miR-188-3p-targeted Regulation of ATG7 Affects Cell Autophagy in Patients With Non-obstructive Azoospermia

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Abstract

Background: Studies have found that ATG7 knockout mice have defects in acrosome development, and the sperm formed by them are similar to human round-headed sperm. miR-188-3p expression is alleviated in the testis tissues of NOA samples. It is unclear whether ATG7 gene is related to non-obstructive azoospermia and whether miR-188-3p and ATG7 have direct target regulation relationship to affect spermatogenesis.

Methods: The testicular tissues of 14 non-obstructive azoospermia (NOA) patients and 13 healthy patients were collected for basic scientific research from October 2017 to June 2018. High-throughput sequencing, qRT-PCR, Western blot, immunohistochemical staining, immunofluorescence, electron microscope, and luciferase experiment were performed to confirm the correlation between miR-188-3p and ATG7 in non-obstructive azoospermia.

Results: ATG7 was highly expressed in NOA group. ATG7 protein was localized in the cytoplasm of spermatogenic cells at various levels, and the expression in NOA group was dramatically higher than that of normal individuals. After overexpression of miR-188-3p, the ATG7 3'UTR-WT luciferase activity was impeded; while the ATG7 3'UTR-MUT luciferase activity had no significant difference. LC3 and Beclin-1 gene and protein expression in the NOA samples were elevated in comparison with the normal samples; LC3 was mainly located in the cytoplasm of spermatogenic cells at all levels. LC3 and Beclin-1 genes expression after miR-188-3p overexpression were prominently in comparison with the normal samples; after miR-188-3p was inhibited, LC3 and Beclin-1 expressions were dramatically higher. The degree of LC3 punctate aggregation in the miR-188-3p mimic NC samples was greater, and that in the miR-188-3p inhibitor group was greater. The number of autophagosomes of the miR-188-3p mimic samples was less compared with the mimic NC group.

Conclusion: We demonstrated here that the ATG7 gene was located in all levels of spermatogenic cells and was highly expressed in the NOA group. The expression of ATG7 gene was negatively correlated with miR-188-3p that may regulate the target gene ATG7 to participate in autophagy and affect the occurrence of NOA.

Introduction

Non-obstructive azoospermia (NOA), known as testicular failure, may cause testicular spermatogenesis dysfunction, resulting in the inability to produce sperm or a very small amount of sperm¹. NOA is a heterogeneous disease caused by complex genetics, including chromosomal abnormalities, gene mutations and environmental factors. Previous research on NOA focused on chromosomal abnormalities², which only explained approximately 25% of azoospermia cases. The aetiology of non-chromosomal NOA is complex, and its pathogenesis is unclear. In particular, the molecular mechanism that determines the amount of spermatogenesis in NOA patients needs further study. Therefore, an in-depth study of the

molecular mechanisms affecting the number of spermatozoa produced in NOA patients will help to find new molecular markers for NOA diagnosis and gene therapy targets.

miRNAs are small non-coding RNAs with a length of approximately 22 nucleotides that are widely found in different organisms. During embryonic development, miRNAs contribute to various processes such as cell proliferation, stem cell differentiation, development time regulation and stem cell division³. In spermatogenesis-related studies, some results supported the hypothesis that various physiological processes such as the synthesis of related proteins are dynamically regulated via some miRNAs, thus indirectly regulating sperm maturation, sperm motility and supporting cell maturation⁴. miR-188 has been implicated as a tumour suppressor and its overexpression in pancreatic cancer inhibited the expression of the target gene BRD4, impeding in the growth of pancreatic cancer cells and an increase in the cytotoxicity of gemcitabine⁵. miR-188-3p is a novel negative regulator of the transmembrane-containing Emp24 domain transporter (TMED3), alleviating the proliferation, migration and invasion of breast cancer cells⁶. Early research indicated miR-188-3p was abundantly monitored in spermatogonia and spermatocytes of human testicular tissue and could be detected during meiosis in mouse cells⁷. Our previous research also confirmed miR-188-3p regulated MLH1 to cause apoptosis of spermatogenic cells, which in turn affected the occurrence of NOA⁸. The above research suggests that miR-188-3p is involved in spermatogenesis.

Autophagy is a process that can exist under both physiological and pathological conditions of the body. Its main metabolic process is the phagocytosis and degradation of damaged organelles and macromolecules by cells. Autophagy begins with the formation of autophagosomes, which are wrapped in a double-layer membrane and swallow part of the cytoplasm and damaged organelles⁹. The whole process is under the regulation of different autophagy-related genes (ATGs), and approximately 30 kinds of ATGs have been discovered. These genes, which are highly conserved in yeast and mammals, are essential molecules for the occurrence of autophagy and participate in different stages of autophagy¹⁰. Studies found that ATG7 gene knockout mice had acrosomal development defects, and the sperm shape was similar to that of human round head sperm¹¹. Furthermore, our previous high-throughput sequencing data indicated ATG7 expression was elevated in the NOA group. Until now, there have been no reports on the ATG7 gene and azoospermia. Whether there is a certain relationship between miR-188-3p and ATG7 and whether the two have a direct targeted regulatory relationship to affect spermatogenesis require further research.

Materials And Methods

1. Sample collection

From October 2017 to June 2018, 14 cases of NOA at the Reproductive Center of the First Affiliated Hospital of Zhengzhou University and 10 NOA testicular paraffin-embedded tissue specimens from the Department of Pathology were selected, as detailed in Table 1. Thirteen male patients who underwent

testicular puncture due to the difficulty in obtaining sperm on the egg retrieval day were selected and 10 normal testicular paraffin-embedded tissues specimens were obtained from the Pathology Department. The inclusion criteria for the NOA group were as follows: the semen or urine after ejaculation was centrifuged at 3000 rpm for 15 minutes; no sperm was found in the sediment by microscopic examination; and the pathological results showed that the number of spermatogenic cells in the seminiferous tubules of the testes decreased, but there were mature sperm.

Table 1
Primers for qRT - PCR (premier 3.0)

Gene name	Primer	Sequences(5'-3')
ATG7	Forward	5'-TCCGGGGATTTCCTTTCACGG-3'
	Reverse	5'-GCTTCATCCAGAGCCGAAGA-3'
LC3	Forward	5'-GATGTCCGACTTATTCGAGAGC-3'
	Reverse	5'-TTGAGCTGTAAGCGCCTTCTA-3'
Beclin-1	Forward	5'-AGCTGCCGTTATACTGTTCTG-3'
	Reverse	5'-ACTGCCTCCTGTGTCTTCAATCTT-3'
GAPDH	Forward	5'-GGTGGTCTCCTCTGACTTCAACA-3'
	Reverse	5'-GTTGCTGTAGCCAAATTCGTTGT-3'
miR-188-3p	Forward	5'-ATTATTGGCTCCCACATGCAGGG-3'
	Reverse	5'-ATCCAGTGCAGGGTCCGAGG-3'
U6	Forward	5'-CGCAAGGATGACACGAA-3'
	Reverse	5'-TGGCCCCTGCGCAAGGAT-3'

The groups in this experiment were as follows: ① Testicular tissue group: NOA group (14 cases) and normal group (male patients with normal spermatogenesis, 13 cases) and ② Cell experiment group: human testicular cancer cell line (NTERA-2) with overexpression/inhibition of miR-188-3p group and without overexpression/inhibition of miR-188-3p group (control group). The exclusion criteria were as follows: patients with past history of mumps, orchitis, tumour, traumatic surgery, exposure to poisons and radiation, obstructive factors and chromosomal abnormalities. All patients were 20–45 years old and had normal chromosomes. All patients signed an informed consent form after approval by the ethics committee. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-78) and was carried out in accordance with the guidelines of ethical management. The human testicular cancer cell line NTERA-2 was donated by Professor Sun Fei's group at the University of Science and Technology of China.

2. High-throughput sequencing of testicular tissue

The testicular tissue was fully ground in a clean mortar with an appropriate amount of liquid nitrogen. After the grinding was complete, TRIzol reagent (Invitrogen, USA) was added for extracting total RNA from testicular tissue (according to the instructions provided by the manufacturer). The sequencing was completed by mRNA enrichment, rRNA removal, end flattening, 3' end plus A, splicing, PCR amplification, library quality control, library standardization and enrichment. Cluster generation and first-way sequencing primer hybridization were completed on the cBot of the Illumina sequencer according to the cBot User Guide. Sequencing reagents were prepared according to the Illumina User Guide, and the flow cell with the cluster was uploaded to the machine. The PAIRED-END programme was selected for double-ended sequencing, and the entire sequencing process was controlled by the DATA COLLECTION software provided by Illumina, with real-time data analysis. Quality control analysis criteria: The sequencing results analysis data volume of each sample was approximately 10 G, and the proportion of each base mass greater than 20 (Q20) was not less than 90%.

3. Cellular transfection

Cells were inoculated at a density of approximately 1.2×10^6 cells per well, with cell confluence of 70%-80%. Preparation of liquid A: Five microlitres of miR-188-3p mimics/control mimics and inhibitors/suppressors at a concentration of 20 μ M were added to 250 μ l Opti-MEM serum-free medium, mixed well, and incubated at 23°C for 5 min. Preparation of liquid B: Liposomal Lipo2000 was mixed before being used, and 5 μ l of Lipo2000 was added to 250 μ l Opti-MEM serum-free medium (according to Opti-MEM: Lipo2000 = 50:1), mixed well, and incubated at 23°C for 5 min. The two liquids were mixed well and incubated at 23°C for 20 min. The mixture was slowly added to a 6-well plate cell culture dish and incubated at 37°C and 5% CO₂ for 4 ~ 6 h. Afterwards, the serum-free medium was replaced with DMEM containing 10% serum.

4. qRT-PCR

TRIzol reagent was used for extracting total RNA; the OD value (OD₂₆₀/OD₂₈₀) and RNA concentration of the extracted RNA were determined. mRNAs were synthesized using primers from the kit, whereas for miRNAs, cDNA synthesis was performed using specific reverse transcription primers. The specific primer for miR-188-3p was as follows:

5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTGCAAA-3'. The RT reaction solution was prepared by adding total RNA at 1 ~ 2 μ g on ice (Supplementary Table SI). Two parallel control wells were set up per sample, and the experiment was repeated at least three times. The relative expression of the genes was calculated according to Eq. $2^{-\Delta\Delta C_t}$, and the endogenous control for microtubule-associated protein 1 light chain 3 (LC3), Beclin-1 and ATG7 was GAPDH, while the endogenous control for miRNA-188-3p was U6. The specific primers were designed by Premier 3.0 and are shown in Table 1. The quantitative real-time PCR configuration is setting as Supplementary Table SII. The set parameters for the PCR conditions are shown in Supplementary Table SIII.

5. Western blot

Total protein was extracted from spermatogenic cells of testicular tissue and NTERA-2 cells. Western blot for ATG7, LC3, Beclin-1 was performed as our previous work⁸. The membrane was incubated with anti-ATG7 antibody (1:1000; Abcam, Cambridge, UK), anti-LC3 antibody (1:1000; Abcam), anti-Beclin-1 antibody (1:1000; Abcam) and anti- β -actin antibody (1:1000; Abcam) overnight at 4°C and incubated with secondary antibodies (1:1000; Sanying Biotechnology, Wuhan, China) for 2 hours at 23°C.

6. Dual luciferase experiment

ATG7 3'UTR:5'..... AUGGGUGAGGGUGGGAC3'

miR-188-3p: 3' ACGUUUGGGACGUACACCCUC 5'

According to the prediction results, the ATG7 3'-UTR dual luciferase reporter wild type (WT) and mutant (MUT) plasmids were constructed as follows: ATG7 3'UTR-WT:

5'-... CCTGTGGGGGCCCTGGGCATGGGTGAGG**GTGGGAC** ...-3'

ATG7 3'UTR-MUT:

5'-... CCTGTGGGGGCCCTGGGCATGGGTGAGG**CACCCTC** ...-3'

The transfected cells were divided into four groups: ①cells transfected with miR-188-3p mimic at a final concentration of 50 nM and 400 ng ATG7 3'UTR-WT plasmid per well in a 24-well plate; ②cells transfected with miR-188-3p mimic NC at a final concentration of 50 nM and 400 ng ATG7 3'UTR-WT plasmid per well in a 24-well plate; ③cells transfected with miR-188-3p mimic at a final concentration of 50 nM and 400 ng ATG7 3'UTR-MUT plasmid per well in a 24-well plate; and ④cells transfected with miR-188-3p mimic NC at a final concentration of 50 nM and 400 ng ATG7 3'UTR-MUT plasmid per well in 24-well plates. After 48 hours of transfection, the cell culture medium was discarded. Subsequently, 100 μ l of 1 $\times$ PLB per well was added to the cells in a 24-well plate, and the cells were lysed at 23°C for 15 min. The sample was centrifuged at 12000 rpm for 10 min at 4°C. The centrifuged supernatant was added to a 96-well plate at 10 μ l per well. Each sample requires 2 parallel wells for a total of 3 wells. Each well sample was increased to 50 μ l/well by adding 40 μ l 1 $\times$ PLB (to avoid sample evaporation affecting the experimental results). Then, 100 μ l LAR II was added to each well to quantify the firefly luciferase activity, and the absorbance value at 480 nm was measured. Subsequently, 100 μ l of 1 $\times$ Stop&Glo® Reagent working solution was added to quench the fluorescence of LARII and simultaneously excite Renilla luciferase fluorescence (internal reference fluorescence). After the treatment, the absorbance value at 480 nm was measured again. The ratio of the two was the relative luciferase activity.

7. Immunofluorescence staining analysis

The cells were fixed with 4% PFA for 15 minutes and then washed with PBS three times for 3–5 minutes each time. The sample was placed in a humidified box containing 0.5% Triton X-100/PBS at 23°C for 20

min and washed with PBS 3 times. Subsequently, the samples were incubated with anti-LC3 antibody (1:100), placed in a wet box, incubated overnight at 4°C, and washed with PBS 3 times. An anti-fluorescence quencher was used to mount the slide, and images were collected under a confocal fluorescence inverted microscope.

8. Electron microscope experiment

After 48 hours of transfection, the discarded cell medium was quickly added to 2.5% glutaraldehyde electron microscopy fixing solution without rinsing, and the cells were scraped off gently to collect the cells into the centrifuge tube. The cells were then collected by centrifugation, added to new electron microscope fixative solution, fixed at 23°C for 2 h, and stored at 4°C. After fixing with 1% osmium acid for 1 to 2 hours, specimens were incubated in 50%, 70%, 80%, and 90% acetone for 5 to 10 minutes and washed in 100% acetone three times, each time for 10 to 15 minutes. Subsequently, the samples were incubated in a mixed solution of 100% dehydrating agent and an equal amount of embedding agent at 23°C for 30 min. Then these samples were placed in pure embedding agent overnight and then embedded. The samples were sliced at 70 nm by an ultrathin microtome and double-stained with 3% uranyl acetate-lead citrate. Finally, two sets of samples were placed under a transmission microscope to observe and film.

9. Statistical analysis

SPSS21.0 software was used for statistical analyses. Measurement data are expressed as the mean $\pm$ standard deviation (mean $\pm$ SD), and the mean between the two groups was compared and analysed by t test. The test standard was $\alpha = 0.05$, and $P < 0.05$ indicated statistical significance. GraphPad Prism5.0 software was used for mapping analysis.

Results

1. Expression and localization of the ATG7 gene in the testis

We selected 3 NOA patients and 3 normal testicular samples for high-throughput sequencing analysis. The obtained P-value was subjected to multiple hypothesis testing and corrected by controlling the false discovery rate (FDR). The Q-value is the corrected P-value. The screening conditions for differentially expressed genes were $Q\text{-value} \leq 0.05$ and $\text{fold-change} \geq 2$. A total of 109 differentially expressed mRNAs were found (of which 93 mRNAs were upregulated and 16 mRNAs were downregulated) (Fig. 1A). According to bioinformatics analysis and prediction, there were 5 mRNAs with potential binding sites to miR-188-3p (Supplementary Table IV). Among them, ATG7 is a key protein for autophagy.

Subsequently, through qRT-PCR detection, ATG7 expression in testicular samples of individuals with non-obstructive azoospermia was prominently higher ($P < 0.05$) (Fig. 1B). It was also observed through immunohistochemistry that the ATG7 protein was mainly localized in the cytoplasm of spermatogenic cells. ATG7 protein staining was brownish yellow, and its expression was detected both in non-obstructive azoospermia and in normal fertility testicular tissues (Fig. 1C). Moreover, ATG7 protein level in non-

obstructive azoospermia testes was dramatically elevated compared with normal testes, detected by integral optical density (IOD) analysis ($P < 0.05$) (Table 2).

Table 2
IOD analysis of ATG7 protein expression in testis tissues of each group

Group	n	IOD	<i>P</i>
Control group	20	47.070 ± 4.7515	0.000
NOA group	20	105.120 ± 8.0311	

2. The effect of miR-188-3p overexpression or inhibition on the expression level of ATG7 and the verification of their interaction sites

Using cell transfection technology, NT-2 cells were transfected with miR-188-3p mimic/mimic NC and miR-188-3p inhibitor/inhibitor NC. As expected, the ATG7 gene expression level was remarkably decreased ($P < 0.05$) (Fig. 2A). The volume of ATG7 protein in the miR-188-3p overexpression samples was detected to be lower by Western blot (Fig. 2B). After miR-188-3p was suppressed, the ATG7 gene and protein expression level were considerably higher ($P < 0.05$) (Fig. 2A, 2C).

Furthermore, evaluation of the targeted relationship between miR-188-3p and ATG7 identified a binding site between the two (Fig. 2D). The ATG7 3'-UTR dual luciferase reporter wild-type (WT) and mutant (MUT) plasmids were constructed according to the prediction results and sequenced (Fig. 2E). The transfection efficiency after miR-188-3p was overexpressed or inhibited checked by qRT-PCR (Fig. 2F). The luciferase activity of the ATG7 3'-UTR wild-type reporter was lower than that of the miR-188-3p group after miR-188-3p overexpression ($P < 0.05$). However, there was no significant difference between the luciferase activity of the mutant ATG7 3'-UTR reporter and the miR-188-3p non-overexpressing group ($P > 0.05$) (Fig. 2G).

3. The expression of LC3 and Beclin-1 and the localization of LC3 in testicular tissue

LC3 protein was mainly localized in the cytoplasm of spermatogenic cells at all levels, and its staining was brownish yellow according to the immunohistochemical results. LC3 protein level in individuals with NOA was elevated in comparison with the normal group, detected by IOD analysis ($P < 0.05$) (Fig. 3A, and Table 3). Moreover, LC3 and Beclin-1 expression levels in the NOA group were enhanced ($P < 0.05$) (Fig. 3B, 3C). Consistent with the genes expression, the proteins levels of the two in the NOA group were higher (Fig. 3D, 3E). In addition, the ratio of autophagosomal membrane type LC3 (LC3-II)/cytoplasmic LC3 (LC3-I) in the NOA group was also considerably elevated (Fig. 3D).

Table 3
IOD analysis of LC3 protein expression in testis
tissues of each group

Group	n	IOD	P
Control group	5	39.478 ± 4.473	< 0.05
NOA group	5	76.241 ± 4.213	

4. The effect of miR-188-3p overexpression or inhibition on autophagy

miR-188-3p expression in each group was monitored after overexpressing or inhibiting miR-188-3p in NT-2 cells. Expression in the miR-188-3p mimic group was higher than that in the miR-188-3p mimic NC group ($P < 0.05$). On the contrary, expression in the miR-188-3p inhibitor samples was lower ($P < 0.05$) (Fig. 2F). Furthermore, after miR-188-3p overexpression in the mimic samples, LC3 and Beclin-1 expression was dramatically alleviated ($P < 0.05$) (Fig. 4A-C, 4F). After miR-188-3p inhibition in the inhibitor samples, the levels of the two were effectively improves ($P < 0.05$) (Fig. 4C-F).

Subsequently, LC3 was found to be localized in the cytoplasm by immunofluorescence. After miR-188-3p was transfected into the mimic NC group, the degree of punctate aggregation of LC3 increased. As expected, the transfected miR-188-3p inhibitor samples had a greater degree of punctate aggregation of LC3. DAPI fluorescence is shown in blue, and LC3 fluorescence is shown in green (Fig. 4G).

After miR-188-3p treatment, the number of autophagosomes in the miR-188-3p mimic samples was alleviated, demonstrated by transmission electron microscopy. Autophagosomes can be distinguished from other cellular structures within the cell because they are characterized by a double-layer or multi-layer membrane, and they contain damaged or ageing organelles and cytoplasmic content (Fig. 4H).

Discussion

miRNAs regulate genes post-translationally by binding to specific mRNA targets and thus participating in various physiological and pathological regulatory processes¹². Preliminary experiments have ascertained that miRNAs expression in spermatogenic cells changes at different stages, which indicates miRNAs contribute to spermatogenic cells development and promote spermatogenesis. Recent studies have confirmed that miRNAs, as key mediators of human and animal testicular development and spermatogenesis, are involved in early male germ cell proliferation and late spermatogenesis to form functional sperm³. Since spermatogenesis is a complex differentiation process, NOA may be caused by abnormal gene expression at any stage during spermatogenesis¹³. Accumulating evidence has confirmed that miR-188-3p is abundantly detected in spermatogonia and spermatocytes in human testicular tissue and during the meiotic cell cycle of mouse cells⁷. Moreover, our previous research data supported miR-188-3p expression in NOA testicular tissue was significantly reduced, indicating the

dysregulated miR-188-3p expression may contribute to the occurrence of NOA. In this experiment, a total of 109 differentially expressed mRNAs were identified by high-throughput sequencing analysis of testicular tissues of patients in the two groups. Among them, 5 mRNAs were predicted by bioinformatics analysis to be targets of miR-188-3p. The identified potential binding sites provided a new target for our subsequent study of the involvement of miR-188-3p in spermatogenesis. Given that previous studies reported that ATG7 is an autophagy gene and an E1-like activating enzyme that is essential for the autophagy coupling system and autophagosome formation. ATG7 can simultaneously control the number and size of autophagosomes¹⁴. Later studies suggested that ATG7 is crucial for the formation of the ATG5/ATG12/ATG16L complex and LC3 complex and contributes to the regulation of the autophagy pathway¹⁵. In animal experiments, Wang et al. found that male mice with ATG7 mutations led to complete infertility and abnormal acrosome formation. This was consistent with human round sperm disease, a severe fertility disorder characterized by abnormal round head sperm¹⁶. Subsequently, it was found that the expression of ATG7 increased in males with infertility due to varicocele, and the level of autophagy and apoptosis increased, which may be related to the elimination of a large number of abnormal sperm and the response to heat and oxidative stress¹⁷. Our results showed that ATG7 was considerably enhanced in NOA testicular samples and was expressed in spermatogenic cells at all levels, suggesting that ATG7 may participate in the development of NOA through high levels of autophagy.

miRNAs regulate genes expression during the post-transcriptional level through base pairing to cause translational inhibition and/or degradation of mRNA, thereby exerting their functions in biological and pathological processes¹². Previous studies have observed that the expression phenotype of testicular tissue in NOA patients is characterized by the loss of normal spermatogenesis, and the upregulated expression of some mRNA genes is caused by the downregulated expression of miRNA genes¹⁸. In addition, Wang K et al confirmed that autophagy promoting factor (APF) regulates autophagy cell death by targeting miR-188-3p and ATG7¹⁹. Previously, studies have shown that proteins related to chromosome structure (such as histones and dislocation repair proteins) may have binding sites with miR-188-3p, and these proteins are crucial for spermatogenesis²⁰. Afterwards, miR-188-3p was proven to be the target of polypyrimidine bundle binding protein (PTBP) 2 in mouse testes, and PTBP2 binds to the 3'-UTR of germ cell-specific phosphoglycerate kinase 2. Therefore, there may be another potential pathway by which miR-188-3p regulates PTBP2 involvement in spermatogenesis²¹. Our previous study detected low expression of miR-188-3p in NOA testes. Furthermore, in this experiment, we detected high expression of the ATG7 gene in NOA testes. After miR-188-3p mimic transfected in the human testicular cancer cell line NTERA-2, the expression level of ATG7 was downregulated. In addition, after miR-188-3p inhibitor transfected, ATG7 expression was upregulated. The above results suggest ATG7 gene expression is impeded by miR-188-3p. However, it has not been reported whether miR-188-3p and ATG7 have a direct targeted regulatory relationship in testicular tissues to affect spermatogenesis. We further predicted through bioinformatics that the 3'-UTR of ATG7 have potential binding sites with miR-188-3p. Dual luciferase reporter assay results showed that after overexpression of miR-188-3p, the luciferase activity of wild-type ATG7 3'-UTR decreased, while the activity of mutant ATG7 3'-UTR showed no

significant difference. Based on the results of these experiments, we speculate miR-188-3p may contribute to autophagy by regulating ATG7 expression, leading to spermatogenesis disorder and then NOA.

Autophagy is a relatively conservative evolutionary mechanism in eukaryotic cells. It begins with the formation of autophagosomes. Autophagy is important for normal cell development and physiological processes. In most cases, autophagy is recognized to protect and destroy cells²². LC3 is a homologous gene of ATG8 in mammals²³ and has two mutually transformable forms, LC3-I and LC3-II. After induction of autophagy, LC3 is cleaved by the cysteine protease ATG4 to form LC3-I. LC3-I is coupled to the substrate phosphatidylethanolamine (PE) to form the autophagosomal membrane-bound form LC3-II. This process is performed by the E1-like enzyme ATG7, the E2-like enzyme ATG3, and the E3-like enzyme ATG5-. ATG12-ATG16L complex together. LC3-II, an important marker molecule for autophagosomes, increases with an increase in autophagosomal membranes²⁴. When autophagy is initiated, LC3-I will enzymatically cleave a small piece of polypeptide and transform into LC3-II. Therefore, the autophagy level can be estimated by the LC3-II/I ratio²⁵. Wei et al. found that the abnormal increase and decrease in LC3-II levels led to autophagy defects, thereby impairing the spermatogenic function of rat testes, leading to testicular developmental disorders²⁶. Beclin-1, as a homologue of ATG6 in mammals, is the core component of the autophagy mechanism²⁷. Beclin-1 is phosphorylated by unc-51-like autophagy activating kinase-1 (ULK1) and acts as an overall scaffold for the phosphoinositide 3-kinase (PI3K) complex, promoting proteins recruitment to autophagy vesicles. When the complex binds to other regulatory proteins, it selectively participates in different stages of autophagy²⁸. In addition, Beclin-1 has an independent mechanism of autophagy and an independent role in autophagy in cancer²⁹. In chronic myeloid leukaemia, miR-21 is upregulated. The expressions of Beclin-1, Vps34, and LC3-II are increased with treatment of anti-miR-21, which ultimately leads to increased autophagy³⁰. In this study, through immunohistochemistry, LC3 protein was found in the cytoplasm of spermatogenic cells at all levels. In addition, as the autophagy marker genes, the expression of LC3 and Beclin-1 in NOA testicular samples was higher than that in normal tissues. In patients with NOA, the expression of the two proteins was shown to increase through Western blot. Highly expressed LC3 and Beclin-1 may participate in the occurrence of NOA through autophagy.

miRNAs are involved in cellular autophagy expression and regulate the target genes expression at the chromatin organization, transcriptional and post-transcriptional levels. miRNAs target the 3' untranslated region of mRNAs, turn on/off the gene expression of key autophagy factors after transcription, and regulate the promotion of autophagy factors to trigger or inhibit the occurrence and development of cellular autophagy. It is known that the interaction between miR-471-5p and autophagy member protein regulates the clearance of apoptotic germ cells through LC3-related phagocytosis. Transgenic mice expressing miR-471-5p in Sertoli cells (SCs) showed increased germ cell apoptosis and impaired male fertility³¹. In our study, autophagy marker genes expression in NOA testicular tissue was detected to be higher than that of the normal group by qRT-PCR and Western blot. Additionally, in cell experiments, after miR-188-3p overexpression, LC3 and Beclin-1 expression was lower. After miR-188-3p suppression, LC3

and Beclin-1 were enhanced in the unsuppressed individuals. The immunofluorescence results showed that after miR-188-3p overexpressed, LC3 spot aggregation elevated in comparison with that in the non-overexpression samples. After miR-188-3p expression was suppressed, LC3 spot aggregation decreased compared with that in the non-inhibition group. Finally, autophagy was further observed through transmission electron microscopy³². The number of autophagosomes in the miR-188-3p overexpression group was observed to be lower than that in the non-overexpression group through transmission electron microscopy. Our results indicated miR-188-3p is related with the occurrence of autophagy. In our study, first, the expression of ATG7 in testicular tissues of the NOA group and normal group was confirmed to be elevated by high-throughput sequencing. It has been proven that ATG7 contribute to autophagy. Wang et al. also confirmed that miR-188-3p impede autophagy by regulating ATG7¹⁹. We further used dual luciferase experiments to verify that miR-188-3p have a mutual regulatory relationship with ATG7 and ATG7 is the target site for miR-188-3p. There may be downregulation of miR-188-3p in the testicular samples by reducing these two genes. The inhibitory effect of ATG7 in turn leads to an abnormal increase in autophagy levels, which affects the spermatogenesis process.

In summary, the results of this study indicate miR-188-3p may regulate its target gene ATG7 to participate in autophagy and thus affect the occurrence and development of NOA. This provides a new experimental and theoretical basis for studying autophagy as a potential target for diagnosis and treatment. Combined with our previous research results that miR-188-3p-targeted regulation of MLH1 affects apoptosis and participates in the occurrence of NOA, it will be possible to explore whether there is an autophagy-apoptosis interaction network affecting NOA.

Declarations

Ethics approval and consent to participate

Informed consent was obtained from each patient, and ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-78) and was carried out in accordance with the guidelines of ethical management.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yingpu Sun, Wenyan Song, Yuan Wang and Chengcheng Tian designed the study and wrote the manuscript. Yingpu Sun and Wenyan Song supervised the research. Chengcheng Tian prepared, analyzed and interpreted protein sequencing data. Yingchun Su and Haixia Jin collected the human samples. Xueshan Ma and Xiangyang Zhang prepared the testicular samples and interpreted sequencing data. Wenbin Niu prepared the testicular samples and validated expression data. Chengcheng Tian and Minrui Liu analysed results and executed qRT-PCR to validate gene expression. Chengcheng Tian and Minrui Liu contributed to Western blot and dual luciferase experiment. All authors approved the final version of the manuscript, and declare that they have no competing interests.

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Figures

Figure 1

Expression and localization of the ATG7 gene in the testis

Figure 2

The effect of miR-188-3p overexpression or inhibition on the expression level of ATG7 and the verification of their interaction sites

Figure 3

The expression of LC3 and Beclin-1 and the localization of LC3 in testicular tissue

Figure 4

The effect of miR-188-3p overexpression or inhibition on autophagy

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