ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(8): 532–540 **CN** 31-1940/Q

Study of a Novel Brain Relatively Specific Gene *LRRC4* Involved in Glioma Tumorigenesis Suppression Using the Tet-on System

Qiu-Hong ZHANG, Li-Li WANG, Li CAO, Cong PENG, Xiao-Ling LI, Ke TANG, Wei-Fang LI, Ping LIAO, Jie-Ru WANG, and Gui-Yuan LI*

Cancer Research Institute, Central South University, Changsha 410078, China

Abstract *LRRC4* is a novel relatively specific gene, which displays significant down-regulation in primary brain tumor biopsies and has the potential to suppress brain tumor growth. In this study, we investigated the growth inhibitory effect of *LRRC4* on tumorigencity *in vivo* and on cell proliferation *in vitro* by a tetracycline-inducible expression system. Results showed that *LRRC4* significantly reduced the growth and malignant grade of xenografts arising from glioblastoma U251MG cells. Cell proliferation was markedly inhibited after U251MG Tet-on-*LRRC4* cell induction with doxycycline. Flow cytometry and Western blot analysis demonstrated that *LRRC4* mediated a delay of the cell cycle in late G_1, possibly through up-regulating the expressions of p21Waf1/cip1 and p27Kip1 and down-regulating the expressions of cyclin-dependent kinase 2, retinoblastoma protein and epidermal growth factor receptors. Together, these findings provide clues to the function of *LRRC4* as a negative regulator of cell growth and underscore a link between the above-mentioned cyclins, cyclin-associated molecules and tumorigenicity.

Key words *LRRC4*; tumorigencity; cell proliferation; cell cycle delay

Gliomas represent only 2% of adult tumors, but they contribute to 10% of all cancer-related deaths [1]. Although there have been significant advances in the treatment of other cancers, there is only modest progress in brain tumor therapy because gliomas grow in an infiltrative fashion. Because substantial genetic heterogeneity exists even within tumors of the same histological subtype, it is generally believed that there are multiple pathways of genetic alterations leading to gliomas [2]. The development of and progression towards gliomas is clearly due to a multistep process that involves functional inactivation of tumor suppressor genes as well as oncogene activation and/or overexpression, related to both cellular proliferation and differentiation processes [3–5]. Some insights into potential future therapies for astrocytomas have been derived from genetic studies [6]. Cancer has always been attributed to an abnormal proliferation of cells, and agents that interfere with cell cycle progression may have potential as anticancer therapeutics. The discovery of inhibitors of the cell cycle such as paclitaxel [7] and olomucine [8] has led to rapid advances in the investigation and design of a variety of anti-mitosis compounds that are used clinically or have the potential for development as anti-proliferative agents.

LRRC4 (GenBank accession No. AF196976), a relatively specific gene cloned from chromosome 7q31-32 [9,10], displays significant down-regulation and expression deletion in primary brain tumor biopsies [9] and has the potential to suppress brain tumor growth [11]. But it is still unclear what mechanisms and molecules are involved in the suppression of glioma growth and cell proliferation.

The gene switch Tet-on system [12,13] can induce gene expression by administrating tetracycline derivatives such

Received: March 20, 2005 Accepted: May 11, 2005

This work was supported by the grants from the National Natural Science Foundation of China (No. 30100191 and No. 30270429) and the Natural Science Foundation of Hunan Province (No. 03JJY3062)

*Corresponding author: Tel, 86-731-480-5090; Fax, 86-731-480-5383; E-mail, Ligy@xysm.net

DOI: 10.1111/j.1745-7270.2005.00079.x

as doxycycline to analyze the relationship between *LRRC4* gene expression and function *in vivo* and *in vitro*.

In this study, we established a stable U251MG Tet-on cell line and two dual-stable U251MG Tet-on cell lines expressing *LRRC4*, and analyzed the inhibitory effect of *LRRC4* on tumorgenesis and cell proliferation in U251MG using tumorigenicity assays, cell growth curves and methylthiazoltetrazolium (MTT) cell proliferation assays. We determined the particular stage when *LRRC4* inhibits cell cycle progression using flow cytometry. In order to illustrate the potential molecular mechanism involved in suppression of glioma tumorigenesis by *LRRC4*, we examined expressions of cell cycle-associated key molecules using Western blot analysis.

Materials and Methods

Cell culture

U251MG cells originally derived from a patient with glioblastoma were obtained from American Type Culture Collection (Rockville, USA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS; BD Biosciences Clontech, Palo Alto, USA).

Generation of U251MG Tet-on cell lines expressing *LRRC4*

U251MG Tet-on cells, which were stably transfected with pTet-on (BD Biosciences Clontech) and a G418-resistance plasmid, were maintained in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 20 U/ml streptomycin and 800 μg/ml G418 (Sigma, St. Louis, USA). Clones were screened by a luciferase-expressing system and counted by scintillation counting.

The doxycycline-inducible *LRRC4* expression plasmid pTRE-2hyg-*LRRC4* was constructed by inserting the *LRRC4* coding sequence into the *Not*I site of vector pTRE-2hyg (BD Biosciences Clontech). To generate stable cell lines, U251MG Tet-on cells were transfected with pTRE-2hyg-*LRRC4*. Each culture was divided and transferred onto three plates 2 days later, grown for an additional 24 h, then subjected to selection with 300 μg/ml hygromycin (Calbiochem, San Diego, USA)/G418 (Sigma). Resulting colonies were screened for *LRRC4* expression by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using avian myeloblastosis virus (AMV) Reverse Transcriptase System (Promega, Madison, USA) and Northern blot. Two positive U251MG Tet-on cell lines expressing *LRRC4* cell clones were selected for the following experiments. Primer and probe sequences were chosen using Primer 3 (http://www-genome.wi.mit.edu/). The *LRRC4* cDNA from stably transfected cell lines was amplified using a forward primer (5′-TTGGCCCACAATAACCTCTC-3′) and a reverse primer (5′-ACAGGCTTGTACTTTCGCGT-3′). As an internal control, β-actin gene was analyzed in parallel using a forward primer (5′-TCCGTGGAGAAG-AGCTACGA-3′) and a reverse primer (5′-GTACTTGAG-CTCAGAAGGAG-3′). Total RNA was extracted from the culture cells using Trizol reagent (Gibco BRL, Grand Island, USA). DNA-free RNA was denatured and transferred onto a nylon membrane (BD Biosciences Clontech) according to the standard procedure. After UV cross-linking, the membrane was hybridized with [α-^{32}P]dATP (Yahui Company, Beijing, China) labeled *LRRC4* cDNA at 68 °C overnight in Express Hyb (BD Biosciences Clontech). The membrane was washed with increasing stringency up to a final wash of 1×SSC, 0.1% sodium dodecyl sulfate (SDS) at 65 °C. The membrane was subsequently reprobed with β-actin. Autoradiograms were exposed after 24 h.

Tumorigenicity assay

We inoculated 5×10^6 parental U251MG and U251MG Tet-on-*LRRC4* cells *s.c.* into the flanks of 4- to 6-week-old male nude mice (BALB/c-nu/nu, Shanghai Cancer Institute, Shanghai, China). Tumorigenicity assay of U251MG and U251MG Tet-on-*LRRC4* cell lines was carried out in the same manner, except that U251MG Tet-on-*LRRC4* cells-treated mice were provided with drinking water containing either 4% sucrose or 4% sucrose plus 2.0 μg/ml doxycycline (Sigma) to induce the pTRE promoter; doxycycline-supplemented water was refreshed every 3 days. Tumor volumes were calculated with the ellipsoid formula: $V=4/3\pi ab^2$, where a and b are the length and width of the tumor, respectively. The mice were killed when the tumors reached a volume of about 1.5 cm^3 or 1 month after inoculation. Experiments were performed in accordance with European Union and Italian animal care regulations.

Cell growth curves and MTT cell proliferation assay

Cell growth curves and MTT cell proliferation assay were conducted. We seeded 1×10^4 parental U251MG and U251MG Tet-on-*LRRC4* cells in 24-well flat-bottom plates (Falcon, BD Labware, Lincoln Park, USA) in 1 ml/well of RPMI 1640 with or without doxycycline. After 24 h, cells were counted for 6 days continuously.

Parental U251MG and U251MG Tet-on-*LRRC4* cells

were plated in 96-well plates (Falcon) at a density of 5×10^3 cells/well in 200 μl/well of RPMI 1640 with or without doxycycline. 48–72 h later, 20 μl 5 μg/ml 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added per well, and the culture was incubated for another 4 h. Then the supernatant fluid was discarded and 150 μl of dimethyl sulfoxide (DMSO) was added per well. The spectrometric absorbance at a wavelength of 570 nm (A_{570}) was measured on a microplate reader (Elx800, Bio-Tek Instruments Inc., Vt., USA). The data were finalized by means of triplicate experiments.

Morphology alteration features

The ultrastructure of the U251MG cells transfected with *LRRC4* was observed with an optical microscope (Olympus, Tokyo, Japan) and a transmission scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

Cell cycle analysis

The cells were plated in 75 cm^2 cell culture flask at a density of 2×10^5 cells/flask. After 24 h, the cells were treated with 0.5 μM nocodazole for 7 or 24 h, then trypsinized, washed, and fixed in 70% ice-cold ethanol at 4 °C for 30 min. The cells were then washed in ice-cold PBS twice and incubated in 100 μg/ml propidium iodide (PI; Sigma) containing 100 μg/ml RNase overnight at 4 °C, then samples were analyzed by flow cytometry using a 488 nm argon laser and FL2-A detection line. DNA content frequency histograms were deconvoluted using ModFit LT software (Verity, Topsham, USA). Data were expressed in mean±SD of three independent experiments.

Western blot analysis

Cells were centrifugated at 12,000 *g* for 10 min, then the pellet was resuspended in lysis buffer (1% Nonidet P-40; 40 mM Tris hydrochloride, pH 8.0, 150 mM NaCl) at 4 °C for 30 min. Protein concentrations were determined with BCA protein assay kit (Pierce, Rockford, USA) on a microplate reader at 570 nm. Cells were lysed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with complete protease inhibitors (Roche Applied Science, Indianapolis, USA), separated by SDS/PAGE and transferred to polyvinylidene fluoride (PVDF; Amersham Biosciences, Piscataway, USA). Blots were incubated with goat anti-epidermal growth factor receptor (EGFR) (sc-03-G; Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-p21 (H-164, sc-756), rabbit anti-p27 (c-19), rabbit anti-retinoblastoma protein (pRb) (C-15, sc-50), and rabbit anti-cyclin-dependent kinase 2 (CDK2) (M2, sc-163) (Santa Cruz Biotechnology), followed by a horseradish peroxidase conjugated anti-goat or anti-rabbit Ab (Santa Cruz Biotechnology), developed using Supersignal chemiluminescence reagents (Pierce), and exposed to X-ray film.

Results

Suppression of tumorigenicity by *LRRC4* in U251MG cell line

Because *LRRC4* displays expression deletion in the U251MG cell line, we chose the U251MG cell line as the host. To determine the effects of *LRRC4* on tumorigenicity *in vivo*, we established the stable U251MG Tet-on cell line and the doxycycline-inducible U251MG Tet-on cell line with the plasmid pTRE-2hyg-*LRRC4*. RT-PCR (data not shown) and Northern blot analysis showed that the levels of *LRRC4* mRNA detected in the stable cell lines P27 and P28 exhibited a significant expression difference in the absence and presence of doxycycline (**Fig. 1**).

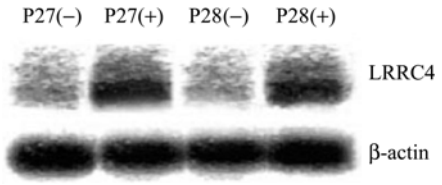

Fig. 1 Different expression of *LRRC4* in U251MG Tet-on-*LRRC4* cell lines revealed by Northern blot analysis

(+), with doxycycline; (–),without doxycycline.

We investigated tumorigenesis in nude mice of the parental U251MG cell line compared with U251MG Tet-on-LRRC4 cell lines (P27 and P28) in the absence of doxycycline (Doxy–) or presence of doxycycline (Doxy+). As shown in **Fig. 2**, inoculation of 5×10^6 cells produced tumors in all mice, with tumor masses detectable after 5–7 d and reaching a volume of 1 cm^3 in approximately three weeks. Like the parental U251MG group, the P27 and P28 Doxy– group produced larger tumors in 100% of the mice. In contrast, the P27 and P28 Doxy+ group produced smaller tumors [**Fig. 2(B)**]; the average weight of xenografts from the Doxy+ group was significantly lighter than any xenografts from the Doxy– group. There was a highly

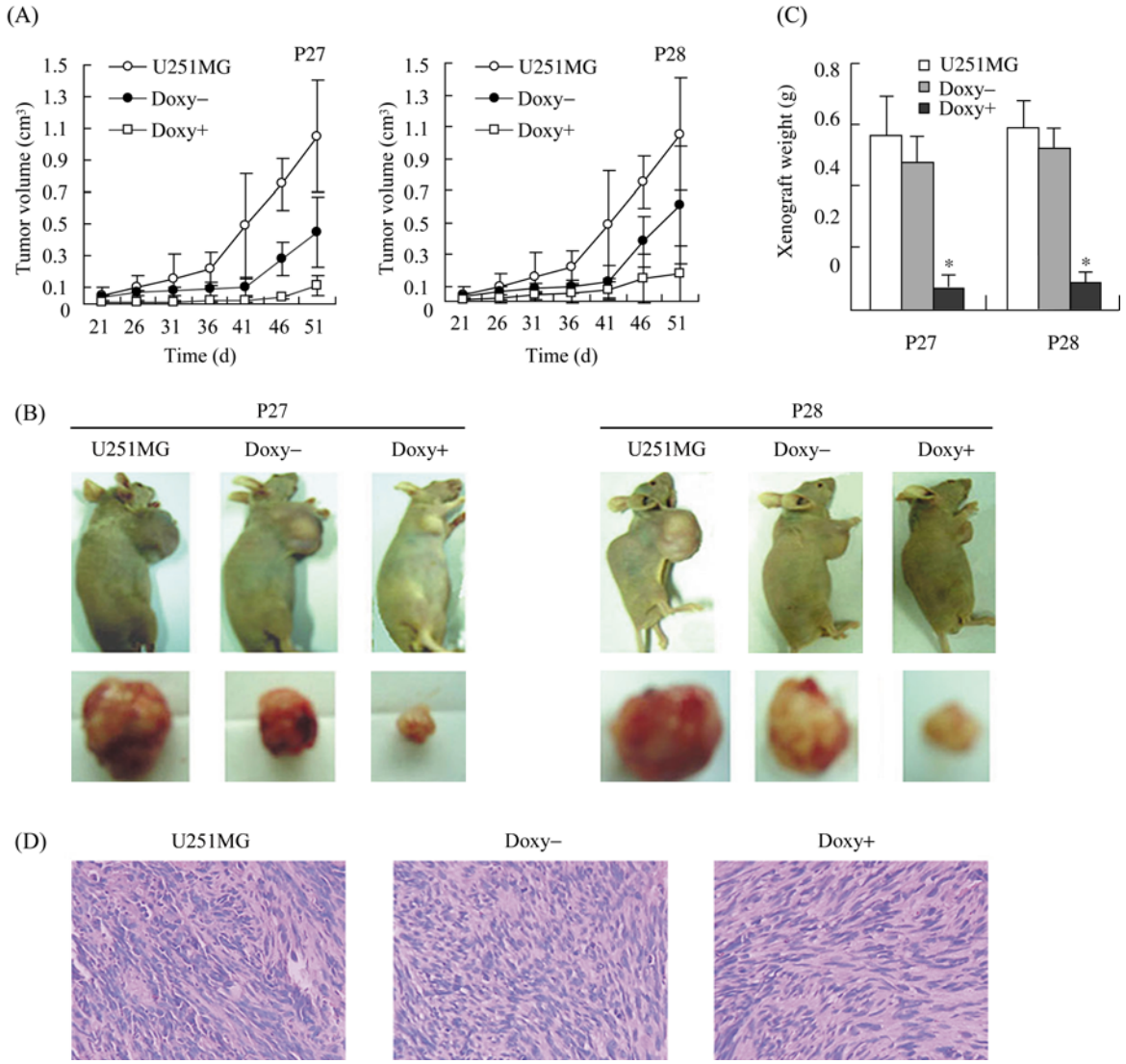

Fig. 2 LRRC4 suppresses tumorigenicity in U251MG cells

(A) Tumor volumes were calculated with the ellipsoid formula $4/3\pi ab^2$, where *a* is the length of the tumor and *b* is the width of the tumor. Doxy+, $P<0.01$; Doxy–, $P>0.05$. (B) Mice were killed when the tumors reached a volume of approximately 1.5 cm^3 or within 1 month of inoculation. (C) Comparison of the weight of xenografts from variable tumor cells. *$P<0.01$. (D) Morphological features of the U251MG cells. Magnification, 200×. Doxy–, U251MG Tet-on cells not induced by doxycycline; Doxy+, U251MG Tet-on cells expressing *LRRC4* induced by doxycycline.

significant difference between the Doxy+ group and the others ($P<0.01$, ANOVA test) [**Fig. 2(C)**]. Consistent with the tumor volume and weight changes, tumors arising from *LRRC4* cell lines treated with doxycycline displayed markedly reduced growth rates compared with control U251MG cells [**Fig. 2(A)**]. Differences in tumor growth rates between the parental U251MG group and the U251MG Tet-on-*LRRC4* (P27, P28) Doxy+ group were highly significant ($P<0.001$, ANOVA test), but there was no significant difference between the parental U251MG and the U251MG Tet-on-*LRRC4* (P27, P28) Doxy– group ($P>0.05$, ANOVA test). One month after the mice had been inoculated, the differences between the groups gradually increased.

Furthermore, the overexpression of *LRRC4* induced significant morphological changes in U251MG cells [**Fig. 2(D)**]. The cells tended to be in a rhombic arrangement, the volume lessened, cytoplasm expanded, nuclei shrinked in a rather regular shape, and the number of nucleoli lowered. Tumor tissue cells, extracted from U251MG Tet-

on-*LRRC4* cell line in the Doxy+ group were regularly arranged like fences and vortices. Nuclear fission in both cell lines was significantly inhibited in the Doxy+ group compared with those in Doxy– group. However, there was marked heteromorphism and more giant malignant cells in the tumor tissues derived from the parental U251MG and U251MG Tet-on cell lines not induced with doxycycline. Among the xenografts from all experiment groups, cells derived from the parental U251MG and U251MG Tet-on cell lines not treated with doxycycline were arranged in a much more disorderly fashion and were accompanied by cell necrosis. RT-PCR analysis confirmed the expression of *LRRC4* in tumors (data not shown). Taken together, the growth rate and malignant grade of tumors arising from the expression of *LRRC4* cells were markedly reduced compared with that of tumors from the other groups of cells.

Inhibition of cell proliferation by *LRRC4*

To investigate the causes underlying the effects of *LRRC4* on tumorigenesis, we compared the proliferation of the U251MG Tet-on-*LRRC4* cells in the absence or presence of doxycycline and the parental U251MG cell line by growth curves and MTT cell proliferation assays. As shown in **Fig. 3**, cell number and viability of the U251MG Tet-on-*LRRC4* positive clones (P27, P28) in the Doxy+ group were lower than that of the same clones in the Doxy– group. Differences in the growth rate and viability of cells in the *LRRC4* group (P27, P28) between the Doxy+ and Doxy– groups were highly significant (P <0.01, t test). However, there was no significant difference between the parental U251MG cells treated with doxycycline and those not (P>0.05, t test).

Transmission scanning electron microscope observation

The transmission scanning electron microscope revealed that the nucleo-cytoplasmic ratio of U251MG Tet-on-*LRRC4* not exposed to doxycycline was relatively larger; the cells showed giant irregular nuclei with scanty and irregularly clumped chromatin and scanty cytoplasm. In a few cells a dense collection of endoplasmic reticulum (ER) was seen around the nucleus and the rest cytoplasm contained several mitochondria. Rough ER was not well developed, and Golgi vesicles were few in number. Part of the ER expanded for compensation (**Fig. 4**, Doxy–). However, after 2.0 μg/ml doxycycline induction, the ultrastructure of U251MG Tet-on-*LRRC4* (P27, P28) also underwent a significant change. The nucleo-cytoplasmic ratio lessened,

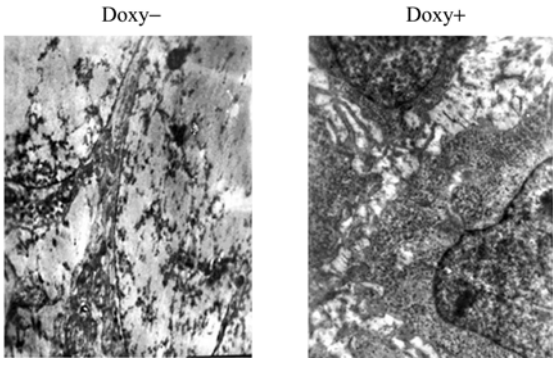

Fig. 4 Alterations in morphology before and after *LRRC4* expression observed using a transmission scanning electron microscope

Magnification, 6000×. Doxy–, U251MG Tet-on cells not induced by doxycycline; Doxy+, U251MG Tet-on cells expressing *LRRC4* induced by doxycycline.

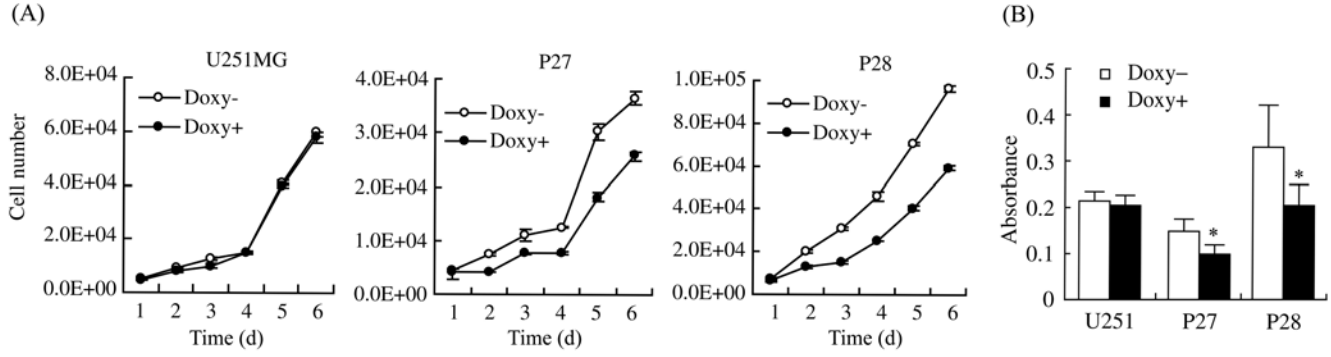

Fig. 3 LRRC4 inhibits U251MG cell growth *in vitro*

(A) Cell proliferation was evaluated by growth curves. (B) MTT assay. *P<0.01 vs. Doxy–. Doxy–, U251MG Tet-on cells not induced by doxycycline; Doxy+, U251MG Tet-on cells expressing *LRRC4* induced by doxycycline.

the nuclear shape became regular, heterochromatin in nuclei decreased while euchromatin increased, the volume of nucleoli lessened, rough ER increased significantly, Golgi apparatus was well-developed and Golgi vesicles increased and were regularly arranged. Most mitochondria were oval, and their crista grew in number and were regularly arranged, polyribosome reduced while free ribosome increased (**Fig. 4**, Doxy+). U251MG Tet-on cell lines expressing LRRC4 showed some ultrastructural characteristics of their normal relevant cells after exposure to doxycycline.

Cell cycle kinetics of *LRRC4* cell lines

To test whether the decreased MTT of the over-expressing *LRRC4* cells reflected a delay at a specific stage in the cell cycle or apoptosis product, we analyzed their DNA content by propidium iodide (PI) staining and flow cytometry. Results showed an increased fraction of cells in G_1 and subsequent decrease in both S- and G_2/M-phase after U251MG Tet-on-*LRRC4* (P27, P28) exposed to doxycycline [**Fig. 5(A)**]. But results failed to reveal significant differences in apoptosis (data not shown). This result suggested that *LRRC4*-mediated growth inhibition probably results from a delay at a particular phase of the cell cycle rather than from apoptosis.

The alteration of cyclins was further analyzed by flow cytometry, and the results showed that cyclin D1 and cyclin E were increased, but cyclin A was decreased when the U251MG Tet-on-*LRRC4* positive clones (P27,P28) were induced by doxycycline [**Fig. 5(B)**]. Together, these observations suggested that *LRRC4* mediates the delay of the cell cycle late in G_1.

Expressions of cell cycle-related key molecules regulated by *LRRC4*

To validate the potential molecular mechanism of *LRRC4*-mediated late G_1 delay in U251MG, we examined the expression alterations of cycle-associated key molecules by Western blot analysis. The results indicated that the expressions of p21Waf1/cip1 and p27Kip1 were up-regu-

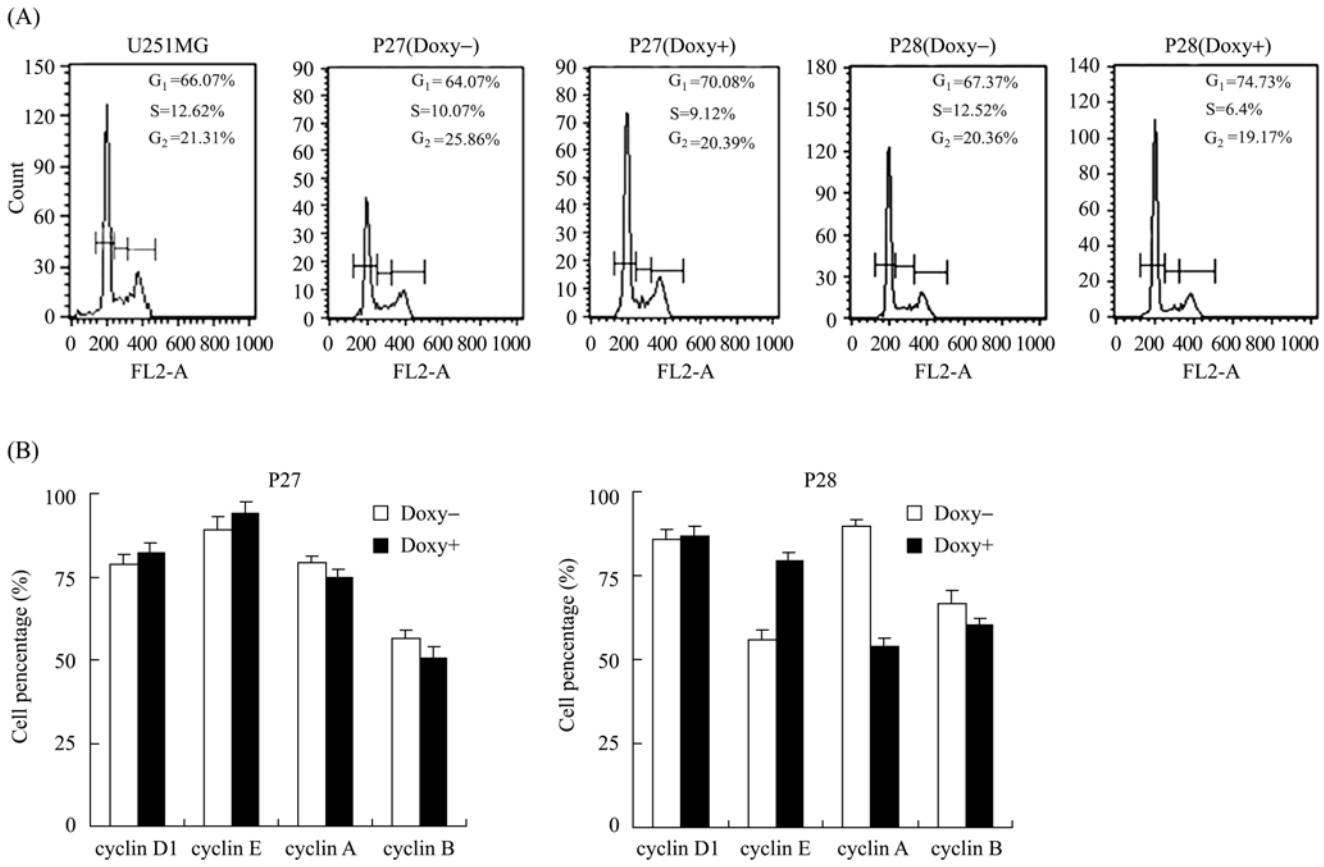

Fig. 5 Flow cytometry analysis of alteration of cell cycle kinetics and cyclins in *LRRC4* cell lines

Doxy–, U251MG Tet-on cells not induced by doxycycline; Doxy+, U251MG Tet-on cells expressing *LRRC4* induced by doxycycline.

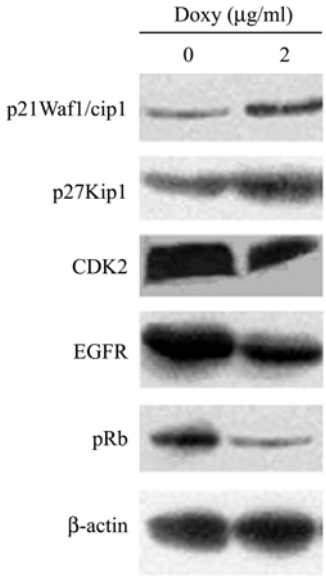

Fig. 6 Western blotting of the expression of cell cycle-related key molecules

CDK2, cyclin-dependent kinase 2; Doxy, doxycycline; EGFR, epidermal growth factor receptor; pRb, retinoblastoma protein.

lated and the expressions of CDK2, EGFR and pRb were down-regulated (**Fig. 6**) after the overexpression of *LRRC4*.

Discussion

Inducibility is desirable for gene function study, since it provides a more flexible control of gene expression. The benefit is obvious: the level of the target gene product can be affected at will. Current examples for inducible gene expression systems include the utilization of tetracycline-regulated transactivation systems [14], metallothionein promoters [15], the yeast Gal14 regulatory region [16], the T7 binary system [17], heat-shock promoters [18], and ecdysone-inducible systems [19]. Among these inducible mammalian gene expression systems, most induction is nonspecific and expression levels can not be precisely regulated. In addition, these systems are generally leaky in the "off" state, and the inducing agent itself may be toxic to the cells. In contrast, regulation of gene expression by the heterogenous bacterial control elements in the Tet systems is very specific, a feature that vastly reduces pleiotropic effects.

Furthermore, the levels of tetracycline or doxycycline required for the full range of gene expression are subtoxic, so the antibiotics have no significant effect on cell growth, even with continuous treatment to keep gene expression off in Tet-off cells [20]. Here we utilized a tetracycline-based inducible system to investigate the correlation between the expression and function of *LRRC4*.

We constructed a stable U251MG Tet-on cell line and two dual-stable U251MG Tet-on-*LRRC4* cell lines. The cell lines exhibited low basal activity and high inducibility. On the basis of the dual-stable U251MG Tet-on cell lines expressing *LRRC4*, we studied the effects and potential molecular mechanisms for suppression of tumorigenesis and cell proliferation of U251MG cells by *LRRC4*.

The tumor suppressive effect of *LRRC4* was demonstrated in two distinct models: drinking water (for mice) supplemented with or without doxycycline. The *in vivo* proliferation defect of *LRRC4*-expressing U251MG Tet-on cells was triggered by the presence of doxycycline (**Fig. 2**). Consistent with these findings, cell cycle and cyclin analysis suggested that *LRRC4* leads to a delay of cell cycle progression at late G_1 [**Fig. 5(A)**], possibly by interrupting the connection between cyclin E and cyclin A [**Fig. 5(B)**]. Therefore, these imply that *LRRC4* is able to suppress tumorigenesis and cell proliferation through mediating a delay in G_1/S transition.

As is well known, the eukaryotic cell cycle transition is regulated by the action of the CDKs, a CDK subunit and a regulatory cyclin subunit [21,22]. Cyclin E is necessary and rate-limiting for the passage of mammalian cells through G_1 of the cell cycle, which is expressed in mid-G_1 and associates with CDK2 [23]. CDK2 accelerates G_1/S transition and S-phase progression by combining cyclin E and activating cyclin A transcription [24]. In addition, progression from G_1 to S requires inactivation of pRb by phosphorylation and the consequent release of a number of factors including the E2F family of transcription factors. These transcription factors then activate transcription of various genes to promote cell cycle progression entry into S phase [25,26]. Aside from being regulated by the activity of cyclins and CDKs, the cell cycle is also regulated by CDK inhibitors, such as p21Waf1/cip1 and p27kip1 [24, 27]. Many antiproliferative factors mediate an arrest in the G_0/G_1-phase by induced expression of p21Waf1/cip1 and p27Kip1, resulting in CDK2 activity inhibition [28–33]. Therefore, it is important to characterize the potential molecular pathway through which LRRC4 mediates its antiproliferative action upon the U251MG cell line. In the absence and presence of doxycycline, we investigated the relation between induced expression of *LRRC4* and cell cycle-associated molecules. Results indicated that the expressions of p21Waf1/Cip1 and p27Kip1 are up-regulated, while the expressions of CDK2 and pRb kinase activity

are down-regulated. These findings suggest that the increase in the expressions of p21Waf1/Cip1 and p27Kip1 and the reduction in CDK2 activity may be a mechanism of cell cycle delay during the early phase of Tet-regulatable *LRRC4* treated with doxycycline. As a result, the expression of pRb and cyclin A are reduced, leading to a delay of the cell cycle in late G_1.

Furthermore, down-regulation of EGFR by *LRRC4* overexpression may be a synergistic effect in the inhibition of cell proliferation and tumorigenesis. EGFR overexpression is observed in a number of diseases, and mediates increased cell proliferation, migration, and aggregation [34]. Inhibition of EGFR signaling could protect human malignant glioma cells from hypoxia-induced cell death [35]. EGFR signaling has become an important target for drug development. Inhibition of EGF-dependent signaling, ERK1/2 and the AKT pathway can result in cell cycle arrest in G_1 and suppression of cell proliferation [36].

In conclusion, our findings demonstrate that *LRRC4* inhibits glioma tumorigenesis and cell growth of U251MG cells mainly by delaying the cell cycle in late G_1, associated with the up-regulation of p21Waf1/Cip1 and p27Kip1 and down-regulation of CDK2, pRb and EGFR. This result may serve as a basis for further study of the role of *LRRC4* in the maintenance of the normal function and inhibition of tumorigenesis in the central nervous system.

References

1 Kleihues P, Cavenee WK. Pathology and Genetics of Tumors of the Nervous System. Lyon: IARC Press 2000

2 Rasheed BK, Wiltshire RN, Bigner SH, Bigner DD. Molecular pathogenesis of malignant gliomas. Curr Opin Oncol 1999, 11: 162–167

3 Ranuncolo SM, Varela M, Morandi A, Lastiri J, Christiansen S, Bal de Kier Joffe E, Pallotta MG *et al*. Prognostic value of *Mdm2*, *p53* and *p16* in patients with astrocytomas. J Neurooncol 2004, 68: 113–121

4 Phatak P, Selvi SK, Divya T, Hegde AS, Hegde S, Somasundaram K. Alterations in tumor suppressor gene *p53* in human gliomas from Indian patients. J Biosci 2002, 27: 673–678

5 Smith JS, Tachibana I, Passe SM, Huntley BK, Borell TJ, Iturria N, O'Fallon RJ *et al.* PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and glioblastoma multiform. J Natl Cancer Inst 2001, 93: 1246–1256

6 Mahaley MS, Mettlin CJ, Natarajan N, Laws ER, Peace BB. National survey of patterns of care for brain tumor patients. J Neurosurg 1989, 71: 826–836

7 Schrijvers D, Vermorken JB. Update on the taxoids and other new agents in head and neck cancer therapy. Curr Opin Oncol 1998, 10: 233–241

8 Meijer L, Kim SH. Chemical inhibitors of cyclin-dependent kinases. Methods Enzymol 1997, 283: 113–128

9 Wang JR, Qian J, Dong L, Li XL, Tan C, Li J, Li GY *et al*. Identification of LRRC4, a novel member of Leucine Repeat (LRR) Superfamily and its expression analysis in brain tumor. Prog Biochem Biophys 2002, 29: 233–239

10 Tan GL, Xiao JY, Tian YQ, Deng LW, Jiang N, Zhan FH, Li GY. Analysis of deleted mapping on chromosome 7q31.3-36 in nasopharyngeal carcinoma. Chin J Otorhinolaryngology-Skull Base 1998, 4: 165–171

11 Wang JR, Li XL, Fan SQ, Tan C, Xiang JJ, Tang K, Wang R, Li GY. Expression of LRRC4 has the potential to decrease the growth rate and tumorigenesis of glioblastoma cell line U251. Ai Zheng 2003, 22: 897–902

12 Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 1992, 89: 5547–5551

13 Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995, 268: 1766–1769

14 Shockett PE, Schatz DG. Diverse strategies for tetracycline-regulated inducible gene expression. Proc Natl Acad Sci USA 1996, 93: 5173–5176

15 Palmiter RD. Molecular biology of metallothionein gene expression. Experientia Suppl 1987, 52: 63–80

16 Sadowski I. Uses for GAL4 expression in mammalian cells. Genet Eng 1995, 17: 119–148

17 Verri T, Argenton F, Tomanin R, Scarpa M, Storelli C, Costa R, Colombo L *et al.* The bacteriophage T7 binary system activates transient transgene expression in zebrafish (*Danio rerio*) embryos. Biochem Biophys Res Commun 1997, 237: 492–495

18 Bienz M, Pelham HR. Heat shock regulatory elements function as an inducible enhancer in the *Xenopus hsp70* gene and when linked to a heterologous promoter. Cell 1986, 45: 753–760

19 No D, Yao TP, Evans RM. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc Natl Acad Sci USA 1996, 93: 3346–3351

20 van Craenenbroeck K, Vanhoenacker P, Leysen JE, Haegeman G. Evaluation of the tetracycline- and ecdysone-inducible systems for expression of neurotransmitter receptors in mammalian cells. Eur J Neurosci 2001, 14: 968–976

21 McDonald ER III, El-Deiry WS. Checkpoint genes in cancer. Ann Med 2001, 33: 113–122

22 Harper JW, Adams PD. Cyclin-dependent kinases. Chem Rev 2001, 101: 2511–2526

23 Koff A, Giordano A, Desai D, Yamashita K, Harper JW, Elledge S, Nishimoto T *et al*. Formation and activation of a cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. Science 1992, 257: 1689–1694

24 Zerfass-Thome K, Schulze A, Zwerschke W, Vogt B, Helin K, Bartek J, Henglein B *et al.* p27KIP1 blocks cyclin E-dependent transactivation of cyclin A gene expression. Mol Cell Biol 1997, 17: 407–415

25 Dyson N. The regulation of E2F by pRb-family proteins. Genes Dev 1998, 12: 2245–2262

26 Nevins JR. Toward an understanding of the functional complexity of the E2F and the retinoblastoma families. Cell Growth Differ 1998, 9: 585–593

27 Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM *et al.* Requirement for *p53* and *p21* to sustain G2 arrest after DNA damage. Science 1998, 282: 1497–1501

28 Rao S, Lowe M, Herliczek TW, Keyomarsi K. Lovastatin mediated G1 arrest in normal and tumor breast cells is through inhibition of CDK2 activity and redistribution of *p21* and *p27*, independent of *p53*. Oncogene 1998, 17: 2393–2402

29 Rao S, Porter D, Chen X, Herliczek T, Lowe M, Keyomarsi K. Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. Proc Natl Acad Sci USA 1999, 96: 7797–7802

30 Chen WJ, Chang CY, Lin JK. Induction of G1 phase arrest in MCF human breast cancer cells by pentagalloylglucose through the down-regulation of CDK4 and CDK2 activities and up-regulation of the CDK inhibitors $p27^{Kip}$

and $p21^{Cip}$. Biochem Pharmacol 2003, 65: 1777–1785

31 Franzén Å, Heldin NE. BMP-7-induced cell cycle arrest of anaplastic thyroid carcinoma cells via $p21^{CIP1}$ and $p27^{KIP1}$. Biochem Biophys Res Commun 2001, 285: 773–781

32 Kano H, Arakawa Y, Takahashi JA, Nozaki K, Kawabata Y, Takatsuka K, Kageyama R *et al*. Overexpression of RFT induces G_1-S arrest and apoptosis via p53/p21Waf1 pathway in glioma cell. Biochem Biophys Res Commun 2004, 317: 902–908

33 Cho JW, Jeong YW, Kim KS, Oh JY, Park JC, Lee JC, Baek WK *et al. p21* (WAF1) is associated with CDK2 and CDK4 protein during HL-60 cell differentiation by TPA treatment. Cell Prolif 2001, 34: 267–274

34 Andl CD, Mizushima T, Nakagawa H, Oyama K, Harada H, Chruma K, Herlyn M *et al.* Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes *in vitro* and *in vivo*. J Biol Chem 2003, 278: 1824–1830

35 Steinbach JP, Klumpp A, Wolburg H, Weller M. Inhibition of epidermal growth factor receptor signaling protects human malignant glioma cells from hypoxia-induced cell death. Cancer Res 2004, 64: 1575–1578

36 Sah JF, Balasubramanian S, Eckert RL, Rorke EA. Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway: Evidence for direct inhibition of ERK1/2 and AKT kinases. J Biol Chem 2004, 279: 12755–12762

Edited by
Da-Fang WAN