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

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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.

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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

reported as mean ± standard deviation (SD). Statistical significance was set as $P < 0.05$ (two-sided).

The study protocols, including the use of tissue specimens, were approved by the Institutional Review Board. The mice used in this experiment were maintained under a specific pathogen-free facility and handled in accordance with NIH Animal Care and Use Committee regulations.

3. Results

3.1. Higher LRRFIP1 expression in GBM correlated with more favorable prognosis upon VM-26 treatment

We have previously shown that miR-21 could directly target LRRFIP1 and contribute to chemoresistance of U373MG cells to VM-26 [14]. We examined the expression of LRRFIP1 in GBM tissue specimens (surgically resected primary GBM patients who were only VM-26-treated) by immunohistochemistry using anti-LRRFIP1 antibody. 33/60 (55%) human GBM tumor samples had high expression levels of LRRFIP1. 27/60 (45%) human GBM tumor samples had low expression levels of LRRFIP1. The Kaplan–Meier survival analysis revealed higher survival rate in the high LRRFIP1 expression group than in the low expression group ($P < 0.05$; Fig. 1B).

3.2. LRRFIP1 sensitized cultured U373MG cells to VM-26

Transient transfection of U373MG cells with pcDNA3/LRRFIP1 increased LRRFIP1 levels by approximately seven folds (vs. the empty pcDNA3 vector) (Fig. 2A). Transient transfection of U373MG cells with pcDNA3/LRRFIP1 also potentiated the inhibitory action of VM-26 on the proliferation and colony-formation of U373MG cells (Fig. 2B and C). Cells overexpressing LRRFIP1 showed a significantly higher rate of apoptosis (31.2%) than the control cells (20.6%) upon exposure to VM-26. U373MG cells overexpressing LRRFIP1 showed a significantly higher rate of apoptosis (31.2%) in response to treatment with VM-26 than that not treated with VM-26 (2.1%, Fig. 2D).

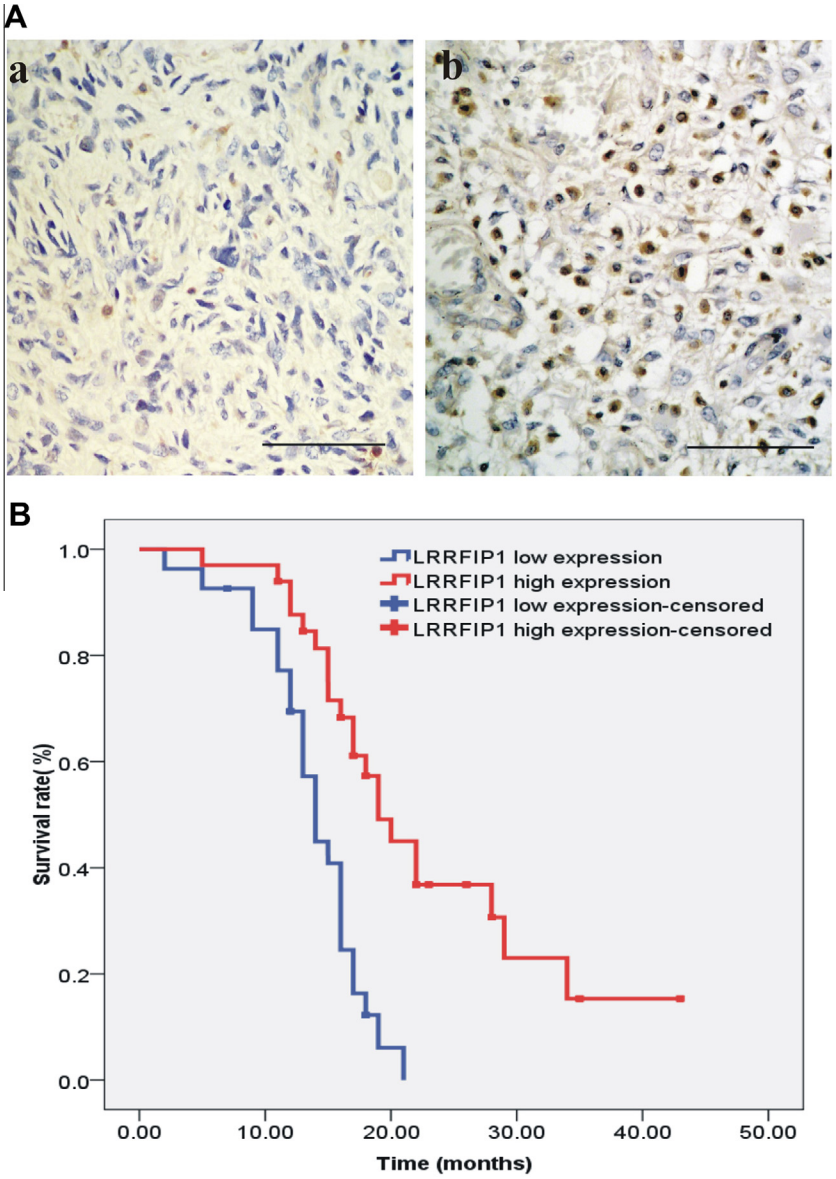

Fig. 1. The relation between LRRFIP1 expression in human GBM tissues and survival of patients with GBM who have been treated with VM-26. (A) The representative IHC staining images of LRRFIP1 in human GBM tissues with low or high LRRFIP1 expression (400×, scale bar = 100 μm). (B) High LRRFIP1 expression is associated with better survival in patients with GBM treated with VM-26 ($P < 0.05$).

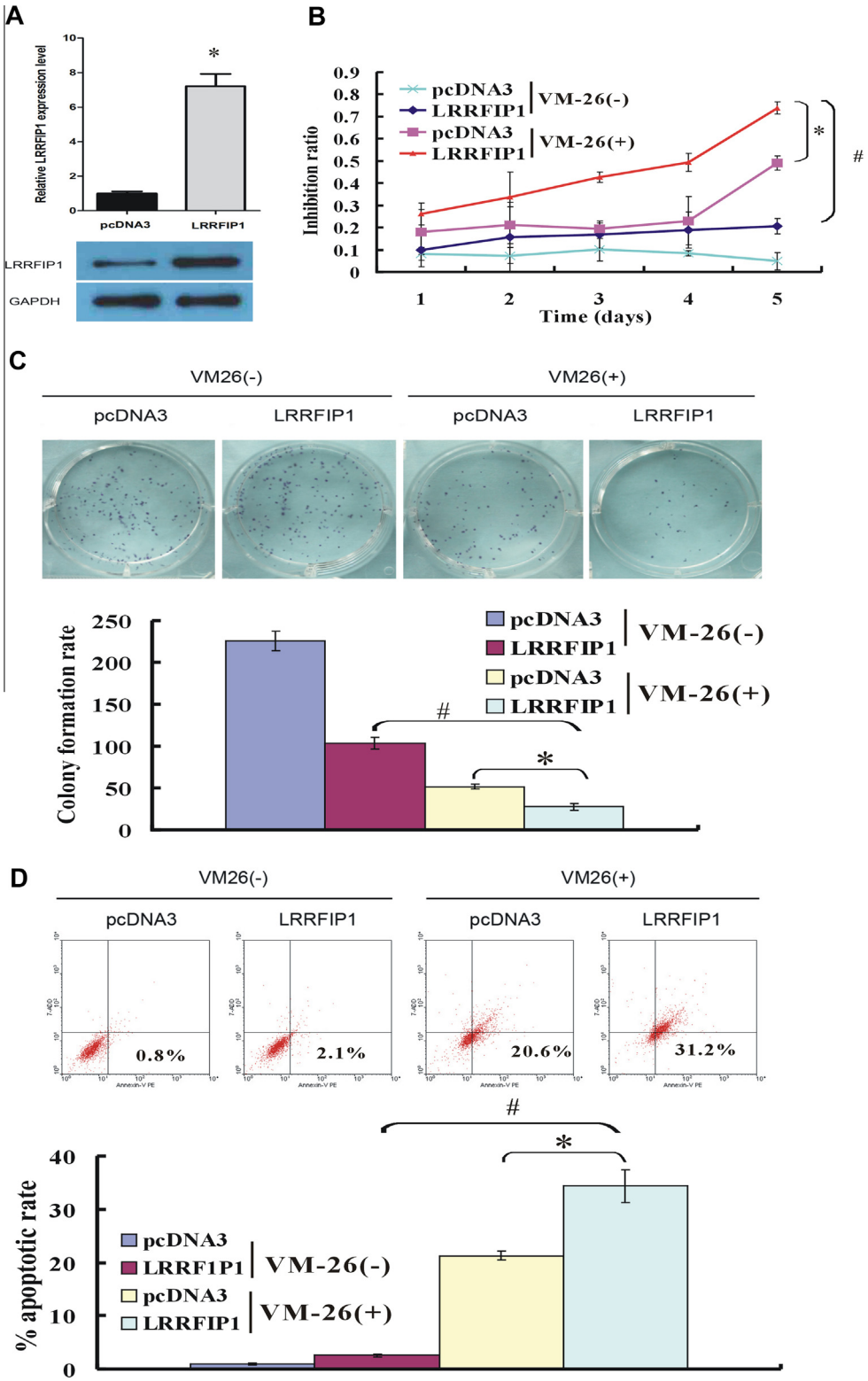

Fig. 2. LRRFIP1 sensitizes U373MG cells to VM-26 by suppressing growth and promoting apoptosis. (A) U373MG cells were transfected with pcDNA3/LRRFIP1 or control vector pcDNA3, and LRRFIP1 level was detected using Western blotting assays. The graph shows the mean ± SD of LRRFIP1 levels normalized against GAPDH (*$P < 0.05$, compared with the cells transfected with the control vector pcDNA3). (B) U373MG cells were transfected with pcDNA3/LRRFIP1 or control vector pcDNA3, followed by treatment with or without VM-26. Cell viability was determined using the MTT assay at different time points after treatment with or without VM-26. The graph shows mean ± SD of A_{570} of inhibition ratio from three independent experiments (*$P < 0.05$, compared with the cells transfected with the control vector pcDNA3; #$P < 0.05$, compared with the cells not treated with VM-26). (C) The colony formation activity of the transfected U373 cells cultured with or without VM-26 was detected using clonogenic assays. The number of colonies was counted from the 7th day after seeding, and the rate of colony formation was calculated. The graph shows mean ± SD of the rate of colony formation from three independent experiments (*$P < 0.05$, compared with the cells transfected with the control vector pcDNA3; #$P < 0.05$, compared with the cells not treated with VM-26). (D) U373MG cells were transfected with pcDNA3/LRRFIP1 or control vector pcDNA3, followed by 48 h of culturing in the medium with or without VM-26. The apoptotic activity of the cells was detected by Annexin V staining. The *X* and *Y* axes represent Annexin V intensity and 7-AAD intensity, respectively (*$P < 0.05$, compared with the cells transfected with the control vector pcDNA3; #$P < 0.05$, compared with the cells not treated with VM-26).

3.3. LRRFIP1 suppressed the growth of U373MG xenografts in athymic nude mice treated with VM-26

We further examined the effect of LRRFIP1 on tumor growth in athymic nude mice bearing U373MG xenografts. We inoculated the athymic nude mice with U373MG cells stably transfected with pcDNA3/LRRFIP1 or the control vector and, seven days after the inoculation, the mice received VM-26 (50 mg/kg) or PBS peritoneally at every three days. Measurement of tumor volume indicated that at the start of and up to one week after the initiation of treatment, athymic nude mice bearing U373MG xenografts overexpressing LRRFIP1 or control xenografts had similar tumor volume.

From two to four weeks after the initiation of VM-26 treatment, the tumor volume of the xenograft tumors transfected with pcDNA3/LRRFIP1 was significantly lower than that transfected with the control vector pcDNA3. Also, for the transplanted tumors transfected with pcDNA3/LRRFIP1, the tumor volume of the xenograft tumors treated with VM-26 was significantly lower than that treated with the PBS solvent control (Fig. 3). The data indicated that LRRFIP1 overexpression was associated with suppressed growth of U373MG xenografts in the presence of VM-26.

3.4. LRRFIP1 sensitized GBM cells transfected with miRNA-21 to VM-26 by suppressing growth

We have previously shown that miR-21 could directly target LRRFIP1 and contribute to chemoresistance of U373MG cells to VM-26. To further demonstrate the role of LRRFIP1 in chemosensitizing GBM cells, we transfected the GBM cells with pcDNA3/miRNA-21 or pcDNA3/miRNA-21/LRRFIP1. The cell viability was further evaluated by MTT to demonstrate the impact of LRRFIP1 on the viability of the cells overexpressing miRNA-21. Growth inhibition in the cells transfected with pcDNA3/miRNA-21 was significantly lower than in the cells transfected with the control vector pcDNA3. Growth inhibition in the cells transfected with pcDNA3/miRNA-21/LRRFIP1 was significantly higher than that in the cells transfected with pcDNA3/miRNA-21 (Fig. 4).

4. Discussion

MiRNAs could regulate the expression of approximately one third of human genes [18]. miR-21 acts as an oncogene in various human cancers [19] and was found dysregulated in GBM [11]. miR-21 overexpression is associated with reduced caspase-3 activity and attenuated apoptosis of GBM cells upon temozolomide exposure [12,13]. A previous study from this laboratory found that miR-21 could sensitize U373MG cells to VM-26 [14]. The same study demonstrated that miR-21 could directly and negatively target *LRRFIP1*, raising the possibility that LRRFIP1 may be involved in miR-21 mediated-chemosensitization of U373MG cells [13]. We hypothesized that overexpression of miR-21 in malignant GBM tissues could downregulate *LRRFIP1*, and increase chemoresistance of U373MG cells to VM-26. Our immunohistochemical analysis revealed that that higher expression of LRRFIP1 in GBM is associated with more favourable prognosis upon VM-26 treatment. Since the patients were not chemotherapy-naive, it is possible that reduced expression of the *LRRFIP1* gene as a consequence of miR-21 overexpression could offer a survival advantage for GBM cells against the insults by chemotherapeutic agents.

LRRFIP1 is a novel protein with largely unknown function. The involvement of LRRFIP1 in GBM development or response to chemotherapeutic agents has not been previously reported. In an earlier study, we identified *LRRFIP1* as a direct target of miR-21, suggesting that LRRFIP1 could be involved in determining the fate of GBM cells in response to chemotherapeutic agents. In this study, we examined the role of LRRFIP1 in GBM response to VM-26. We found that LRRFIP1 overexpression increases the sensitivity of U373MG cells to VM-26 *in vitro* as manifested by a reduced rate of proliferation, impaired clonogenicity, and an increased rate of apoptosis. Increased levels of miR-21 suppresses p53-mediated apoptosis in GBM U251 cells [19] and could reduce caspase-3 activity and attenuate apoptosis of GBM cells exposed to temozolomide [12,13]. We speculate that, as a target of miR-21, LRRFIP1 could function downstream of miR-21 and mediate many of the anti-apoptotic effects of miR-21, probably through a network of interacting proteins. LRRFIP1 binds to a variety of proteins, including the TRAF-interacting protein (TRIP) [20]. TRIP interacts with the receptor/TRAF signaling complex, and inhibits the TRAF2-mediated activation of nuclear factor kappa-B (NF-κB). Activation of NF-κB is required for cell activation and also for protection against apoptosis and underlie tumor chemoresistance primarily due to its anti-apoptotic activities [21–23]. However, the interconnection of LRRFIP1 with TRIP or the NF-κB signaling network in determining the fate of GBM cells to chemotherapeutic agents like VM-26 remain to be established.

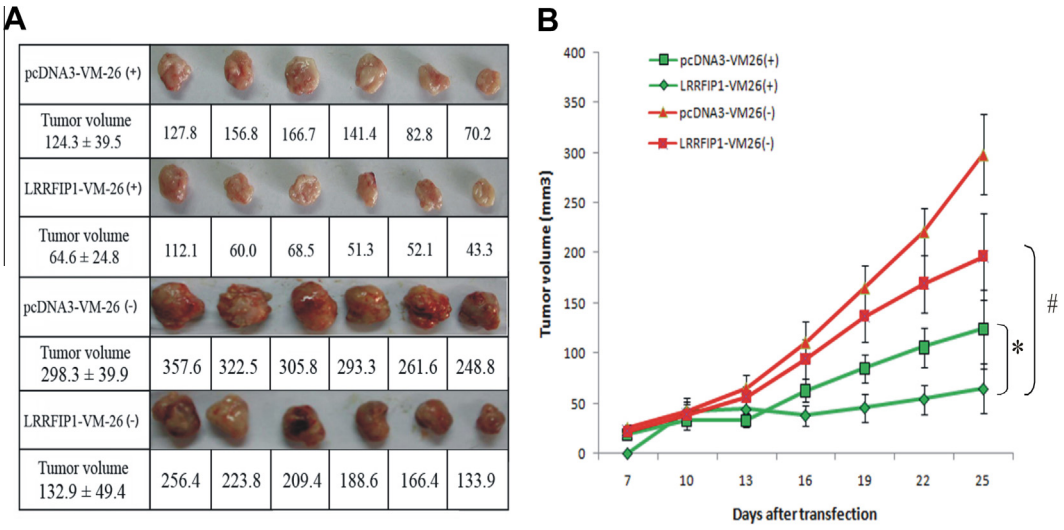

Fig. 3. LRRFIP1 suppressed U373MG xenograft growth in athymic nude mice exposed to VM-26. (A) Representative photographs of xenograft tumors. (B) The tumor size is shown in the tumor growth curve ($^{*}P < 0.05$, compared with the tumors transfected with the control vector pcDNA3; $^{\#}P < 0.05$, compared with the tumors treated with the PBS solvent control).

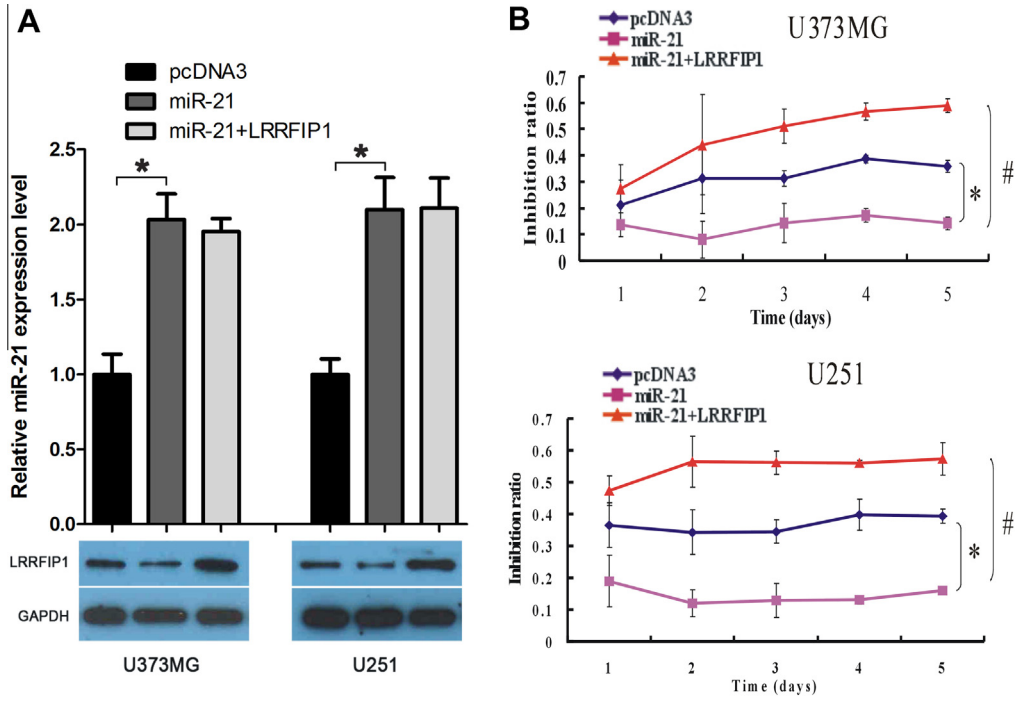

Fig. 4. LRRFIP1 sensitizes GBM cells transfected with miRNA-21 to VM-26 by suppressing growth. (A) U373MG and U251 cells were transfected with the control vector pcDNA3, pcDNA3/miRNA-21 or pcDNA3/miRNA-21/LRRFIP1. The upper panel showed the miRNA-21 expression levels were detected by quantitative real-time PCR and the lower panel showed the LRRFIP1 level was detected using Western blotting assays. The graph shows the mean ± SD of miRNA-21 levels normalized against that of the cells transfected with the control vector pcDNA3 ($^{*}P < 0.05$, compared with the cells transfected with the control vector pcDNA3). (B) U373MG and U251 cells were transfected with the control vector pcDNA3, pcDNA3/miRNA-21 or pcDNA3/miRNA-21/LRRFIP1, followed by treatment with VM-26. Cell viability was determined using the MTT assay at different time points after treatment with VM-26. The graph shows mean ± SD of A_{570} of inhibition ratio from three independent experiments ($^{*}P < 0.05$, compared with the control vector pcDNA3; $^{\#}P < 0.05$, compared with the cells transfected with pcDNA3/miRNA-21).

Defining the mechanisms of chemoresistance could lead to improved therapeutic outcome for GBM. O^6-methylguanine-DNA methyltransferase (MGMT) mediates resistance to chloroethylating and methylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine) and temozolomide, respectively [24] and contribute to the poor outcome of GBM patients. O^6-benzylguanine, a suicidal MGMT inhibitor, has been used to modulate MGMT activity in an attempt to improve the outcome of GBM patients [25,26]. We showed here that LRRFIP1 overexpression could inhibit GBM cell growth *in vitro* and increase the rate of apoptosis of GBM cells in response to VM-26. Our *in vivo* studies using nude mice bearing U373MG xenografts further showed that LRRFIP1 overexpression could hinder the growth of GBM xenografts. Our findings indicate that modulating LRRFIP1 levels or activities could be explored to suppress GBM growth and encourages using LRRFIP1 as a therapeutic target to improve the treatment of GBM.

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