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microRNA-383 impairs phosphorylation of H2AX by targeting PNUTS and inducing cell cycle arrest in testicular embryonal carcinoma cells ☆

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ABSTRACT

Male germ cells with aberrant DNA damage are the weighted factor contributing to male infertility. Mounting evidence shows that DNA damage in male germ cells impairs spermatogenesis and lowers fecundity. MicroRNAs (miRNAs) regulating expression of multiple genes play a significant role in spermatogenesis. Our previous results have shown that microRNA-383 (miR-383) is one of the notable down-regulated microRNAs in the testes of sterile males with maturation arrest (MA) and is located predominantly in spermatogonia and primary spermatocytes. However, the role that miR-383 plays in DNA damage during spermatogenesis remains unknown. In this study, we found that miR-383 inhibited the focal formation and abundance of γH2AX, which is the major marker of sites of DNA damage, with or without ultraviolet irradiation and cisplatin in testicular embryonal carcinoma (NT-2) cells. In addition, NT-2 cells were remarkably sensitized to DNA damage reagent (cisplatin) by forcing expression of miR-383 and silencing expression of protein phosphatase 1, regulatory subunit 10 (PNUTS). By constructing *Renilla* luciferase reporters and co-transfecting miR-383 and reporters in NT-2 cells, we identified that PNUTS was a valid target of miR-383. Further results demonstrated that the repression of the phosphorylated form of H2AX by miR-383 was due to independent depletion of PNUTS and cell cycle arrest. In conclusion, we found a novel function of miR-383 in the DNA damage pathway. miR-383 impairs the phosphorylation of H2AX by targeting PNUTS and inducing cell cycle arrest independently, as well as sensitizing NT-2 cells to cisplatin.

1. Introduction

Ten to fifteen percent of couples of childbearing age are infertile, and half of the cases are due to male factors, which affect one out of 20 men in the general population [1,2]. Furthermore, human patients with non-obstructive azoospermia (NOA), which accounts for a considerable proportion of male factor infertility, possess dramatically lower sperm retrieval rate and clinical pregnancy rate and higher rates of DNA fragmentation [3,4]. Many studies have shown that DNA damage in sperm is associated with infertility and low fecundity [5–7]. However, the molecular mechanism of DNA damage occurring during spermatogenesis, especially in the early stage of spermatogenesis, remains obscure.

Spermatogenesis is a well-tuned and dynamic process involving the transformation of spermatogonial stem cells to mature spermatozoa, including the stages of mitotic spermatogonia and meiotic spermatocytes. Five most common types of DNA damage occur during spermatogenesis due to the remodeling of chromatin [6]. Normally, these sites of DNA damage are associated with phosphorylation of H2AX(H2A histone family, member X) at serine 139, referred to as γH2AX, and are resolved by DNA damage repair regulation before spermatozoa production during spermatogenesis [8]. The mechanism underlying the aberrant DNA damage observed during spermatogenesis of testes of sterile patients is little known.

MicroRNAs (miRNAs), a family of small noncoding RNAs, are generated from processing of stem-loop structures of 70 nucleotides by several specific endonucleases and are ultimately delivered as mature single-stranded small RNAs of 19–23 nt that post-transcriptionally regulate gene expression through base pairing with the 3′-untranslated region (3′UTR) of target mRNAs [9]. Numerous studies have suggested that miRNAs play a crucial role in regulating the DNA damage pathway activated by radiation and DNA damage reagents. Inhibition of miR-34-c impairs DNA damage-induced S-phase arrest, whereas forced expression of miR-34 represses expression of c-Myc, leading to genomic instability [10]. miR-138 [11] and miR-24 [12] modulate the DNA damage response by targeting H2AX directly in cancer cell lines and terminally differentiated blood cells, respectively. However, the role miRNAs play in DNA damage during spermatogenesis is poorly understood.

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Our previous study has identified the differential expression of miRNAs in the testes of NOA patients with maturation arrest (MA) [13]. In particular, miR-383 was notably down-regulated in spermatogonia and spermatocytes of MA patients. These findings indicate that miR-383 may be involved in events occurring in the early stage of spermatogenesis where the DNA damage response is activated during meiosis. To investigate the possible role of miR-383 in DNA damage signaling in spermatogenesis, we studied the correlation between miR-383 and γH2AX in the testicular embryonal carcinoma cell line NTERA-2 (NT-2) and explored the potential targets of miR-383 that may play roles in the DNA damage pathway.

2. Material and methods

2.1. Human testicular samples

Human testicular tissue samples from nine patients (aged 21–38 years) with MA and from three control patients (aged 31–37 years) were obtained from the First Affiliated Hospital of Anhui Medical University (Hefei, China). Samples were dissected into several portions for different experiments. The volumes of testicular tissues from patients varied greatly. As a result, some specimens were not enough to be used in all experiments. Ideal normal controls would be obtained from volunteers of known fertility; however, difficulties in acquiring testicular material from this demographic group made this impractical. Therefore, we obtained testicular tissues from patients undergoing orchiectomy for prostate carcinoma before chemotherapy, who had a history of normal spermatogenesis and fertility. All specimens were taken with patient informed consent and with ethical approval from the Institutional Review Boards of the University of Science and Technology of China and the Anhui Medical University.

2.2. Cell culture and transfection

NT-2 cells are derived from human embryonal carcinomas. Cells (NT-2, 293T, MCF-7, and H1299) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Life Technology Inc., Grand Island, NY, USA) and 1% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin, Life Technology Inc.). Cells were incubated at 37 °C in a humidified incubator with 5% carbon dioxide. Different transfection reagents were used for different cell lines and nucleotides. Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection of 293T cells and Lipofectamine RNAiMAX (Invitrogen) and Fugene HD (Roche) were used in transfection of oligonucleotides and plasmids into NT-2 cells, respectively. All transfection procedures were performed according to the protocols supplied by manufacturers.

2.3. Oligonucleotides and vectors

siRNA duplex homologs in sequences with miR-383 mimics and miR-383 inhibitor, as well as negative control (NC) and inhibitor NC were chemically synthesized and optimized by Shanghai Gene-Pharma Co. (Shanghai, China). The inhibitor of miR-383 is single-stranded, sequence-specific, and chemically modified to specifically target and knock down miR-383 molecules. PNUTS siRNA (si.PNUTS) was designed and chemically synthesized by Shanghai Gene-Pharma Co. The sequence of si.PNUTS was: sense, GACCCGUUCACCAGAAAUATT; antisense, UAUU UCUGGUGAACGGGUCTT.

The psiCHECK-2 dual luciferase reporter plasmid was kindly provided by Biliang Zhang (Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, China), and the pcDNA3.1 + vector was kindly provided by Mian Wu (USTC, China). Wild-type (WT) plasmids of PNUTS 3′ UTR and H2AX 5′ UTR plus Coding DNA Sequence (CDS) were constructed by amplifying a 848-bp 3′ UTR fragment of PNUTS mRNA and 505-bp 5′ UTR plus CDS fragment of H2AX mRNA harboring the miR-383 binding site predicted by miRanda (http://www.microrna.org) and DNAman, respectively, whereas mutated (MT) PNUTS 3′ UTR was generated by PCR-based site-directed mutagenesis. The fragments of WT and MT PNUTS 3′ UTR were fused with the psiCHECK-2 reporter vector at the XhoI and NotI sites. The primers were as follows:

WT PNUTS 3′ UTR
Forward primer: 5′-CTACTCGAGCTGTGGACTGCAGCCTCGCTCTT-3′
Reverse primer: 5′-CTAGCGGCCGCTTCACGCTGGCAAGGTTC-3′
MT PNUTS 3′ UTR
Forward primer: 5′-CTACTCGAGTTCTGTAGACTGGTGCGCATTG-3′
Reverse primer: 5′-CTAGCGGCCGC CAATGCGCACCAGTCTACAG AA-3′
WT H2AX 5′ UTR plus CDS
Forward primer: 5′-CGCCTCGAGACAGCAGTTACACTGCGGCG-3′
Reverse primer: 5′-CGC GCGGCCGCTTAGTACTCC TGGGAGGCCTGG GTG-3′

2.4. Luciferase reporter assay

Either 40 nM miR-383 mimics or miR-383 inhibitor were co-transfected with 200 ng psiCHECK-2 vector into 293 T cells in 24-well plates, with three replicate wells for each condition. Cells were harvested 30 h after transfection using the Dual Luciferase Reporter Assay System (Promega).

2.5. Western blotting

Western blotting experiments were performed as previously described [14]. The following antibodies were used for Western blotting analysis: anti-PNUTS (BD Transduction Laboratories™, USA); anti-γH2AX (Abcam, Cambridge, MA, USA); anti-cyclin D1 (Santa Cruz Biotechnology, Inc., CA, USA); anti-CDK4 (Santa Cruz Biotechnology, Inc.); and anti-actin (Abcam). Protein levels were normalized to actin and quantified using ImageJ system (USA).

2.6. Cisplatin sensitivity assay

cis-Diamminedichloroplatinum(II) (cisplatin or CDDP) was purchased from Sigma-Aldrich (Dorset, UK). It was dissolved in 0.9% (w/v) saline before each experiment at a stock concentration of 1 mM, and then further diluted into the final concentrations used in experiments.

NT-2 cells were collected at the phase of exponential growth and then seeded into 12-well plates at 70–80% confluence. They were cultured overnight in an incubator. Cells were transfected with miR-383 mimics/negative control or si.PNUTS/siRNA negative control the next day. After 24 h, cells were collected and re-seeded into 96-well plates at a density of 4000 cells per well within 100 μl of growth medium. Cells were allowed to adhere overnight and then treated with different concentrations of cisplatin, ranging from 0 μM to 15 μM for a continuous 72 h. Cisplatin sensitivity was detected with the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) as previously described [15]. The numbers of cells were determined by measuring the absorbance at 450 nm using a 96-well format plate reader (ELX 800 universal microplate Reader; BioTek, Inc., Highland Park, IL, USA).

2.7. Immunocytochemistry (ICC)

After NT-2 cells were exposed to ultraviolet (UV) irradiation for 12 h and transfected with miR-383 mimics/negative control, cells were fixed with 4% paraformaldehyde (PFA) for 20 min, and then permeabilized for 10 min with 0.2% Triton/PBS, and blocked with 1% BSA/TBS for 1 h. The cells were then incubated with anti-γH2AX monoclonal antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. Then they were incubated with donkey anti-mouse IgG (H + L) secondary antibody (Alexa Fluor 555, Invitrogen) for 30 min in the dark at

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