Translational Oncology
www.transonc.com

Volume 9 Number 6 December 2016 pp. 548–556 548

Cyclin-Dependent Kinase 2 Promotes Tumor Proliferation and Induces Radio Resistance in Glioblastoma

Jia Wang*,†, Tong Yang*,†, Gaofeng Xu*, Hao Liu*, Chunying Ren*, Wanfu Xie* and Maode Wang*

*Department of Neurosurgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China; †School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China

Abstract

Accumulating evidence indicates that CDK2 promotes hyperproliferation and is associated to poor prognosis in multiple cancer cells. However, the physiological role of CDK2 in GBM and the biological mechanism still remains unclear. In this study, we identified that CDK2 expression was significantly enriched in GBM tumors compared with normal brain. Additionally, CDK2 was functionally required for tumor proliferation and its expression was associated to poor prognosis in GBM patients. Mechanically, CDK2 induced radio resistance in GBM cells and CDK2 knock down increased cell apoptosis when combined with radiotherapy. Therapeutically, we found that CDK2 inhibitor attenuated tumor growth both *in vitro* and *in vivo*. Collectively, CDK2 promotes proliferation, induces radio resistance in GBM, and could become a therapeutic target for GBM.

Translational Oncology (2016) 9, 548–556

Introduction

Glioblastoma (GBM) is one of the most common malignant primary brain tumors in human adults with the median survival in the range of 14 to 18 months despite multimodality treatment comprising maximal safe resection, radiotherapy, and concomitant and adjuvant chemotherapy [1,2]. During their clinical course, almost all patients suffer a post-treatment recurrence then become resistant to both radiotherapy and chemotherapy. Therefore, the drug resistance, high recurrence and poor prognosis of GBM promote us to identify novel biomarkers and explore novel therapeutic targets for the available clinical treatment of GBM.

Cyclin-dependent kinases (CDKs) are heterodimeric complexes composed of a catalytic kinase subunit and a regulatory cyclin subunit, and comprise a family divided into two groups based on their roles in cell cycle progression and transcriptional regulation [3,4]. There are totally 13 CDKs in human cells and five of them, named CDK1, CDK2, CDK3, CDK4, and CDK6, respectively, are involved in the regulation of cell cycle [5]. Cyclin-dependent kinases 2 (CDK2) is one of the most essential regulators for the transition and progression in a cell-division cycle and plays a crucial role in regulating multiple events of cell division cycle including centrosome duplication, DNA synthesis, G1-S transition, and modulation of G2 progression [6–9]. Accumulating evidence has been shown that CDK2 is functionally associated with hyperproliferation in multiple cancer cells and could be regarded as a potentially therapeutic target for cancer therapy [6]. Recent study indicated that CDK2 expression level was elevated in human cholangiocarcinoma tissues and apoptosis-related protein-1 dependent suppression of CDK2 induced cell cycle arrest then restrain tumor growth in cholangiocarcinoma [10]. Another study showed that inhibition of CDK2 kinase activity reduced tumor proliferation *via* selectively targets the $CD44^{+}/CD24^{-/Low}$ stem-like cells in triple-negative breast cancer when combined with conventional chemotherapy [11]. Additionally, a newly published study demonstrated that CDK2 inhibitor exhibits anti-cancer effect in human hepatoma HepG2 and Huh7 cells and significantly inhibited tumor growth [12]. Lim et al. identified CDK2 as a direct therapeutic target of curcumin in colon cancer cells *via* cell cycle arrest in HCT116 cells [13]. Taken together, CDK2-dependent cell cycle regulation plays an essential role in tumor growth and might be a potential therapeutic target for cancer treatment. However,

Address all correspondence to: Wanfu Xie or Moade Wang, Department of Neurosurgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China.
E-mail: wanfu67@aliyun.com
Received 4 July 2016; Revised 18 August 2016; Accepted 18 August 2016

1936-5233/16
http://dx.doi.org/10.1016/j.tranon.2016.08.007

despite these findings, the physiological role of CDK2 and the biological mechanism in GBM still remains unclear.

In this study, we identified that CDK2 expression was significantly enriched in GBM tumors and was functionally required for tumor proliferation both *in vitro* and *in vivo*. Furthermore, high CDK2 expression was associated to poor prognosis in GBM patients. Mechanically, CDK2 induced radio resistance in GBM cells and CDK2 knock down increased apoptosis in GBM cells when combined with radiotherapy. Therapeutically, we found that CDK2 inhibitor attenuated tumor growth both *in vitro* and *in vivo*. Collectively, CDK2 promotes proliferation, induces radio resistance in GBM, and could be a therapeutic target for GBM.

Materials and Methods

Ethics

All the usage of experimental animals (nude mice) in this study is approved by the Scientific Ethics Committee of School of Medicine, Xi'an Jiaotong University, Xi'an, China (No. 2016-085). The collection and usage of the human tumor samples and patient information are approved by the Scientific Ethics Committee of First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China (No. 2016-18).

Reagents and Antibodies

The following reagents and primary antibodies are used in this study: DMEM-F12 (Gibco, 10,565-018), Fetal bovine serum (Gibco, 10,082-147), Accutase solution (Sigma, A6964-100), Alamar Blue (Invitrogen, DAL1100), RIPA buffer (Sigma, R0278), Phosphatase inhibitor cocktail (Sigma, P0044), Protease inhibitor cocktail (P8340), Bradford (BIORAD, 500-0006), BSA used in Bradford assay (BioLabs, B9001S), PageRuler plus prestained protein (Thermo scientific, 26,619), iScript Reverse Transcription supermix for qRT-PCR (Bio-rad, 170-8841), shCDK2 lentivirus particles (Origene, TL320291V), CDK2 Inhibitor II (Santa Cruz Biotechnology, CAS 222035-13-4), Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific,V13241). Anti-CDK2 (Abcam, ab54513, Mouse, used for WB), anti-CDK2 (Abcam, ab32147, Rabbit, used for IHC), β-actin (Sigma, A5316, Mouse, used for WB).

In Vitro Cell Cultures

Glioblastoma cells are cultured in DMEM/F12 medium containing 10% FBS supplement (vol%). The culture medium is replaced every 5 to 10 days. Normal Human Astrocytes (NHA, Lonza) are used as a control sample in this study.

RNA Isolation and Quantitative Real-Time PCR

RNA is isolated by using RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. RNA concentration is determined using a Nanodrop 2000 (Thermo scientific). cDNA is synthesized by using iScript reverse transcription supermix for qRT-PCR (Bio-rad) according to the manufacturer's protocol. The reverse-transcribed cDNA is analyzed by quantitative RT-PCR (qRT-PCR), and GAPDH or 18 s is used as an internal control. Each qRT-PCR includes a 10 μl reaction mixture per well that includes 2.5 μl cDNA, 0.5 μl forward primer (0.5 μM), 0.5 μl reverse primer (0.5 μM), 1.5 μl of DNase/RNase-free distilled water, and 5 μl SYBR green reagent (QIAGEN). The following cycles are performed during DNA amplification: 94 °C for 2 min, 40 cycles of 94 °C (30 s), 60 °C (30 s), and 72 °C (40 s).18S is used as an internal control. The primer sequences are showed below: CDK2-Forward: GAATCTCCAGGGAATAGGGC, CDK2-Reverse: CTGAAATCCTCCTGGGCTG, 18S-Forward: GGCCCTGTAATTGGAATGAGTC and 18S-Reverse: CCAAGAT CCAACTACGAGCTT.

Western Blotting

The cell lysates are prepared in RIPA buffer containing 1% protease and 1% phosphatase inhibitor cocktail (Sigma Aldrich) on ice. The sample protein concentrations are determined by the Bradford method. Equal amounts of protein lysates (10 μg/lane) are fractionated on NuPAGE Novex 4% to 12% Bis-Tris Protein gel (Invitrogen) and transferred to a PVDF membrane (Invitrogen). Subsequently, the membranes are blocked with 5% skimmed milk for 1 h and then treated with the relevant antibody at 4 °C overnight. Protein expression is visualized with Amersham ECL Western Blot System (GE Healthcare Life Sciences). β-Actin serves as a loading control.

Flow Cytometry Analysis

Harvest the cells after the incubation period and wash in cold phosphate-buffered saline for 3 times (PBS). Re-centrifuge the washed cells (from step 2), discard the supernatant and suspend 5×10^5 cells in 100 μl 1X Annexin-binding buffer. Add 5 μl Alexa Fluor® 488 annexin V and 1 μl 100 μg/ml PI working solution thatwas prepared according to the protocol. Incubate the cells at room temperature for 15 minutes then add 400 μl 1× annexin -binding buffer, mix gently and keep the samples on ice. Analyze the stained cells by measuring the fluorescence emission at 530 nm and 575 nm with 488 nm excitation.

Immunohistochemistry Staining

For IHC, experimental mice are sacrificed and perfused with ice-cold PBS followed by 4% (wt/ vol) paraformaldehyde (PFA). Then brains are harvested and fixed in 4% (wt/vol) PFA for 24 h and then transferred into 10% formalin. Brain sections are incubated with the indicated primary antibodies overnight at 4 °C, followed by incubation with an HRP-conjugated secondary antibody for 1 h at room temperature. Signals are detected using DAB substrate kit (Vector). Nuclei are counter stained with hematoxylin or Hoechst, respectively. Samples incubate without primary antibodies are used as negative controls.

German Immunohistochemical Score

Percentage of positive cells is classified as 0 (negative), 1 (up to 10%), 2 (11% to 50%), 3 (51% to 80%), or 4 (>80% positive cells), staining intensity is classified as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). The final immunoreactivity score is defined as the multiplication of both grading results (percentage of positive cells × staining intensity).

In Vivo Intracranial Xenograft Tumor Models

Six-week-old nude mice are used for GBM intracranial implantation. All animal experiments are carried out in Xi'an Jiaotong University. The GBM suspension (1×10^5 cells in 5 μl of PBS) transduced with non-target or shCDK2 lentivirus is injected into the brains of nude mice after anesthesia. At least six mice are used for each group. Drug treatment is done through tail vein injection and starts from 5 days after tumor cells are implanted. Mice were monitored once a day for symptoms related to tumor growth including an arched