Cellular Signalling 54 (2019) 170–178

Contents lists available at ScienceDirect

Cellular Signalling

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

The RNA helicase DHX33 is required for cancer cell proliferation in human glioblastoma and confers resistance to PI3K/mTOR inhibition

Hongzhong Wang[a,1], Junyan Yu[a,1], Xingshun Wang[a,b], Yandong Zhang[a,*]

[a] Department of Biology, Southern University of Science and Technology, Shenzhen, Guangdong, China
[b] Southern University of Science and Technology - University of Macau Joint Ph.D Program, Shenzhen, Guangdong, China

ARTICLE INFO

Keywords:
DHX33
RNA helicase
PI3K/Akt/mTOR
Glioblastoma

ABSTRACT

Human Glioblastoma is one deadly disease; the median survival time is reported to be 13.9 months after treatment. In the present study, we discovered that DHX33 is highly expressed in 84% of all Glioblastoma multiforme (GBM). Knockdown of DHX33 led to significant reduced proliferation and migration in glioblastoma cells *in vitro* and *in vivo*. Mechanistically, DHX33 regulated a set of critical genes involved in cell cycle and cell migration to promote glioblastoma development. Additionally, DHX33 was found to be induced by inhibitors of PI3K and mTOR whose activation has been detected in 50% of glioblastoma. Overexpression of wild type DHX33 protein, but not the helicase dead mutant, confers resistance to mTOR inhibitors in glioblastoma cells. DHX33 probably functions as a critical regulator to promote GBM development. Our results highlight its therapeutic potential in treating GBM.

1. Introduction

Glioblastoma originates from glial cells, astrocytes, which normally support the function and growth of neurons in the brain [1]. Glioblastoma multiforme (GBM) represents astrocytoma stage IV; it is the most common type of brain cancer and is highly aggressive, with a median patient survival of about 13.9 months post-diagnosis [2]. Systematic analysis for hundreds of glioblastoma patients demonstrates that three major core pathways were found to be frequently deregulated and were highly associated with glioblastoma development. These core pathways include Receptor Tyrosine Kinase/PI3K/Akt pathway, retinoblastoma cell cycle pathway and p53 pathway [2,3]. Currently there is no drug that could precisely target these altered signaling pathways in clinics. GBM patients were normally treated by combinational radiotherapy and chemotherapy following surgery [3,4], two commonly used chemotherapeutic drugs are carmustine and temozolomide (TMZ) which cause DNA alkylation [5]. A monoclonal antibody drug that targets EGFR has also been utilized for GBM treatment [6,7]. Despite these efforts, GBM remains incurable up till now. Inhibitors for PI3K pathway have been explored as potential therapeutic drugs [8]. However, due to the rapid reprograming of transcriptome after PI3K inhibitor treatment, the critical downstream kinase, mammalian target of rapamycin (mTOR), still remains active [9]. Therefore PI3K inhibitors have limited effects in halting glioblastoma development. The Ser/Thr kinase mTOR is a master regulator of cell growth, it mainly functions in two different complexes: mTORC1 interacts with RAPTOR to regulate cell size and cell growth, while mTORC2 interacts with RICTOR to regulate cytoskeleton and Akt activation [10]. Recently, the third-generation inhibitors targeting mTOR have been developed, however, it remains unknown whether mTOR inhibitors could effectively block glioblastoma progression clinically despite they have been shown to inhibit GBM development in mouse models [11].

Given the above reasons, it is necessary to further unravel the molecular mechanisms in glioblastoma and to identify novel therapeutic drug target in treating this deadly disease. Recent studies suggested that several RNA helicases, such as DHX9, DDX3, DDX6, DDX53, DDX5, play important roles in tumor cell proliferation in various human cancers [12–16]. These RNA helicases contain eight conserved motifs in their primary amino acid sequences. They hydrolyze ATP or NTP to remodel the conformation of RNA/DNA-protein complexes, thereby influencing all aspects of RNA/DNA metabolism [17].

In this study, we discovered that one of these RNA helicases, a DEAH box protein DHX33, is highly expressed in 84% of GBM. The expression of DHX33 is correlated to the progression of this disease. DHX33 promotes glioblastoma cell proliferation through multiple mechanisms. DHX33 transcriptionally controls the expression of critical

* Corresponding author at: Department of Biology, Southern University of Science and Technology, 1088 Xueyuan Blvd, Xili, Nanshan District, Shenzhen, Guangdong, China.
E-mail address: zhangyd@sustc.edu.cn (Y. Zhang).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.cellsig.2018.12.005
Received 22 October 2018; Received in revised form 11 December 2018; Accepted 11 December 2018
Available online 12 December 2018

genes involved in cell cycle progression and cell migration. We further found that the helicase activity of DHX33 is required in the process, as DHX33 helicase dead mutant could not promote cell proliferation while wild type DHX33 can. Most notably, we found that DHX33 can be induced by PI3K and mTOR inhibitors in glioblastoma cells. Higher expression of DHX33 confers resistance of glioblastoma cells to mTOR inhibition. Our study highlights this critical DHX33 protein in glioblastoma cell proliferation and its potential as a novel therapeutic drug target.

2. Materials and methods

2.1. Cell lines

293 T cells were purchased from ATCC (www.atcc.org) in Dec, 2015. U87-MG, U118-MG and U251-MG were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China) in November 2016. Cells were thawed upon arrival and cultured following recommended protocol. Cells at early passages (within 10 passages or within two months after original resuscitation) were used for experiments. The authenticity of cell lines in our study was verified by DNA sequencing using the Applied Biosystems AmpF/STR Identifier kit in 2017 and 2018. U87-MG, U-251MG glioblastoma cell lines were maintained in Minimum Essential Medium containing 10% fetal bovine serum (FBS), 2 mM L-Glutamine, non-essential amino acids, streptomycin and penicillin. U118-MG cells were maintained in DMEM medium containing 10% FBS, streptomycin and penicillin. HEK293 T cells were maintained in DMEM medium with 10% FBS and streptomycin/penicillin. All cells were cultured in a sterile incubator with 5% CO_2 at 37 °C with humidity.

2.2. Lentivirus packaging and virus infection

The targeting sequences of shRNAs for human DHX33 are as follows: (5′-3′): sh-DHX33–1: CTCGGGAAACTTCCTCTGAAA; sh-DHX33–2: GCTATCGCAAAGTGATCATTT; sh-DHX33–3: CATTTCCTTT AGAACCCAAAT. A pLKO.1 vector encoding shScrambled was purchased from Addgene. To produce knockdown virus, 293 T cells were transfected by pLKO.1-shRNA, pCMV-VSV-G, pCMVΔR8.2 by Lipofectamine 2000 (Life Technologies) for virus packaging. To produce lentivirus encoding DHX33 protein, 293 T cells were transfected by pLVX-FLAG-DHX33 (wild type or helicase dead K94 N or K94R), pCMV-VSV-G, p CMVΔR8.2 by Lipofectamine 2000 (Life Technologies) for virus packaging. Culture supernatants were harvested 24 h and 48 h after transfection and then centrifuged at 2000 rpm for 5 min. After virus titering, viruses were infected onto target cells at a MOI of 10 in the presence of 10 μg/ml of polybrene.

2.3. Western blotting and antibodies

Whole cell lysates were prepared by incubation with whole cell lysis buffer including 0.5% NP40 and 1% SDS supplemented with HALT protease and phosphatase inhibitors (Sigma). Lysates were sonicated and then cleared by centrifugation. Protein concentration was tested by DC assay (Bio-Rad). Lysates were boiled with SDS sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% nonfat dry milk TBS-T [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] buffer and incubated in primary antibodies diluted in blocking buffer at 4 °C overnight. Blots were washed with TBS-T buffer and incubated with horseradish peroxidase–conjugated secondary antibodies (1:10,000; Life Technologies) in blocking buffer at room temperature. Immune complexes were visualized with an enhanced chemiluminescence kit (Pierce). Primary antibodies for immunodetection were sourced as follows: anti-tubulin (goat, Santa Cruz), anti-DHX33 (DHX33, B4), anti-GAPDH (Absin), anti-cyclins (Santa Cruz), anti-cdc20 (Santa Cruz), anti-MCMs (Abcam and Santa Cruz). Anti-S6, anti-pS6, anti-Akt, anti-pAkt (S473), anti-ERK1/2, anti-pERK1/2, anti-4EBP1 and anti-p4EBP1 were all purchased from Cell Signaling Technologies (CST).

2.4. Quantitative PCR

The primers used in this study were purchased from GenScript as shown previously [18,19]. Total RNA was extracted by NucleoSpin II (Clontech) RNA isolation kit and was reverse transcribed into cDNA by PrimeScript One Step RT-PCR Kit (Takara). PCRs were performed with a Step one plus thermal cycler. SYBR green mix from Bio-Rad was used for all quantitative real-time PCR (qRT-PCR) analyses. Transcript quantification was calculated based on the $_{\Delta\Delta}$CT value after normalization to GAPDH values. Melt curve analysis confirmed that single product was amplified.

2.5. Inhibitor studies

Kinase inhibitors were all purchased from Selleck (Guangzhou, China). Wortmannin was used as an inhibitor for PI3K kinase; U0126 was used as an inhibitor for MEK kinase; Rapamycin was used as an inhibitor for mTORC1. Torin1 was used as pan inhibitor for mTORC1 and mTORC2. Inhibitors were added onto sub-confluenced glioblastoma cells for 48 h before cell harvesting.

2.6. Immunohistochemistry staining

Human tumor tissue microarrays were purchased from US Biomax. Three types of tissue microarray plates were used for analysis of glioblastoma. One contains 60 cases of astrocytoma stage I to astrocytoma stage IV, with 9 normal cerebral tissues as control samples. The second tissue array contains 35 cases of GBM, 2 tumor adjacent normal tissues and 3 normal brain tissues as controls. The third tissue array contains 25 cases of normal brain tissues. A mouse monoclonal anti-human DHX33 antibody (Santa Cruz, S15) was used at 1:50 dilution ratio. Tissue slides were deparaffinized in xylene and rehydrated in a series of alcohol solutions with decreasing concentration and the antigen was retrieved in Tris buffer (pH 9.0) using a steamer. The sections were then treated with 1% hydrogen peroxide in methanol for 30 min to exhaust endogenous peroxidase activity. After pre-incubation in 10% normal fetal bovine serum for 1 h to prevent nonspecific staining, the samples were incubated with primary antibody at room temperature for 2 h. Standard protocol was then followed based on DAKO envision kit using polymer to amplify signals. Typical images were photographed at 4× and 10× magnifications. Brownish staining in > 50% of total cell nuclei was regarded as positive staining, while little < 50% or no brownish staining in tissues was regarded as negative staining. Whole tissue section was photographed.

2.7. Focus analysis

Cells were plated at a density of 10^4 per 100-mm dish and grown for 10 to 20 days. Colonies were washed with cold phosphate-buffered saline twice and fixed with 100% methanol for 10 min at room temperature. Colonies were then stained with Giemsa stain for 1 h at room temperature and washed with water before air-dried and photographed.

2.8. Soft agar analysis

Approximately 1.0×10^4 cells were mixed in 4.0 ml 0.3% agar/MEM/10% FBS as the top agar and plated into 60 mm plates with 4.0 ml 0.6% agar/MEM/10% FBS as the base agar. Plates were incubated at 37 °C, checked every 3 days, and fed with 2.0 ml 0.3% agar/ MEM/10% FBS every week. Colonies were photographed and counted 2–3 weeks later.

2.9. Growth curve analysis

Approximately 1.0×10^4 cells were plated onto 12-well dishes. Cells were then counted by a hemocytometer on a daily basis, each condition were counted three times.

2.10. Transwell cell migration analysis

Transwell Migration assay plates (8 μm) were purchased from Corning (Shenzhen, China), standard protocol was followed according to manufacturer's protocol. Briefly, 1.0×10^4 cells were suspended in MEM media containing 0.25% FBS on the top container which was submerged into complete MEM media containing 10% FBS. Cells were allowed to culture overnight. The next morning, membrane was removed; all the cells inside the well was rubbed off, the cells that penetrate the membrane was either stained by Giemsa or by DAPI before photographing under the microscope.

2.11. Xenograft mouse studies

The Animal Ethics Committee of Southern University of Science and Technology has approved the use of animals in this study under the protocol SUSTC-JY2018040. All mouse experiments followed the standard guidelines. NUDE female mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and received standard institutional care. They were at 8-week old at the time of experiments. For NUDE mice injection, U87-MG glioblastoma cells were infected as indicated. Cells were trypsinized and re-suspended in MEM complete media at a concentration of 5×10^7 cells/ml. Eight-week old NUDE mice were injected subcutaneously with 5×10^6 cells along their flank, with sample sizes of 3 mice per condition. Two weeks later when tumor was palpable, mice were sacrificed and tumors were dissected, photographed and weighed.

For the mTOR inhibitor treatment experiment, 8 NUDE mice were injected similarly with U87-MG cells overexpressing either wild type or K94R mutant DHX33. Two weeks later when tumor volume reached nearly to 100 mm^3, Torin1 was administered into these mice 3 times per week through introperitoneal injection at a dose of 20 mg/kg. Tumor size and mouse weight were monitored three times per week. To calculate tumor volume, the length and the width of the tumor were evaluated by a caliber, and the tumor volume is calculated as $1/2(\text{length} \times \text{width}^2)$. Three weeks later, the data were analyzed.

2.12. Statistical analysis

Data is presented as the mean ± SD. Statistical significance was determined using the Student's *t*-test, with a P value < .05 considered significant.

3. Results

3.1. Overexpression of DHX33 in glioblastoma but not in normal brain tissues

DHX33 protein has been found to be highly expressed in lung cancer, lymphoma, liver cancer [18–20], but it remains unknown whether DHX33 could be overexpressed in glioblastoma. To investigate the potential role of DHX33 in glioblastoma, we firstly used a specific DHX33 antibody to perform immunohistochemistry staining for glioblastoma tissues. We compared the expression level of DHX33 in normal cerebral tissues and glioblastoma tissues. Totally, we analyzed 95 cancer tissues, 39 normal and tumor adjacent normal tissues. As shown in Fig. 1 and Supplemental Fig. S1, we found that DHX33 is highly expressed in glioma tissues, but not in normal cerebral tissues. All the 39 normal cerebral tissues have little or no staining for DHX33. At the cellular level, DHX33 protein primarily localized to the nuclei of glioblastoma cells, though some cancer cells have DHX33 expression throughout the cell. Statistical analysis for DHX33 staining intensity in these clinical samples revealed that DHX33 expression correlates strongly with clinical cancer stages. DHX33 is positively expressed in 52% of astrocytoma stage I/II (16 out of 31), but in 84% of all cases of astrocytoma stage III/IV (54 out of 64). Fig. 1 shows the IHC images for all cases of astrocytoma that have positive DHX33 staining in the first tissue microarray, with 8 normal brain tissues as controls. Supplemental Fig. S1 shows the representative cases of DHX33 positive GBM tissues with tumor adjacent normal and normal cerebral tissues for controls in the second tumor microarray.

3.2. DHX33 is required for glioblastoma cell proliferation in vitro and in vivo

Given the fact that DHX33 is highly expressed in cancer tissues but not in normal tissues, we speculate that DHX33 might play an important role to promote glioblastoma development. We firstly choose established glioblastoma cell lines for DHX33 functional studies. In three different glioblastoma cell lines, U118-MG, U87-MG and U251-MG, we stably knocked down DHX33 through lentiviral delivery of two different shRNAs targeting DHX33. We then analyzed various cancer cell phenotypes including cell proliferation, anchorage-independent cell growth, cell migration and cell morphology. The knockdown efficiency of DHX33 in these cancer cell lines was examined by western blot and was shown in Fig. 2A, D and G, respectively. In all three different cell lines, DHX33 protein was knocked down markedly. Deficiency of DHX33 resulted in dramatically reduced cell proliferation, cell migration and/or reduced anchorage-independent cell growth (in U118-MG). These results indicate that DHX33 is required for glioblastoma cell proliferation and migration, and it is pivotal for maintaining cancer cell phenotype (Fig. 2B, E, H). The quantitation for these analyses was shown in Fig. 2C, Fig. 2F and Fig. 2I. To confirm these results were not due to shRNA off-target effects, we performed knockdown rescue analysis as shown in supplemental Fig. S2. By expressing the shRNA resistant mouse DHX33 gene in U87-MG cells, we were able to reconstitute the protein level of DHX33 after DHX33 knockdown. We found that both cell proliferation and cell migration were rescued to a significant degree. To investigate the role of DHX33 in glioblastoma development *in vivo*, we performed xenograft mouse models with U87-MG cells. This cell line is highly tumorigenic. After DHX33 was stably knocked down by lentivirus encoding shRNAs, cells were injected into NUDE mice subcutaneously. As shown in Fig. 2J, we found that DHX33 deficiency greatly decreased the tumorigenic property for the cells. In control group which was transduced by shScrambled control virus, tumors grew into large sizes. In DHX33 knockdown group, none of the mice develop tumors.

3.3. DHX33 promotes the expression of important genes involved in cell cycle progression and migration

We have previously discovered that DHX33 plays a key role in controlling the transcription of a set of genes involved in cell cycle progression and migration in lung cancer cells [18,19]. To investigate whether DHX33 is involved in glioblastoma development *via* regulating these genes, we analyzed protein expression for these genes in glioblastoma cells after DHX33 knockdown. We firstly performed protein expression studies after delivering two different shRNAs targeting DHX33 in U87-MG cells. As shown in Fig. 3A, we found that DHX33 deficiency reduced the expression of E2F1, cyclin E2, cyclin D1, MMP9, MCMs, CDC6 and CDC20 significantly. We further performed a similar analysis in U251-MG cells with three different shRNAs. As shown in Fig. 3B, we found that DHX33 knockdown reduced most of the designated genes' expression. These include E2F1, cdc20, cyclins, MMP9 and MCMs. To investigate whether reduced expression of these proteins was due to transcription downregulation, we analyzed the mRNA levels for

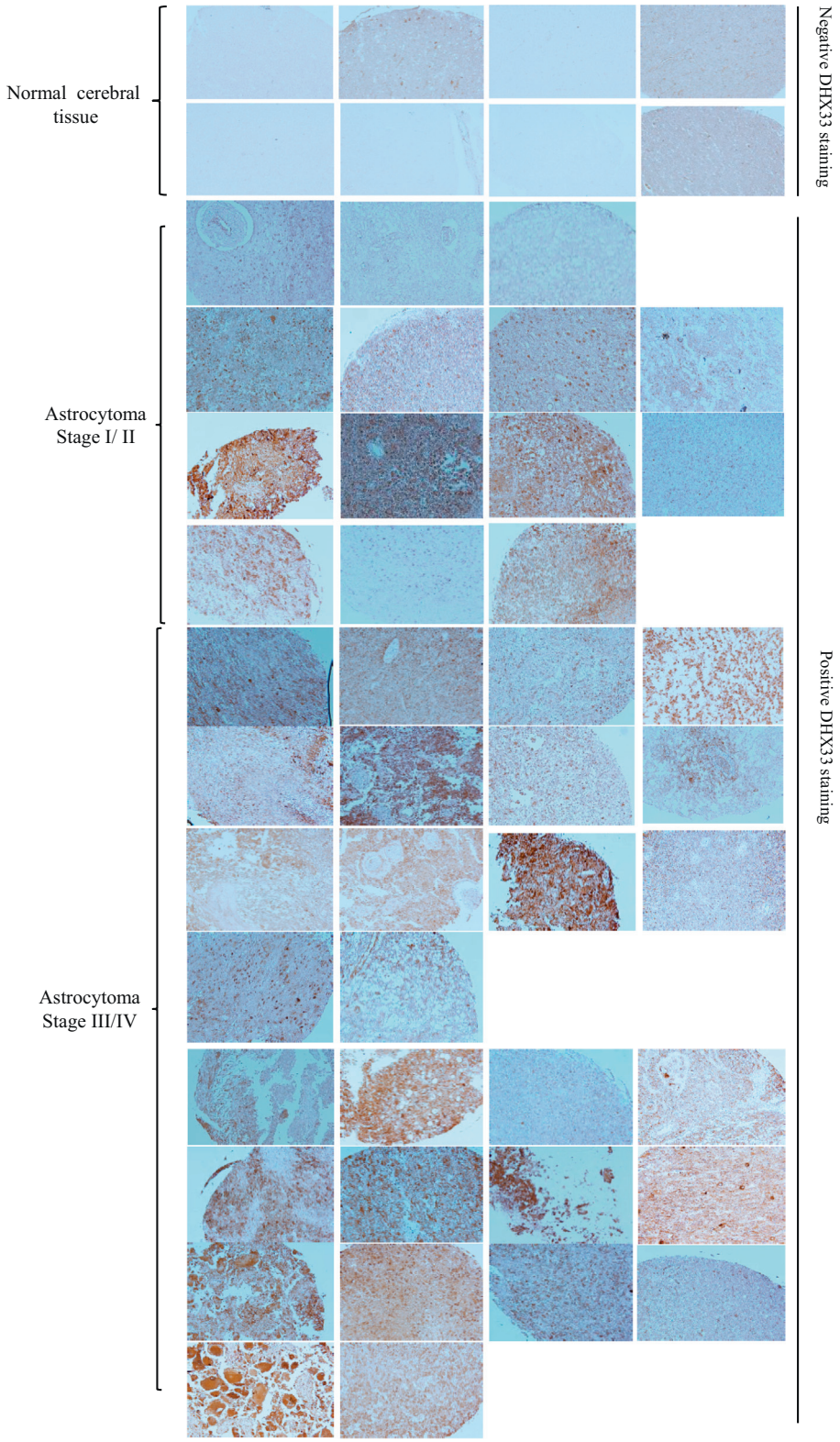
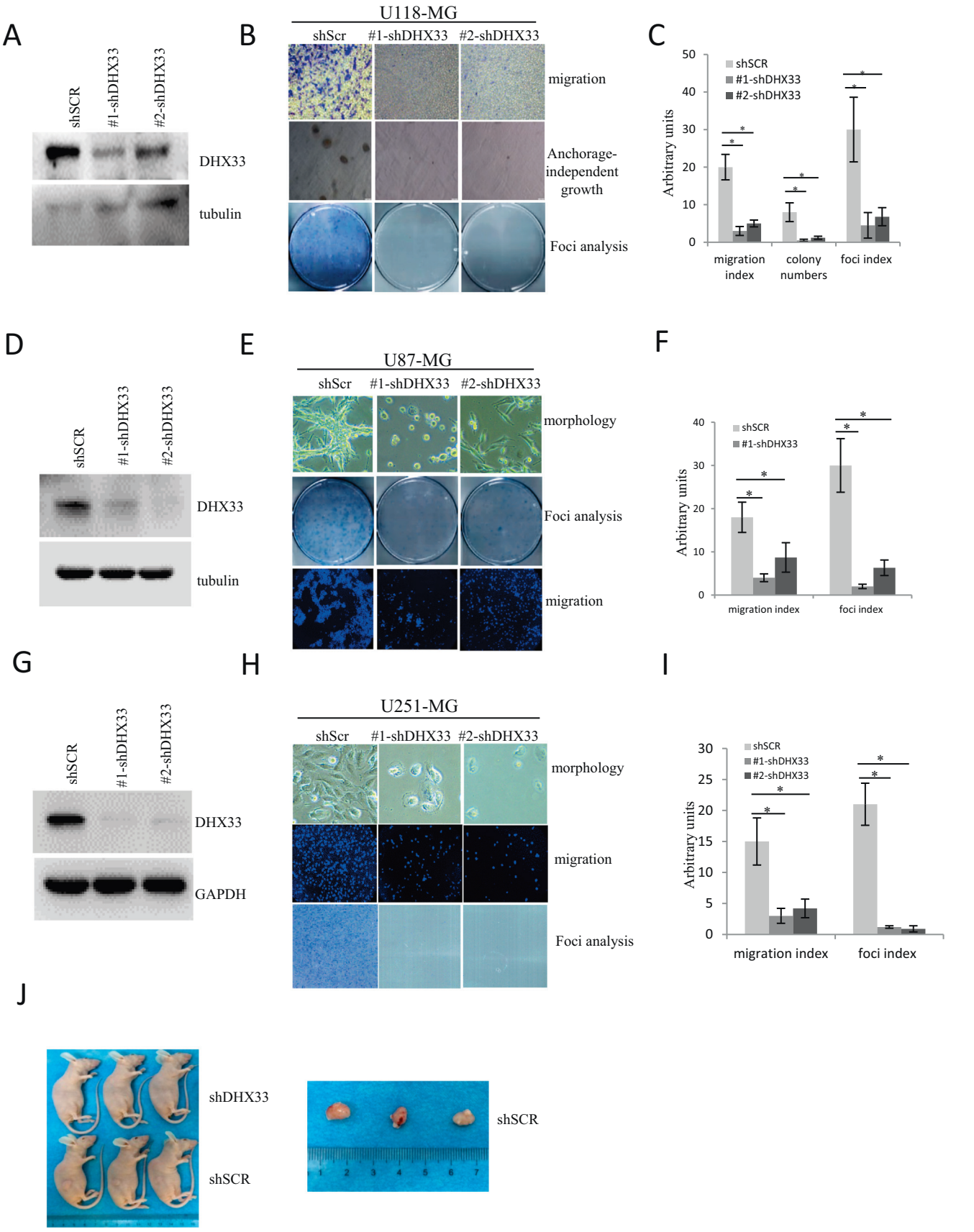


Fig. 1. DHX33 is highly expressed in glioblastoma tissues.
A glioblastoma tissue array was used to analyze the expression of DHX33 by immunohistochemistry. Eight normal cerebral tissues were used as negative control. Astrocytoma stages from I to IV were analyzed for DHX33 expression. All DHX33 positive tissues as demonstrated by brownish staining in the nuclei were categorized into different tumor stages. Images shown are in 4× magnification.

a few genes after DHX33 knockdown. We found that cyclin D1, MMP9 and CDC20 were all downregulated in DHX33 deficient cells (Fig. 3C). Cyclins, CDCs and MCMs, E2F1 are important to promote cell cycle progression and DNA replication, while MMP9 is pivotal in cell migration. Downregulation of these genes significantly reduced cancer cell proliferation and cancer cell invasion as expected. Taken together, these results suggest that DHX33 overexpression in glioblastoma cells promotes expression of critical genes to maintain cancer cell phenotype.



(caption on next page)

Fig. 2. DHX33 is important to drive glioblastoma cell proliferation and cell migration *in vitro* and *in vivo*.
(A). U118-MG cells were infected by lentivirus encoding shRNAs targeting DHX33 with shScrambled as a control. Whole cell extracts were then analyzed by western blot with the indicated antibodies.
(B). Above-mentioned cells were plated for Transwell cell migration analysis, anchorage-independent growth and foci analysis. Equal numbers of each cell samples were plated.
(C&F&I). Migrated cells were counted in 5 different fields under the microscope and quantitated. Data shown represent the average ± S.D. from three different experiments.
Colonies in the soft agar plates were quantitated in 5 separate fields. Data represent the average ± S.D. from three different experiments. Quantitation of Foci analysis was based on the foci numbers in 5 typical fields. Data shown represent the average ± S.D. from three different experiments. *, $P < .05$, $n = 5$.
(D. U87-MG cells were infected by lenvirus encoding shRNAs targeting DHX33 with shScrambled as a control. Whole cell extracts were then analyzed by western blot with the indicated antibodies.
(E). Above-mentioned cells were examined under the microscope for cell morphology, plated for foci analysis, transwell cell invasion assay (DAPI demarked nuclei, blue). Typical images were shown.
(G).U251-MG cells were infected by lenvirus encoding shRNAs targeting DHX33 with shScrambled as a control. Whole cell extracts were then analyzed by western blot with the indicated antibodies.
(H). Above-mentioned cells were examined under the microscope for cell morphology, plated for foci analysis, transwell cell invasion assay (DAPI demarked nuclei, blue). Typical images were shown.
(J). U87-MG cells were infected by lenvirus encoding shRNAs targeting DHX33 with shScrambled as a control. Approximately $5X10^6$cells were injected into the flanks of nude mice subcutaneously. Three weeks later, mice were sacrificed for imaging. Tumors were dissected and photographed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Overexpression of wild type DHX33 confers resistance to mTOR in vitro and in vivo

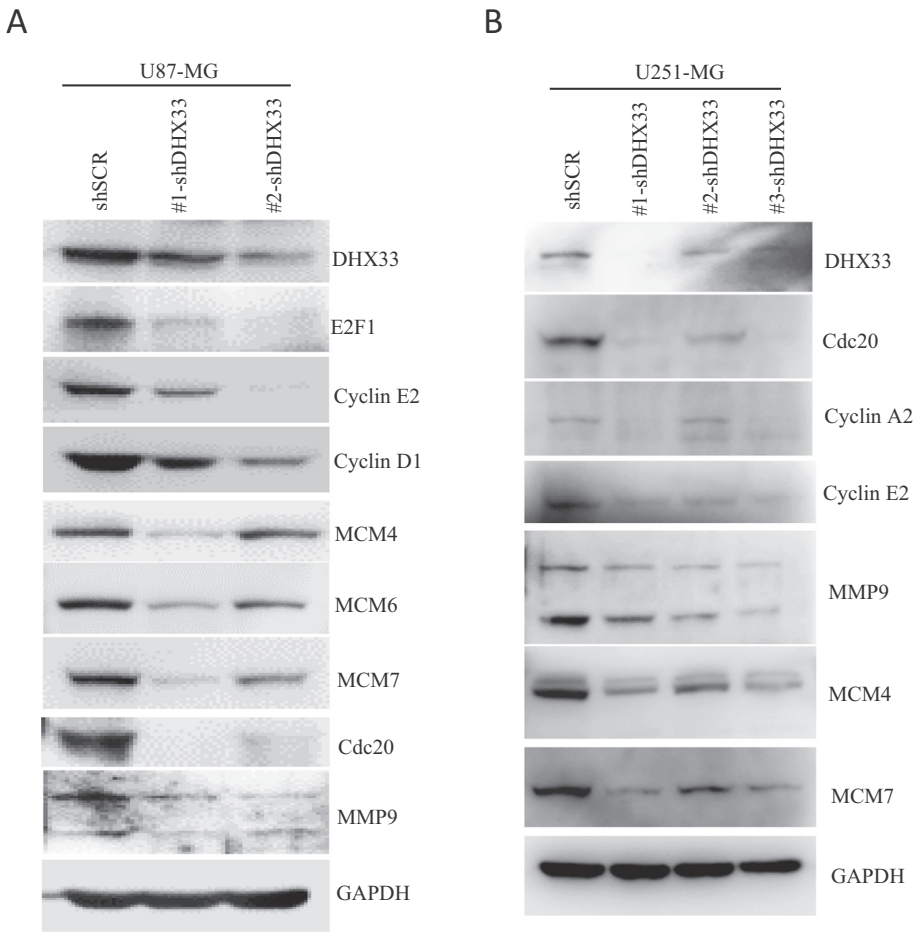
It has been reported that the PI3K/Akt signaling activation occurs in 89% of all glioblastoma [21]. However, PI3K inhibitors produce limited response [9]. Other parallel pathways cause bypass of PI3K inhibition to maintain PI3K downstream signaling. To unravel the molecular mechanisms for the resistance to PI3K/mTOR inhibitors in GBM, we analyzed whether upregulation of DHX33 would confer this resistance. We stably expressed wild type DHX33 in U87-MG cells, with DHX33 helicase dead mutant K94 N and empty vector as controls. Then we analyzed cell proliferation and cell migration after treating cells with mTOR inhibitors. Normally Glioblastoma cells are sensitive to the mTORC1 inhibitor, rapamycin. However, when cells expressed wild type DHX33 they developed resistance, DHX33 helicase dead mutant failed to do so (as shown by the growth curves in Fig. 4A). We also examined the cell morphology under the microscope. Normally, U87-MG cells were highly motile; they tended to migrate and huddled together in cohorts. When cells overexpressed K94 N DHX33, they appeared to be dispersed (Fig. 4B). We further analyzed cell migration, as shown in Fig. 4C, rapamycin treatment decreased cell motility of these cells. When cells overexpressed wild type DHX33, they migrated as well as the normal cells. The expression levels of DHX33 and other proteins were shown in Fig. 4D, the phosphorylation of status of S6 and Akt demonstrated that these inhibitors worked well. To study whether DHX33 would confer resistance to mTOR inhibition *in vivo*, we injected U87-MG cells that overexpressed either DHX33 wild type or helicase dead mutant K94R into the flanks of NUDE mice. When tumors grew into approximately $100mm^3$, we treated these mice with Torin1. As shown in Fig. 4F, wild type DHX33 expression caused the tumor to grow quickly despite Torin1 administration, while tumors with DHX33 K94R expression grew much slowly. The weight of the mice in the two groups had no significant change (Fig. 4G). The expression of the exogenous DHX33 was evaluated by western blot in Fig. 4H. Similar analysis was performed on U251-MG cells (Supplemental Fig. S3); we added two more inhibitors, U0126 to inhibit MEK in MAP kinase pathway and wortmannin to inhibit PI3K. We found that wild type DHX33 caused cells to develop resistance not only to rapamycin but also to U0126. Wortmannin itself did not significantly decreased cell proliferation for U251-MG cells.

3.5. DHX33 can be induced by PI3K and mTOR kinase inhibitors in U251-MG glioblastoma cells

Previously we have found that PI3K/mTOR pathway regulated DHX33 expression [22], to investigate whether this is the case in glioblastoma cells, we decided to treat these cells with inhibitors targeting PI3K and mTOR kinases. We treated U251-MG cells with each of the inhibitors for 48 h and then analyzed DHX33 protein expression. To make sure that these inhibitors functioned properly, we also analyzed the phosphorylation status of the designated kinases. As shown in Fig. 5A, in U251-MG cells, we found that PI3K inhibitor and mTOR inhibitors increased DHX33 protein expression, while MEK inhibitor has minimal effect on DHX33 expression level. The phosphorylation status of pAkt (473), pERK, and pS6 confirmed that each inhibitor worked properly. Our results indicate that none of the PI3K, mTOR or MAP kinase pathway plays a major role in causing DHX33 overexpression in glioblastoma tissues. This result implicated that other factors might regulate DHX33 protein levels in glioblastoma cells. Despite this was observed in U251-MG cells, we did not observe the same result in U87-MG cells (data not shown). Because rapamycin only inhibits mTORC1, to exclude the possibility of mTORC2 in regulating DHX33 protein, we further treated cells with Torin1, which inhibits both mTORC1 and mTORC2 effectively. As shown in Fig. 5B, we found that Torin1 induced DHX33 expression even more dramatically. The reduction of phosphorylation on 4EBP1 indicates that Torin1 functioned well.

4. Discussion

Astrocytes make up one half of the total brain cells; the mechanism of its carcinogenesis is not fully understood [1]. There are two types of glioblastoma, primary glioma and secondary glioma. The first one originates from normal brain astrocytes while the other type is triggered by pre-existing low-grade astrocytoma [1]. Glioblastoma is the first type of cancers that was deep-sequenced at the genome level [21,23]. Molecular mechanism of GBM has been investigated extensively. Existing data show that multiple cancer critical genes have been found to be mutated and to be causal factors in GBM progression [2]. Among them, two prominent genes are EGFR and PTEN, whose mutation would impact PI3K/Akt pathway [2]. Although results demonstrate that PI3K/Akt signaling activation has been detected in 80% of all glioblastoma, clinical trials with PI3K and mTOR inhibitors resulted in limited effect [11]. Multiple factors have been found to be responsible for the resistance to PI3K/mTOR inhibitors in GBM. Several studies have shown that insulin-like growth factor 1 receptor, S6 kinases and Wee1 kinase can be activated by PI3K/mTOR blockade in different situations [9,24–26]. The activation of these factors causes bypass of PI3K/mTOR inhibition to enhance cell proliferation and survival. For reasons stated above, this cancer remains incurable despite extensive efforts [3].



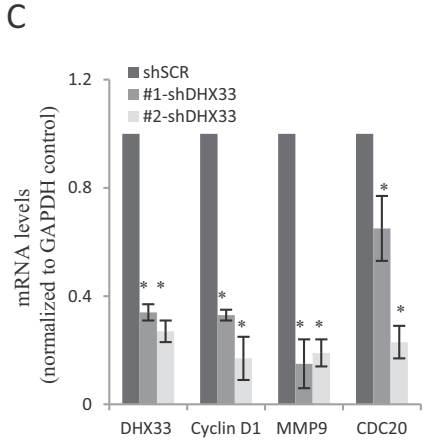


Fig. 3. DHX33 promotes the expression of important genes involved in cell cycle progression and migration.
(A). U87-MG glioblastoma cells were transduced with either shSCR, #1 or #2-shRNA-DHX33 lentivirus. Four days post lentiviral infection, cells were harvested. Whole cell lysates were subjected to western blot analysis with the indicated antibodies, with anti-GAPDH antibody for a control.
(B). U251-MG cells were transduced with lentivirus encoding either shSCR or three different shRNAs targeting DHX33. Four days post lentiviral infection, cells were harvested. Whole cell lysates were subjected to western blot analysis with the indicated antibodies.
(C). U87-MG cells were infected with lentiviruses targeting two different shRNAs targeting DHX33, with shSCR as a control. Four days post lentiviral infection, cells were harvested. Total RNA was extracted and reverse-transcribed into cDNA, qPCR was performed on these cells with indicated primers. Bars stand for standard deviation from 3 independent analyses. All demonstrated statistically significant changes as compared to scrambled control. *, $P < .05$, $n = 3$.

In this study, we present original evidence on the role of DHX33 in human glioblastoma. For the first time, we discovered that DHX33 is highly expressed in human glioblastoma tissues. Its expression is correlated to disease progression. In glioblastoma, DHX33 is overexpressed in approximately 84% of all cases. High level of DHX33 expression is pivotal for its functions in promoting cell proliferation, migration and tumorigenesis.

Interestingly, though we have found that DHX33 can be regulated by PI3K/mTOR pathway before [22], we found that DHX33 protein is not regulated by this pathway in GBM. On the contrary, inhibitors of PI3K/mTOR induced DHX33 expression significantly in certain glioblastoma cell lines. Simultaneous inhibition of both mTORC1 and mTORC2 caused DHX33 protein levels to be even enhanced. This implicates a possible feedback response to PI3K/mTOR inhibition, highlighting the activation of other signaling pathways in sensing mTOR inhibition. MAP kinase pathway did not regulate DHX33 in GBM either. DHX33 has been found to be a converging downstream target of many oncogenes and tumor suppressors. Other than Ras, Akt, mTOR, Myc also regulates DHX33 through direct binding to DHX33 promoters [18]. It should be worthwhile to further delineate the regulatory mechanism for DHX33 protein in GBM.

Overexpression of the wild type DHX33 endowed cells with proliferative and migration potential in the presence of mTOR inhibitors. Our result clearly indicates that DHX33 is an important regulator to

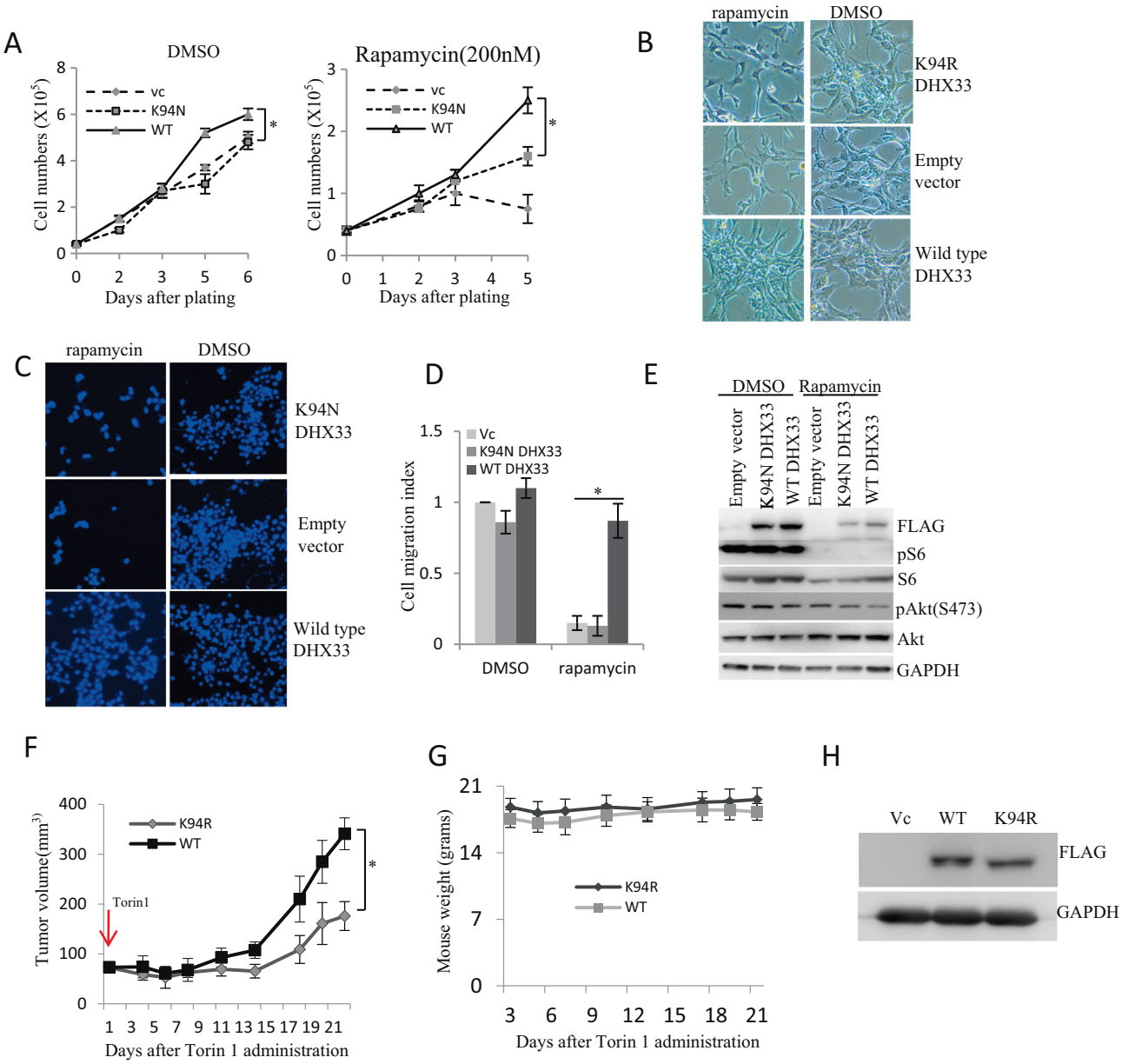


Fig. 4. Overexpression of wild type DHX33 confers resistance to mTOR inhibition in U87-MG cells *in vitro* and *in vivo*.
(A). U87-MG cells were infected by lentivirus encoding vector only, wild type DHX33 and K94 N mutant DHX33. Cells were treated with the rapamycin or wortmannin inhibitors, with DMSO as a control. Growth curves were generated over a 6-day period; bars represent standard deviation from three separate counts for each sample. *, $P < .05$.
(B). Above-mentioned cells were photographed under the microscope for cell morphology. Typical images were shown.
(C). Above-mentioned cells were plated for transwell cell invasion assay. Typical images were shown.
(D). Quantitation for the migrated cells in each samples. Migrated cells were counted in 5 different fields under the microscope and quantitated. Data shown represent the average ± S.D. from three separate experiments. *, $P < .05$, $n = 5$.
(E). Above-mentioned U87-MG cells were harvested after inhibitor treatment. Whole cell extracts were then analyzed by western blot with the indicated antibodies.
(F–H). Eight NUDE mice were injected subcutaneously with approximately 1×10^7 U87-MG cells overexpressing either wild type or K94R DHX33. When tumor size reached to nearly 100mm^3, they were treated by Torin1 at a dose of 20 mg/kg in DMSO through introperitoneal injection three times per week. Tumor volume curve (Fig. 4F) and mice weight curve (Fig. 4G) were generated over a 3-week period.
(H)The expression of exogenous DHX33 was analyzed by western blot with anti-FLAG antibody. GAPDH served as an internal control. *, $P < .05$.

promote cell proliferation, which bypasses the upstream inhibitory signals in the PI3K/mTOR pathway. The fact that DHX33 can be induced by PI3K and mTOR inhibitor implicates that DHX33 induction might partially explain the molecular mechanisms for the failure of PI3K/mTOR inhibitors to halt GBM development in the long run, though this occurs in certain cell types, but not all. These results implicate that DHX33 inhibition might be a feasible option in glioblastoma treatment.

To support its potential as a therapeutic target in GBM, significant results were observed after DHX33 knockdown in glioblastoma cells. These cells demonstrate markedly reduced cell proliferation, cell migration. This is partially mediated through DHX33 regulation of critical genes involved in cell cycle progression and cell migration.

In summary, our data provide strong evidence for a previously unappreciated role of DHX33 in glioblastoma. We clearly show that DHX33 promotes cancer cell proliferation and cell migration through

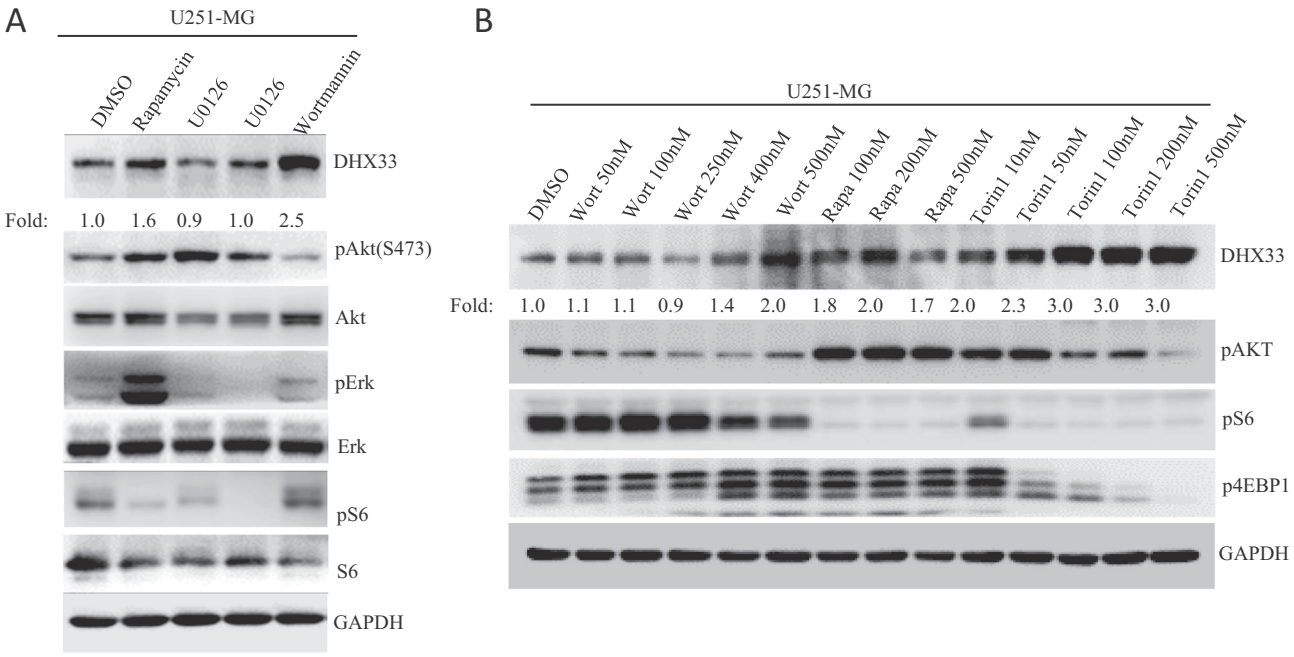


Fig. 5. DHX33 expression can be induced by PI3K and mTOR kinase inhibitors.
(A). U251-MG cells were treated with rapamycin, U0126 or wortmannin inhibitors at the indicated concentration for 48 h, with DMSO as a control. Whole cell extracts were subjected to western blot analysis with the designated antibodies, GAPDH served as an internal control.
(B). Similarly U251-MG cells were treated with wortmannin, rapamycin and Torin1 at different concentrations for 48 h. Torin1 dramatically induced DHX33 expression.

transcriptionally controlling critical genes involved in cell cycle and migration. Finally, DHX33 is expressed only by glioblastoma cells but not by normal cerebral cells, these results suggest that cancer therapies targeting DHX33 helicase might provide a novel way in treating DHX33 positive glioblastoma.

Grant support

This work was supported by Science and Technology Innovation Committee of Shenzhen Municipality (Grant Number: JCYJ20170307110713487) and Guangdong Provincial Science and Technology Department (Grant number: 2017A020211024).

Conflict of interest

We declare that we have no competing financial interests.

Acknowledgement

The authors thank members of the Zhang lab for their technical input and suggestions; thank the staff in Department of Biology in SUSTC who provided support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.12.005.

References

[1] F.B. Furnari, et al., Malignant astrocytic glioma: genetics, biology, and paths to treatment, Genes Dev. 21 (21) (2007) 2683–2710.
[2] C.W. Brennan, et al., The somatic genomic landscape of glioblastoma, Cell 155 (2) (2013) 462–477.
[3] M. Paolillo, C. Boselli, S. Schinelli, Glioblastoma under Siege: an overview of current therapeutic strategies, Brain Sci. 8 (1) (2018).
[4] A.O. Sasmita, Y.P. Wong, A.P.K. Ling, Biomarkers and therapeutic advances in glioblastoma multiforme, Asia-Pacific J. Clin. Oncol. 14 (1) (2018) 40–51.
[5] V.A. Venur, D.M. Peereboom, M.S. Ahluwalia, Current medical treatment of glioblastoma, Cancer Treat. Res. 163 (2015) 103–115.
[6] B.M. Ellingson, et al., Post-chemoradiation volumetric response predicts survival in newly diagnosed glioblastoma treated with radiation, temozolomide and bevacizumab or placebo, Neuro-Oncology 20 (11) (2018) 1525–1535.
[7] S. Ohba, Y. Hirose, Current and future drug treatments for glioblastomas, Curr. Med. Chem. 23 (38) (2016) 4309–4316.
[8] T.F. Cloughesy, et al., Antitumor activity of rapamycin in a phase I trial for patients with recurrent PTEN-deficient glioblastoma, PLoS Med. 5 (1) (2008) e8.
[9] Q.W. Fan, et al., EGFR signals to mTOR through PKC and independently of Akt in glioma, Sci. Signal. 2 (55) (2009) ra4.
[10] R. Loewith, et al., Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control, Mol. Cell 10 (3) (2002) 457–468.
[11] Q. Fan, et al., A kinase inhibitor targeted to mTORC1 drives regression in glioblastoma, Cancer Cell 31 (3) (2017) 424–435.
[12] Z.X. Chen, et al., RNA helicase a is a downstream mediator of KIF1Bbeta tumor-suppressor function in neuroblastoma, Cancer Dis. 4 (4) (2014) 434–451.
[13] M.R. Heerma Van Voss, P.J. van Diest, V. Raman, Targeting RNA helicases in cancer: the translation trap, Biochim. Biophys. Acta 1868 (2) (2017) 510–520.
[14] A. Mazurek, et al., DDX5 regulates DNA replication and is required for cell proliferation in a subset of breast cancer cells, Cancer Dis. 2 (9) (2012) 812–825.
[15] M. Sarkar, V. Khare, K.K. Guturi, N. Das, M.K. Ghosh, The DEAD box protein p68: a crucial regulator of AKT/FOXO3a signaling axis in oncogenesis, Oncogene 34 (47) (2015) 5843–5856.
[16] K. Taniguchi, et al., Oncogene RNA helicase DDX6 promotes the process of c-Myc expression in gastric cancer cells, Mol. Carcinog. 57 (5) (2018) 579–589.
[17] P. Linder, F. Fuller-Pace, Happy birthday: 25 years of DEAD-box proteins, Methods Mol. Biol. 1259 (2015) 17–33.
[18] J. Fu, Y. Liu, X. Wang, B. Yuan, Y. Zhang, Role of DHX33 in c-Myc-induced cancers, Carcinogenesis 38 (6) (2017) 649–660.
[19] B. Yuan, et al., DHX33 transcriptionally controls genes involved in the cell cycle, Mol. Cell. Biol. 36 (23) (2016) 2903–2917.
[20] Q.H. Tian, et al., DHX33 expression is increased in hepatocellular carcinoma and indicates poor prognosis, Biochem. Biophys. Res. Commun. 473 (4) (2016) 1163–1169.
[21] Cancer Genome Atlas Research N, Comprehensive genomic characterization defines human glioblastoma genes and core pathways, Nature 455 (7216) (2008) 1061–1068.
[22] Y. Zhang, A.J. Saporita, J.D. Weber, P19ARF and RasV(1)(2) offer opposing regulation of DHX33 translation to dictate tumor cell fate, Mol. Cell. Biol. 33 (8) (2013) 1594–1607.
[23] D.W. Parsons, et al., An integrated genomic analysis of human glioblastoma multiforme, Science 321 (5897) (2008) 1807–1812.
[24] V. Serra, et al., RSK3/4 mediate resistance to PI3K pathway inhibitors in breast cancer, J. Clin. Invest. 123 (6) (2013) 2551–2563.
[25] S. Wu, et al., Activation of WEE1 confers resistance to PI3K inhibition in glioblastoma, Neuro-Oncol. 20 (1) (2018) 78–91.
[26] S. Wu, et al., MSK1-mediated beta-catenin phosphorylation confers resistance to PI3K/mTOR inhibitors in glioblastoma, Mol. Cancer Ther. 15 (7) (2016) 1656–1668.