822	0.1221	0.0028*	0.0045*
<i>r</i>	0.1503	0.1182	0.3243	0.5830	0.5588

*n*=24; \**P*<0.05, Spearman rank correlation. CRISP2: cysteine-rich secretory protein 2

**Supplementary Table 4a: Numbers of study subjects from the Norm group and ATZ group with infertility or fertility**

	Infertility	Fertility	Total	Infertility rate (%)
Norm	4	13	17	23.53**
ATZ	12	3	15	80.00
Total	16	16	32	-

\*\**P*<0.01, Fisher's exact test, Norm versus ATZ. Norm: normozoospermic control group; ATZ: asthenoteratozoospermic group

**Supplementary Table 4b: The individual correlation between miR-27a expression and fertility**

	Infertility	Fertility	Total	Infertility rate (%)
Relatively high miR-27a expression group	9	2	11	81.82*
Relatively low miR-27a expression group	7	14	21	33.33
Total	16	16	32	-

\**P*<0.05, Fisher's exact test, relatively low miR-27a expression group versus relatively high miR-27a expression group. miR-27a: microRNA-27a

**Supplementary Table 4c: The individual correlation between CRISP2 protein expression and fertility**

	Infertility	Fertility	Total	Infertility rate (%)
Relatively high CRISP2 expression group	2	6	8	25.00*
Relatively low CRISP2 expression group	12	4	16	75.00
Total	14	10	24	-

\**P*<0.05, Fisher's exact test, relatively low CRISP2 expression group versus relatively high CRISP2 expression group. CRISP2: cysteine-rich secretory protein 2