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MiR-22-3p targeting alpha-enolase 1 regulates the proliferation of retinoblastoma cells

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ABSTRACT

Objective: This study sought to explore the role of alpha-Enolase 1 (ENO1) in retinoblastoma (RB) which remains uncertain.
Methods: The expression of ENO1 in RB cell lines was examined by RT-qPCR and western blot. The biological function of ENO1 on cell proliferation in RB was determined *in vitro*. The predicted target of ENO1 was validated by dual-luciferase reporter assay and rescue experiment. The validation of clinical tissue samples was performed by RT-qPCR.
Results: Elevated ENO1 could promote the proliferation of RB cells. The dual luciferase reporter assay confirmed that ENO1 is the target for miR-22-3p. In rescue experiment, the result also indicated that miR-22-3p inhibits the proliferation of RB cells by negatively regulating the expression of ENO1. These differences were statistically significant ($P < 0.05$).
Conclusion: ENO1 functions as an oncogene in RB and inhibiting ENO1 by miR-22-3p suppresses the proliferation of RB cell lines. miR-22-3p/ENO1 pathway may serve as a novel target in RB.

1. Introduction

Retinoblastoma (RB) is a common intraocular malignancy in infants and young children, with a prevalence of 1 per 15,000–20,000 newborns. The number of new RB cases diagnosed in China accounts for approximately 20% of the world's new cases each year. RB is highly malignant and tends to lead to intracranial metastasis and death; RB-related deaths account for 1% of all deaths in infants and young children [1]. Based on hereditary characteristics, RB can be divided into hereditary cases and sporadic cases, which account for 40% and 60% of the total cases, respectively. Hereditary RB can occur in one or both eyes, and sporadic RB usually occurs in only one eye. RB is often associated with secondary malignant tumors such as osteosarcoma, fibrosarcoma, and malignant reticulocytoma. RB has received worldwide attention because it is a serious threat to vision and life [2]. RB is the first tumor found to be caused by RB1 (a tumor suppressor gene) mutations; however, some patients do not have RB1 mutations, and in some cases, RB even shrinks spontaneously [3]. The development and progression of RB is a complex process that is often associated with multiple abnormal genes, such as RB1, MYCN, murine double minute 4 (MDM4), KIFl4, DEK, E2F3, and CDH11. Also, several microRNA has been proved to be involved in RB occurrence and progression. miR-138-5p, miR-29a, miR-503-5p, miR-497 and miR-200c were reported as tumor suppressor in RB [4–8]. MiR-498 may act as an oncogene in RB [9]. Further discovery of abnormal oncogenes may help researchers better understand the mechanism of RB development and progression and guide clinical treatment.

α-Enolase 1 (ENO1) is a multifunctional protease located at 1p36.3–1p36.2. ENO1 is a bidirectional gene that encodes both ENO1 and c-myc promoter binding protein 1 (MBP1). Found in many tissues, ENO1 is involved in a variety of physiological and pathological processes in addition to catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glucose metabolism. ENO1 is closely associated with the development and progression of various head and neck squamous cell carcinomas [10], thoracic tumors [11,12], digestive tumors [13,14], and gynecological tumors [15]. Its function and mechanism in RB are still unknown. In this study, we investigated the function and regulatory mechanism of ENO1 in RB cell lines *in vitro*.

2. Materials and methods

2.1. Tissue samples

A total of 31 Pathological retinal tissue samples were obtained from

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RB patients who underwent surgical resection at Third Affiliated Hospital of Soochow University. No preoperative treatment subjected to these patients. Another 10 Normal retina tissues were collected from patients with traumatic ruptured. All tissue samples were stored in liquid nitrogen at −80 °C. Informed consent was obtained from each participant in order for tissue donation to occur for research purpose. The use of human clinical tissues was approved by the Institutional Human Experiment and Ethics Committee of Third Affiliated Hospital of Soochow University. The Declaration of Helsinki was strictly followed during experiments.

2.2. Cell lines

Three RB cell lines (Y79, Weri-RB1, and SO-RB50) and one human normal retinal pigmented epithelium cell line (APRE-19) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Human RB cell line HXO-RB44 was purchased from Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Y79, Weri-RB1, SO-RB50, and HXO-RB44 cell lines were cultured in modified Roswell Park Memorial Institute (RPMI)-1640 medium (10% FBS, 100 μg/L penicillins, 100 μg/L streptomycin) at 37 °C and 5% CO_2. ARPE-19 cell line was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM: F12) (ATCC)

2.3. Real-time quantitative polymerase chain reaction (RT-qPCR)

Cells in exponential growth phase were harvested to extract total RNA according to the instructions of an RNA extraction kit as well as tissue samples. The RNA stock was diluted 10-fold in 0.1% DEPC water (*i.e.*, 2 μL + 18 μL 0.1% DEPC), and optical density (OD) was determined at A260 nm, A280 nm, and A260 nm/A280 nm with a microplate reader (three measurements each). The concentration of total RNA in the sample = A260 nm × 40 mg/ml × 20. (Note: A260 nm = 0.15–0.1 and A260 nm/A280 nm = 1.8–2.2 indicated reliable concentration value and purity). The primers were synthesized by Shanghai Jierui Biological Engineering Co., Ltd., as follows: GAPDH: Forward primer: 5′-GAAGGTGAAGGTCGGAGTC-3′, Reverse primer: 5′-GAAGATGGTGATGGGATTTC-3′; ENO1: Forward primer: 5′-AGCUG GUGCCGUUGAGAAGTT-3′, Reverse primer: 5′-CUUCUCAACGGCACC AGCUTT-3′, miR-22-3p: Forward primer: 5′-AAGCTGCCAGTTGAAGA ACTGTA-3′, Reverse primer: 5′-GCTGTCAACGATACGCTACGTAAC-3′. U6: Forward primer: 5′-ACTTCAGCAGCACATATACTAAAAA-3′, Reverse primer: 5′-CGCTTCACGAATTTGCATGTCAT-3′. The results are expressed as $2^{-\Delta\Delta Ct}$, which indicates the relative expression level of the target mRNA. The experiment was repeated three times.

2.4. Western blot

Cells in exponential growth phase were harvested, and total protein was extracted with RIPA lysis buffer and quantitated to 100 μg per sample. ENO1 content was determined by Western blotting, and β-actin was used as an internal control. Western blots were performed as follows: the protein sample was added to an equal volume of 2 × loading buffer, boiled at 95 °C for 10 min, and then loaded onto the gel (total volume: 30 μl). After electrophoresis, the protein bands were transferred to a membrane, which was blocked in TBST buffer containing 5% skim milk at room temperature for 2 h. Next, the membrane was incubated with ENO1 antibodies (1:1000 in 1% BSA) and β-actin antibodies (1:5000 in 1% BSA) at 4 °C overnight. On the next morning, after three TBST washes (10 min each), the membranes were incubated with secondary antibody (diluted in TBST) at room temperature for 1 h. After three washes with TBST, ECL agents were added, and the protein bands were observed after one minute. Quantity one was used to measure the gray value of the target protein bands, and the results are expressed as the ratio of the gray value of the target band to the gray value of the corresponding internal control. The experiment was repeated three times.

2.5. Flow cytometry

Cells were collected and prepared as single-cell suspensions. After two PBS washes and centrifugation, the supernatant was discarded. Apoptosis was determined with the Annexin V-FITC early apoptosis kit and a flow cytometer. Specifically, 0.5 ml of 70% pre-chilled ethanol (−20 °C) was added to each group of cells to fix the cells at 4 °C overnight, followed by centrifugation (1000 rpm, 4 °C) for 5 min and two PBS washes. Next, 1 ml of propidium iodide staining solution was added to the cells and kept in the dark at 4 °C for 30 min, followed by analysis with a flow cytometer to determine cell cycle distribution.

2.6. Cell counting kit-8 (CCK8) assay

To analyze cell proliferation, cells (2×10^4 cells/ml) were seeded onto 96-well plates, with 100 μL in each well. The cells were placed in an incubator at 37 °C and 5% CO_2 for 24 h. After the cells were cultured for an additional 0–4 days (five-time points), 10 μl of CCK8 solution was added to each well, and the cells were cultured in an incubator for 1 h. Next, A450 nm was measured with a microplate reader.

2.7. Construction of overexpression and interference vectors and packaging of viral particles

A cDNA template of ENO1 was synthesized and digested with BamHI and EcoRI. The ENO1 interference sequence or mock sequence was annealed and ligated with BamHI- and EcoRI-digested pLVX-U6-GFP-Puromycin/pLVX-CMV-GFP-Puromycin (Promega, USA), and the sequences were verified. The viruses were packaged in the renal epithelial cell line 293 T. The cells were digested on the previous day. Once the cells reached 80% confluency, pLVX-U6-ENO1 shRNA/pLVX-CMV-ENO1 (containing the ENO1 interference sequence or mock sequence), pVSV-G, and pMD2.G were added; the viruses were packaged according to the Lipofectamine2000 manufacturer's instructions. The viruses were harvested every 24 h, filtered, centrifuged at 50,000 *g* at 4 °C for 2 h, and concentrated. After the titer was calculated, the virus was aliquoted and stored at −80 °C.

2.8. Dual-luciferase reporter assay

The wild-type or mutant ENO1 3′-UTR fragments which contained predicted miR-22-3p binding sites were cloned into psiCHECK-2 vector (Promega, Madison, WI, USA). The recombinant vector and miR-22-3p mimics or miR-22-3p inhibitors were co-transfected into Y79 cells using Lipofectamine 2000 (Invitrogen) and incubated for 48 h. The luciferase activity was evaluated 48 h later using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocols.

2.9. Statistics analysis

All data were represented as mean ± standard deviation (SD). Differences were analyzed using Student's *t*-test or one-way ANOVA analysis of variance using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Endogenous ENO1 expression in RB cell lines

To investigate the potential role of ENO1, we firstly measured the expression of ENO1 in 31 pathological retinal tissues and 10 normal retinal tissues by RT-qPCR. The relative expression of ENO1 in tumor tissues normalized to GAPDH was 2.03 ± 0.31 (mean ± SD), while