

Role of lncRNA NEAT1 mediated by YY1 in the development of diabetic cataract *via* targeting the microRNA-205-3p/MMP16 axis

Y. LI^{1,2}, S.-H. JIANG², S. LIU², Q. WANG²

¹Shandong University, Jinan, China

²Department of Ophthalmology, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, China

Abstract. – OBJECTIVE: We aimed at investigating the possible role and mechanism of NEAT1 in the pathogenesis of diabetic cataract.

PATIENTS AND METHODS: YY1 and NEAT1 expressions in anterior lens capsule collected from diabetic cataract (DC) patients and normal controls were examined by quantitative real-time polymerase chain reaction (qRT-PCR) analysis, and their correlation was analyzed. The binding site between YY1 and NEAT1 sequences was predicted by JASPAR and detected by Dual-Luciferase reporter assay and chromatin immunoprecipitation (ChIP) assay. The proliferation and apoptosis of high-glucose-induced cells with NEAT1 knockdown were detected. Potential downstream targets of NEAT1 were predicted by bioinformatics and detected by Dual-Luciferase reporter assay.

RESULTS: YY1 and NEAT1 expressions in the anterior capsule tissue of DC lens were remarkably reduced and positively correlated. Dual-Luciferase reporter assay and ChIP confirmed that YY1 could bind to locus 2 of NEAT1. Knockdown of NEAT1 inhibited proliferation while promoted apoptosis under high glucose conditions. Further mechanism studies revealed that knockdown of NEAT1 could upregulate microRNA-205-3p, and MMP16 was a potential target of miR-205.

CONCLUSIONS: The low expression of YY1 induces NEAT1 downregulation, which regulates microRNA-205-3p/MMP16 axis and thus participates in the development of DC.

Key Words:

Diabetic cataract, YY1, NEAT1, MicroRNA-205-3p, MMP16.

abetes and its complications (diabetic retinopathy, cataracts, etc.) has also increased remarkably. Notably, the incidence of cataract in diabetic patients is about five times higher than that of non-diabetic populations^{1,2}. Diabetic cataract (DC) and age-related cataract with diabetes are the main types of cataract. So far, surgical treatment is the only effective treatment for DC, but its unavoidable complications and medical costs required also bring a heavy burden on the medical systems of countries around the world^{3,4}. Therefore, it is of great social and economic significances to study the pathogenesis of DC, thus preventing and delaying the progress of cataract in the early stage.

It is estimated that 98% of the human genome transcripts are non-coding RNAs (ncRNAs), which constitute a highly complex structural regulatory network with multiple biological functions⁵. There are five classic biological types of long non-coding RNA (lncRNA) as follows: antisense lncRNA, intronic transcript, large intergenic ncRNA, promoter-associated lncRNA and UTR-associated lncRNA. However, their quantities and functions are still not very clear⁶. Although lncRNAs were originally considered as “transcription noise”, they are now known to affect gene expressions and cell phenotypes⁷. Moreover, lncRNAs are involved in the diagnosis, treatment and progression of many diseases, including eye diseases. Shen et al⁸ has shown that lncRNA MIAT can regulate the survival, proliferation, apoptosis and migration of LECs *via* regulating protein kinase B (Akt) activation.

lncRNA NEAT1 has been found to be abnormally highly expressed in a variety of malignant tumors, including glioblastoma, breast cancer, gastric cancer, liver cancer, colorectal cancer, osteosarcoma, ovarian cancer, etc.⁹⁻¹¹. Its potential role in the progression of DC has not been reported, which will be clarified in the present study.

Introduction

At present, cataracts still rank first among blinding eye diseases in the world. In recent years, with the improvement of people's living conditions and the aging of population, the incidences of di-

Patients and Methods

Sample Collection

The anterior lens capsule tissues were collected from patients with diabetes during lensectomy from December 2015 to December 2018. Inclusion criteria for DC were as follows: patients were diagnosed with type 2 diabetes mellitus, and traumatic cataract, concurrent cataract, drug-induced cataract and other influencing factors were excluded. All procedures were conducted in accordance with the principles of the Helsinki Declaration and were approved by the Yantai Affiliated Hospital of Binzhou Medical University Research Ethics Committee. Written informed consents were obtained from all donors.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform, and isopropanol. After the concentration of the extracted RNA was determined, it was stored at -80°C until use. Complementary deoxyribose nucleic acid (cDNA) was obtained by reverse transcription, and 1 µL of cDNA samples were taken for detection. SYBR Green method was used for PCR detection (TaKaRa, Komatsu, Japan). The primer sequences were: NEAT1 (F: 5'-GCTGGAGTCTTGGGCACGGC-3', R: 5'-TCAACCGAGGCCGCTGTCTC-3'); YY1 (F: 5'-ACGGCTTCGAGGATCAGATTC-3', R: 5'-TGACCAGCGTTTGTTCATGT-3'); MMP16 (F: 5'-AGCACTGGAAGACGGTTGG-3', R: 5'-CTCCGTTCCGCAGACTGTA-3'); GAPDH (F: 5'-CCTCGTCTCATAGACAAGATGGT-3', R: 5'-GGGTAGAGTCATACTGGAACATG-3'). U6 primer (F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', downstream of the kit universal primer), miR-205 (F: 5'-CGGCTAGCCGAGGTCCTTGACATCT-3', R: 5'-CCCTCGAGGGCCTAAGTCAGAGTTA-3').

Cell Culture

The human LEC cell line (HLE B-3) purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 U/mL penicillin, 100 µg/mL streptomycin in an incubator with 5% CO₂ at 37°C. Plasmids including pcDNA-YY1, NEAT1-shRNA1, NEAT1-shRNA2, NEAT1-shRNA3, pcDNA-NEAT1, microRNA-205-3p mimics and inhibitors (GenePharma, Shanghai, China) and the transfection reagent Lipofectamine RNAiMAX

(Invitrogen, Carlsbad, CA, USA) were diluted with Opti-MEM respectively for transfection.

Dual-Luciferase Reporter Assay

Cells were seeded into a 48-well plate at an appropriate density and transfected with Lipofectamine RNAiMAX on the next day when the cell density was basically 40-60%. Luciferase vectors were designed based on JASPAR prediction. 24 hours after transfection, Luciferase activity of each group was measured using the dual-report detection system (Promega, Madison, WI, USA).

Chromatin Immunoprecipitation (ChIP)

Approximately 1×10^6 cells were cross-linked with formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 1% at 37°C for 10 minutes, and DNA was collected. The DNA was treated with anti-YY1 antibody or IgG antibody, and then rotated with protein A agarose beads at 4°C overnight. The precipitated DNA was subjected to qPCR using specific primers and 2 × PCR Master Mix (Promega, Madison, WI, USA).

Cell Counting Kit-8 (CCK-8) Test

Cells were collected 24 h after transfection and seeded into 96-well plates (2×10^4 cells/well), with 6 replicates in each group. CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) was conducted based on recommended instructions.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cells in logarithmic growth phase were plated into 96-well plates. EdU assay (Sigma-Aldrich, St. Louis, MO, USA) was conducted based on manufacturer's instructions. Images were taken with a fluorescence microscope.

Apoptosis detection

Cells were digested to prepare single cell suspension at 1×10^6 /mL. Cells were incubated with 10 µL of Annexin-V and 10 µL of Propidium Iodide (PI) at 4°C for 15 minutes in the dark. Flow cytometry (Partec AG, Arlesheim, Switzerland) was conducted for detection of cell apoptosis.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA). Measurement data were presented as $\bar{x} \pm SD$, and data comparison between groups was performed by the *t*-test. The Spearman rank method was used to calculate the

correlation between two gene expressions, and a difference of $p < 0.05$ was statistically significant.

Results

YY1 and LncRNA NEAT1 Are Down-Regulated in HLE B-3 Cells Treated with High Glucose (HG)

We first tested NEAT1 expression in lens of patients with DC ($n = 15$) and normal subjects. NEAT1 level was remarkably reduced in the former group (Figure 1A). According to JASPAR prediction, the transcription factor YY1 may be combined with the NEAT1 promoter region (Figure 1B). Luciferase vectors (wild-type and mutant-type ones) were constructed based on their binding sequences (Figure 1C). YY1 expression was remarkably downregulated in a high glucose-treated human LEC cell line (HLE B-3) (Figure 1D). Meanwhile, in tissue samples, we found a significant reduction of YY1 expression in patients with DC (Figure 1E) and a positive correlation was detectable between YY1 and NEAT1 ($p = 0.0072$) (Figure 1F). Results of Dual-Luciferase reporter assay showed that transcription of NEAT1 was not activated after mutating binding site 2, indicating the binding between YY1 and NEAT1 (Figure 1G). Furthermore, ChIP experiments also showed that YY1 can target binding site 2 of NEAT1 (Figure 1H). Additionally, over-expression of YY1 remarkably increased NEAT1 expression (Figure 1I). The above data proved the direct binding between NEAT1 and YY1 in DCs.

Knockdown of NEAT1 Promotes Apoptosis and Inhibits Proliferation of HLE B-3 Cells In HG

To further explore the role of NEAT1 in DC, knockdown of NEAT1 in HLE B-3 cells was achieved by transfection of NEAT1 shRNA, and shRNA1 had the most evident effect (Figure 2A). Subsequently, CCK-8 assay indicated that induction of high glucose inhibited cell activity, which could be further reduced by downregulation of NEAT1 (Figure 2B). The results of EdU assay were consistent with those of CCK-8 assay (Figure 2C). Flow cytometry revealed that high glucose promoted cell apoptosis, which was further enhanced by knockdown of NEAT1 (Figure 2D). The above results suggested that NEAT1 may be engaged in the occurrence of DC.

LncRNA NEAT1 Modulates MicroRNA-205-3p Expression

Since lncRNAs usually display biological functions through binding miRNAs, we predicted the miRNA that may bind to NEAT1 through bioinformatics analysis (Figure 3A). Dual-Luciferase reporter assay results showed that microRNA-205-3p remarkably attenuated the fluorescence activity of wild-type plasmids but not changed that of mutant type plasmids (Figure 3B). In addition, knockdown of NEAT1 enhanced microRNA-205-3p expression (Figure 3C), while overexpression of NEAT1 resulted in an opposite effect (Figure 3D). After detecting the expression of microRNA-205-3p in the population samples, a negative correlation was found between NEAT1 and microRNA-205-3p (Figure 3E). These results suggested that NEAT1 may play a role through modulating microRNA-205-3p expression.

MicroRNA-205-3p Regulates MMP16 Expression

Bioinformatics predicted a potential target gene MMP16 that may be bound to microRNA-205-3p (Figure 4A). The wild-type (WT) and mutant (MT) plasmids were constructed based on the binding sequence. It is shown that the fluorescence value in MMP16-WT was remarkably attenuated by overexpression of microRNA-205-3p, while no difference was detectable in MMP-MT (Figure 4B). In addition, overexpression of microRNA-205-3p inhibited MMP16 expression, while the opposite effect was observed after knockdown of microRNA-205-3p (Figure 4C). However, downregulation of NEAT1 inhibited MMP16 expression, and MMP16 was upregulated by overexpression of NEAT1 (Figure 4D). Meanwhile, we found a positive correlation between the above two gene expressions in tissue samples (Figure 4E). It was concluded that NEAT1 may affect the occurrence of DC by regulating the expression of MMP16 through binding microRNA-205-3p.

Discussion

Cataract is a major blinding eye disease worldwide¹. DC is an early ocular complication in diabetic patients and one of the main causes of blindness. Age-related cataracts in diabetic patients are characterized by early onset age, rapid progression of lens opacity and easy maturation¹²⁻¹⁴. Despite the great development and improvement of

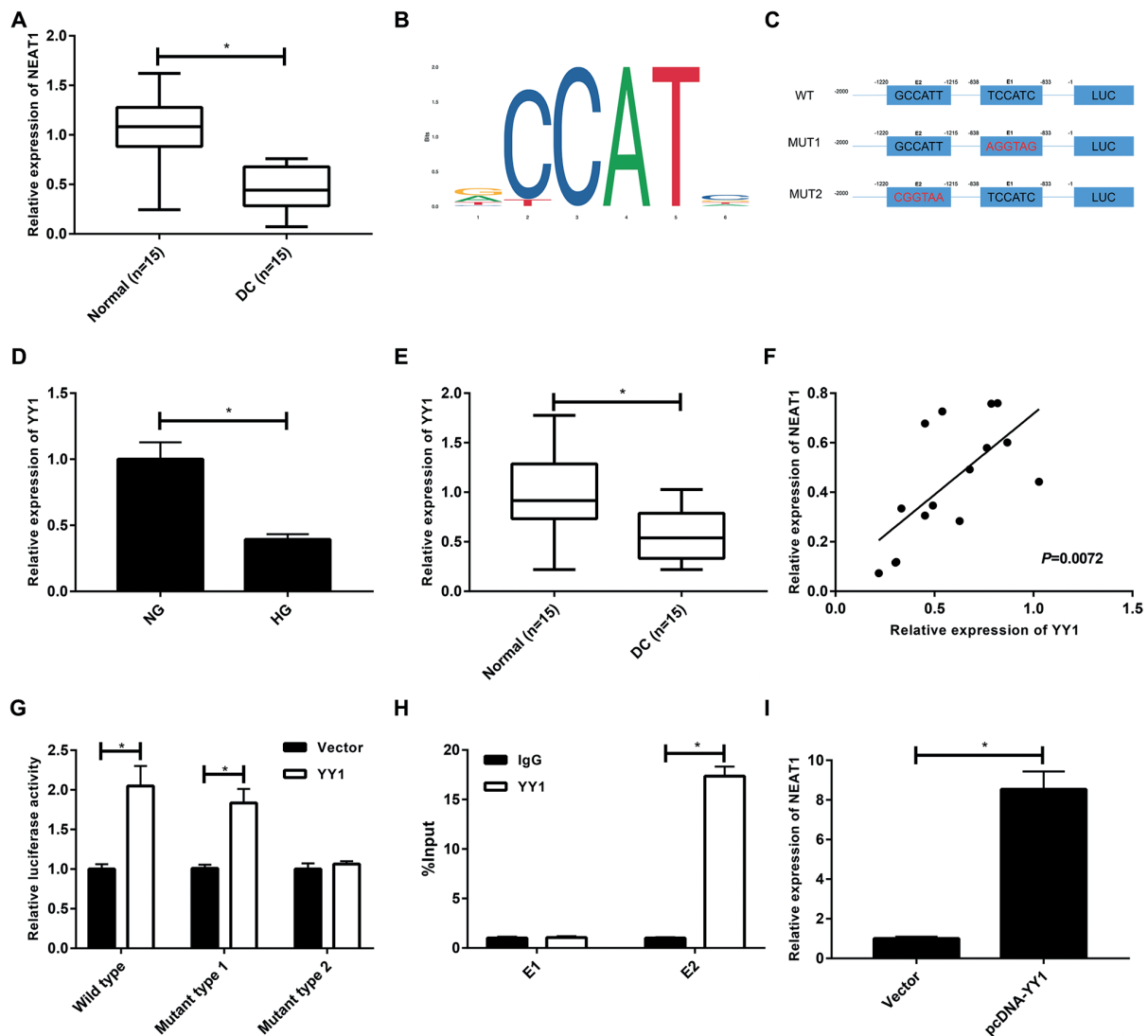


Figure 1. YY1 and lncRNA NEAT1 are down-regulated in high glucose-treated HLE B-3 cells. **A**, QRT-PCR was used to detect the expression of lncRNA NEAT1 in lens of DC patients and normal control. **B**, The binding motif of YY1 from JAS-PAR. **C**, Schematic diagram of Luciferase reporter gene containing two binding sites of YY1 and NEAT1 promoters. **D**, The mRNA expression level of YY1 in HLE B-3 cells treated with normal glucose (NG) and high glucose (HG) was detected by qRT-PCR. **E**, qRT-PCR was used to detect the expression level of YY1 in lens of DC patients and normal control. **F**, Spearman rank method was used to calculate the correlation between YY1 and NEAT1 expression in DC samples. **G**, Luciferase assay was used to confirm the interaction between the NEAT1 promoter and YY1 binding site 1 or site 2. **H**, Chromatin immunoprecipitation (ChIP) detected the binding relationship between the NEAT1 promoter and YY1 binding site 1 or site 2. **I**, qRT-PCR detection of NEAT1 expression level after overexpression of YY1. * $p < 0.05$

surgical equipment and technology, the incidences of surgical complications in diabetic patients are relatively high^{15,16}. With the increasing incidence of diabetes, DC has been well concerned, but its pathogenesis still remains unclear.

Although lncRNA was previously considered to barely have biological effects, recent studies have shown that lncRNA plays an important role

in a variety of processes. MALAT1 is not only abnormally expressed in the lens capsule tissue before DC and in HG-induced HLEC, but also up-regulated by HG to promote HLEC apoptosis and oxidative stress¹⁷. lncRNA PVT1 expression was up-regulated in HG-induced HLE B-3 cells, and PVT1 knockdown could inhibit proliferation and promote apoptosis of HLE b-3 cells¹⁸. In

this study, lncRNA NEAT1 was a key structural component of secondary spots, which played an indispensable role in the formation of secondary spots. As nuclear structures located in the chromatin internuclear region, the secondary spots are engaged in the regulation of gene expression through various mechanisms, including mRNA retention, mRNA rupture, A-to-I editing and

protein capture¹⁹. NEAT1 is upregulated in most cancerous tissues and down-regulated in leukemia and multiple myeloma²⁰. It is reported that knockdown of NEAT1 suppresses the progress of diabetic retinopathy via inactivating TGF- β 1 and VEGF signaling pathways²¹. Moreover, NEAT1 is involved in the protective effect of Klotho on renal tubular epithelial cells in diabetic kidney dis-

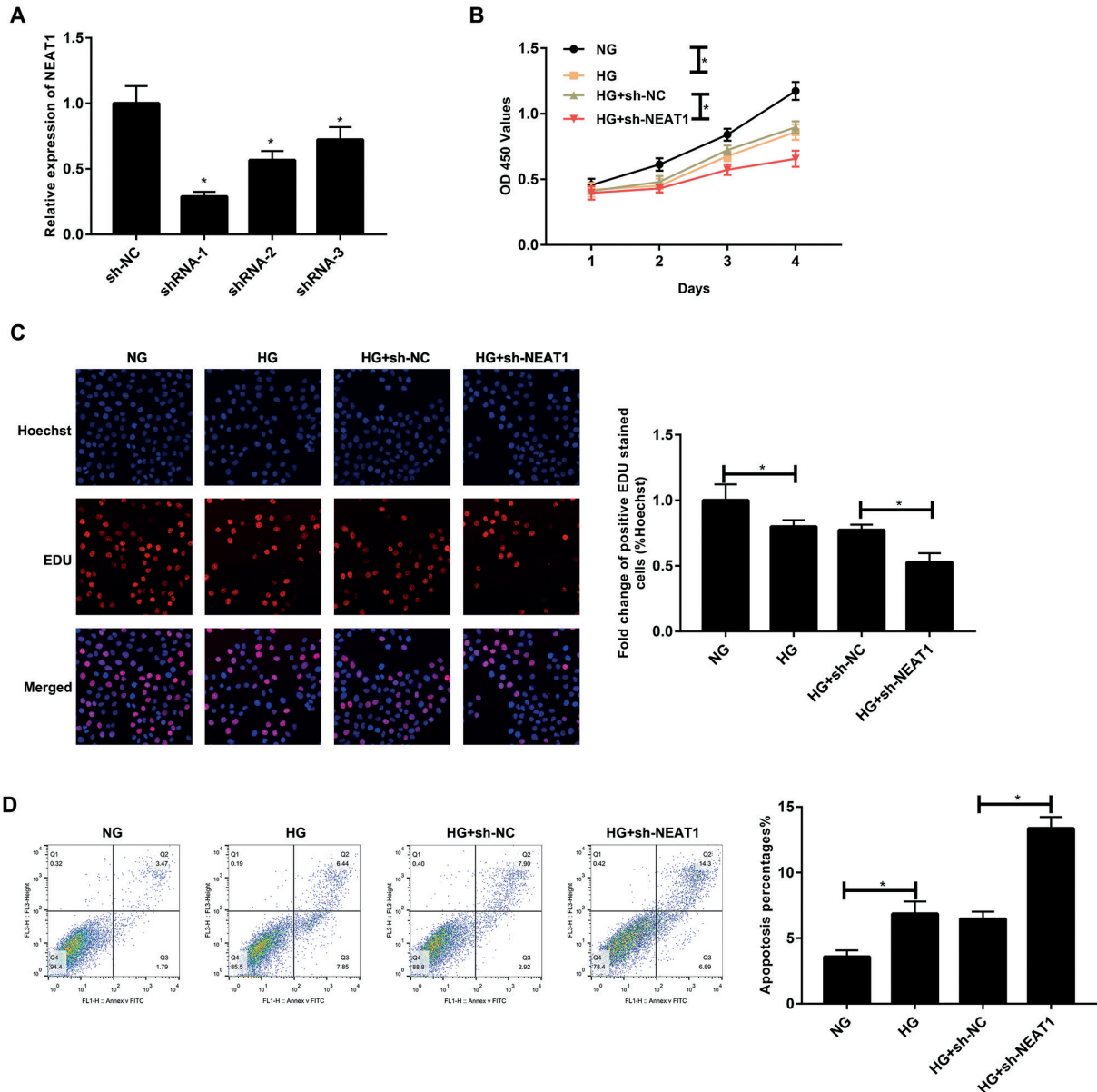


Figure 2. Low expression of NEAT1 promotes apoptosis and inhibits proliferation of HLE B-3 cells in HG. **A**, Short hairpin RNA (shRNA) was synthesized to silence NEAT1 expression. **B**, CCK-8 detected the proliferation of HLE B-3 cells treated with normal glucose (NG), high glucose (HG) and transfection of sh-NEAT1. **C**, EDU detected the proliferation of HLE B-3 cells treated with normal glucose (NG), high glucose (HG), and transfection of sh-NEAT1 (magnification: 40 $\times$). **D**, Flow cytometry detected the apoptosis of HLE B-3 cells treated with normal glucose (NG), high glucose (HG) and transfection of sh-NEAT1. * $p < 0.05$

ease through the ERK1/2 signaling pathway²². In our study, we found that NEAT1 expression in the lens of DC patients was remarkably decreased, which promoted the apoptosis and inhibited the proliferation of HLE B-3 cells in HG.

Yin Yang 1 (YY1) is an important zinc finger structural protein transcription factor with dual functions of transcriptional activation and transcriptional inhibition²³. The N-terminal of YY1 protein is enriched in glycine and alanine²³ and has transcriptional activation. The C-terminal has four zinc finger structures that bind to the CART and ACAT promoter regions of DNA, and the C-terminal can mask the activation domain of the N-terminal to play transcriptional repression. In addition, once the C-terminal is removed, the activation domain of the N-terminal will be exposed and YY1 will be transformed into a transcriptional activator²⁴. In the human genome, YY1 has a large number of target genes, which are involved in pathophysiological processes such as cell proliferation, differentiation, invasion and metastasis of cancer cells, and plays an essential part in human diseases²⁵. In addition, YY1 protein ameliorates

diabetic nephropathy pathology through transcriptional repression of TGFβ1²⁶. YY1 can be involved in the regulation of expression of a great number of lncRNAs²⁷⁻²⁹. In this study, YY1 was remarkably decreased in the lens of DC patients and could regulate NEAT1 expression through directly binding to the promoter area to regulate its expression.

MiRNAs play a pivotal role in eye diseases and may be involved in the pathogenesis of various types of cataracts, such as congenital cataracts, senile cataracts, posterior cataracts and DCs³⁰⁻³². The current study indicated that lncRNAs could act as competing endogenous RNA (ceRNA) and competitively bind to miRNAs, thus regulating the transcription level of mRNA and modulating gene expression³³. It is reported that NEAT1 act as a ceRNA and bind to miR-193a-3p to accelerate deterioration of lung adenocarcinoma³⁴. In this study, the expression of microRNA-205-3p in DC tissue samples was negatively regulated by NEAT1, and it was responsible for NEAT1-regulated DC progression.

In this research, we firstly confirm the regulatory axis of YY1/NEAT1/microRNA-205-3p in DC,

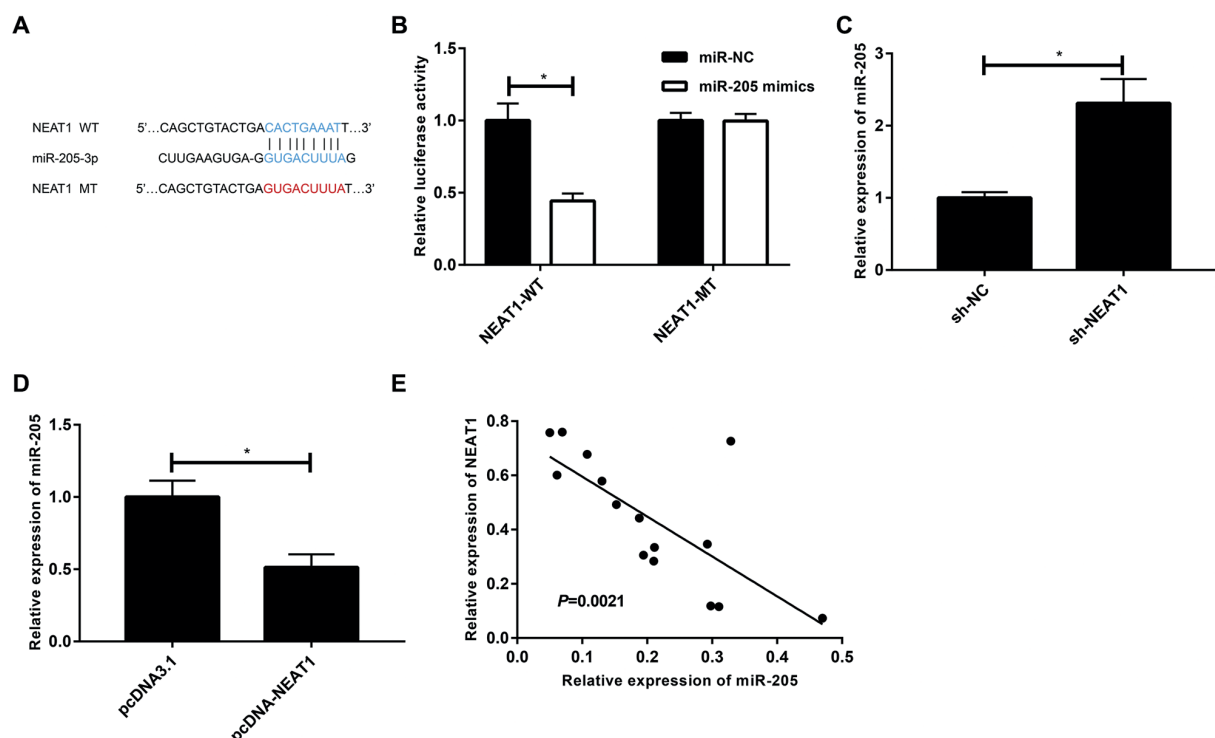


Figure 3. lncRNA NEAT1 regulates miR-205-3p expression. **A**, Bioinformatics analysis indicates that miR-205-3p may bind to the promoter of NEAT1. **B**, Luciferase reporter gene shows Luciferase activity after co-transfection of NEAT1 wild type and miR-205-3p. **C**, qRT-PCR detection of miR-205-3p expression level after knockdown of NEAT1. **D**, qRT-PCR detection of miR-205-3p expression level after knockdown of NEAT1. **E**, Analysis of correlation between miR-205-3p and NEAT1 in DC samples using Spearman rank method. * $p<0.05$

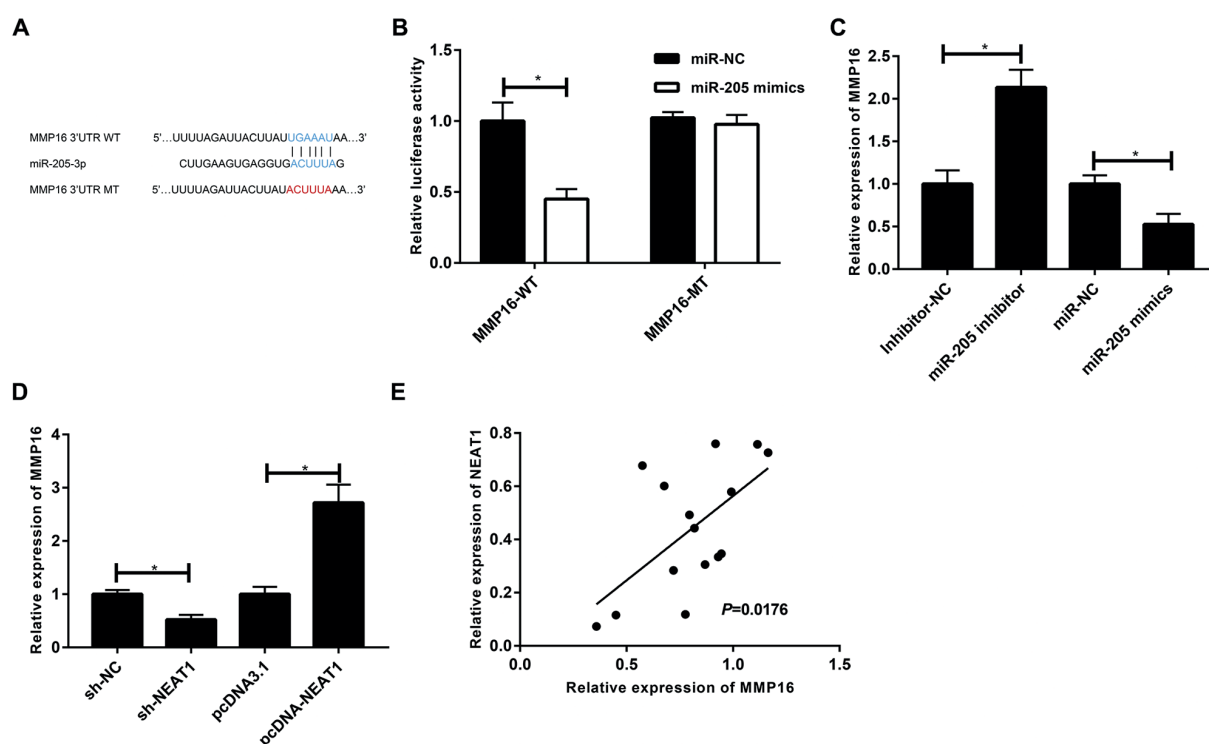


Figure 4. miR-205-3p regulates MMP16 expression. **A**, Bioinformatics shows that there is a binding site of miR-205-3p in MMP16 promoter. **B**, Luciferase reporter gene assay confirmed the interaction between MMP16 and miR-205-3p. **C**, qRT-PCR detected the expression level of MMP16 after miR-205-3p is lowly-expressed or overexpressed. **D**, qRT-PCR detection of MMP16 expression level after over-expression or over-expression of NEAT1. **E**, Spearman rank method was used for correlation analysis of MMP16 and NEAT1 expression in DC samples. * $p < 0.05$.

which provides a novel therapy target in treating DC. However, there are still some deficiencies in this study. The specific molecular mechanism of MMP16 involved in DC progression should be explored. In addition, our conclusion should be further validated in an animal model.

Conclusions

This study detected that the lowly-expressed YY1 inhibits NEAT1 expression, which then affects the progression of DC by regulating MMP16 through binding microRNA-205-3p.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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