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Long non-coding RNA CASP5 promotes the malignant phenotypes of human glioblastoma multiforme

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ABSTRACT

Background: Long non-coding RNAs (lncRNAs) have been demonstrated to be intensively involved in the development of various carcinomas, including glioblastoma multiforme (GBM). However, only a few of them have been well characterized. LncRNA CASP5 have been found to be up-regulated in GBM tissues compared with normal tissues in a microarray-based lncRNA profiling study. In the present study, we further explored the biological role of lncRNA CASP5 in GBM.

Methods: We examined the expression level of lncRNA CASP5 in GBM tissues as well as GBM cell lines. CCK-8 assay, flow cytometric analysis, western blotting, orthotopic GBM model as well as transwell assay were performed to investigate the biological role of CASP5.

Results: We observed that lncRNA CASP5 was highly expressed in GBM tissues and cell lines. Knockdown of CASP5 greatly inhibited GBM proliferation and resulted in G1 cell cycle arrest along with higher apoptosis ratios *in vitro* and *in vivo*, while overexpression led to the opposite phenomenon. Furthermore, the migration and invasion ability of GBM cells were significantly decreased after CASP5 down-regulation, while increased migration and invasion can be observed after CASP5 up-regulation.

Conclusion: We demonstrate for the first time the potential oncogenic role of lncRNA CASP5 which may be helpful for identifying novel therapeutic targets in GBM.

1. Introduction

Glioblastoma multiforme (GBM, WHO grade IV) is the most common and most malignant type of primary brain tumor. It is characterized by remarkably cell proliferation, migration and invasion abilities. Even with optimal treatment, the prognosis of GBM patients is dismal with median survival less than 15 months [1]. Due to this high morbidity and mortality, it is critical to elucidate the molecular mechanisms underlying GBM pathogenesis.

Long non-coding RNAs (lncRNAs) are one type of non-coding transcripts, that are transcribed by RNA polymerase II and are longer than 200 nucleotides. With little or no protein-coding potential, they still account for approximately 70% of the human transcriptome [2,3]. Emerging evidence indicates that lncRNAs are involved in many important processes like protein function regulation, chromatin modification, transcriptional and post-transcriptional regulation etc [4–6]. Dysregulated lncRNAs are often found in diverse human diseases, especially cancers [7–9]. Many aspects of lncRNA regulatory influence on cancer cells have been reported, such as cell proliferation, migration and invasion [10,11]. It is therefore essential to identify key oncogenic lncRNAs for design of better GBM therapy.

Elevated expression of lncRNA CASP5 (NR_036562.2) was identified in our preliminary work (GSE 100675) in which transcriptome profiles of lncRNAs and mRNAs from GBM and paired non-tumor tissues were analyzed, suggesting that lncRNA CASP5 was involved in the development of GBM. In this study, the biological role of lncRNA CASP5 was further explored. We found that CASP5 was up-regulated in GBM tissues and cell lines. Elevated expression of CASP5 promoted GBM proliferation *in vitro* and *in vivo*, and facilitated GBM cell migration and invasion. Collectively, the results of this study showed the oncogenic role of CASP5 in GBM, and it might be helpful for identifying novel therapeutic target in GBM.

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2. Materials and methods

2.1. Clinical samples and cell lines

Forty pairs of glioblastoma multiforme (GBM) tissues and paired non-tumor tissues were collected from patients who underwent surgical resection at Jinling Hospital. Paired adjacent non-tumor tissues were isolated from the tumor border and were shown to lack tumor cells by microscopy. All specimens were collected directly after resection and quickly frozen in liquid nitrogen. The patients received no anti-cancer treatments before surgery and informed consent forms were signed. This study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board, Nanjing University.

The human GBM cell lines A172, U87MG, U251MG, T98G, U118MG and the human astrocyte cell line HA were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM with 10% FBS at 37 °C and 5% CO_2.

2.2. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from tissue samples or cultured cells using Trizol reagent (Invitrogen) following the manufacturer's protocol and reverse transcribed cDNA was amplified immediately. The gene-specific primers were listed as follows: lncRNA CASP5, 5′-ACGCCTGGCTCTCATCATATGC-3′ and 5′-CGTGCTGTCAGAGGACTTGTGC-3'; β-actin, 5′-CACCCAGCACAATGAAGATCAAGAT-3′ and 5′-CCAGTTTTTAAATCCTGAGTCAAGC-3'. The PCR reaction was carried out using the ABI 7500 real-time PCR system (Applied Biosystems) and the FastStart universal SYBR green master (Roche). β-actin was used for normalization and the $2^{-\Delta\Delta CT}$ method was applied to calculate relative concentrations of target genes.

2.3. Cell transfection

CASP5 specific shRNA and a non-targeting sequence was cloned into the pGCSIL-GFP vector to construct the lentiviral vector. Lentiviruses were used to knock down lncRNA CASP5 or as a negative control. The primer sequence of CASP5 shRNAs were listed as follows: shCASP5-1, 5′-ATTACGGAACTCATCACAT-3'; shCASP5-2, 5′-CCATAGAACGAGCAACCTT-3'. The lncRNA CASP5 sequence (NR_036562.2) was obtained from NCBI and subcloned into a plasmid lentiviral vector (pLV-GFP) for the overexpression of lncRNA CASP5. An empty vector was used as a negative control. Cells were transfected following the manufacturer's instruction. For further use, the cells were harvested 48 h after transfection.

2.4. Cell growth assay

Cell Counting Kit-8 (CCK-8) assay (Dojindo) was used to determine cell viability. Transfected cells were plated into 96-well plates (2000 cells/well) and absorbance was measured at 450 nm every 24 h according to the manufacturers' instruction.

2.5. Flow cytometric analysis

In cell cycle analysis, transfected cells were fixed with 75% ethanol and then stained with PI (BD Biosciences). The stained cells were analyzed with FACScan and the percentage of cells in the G0/G1, G2/M and S cell cycle phases were counted and compared.

For analysis of apoptosis, transfected cells were stained with Annexin V-APC/PI apoptosis detection kit (KeyGEN) according to the manufacturer's protocol and then analyzed with FACScan.

2.6. Western blotting

Cells were lysed in RIPA buffer with 1% PMSF and protein concentration was measured by BCA assay. Extracted proteins were separated by SDS-PAGE and electro-transferred onto PVDF membranes. The membranes were blocked with 5% nonfat milk and incubated with specific antibodies. β-actin protein was used as the loading control. The primary antibodies (Cell Signaling Technology, 1:1000 dilution) were: Cyclin D1 (#2978), Cleaved Caspase-3 (#9661), Caspase-3 (#9665), MMP-9 (#13667), MMP-2 (#87809), E-Cadherin (#3195), N-Cadherin (#13116), Vimentin (#5741).

2.7. Orthotopic GBM model

All experimental procedures and animal care were approved by the Animal Care Committee of Nanjing University. Athymic BALB/c nude mice (4-week-old, male) were used to establish orthotopic GBM model and randomly divided into 5 groups (U87MG-Luc-shCtrl, U87MG-Luc-shCASP5-1, U87MG-Luc-shCASP5-2, U87MG-Luc-pLV-Ctrl, U87MG-Luc-pLV-CASP5; each group $n = 5$). Transfected U87MG-Luc cells (500,000 cells/mouse) were implanted stereotactically 1.0 mm anterior and 2.5 mm lateral to the bregma and at 3.5 mm depth from the skull surface [12]. Caliper IVIS Spectrum was applied for *in vivo* bioluminescence imaging and luciferin was used as substrate for luciferase.

2.8. Hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) analysis

Tumor tissues from nude mice were fixed in 4% paraformaldehyde and then embedded in paraffin. Sections were used for analysis of HE, Ki-67 (Cell Signal Technology, 1:200 dilution), and TUNEL with an *in situ* cell death detection kit (TMR red, Roche).

2.9. Transwell assay

Transwell chambers (24-well insert, 8 μm pore size, Corning) were used for transwell migration assay. Transfected cells (100,000 cells/well) resuspended in 100 μL serum-free medium were added to the upper chambers and 500 μL medium with 10% FBS was added to the lower chamber. After incubation, cells that did not migrate were removed softly with cotton swab, whereas the cells migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. For each chamber, five random fields were recorded and counted.

The transwell invasion assay was essentially the same as the migration assay, except that Matrigel (BD Biosciences) were precoated before transfected cells were seeded.

2.10. Statistical analysis

SPSS 23.0 was used to perform statistical analyses. Student's t-test was used to compare differences between 2 groups. P-value <0.05 was considered to be significant.

3. Results

3.1. LncRNA CASP5 was up-regulated in GBM tissues and cell lines

To clarify whether lncRNA CASP5 was differentially expressed in GBM, we performed qRT-PCR to assess CASP5 expression level in GBM tissues and paired adjacent non-tumor tissues from 40 GBM patients. Our results showed that CASP5 was significantly up-regulated in 75% (30/40) GBM tissues with an average fold-change of 3.417 (p-value<0.01, Fig. 1A and B) compared with

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