Biochemical and Biophysical Research Communications 446 (2014) 1261–1267

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Higher LRRFIP1 expression in glioblastoma multiforme is associated with better response to teniposide, a type II topoisomerase inhibitor

Wei-Qing Li [a,1], Hong-Yu Yu [a,1], Yi-Ming Li [b,*], Xiang Wang [b], Jin He [a], Hong-Zhu Yan [a], Dao-Hua Yang [a], Xiao-Jun Wu [b], Li-Jun Hou [b], Hui-Min Liu [a], Chun-Yan Xia [a], Yi-Cheng Lu [b,*]

[a] Department of Pathology, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai, China
[b] Department of Neurosurgery, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai, China

ARTICLE INFO

Article history:
Received 10 March 2014
Available online 29 March 2014

Keywords:
Glioblastoma multiforme
Teniposide
VM-26
LRRFIP1

ABSTRACT

Previous studies from this laboratory indicated that microRNA-21 (miR-21) contributes to chemoresistance of glioblastoma multiforme (GBM) cells to teniposide, a type II topoisomerase inhibitor. We also showed that *LRRFIP1* is a target of miR-21. In this study, we found that higher baseline LRRFIP1 expression in human GBM tissue ($n = 60$) is associated with better prognosis upon later treatment with teniposide. Experiments in cultured U373MG cells showed enhanced toxicity of teniposide against U373MG cells transfected with a vector that resulted in LRRFIP1 overexpression (vs. cells transfected with control vector). Experiments in nude mice demonstrated better response of LRRFIP1 overexpressing xenografts to teniposide. These findings indicate that high baseline LRRFIP1 expression in GBM is associated with better response to teniposide, and encourage exploring LRRFIP1 as a target for GBM treatment.

1. Introduction

Glioblastoma multiforme (GBM) is the most malignant and recalcitrant form of astrocytomas [1,2]. GBM is a rapidly growing and infiltrative tumor, and is often unresectable at the time of diagnosis. Current standard therapy includes maximal surgical removal, followed by focal radiotherapy and adjuvant chemotherapy [2]. Nitrosourea-based chemotherapy is commonly used to treat GBM, but could only marginally improve patient survival despite of severe toxicity [3,4]. Temozolomide, the current chemotherapeutic agent of choice for GBM, also produces only modest impact on patient survival [1,5]. Resistance to chemotherapeutic drugs can be either *de novo* (already present at diagnosis) or acquired (developed upon chemotherapy). Indeed, the poor prognosis in GBM patients could largely be attributed to chemoresistance to anticancer drugs and recurrence after the treatment [6]. Many factors contribute to the response of GBM to chemotherapeutic agents [7–10].

MicroRNA-21 (miR-21) has been recently found to be dysregulated in GBM [11]. More specifically, miR-21 overexpression could attenuate temozolomide-induced apoptosis of GBM cell line U87MG by reducing caspase 3 activity [12,13]. In a recent study [13], we showed that miR-21 is overexpressed in GBM cell line U373MG and contribute to the chemoresistance to VM-26 [14]. In patients with malignant glioma, VM-26 as a semisynthetic podophyllotoxin derivative and a topoisomerase II inhibitor produce survival benefit in combination with the DNA bifunctional alkylating agent 1,3-bis(2-chloroethyl-1-nitrosourea) (BCNU) [15].

The leucine-rich repeat (in Flightless I) interacting protein-1 (LRRFIP1) gene encodes a protein that participates in type I interferon response [16,17]. Its product is also known as the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) interacting protein (TRIP) and is a component of the TNFR superfamily. An earlier study from this laboratory demonstrated that the LRRFIP1 gene is a direct target of miR-21 [14], suggesting that LRRFIP1 gene could be involved in GBM response to chemotherapeutic agents. Based on these previous findings, we hypothesized that LRRFIP1 expression is decreased in malignant glioma tissues expressing high levels of miR-21. We also speculated that decreased LRRFIP1 expression contributes to chemoresistance of GBM cells.

In the current study, we first examined the expression of LRRFIP1 in 60 human GBM tissue specimens and found higher expression of LRRFIP1 in the cases that responded more favorably to VM-26 treatment. In the next step using cultured U373MG GBM cells and a xenograft mouse model, we found that LRRFIP1 overexpression could sensitize GMB cells to VM-26.

* Corresponding authors. Address: Department of Neurosurgery, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai 200003, China.
E-mail addresses: yiyimig@163.com (Y.-M. Li), luyicheng10@gmail.com (Y.-C. Lu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.03.105
0006-291X/

2. Materials and methods

2.1. Human GBM tissue samples

The current study included formalin-fixed and paraffin-embedded tumor sections obtained with surgical resection in 60 patients with primary GBM during a period from January, 1996 to December, 2009. All 60 cases were treated only with VM-26 and not any other chemotherapeutic agents after the surgical resection. Two experienced pathologists reviewed the tissue sections independently using the World Health Organization (WHO) classification of tumors in the central nervous system (CNS).

2.2. Immunohistochemistry assay

Formalin-fixed, paraffin-embedded tissue sections (3-μm) were deparaffined in xylol and rehydrated in gradient ethanol. Antigen retrieval was performed by microwave heating for 20 min in 1-mM EDTA buffer (pH 8.0). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 30 min. Sections were incubated with an anti-LRRFIP1 antibody (Abcam, Hong Kong) overnight at 4 °C. After washing in Tris-buffered saline with Tween-20, the sections were incubated with a biotin-conjugated secondary antibody for 20 min at room temperature followed by 20-min incubation with peroxidase-conjugated biotin-streptavidin complex (Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin and visualized by staining with 3,3′-diaminobenzidin.

To quantify LRRFIP1 immunoreactivity, two experienced pathologists examined representative visual fields (400× magnification; 5 fields per specimen) independently. The intensity of positive staining in tumor cells was scored using a scale from 0 to 3 (0 for no immunostaining, 1 for light-brown color, 2 for medium-brown color, and 3 for dark dark-brown color). The percentage of positive staining cells was also scored (0, no staining; 1, positive staining in <25% of the tumor cells; 2, positive staining in 25–75% of the tumor cells; and 3, positive staining in >75% of the tumor cells.). The two scores were then multiplied, and the results were used to reflect the expression. All discrepancies in scoring were reviewed, and a consensus was reached. Staining was classified as: strong (+++, total score = 6), moderate (++, total score = 4–6), weak (+, total score = 1–3), and null (–, total score = 0). Expression was defined as high (++ and +++), low (+ and negative).

2.3. Overexpressing LRRFIP1 in U373MG cells

For vector construction, the coding sequence of LRRFIP1 mRNA was amplified from cDNA clone (Origene, Beijing, China) using the following primers: LRRFIP1-F, 5′-CGGGGTACCATGACCAGCCCCGCGGCCGCTC-3′ and LRRFIP1-R, 5′-CGCGGATCCTTAGGACATGGTACAGTCTTC-3′. The PCR fragment was cloned into the *Kpn*I and *Bam*HI sites of pcDNA3 vector. The resulting vector construct was referred to as pcDNA3/LRRFIP1. U373MG cells were cultured in α-minimal essential medium (MEM) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin at 37 °C with 5% CO_2, and transfected with pcDNA3/LRRFIP1 or the control vector pcDNA3 with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfected cells were subjected to selection with 800-μg/ml G418 (Invitrogen) in complete medium for 20–30 d. U373MG cells with stable transfection were maintained in α-MEM containing 10% FBS and 800-μg/ml G418.

2.4. DNA construction

For miR-21 expression plasmid construction, DNA fragment containing miR-21 precursor sequence from HEK293 cell genome was amplified with PCR using the following primers: miR-21-F, 5′-TCCATGGCTGTACCACCTTG-3′, miR-21-R, 5′-CTCTAAGTGCCACCAGACAG-3′. Then resulting product was inserted into pcDNA3 clone vector at the *Bam*HI and *Eco*RI sites. DNA fragment containing full length LRRFIP1 coding sequence was amplified by PCR using the following primers: LRRFIP1-F, 5′-CGGGGTACCATGACCAGCCCCGCGGCCGCTC-3′, LRRFIP1-R, 5′-CGCGGATCCTTAGGACATGGTACAGTCTTC-3′. PCR products were digested with the *Kpn*I and *Bam*HI restriction enzymes and then cloned into pcDNA3 plasmid.

2.5. Western blot analyses

Cell lysate was prepared using the RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.2), 1% Triton X-100, and 0.1% SDS. Immunoblotting was performed as previously described using a rabbit polyclonal anti-LRRFIP1 antibody [8]. GAPDH was used as an internal control. Protein bands were visualized by enhanced chemiluminescence and analyzed using the LabWorks™ Image Acquisition and Analysis Software (UVP) and normalized against GAPDH.

2.6. Proliferation and clonogenic assay under VM-26 exposure

Cells in the logarithmically growing phases were plated at a density of 3.5×10^4 cells per well in 6-well plates and transfected 24-h later. Twenty-four hours after the transfection, the cells were plated at a density of 8×10^3 cells per well in 96-well plates in the presence or absence of VM-26 (0.5PPC = 22.5 μg/mL). Cell viability was assessed 1, 2, 3, 4 or 5 days later using a tetrazolium-based semi-automated colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays at 570 nm (Nanjing Keygen Biotech, Nanjing, China). The inhibition was calculated as: inhibition ratio = $(OD_{control} - OD_{VM\text{-}26})/(OD_{VM\text{-}26} - OD_{background}) \times 100\%$. For clonogenic assay, U373MG cells were seeded in 12-well plates at a density of 100 cells per well in the presence of 0.5 PPC of VM-26. The number of colonies (at least 50 cells under crystal violet staining) was counted 7 days later. The rate of colony formation was calculated as: (number of colonies/number of seeded cells) × 100%.

2.7. Annexin V staining

U373MG cells were stained with Annexin V and 7-AAD using an ApoScreen Annexin V apoptosis kit (Southern Biotech, Birmingham, Alabama), and analyzed using a Beckman coulter flow cytometer.

2.8. Xenograft studies

U373 cells (5×10^6) stably expressing LRRFIP1 or the control cells were inoculated subcutaneously at the axillary fossae of female athymic nude mice (age, 6–8 weeks). Starting from the 7th day after inoculation, the mice received either VM-26 (50 mg/kg) or PBS by peritoneal injection every 3 days. The tumor size was monitored every 3 days by measuring the length and width with a caliper. Tumor volume was calculated as: $(L \times W^2) \times 0.5$ mm^3, where L is the length and W is the width of each tumor. At the 25th day after inoculation, the mice were sacrificed and the tumors were dissected, measured and photographed.

2.9. Statistical analysis

All the experiments were performed in triplicate. Kruskal–Wallis test was used to analyze the immunohistochemistry results. For all other experiments, Student's *t*-test was used. Data are