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Long noncoding RNA BDNF-AS is a potential biomarker and regulates cancer development in human retinoblastoma

Weiwei Shang [1], Yanyan Yang [1], Jingyi Zhang*, Qizheng Wu

Department of Ophthalmology, Liaocheng City People's Hospital, Liaocheng City, Shandong Province, 252000, China

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ABSTRACT

Background: Long non-coding RNAs (lncRNA) have been shown to play important roles in human cancer. We examined expression, prognostic potential and functional roles of lncRNA, brain-derived neurotrophic factor antisense (BDNF-AS) in human retinoblastoma (RB).

Methods: BDNF-AS expression in RB tumors was characterized according to the clinicopathological parameters of patients. BDNF-AS mRNA level was compared between RB tumors and normal retinas, as well as RB cell lines and normal retinal epithelial cells. RB patients' overall survival was compared between those with low and high BDNF-AS tumor expressions. Statistical analysis was performed to examine the independence of BDNF-AS being cancer biomarker in RB. In Y79 and WERI-Rb-1 cells, BDNF-AS was upregulated. It's effect on cancer proliferation, migration and cell-cycle transition were assessed.

Results: BDNF-AS is downregulated in RB tumors and cell lines. Low BDNF-AS expression in RB tumors is correlated with patients' advanced clinical stage and tumor differentiation status. Low BDNF-AS expression is associated with shorter overall survival and may be acting as an independent marker in RB. In Y79 and WERI-Rb-1 cells, forced overexpression of BDNF-AS inhibited cancer proliferation and migration. It also induced cell-cycle arrest at G0/G1 phase by downregulating CDC42, Cyclin E and BDNF.

Conclusion: BDNF-AS is lowly expressed, and may be used as a prognostic biomarker in RB. Upregulating BDNF-AS has inhibitory effect on RB development, probably through the suppression of cell-cycle transition.

1. Introduction

Retinoblastoma (RB) is one of the most common and aggressive eye caners in children [1–3]. In developing countries (including China), patients with RB were often diagnosed at advanced stages, and the survival rates were much worse than the survival rates of patients in developed countries [4,5]. While surgical procedure, such as enucleation, remains the standard and major therapeutic strategy for patients with RB, various chemotherapeutic approaches, including intraarterial chemotherapy, intravenous chemotherapy or periocular chemotherapy have been developed in recent decades to relieve the physical and psychological burden of patients who were at risk of losing their eyes at young ages [6–9]. Therefore, understanding the molecular signaling pathways and therapeutic targets of RB is critical for developing the optimal treatment plan for chemotherapy or other targeted therapy for RB patients.

Recently, more and more studies had demonstrated that, families of long non-coding RNAs (lncRNAs) were either upregulated or downregulated in certain diseases, and can epigenetically regulate coding DNAs to exert profound biological functions relating to human health [10–13]. In human cancers, lncRNAs had been identified as critical tumor modulators or potential cancer biomarkers [14–16]. For instance, a natural hypoxia-inducible factor 1-alpha (HIF-1α) antisense lncRNA, HIF-AS was discovered to be aberrantly expressed in breast cancer, and functioning as cancer regulator and biomarker [17–19]. In addition, lncRNA of highly upregulated in liver cancer (HULC) was shown to be upregulated by CREB and interact with microRNA-372 to regulate chromatin accessibility and histone acetylation in human liver cancer [20]. Most specifically, in human RB, it was recently discovered that lncRNA BRAF-activated non-coding RNA (BANCR) can be closely

* Corresponding author. 67 Dongchang West Road, Department of Ophthalmology, Liaocheng City People's Hospital, Liaocheng City, Shandong Province, 252000, China.
E-mail address: zhjyy4@aol.com (J. Zhang).
[1] These authors contributed equally.

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associated with RB patients' prognosis, and modulate RB cancer development and metastasis [21].

LncRNA of brain-derived neurotrophic factor antisense (BDNF-AS) is a natural non-coding antisense of neuronal transcriptional factor BDNF [22]. It was discovered that BDNF-AS was acting as a reverse regulator of BDNF, thus having profound effects in neuronal system [23,24]. Interestingly, as more and more studies showed that BDNF could act as a predominant oncogenic factor in human cancers [25–27], little is known whether BDNF-AS may also act a functional regulator or biomarker in human cancer. Thus, in this work, we explored the expression, prognostic potential and functional mechanisms of BDNF-AS in RB. We examined BDNF-AS mRNA level in both RB biopsy specimens and *in vitro* RB cell lines, and statistically analyzed the correlation between BDNF-AS tumor expression and RB patients' clinicopathological parameters and overall survival. In addition, in *in vitro* RB cell lines Y79 and WERI-Rb-1 cells, we overexpressed BDNF-AS to further examine the functional mechanisms of BDNF-AS in regulating RB proliferation, migration and cell-cycle transition.

2. Materials and methods

2.1. Ethic approval

In this work, all procedures were reviewed and approved by the Clinical Study & Ethic Committee at Liaocheng City People's Hospital at Liaocheng City, Shandong Province, China. All enrolled patients signed consent forms before taking any procedures in the study. All protocols were performed in compliance with the Declaration of Helsinki, and Good Clinical Practice by China Food and Drug Administration (CFDA).

2.2. Patients

Between February 2009 and September 2013, 131 patients with retinoblastoma (RB) were enrolled in this study. Their biopsy specimens were obtained during enucleation, which was performed in the Department of Ophthalmology at Liaocheng City People's Hospital in Liaocheng City, Shandong Province, China. All tumors were examined by independent histopathologists at Liaocheng City People's Hospital, and graded in accordance with the guideline published by the 7th edition of American Joint Committee on Cancer [28]. Normal retinal specimens were obtained from 35 patients who died from other causes at Liaocheng City People's Hospital between 2010 and 2013. Consent forms were signed directly by the donors or their family members. All biopsy specimens were immediately frozen in liquid nitrogen and stored at −70 °C before further processing.

2.3. Retinoblastoma cell lines

A human retinal pigment epithelial cell line, ARPE-19, a human telomerase-immortalized retinal pigmented epithelial cell line, hTERT-RPE1, and human retinoblastoma cell lines, Y79 and WERI-Rb-1 cells were obtained from American type culture collection (ATCC, USA). Rb116 and Rb143 cells were obtained from KaraFAST (KaraFAST, USA). SO-RB50 and HXO-RB44 were obtained from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (CTCCC, China). All cells were maintained in RPMI-1640 medium (ThermoFisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Scientific, USA) and 1% antibiotic cocktail (10 mg/mL streptomycin, 10,000 U/mL penicillin, and 25 μg/mL Amphotericin), in a tissue-culture chamber with 5% CO_2 at 37 °C.

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted from biopsy specimens or *in vitro* RB cell lines using the Qiazol reagents and RNeasy mini kit (Qiagen, USA) according to the manufacturer's protocol. RNA product was quantified using a NanoDrop™ 3000 Spectrophotometer (ThermoFisher Scientific, USA), and reverse-transcribed into complimentary DNA (cDNA) using a TaqMan Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real-time PCR (qRT-PCR) was conducted using a Brilliant II SYBR® Green qPCR Mastermix Kit (Stratagene, USA) according to the manufacturer's protocol. U6 small nuclear RNA was used as house keeping gene and relative expression levels of BDNF-AS cDNA were quantified as folder changes using the $2^{-\Delta\Delta Ct}$ methods.

2.5. Forced-overexpression of BDNF-AS in retinoblastoma cells

Whole sequence of BDNF-AS gene was amplified from a human cDNA library, and inserted between BamHI and XhoI restriction sites of a pCDNA3/+ vector (Takara, China), resulting in a BDNF-AS+ overexpression plasmid. The empty pCDNA3/+ vector was used as Control plasmid. Two retinoblastoma cell lines, Y79 and WERI-Rb-1 cells were transfected with BDNF-AS+ or Control plasmid, using Lipofectamine 2000 reagent (ThermoFisher Scientific, USA), and then selected in tissue-culture medium containing G418 (0.8 mg/mL). Transfection efficiency was verified by qRT-PCR.

2.6. Viability assay

Transfected Y79 and WERI-Rb-1 cells were trypsinized by 0.5% trypsin-EDTA (ThermoFisher Scientific, USA), and then stained by 0.2% trypan blue solution for 15 min at room temperature. Both viable (white) cells and non-viable (blue) cells were counted. Cell viability was then estimated by the percentage of viable cells among all cells (viable & non-viable).

2.7. Proliferation assay

Transfected Y79 and WERI-Rb-1 cells were suspended and re-seeded in 96-well plate at density of 5000 cells per well. Their *in vitro* proliferation was estimated using a Vybrant MTT proliferation kit (MilliporeSigma, USA) according to the manufacturer's protocol. Every day, 20 μL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide (MTT) solution (12 mM) was added into tested wells for 4 h, followed by another 4 h incubation of HCl-SDS at room temperature to solubilize the formazan product. Cancer cell proliferation was estimated by measuring fluorescent absorbance at 570 nm using a Beckman Coulter Multimode DTX 880 microplate reader (BD, USA).

2.8. Migration assay

In a transwell migration assay, transwell insert (8-μm pore-size, BD, USA) was coated with matrigel and placed in a 24-well plate. Transfected Y79 and WERI-Rb-1 cells were seeded in the upper chamber at density of 10,000 cells per well in tissue-culture medium with 1% FBS. The lower chamber was filled with regular culture medium with 10% FBS, acting as chemoattractant. 24 h later, the upper chamber was discarded. Cancer cells migrated into lower chamber were quickly fixed by 4% PFA for 5 min at room temperate, and then stained by 0.1% crystal violet. Relative migration was estimated by counting cells in all lower chambers and normalizing them against the number in control chamber.

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