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ORIGINAL RESEARCH

KDELR2 Promotes Glioblastoma Tumorigenesis Targeted by HIF1a via mTOR Signaling Pathway

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Abstract

The KDEL (Lys-Asp-Glu-Leu) receptors (KDELRs), proteins with seven transmembrane domains, are primarily responsible for endoplasmic reticulum (ER) homeostasis. Recent studies have found additional function of KDELRs in growth, cellular secretory traffic, immune response, and autophagy; however, its role in tumorigenesis is still poorly understood. Here, we showed that KDELR2 is highly expressed in glioblastoma (GBM) tissues. Reviewing the expression of KDELR2 in TCGA and REMBRANDT database, we found that higher expression of KDELR2 is associated with shorter survival of GBM patients. We explored the effect of KDELR2 on tumorigenesis in GBM cells and animal model (nude mice), and identified KDELR2 as oncogene promoting cell proliferation. Additionally, KDELR2 expression in GBM cells correlated positively with HIF1alpha (HIF1α) expression, and we demonstrated by ChIP-qPCR and luciferase reporter assay that the upstream region of the KDELR2 gene is directly targeted by HIF1alpha. Taken together, our data suggest that KDELR2 is a target gene downstream of HIF1-alpha driving the malignancy of GBM and could eventually serve as a therapeutic target for the treatment of GBM patients.

Keywords KDELR2 · Glioblastoma · HIF1α · mTOR

Introduction

Glioblastoma (GBM) is a common malignant phenotype in glioma (Touat et al. 2017). The characteristics of strong proliferative capacity and abundant low-functional neoplastic angiogenesis often result in necrosis and hypoxia (Barker et al. 1996; Kaur et al. 2005). In turn, hypoxia induces a variety of stress responses to adapt to harsh environments, thus providing greater viability.

The KDEL receptors (KDELRs) family is primarily responsible for the retrieval of soluble endoplasmic reticulum (ER)-resident proteins from the Golgi complex (Capitani and Sallese 2009). In mammals, the KDELRs family consists of three subtypes, KDELR1, KDELR2, and KDELR3, all of which localize around the ER and Golgi apparatus (Lewis and Pelham 1990; Hsu et al. 1992; Lewis and Pelham 1992; Collins et al. 2004). There are about 20% differences in the amino acid sequence of the three distinct KDELR subtypes, leading to differences in the function (Capitani and Sallese 2009). Recent evidence shows that KDELR2 and KDELR3 are upregulated in the unfolded protein response (UPR) that is an adaptive response enhancing cell survival in harmful environment, such as hypoxia glucose deprivation and so on, but KDELR1 is not (Trychta et al. 2018). In addition, the three human KDELRs have different binding substrates, with KDELR2 being specific and the other subtypes being general (Raykhel et al. 2007).

KDELR2 binding to ligands depends on the pH level and exert more efficiency at acidic or weaker basic PH level (Wilson et al. 1993). Because of the different PH environments, KDELR2 could bind the substrates at an acid pH and release them at neutral pH to complete the transporting

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of protein. Previous findings have suggested that binding of ligands to KDELR can activate several signaling cascades regulating the ER stress and autophagy (Yamamoto et al. 2003; Wang et al. 2011), cellular secretory traffic (Giannotta et al. 2012), cell proliferation and immune responses (Kamimura et al. 2015), and invasiveness. However, the roles of KDELRs family remain unclear, especially in tumor progression.

In this study, we identified KDELR2 as a poor prognostic factor in GBM and determined a key role of KDELR2 in GBM tumorigenesis and progression.

Materials and Methods

Clinical Samples and Cell Lines

GBM tissues and matched para-cancerous tissues ($n = 10$ pairs) were obtained from the department of Neurosurgery, Tianjin Medical University Cancer Institute & Hospital (China). All patients signed the informed consent and the study was approved by the Hospital Ethics Committee.

The LN229 and T98G human GBM cell lines were obtained from the American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2. All of the cells were tested by specialized technology resources (STR) and were excluded with mycoplasma contamination.

Bioinformatics Analysis of Databases

The KDELR2 transcript expression data of 136 patients with GBMs and 11 cases normal tissues were downloaded from The Cancer Genome Atlas (TCGA) website (http://cancergenome.nih.gov/). The Gene Expression Profiling Interactive Analysis website (http://gepia.cancer-pku.cn/) (Tang et al. 2017) is used for survival analysis.

The KDELR2 transcript expression data of 446 patients (214 cases GBM, 145 cases astrocytoma, 66 cases oligodendroglioma, 21 cases normal) from the REMBRANDT database were analyzed using the Betastasis website (www.betastasis.com) and figures generated.

qRT-PCR

Total RNA was extracted from GBM cells or tissues with TRIzol reagent (Invitrogen) following the manufacturer's instructions, and then was reversely transcribed to single-stranded cDNAs using the reverse-transcription PCR (RT-PCR) system. The cDNA samples were quantitatively analyzed by real-time PCR. The sequences of the primers are listed in Table 1.

Table 1 The primers used in qRT-PCR and ChIP PCR (from 5′ to 3′)

qRT-PCR	
KDELR2	Forward: CTTCTGTTTGCACTGGTCT
	Reverse: TTTCCATCGTAGGTTGCCTT
HIF1A	Forward: GAAGAACTTTTAGGCCGCTCA
	Reverse: AGTCGTGCTGAATAATACCACT
ChIP PCR	
KDELR2	Forward: CGTCCCGCAAATTATGTAGCAGA
	Reverse: GGTGAAACCCCGTCTCTACTGA

Western Blotting Analysis

Western blotting experiment was performed as described previously (Li et al. 2016). Protein lysates (30 μg) were separated by 10–15% SDS-PAGE, and target proteins were detected by Western blot analysis with antibodies against ELP1 (KDELR2, ab211470, Abcam, USA), mTOR (#2983, Cell Signaling Technology, USA), HIF1α (ab113642, Abcam, USA), p-mTOR (#5536, Cell Signaling Technology, USA), p70s6k1(ab59208, Abcam, USA), and GAPDH (60004-1-Ig, Proteintech, China).

Cell Counting Kit-8

The CCK-8 assay was performed using the CCK-8 kit (KGA317, KeyGen Biotech, China). Briefly, A total of 2000 LN229 cells (scramble, sh#1, sh#2) and T98G cells (vector, KDELR2) were, respectively, seeded in 96-well plate filled with 100 μl of medium per well and cultured for the indicated time (24 h, 48 h, 72 h). At each timepoint, 10 μl of CCK-8 solution was added to the plates. After 4 h, the absorbance in each well was evaluated using a microplate reader (Bio-Rad, USA) at a wavelength of 450 nm and then cell viability was calculated.

EdU Assays

EdU assay was performed to detect the DNA synthesis activities using an EdU kit (KGA331-100, KeyGen Biotech, China) as per the manufacturer's instructions. Briefly, 5×10^3 LN229 stable cells (scramble/sh#1) or T98G cells (vector/KDELR2) were, respectively, seeded in triplicate in 96-well plate. After cultured for 24 h, the cells were exposed to 50 μM of EdU for 2 h at 37 °C, and then cells were fixed with 4% formaldehyde for 10 min at room temperature. Subsequently, after washing with PBS for 3×, each well was added with 100 μl of 1 × Apollo® reaction cocktail for 30 min, the DNA contents of cells

were stained with 100 µl of Hoechst 33342 (5 µg/ml) for 30 min and visualized under a fluorescent microscope.

Plate Colony Formation Assay

About 1000 cells of LN229-KDELR2 sh#1 or T98G-KDELR2 were cultured in 6-well plates. Two weeks later, the cells were fixed with paraformaldehyde solution after removing the medium. Subsequently, the plates were stained with 1% crystal violet for 30 min at room temperature. Colonies of > 50 cells were counted in five microscopic fields at × 4 magnification. In the rescue experiment, T98G-KDELR2 cells were treated with 100 nM Rapamycin (S1039, Selleck, China) for 48 h and then seeded in 6-well plates at a density of 1000 cells per well.

Chromatin Immunoprecipitation (ChIP) and Dual-Luciferase Reporter Analysis

ChIP assays were performed using the ChIP kit (Millipore) according to the manufacturer's instructions. LN229 cells were incubated at the normoxic or hypoxic conditions for 12 h and then were harvested for ChIP analysis. The chromatin immunoprecipitation pulled down by the anti-HIF1A antibody was tested by RT-PCR. The primers for the PCR are listed in Table 1.

Luciferase analysis was performed as described previously (Li et al. 2016). Briefly, LN229 cells with pcDNA-HIF1A or pcDNA-vector were transfected with the pGL3-empty vector (pGL3.1 EV), pGL3- KDELR2-promoter, or pGL3- KDELR2-promoter mutation (MUT) constructs. After 48 h, the relative luciferase activity was tested by a dual-luciferase reporter kit (Promega).

Cell Transfected with siRNA and Plasmids

siRNAs targeting HIF1A and pcDNA-HIF1A plasmids were designed as previous described (Zhao et al. 2012; Li et al. 2016). The GBM cells were plated in 6-well plates with 2×10^5 cells per well. At 60–70% confluence, about 10 nmol/l siRNAs or 2 µg plasmids were transfected into the cells using Lipofectamine 2000 (Invitrogen) for 6 h, and then cells were cultured in a common medium for 24–48 h.

The complete coding sequences of human KDELR2 genes were cloned into pLV-EF1-MCS-IRES-Bsd vectors (Biosettia). Lentiviral infections were conducted following the standard protocol. The viral supernatants were used to infect the GBM cells for 24 h. 48 h after infection, blasticidin (Invitrogen) was supplied to the cells to select blasticidin-resistant cells.

Tumor Model

All animal studies were carried out with an approved protocol by Tianjin Cancer Institute and Hospital according to the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A total of 3 × 106 LN229 shKDELR2 #1 or LN229-scr cells were subcutaneously injected into the right flank of six nude mice. Tumor growth was observed every 7 days for 28 days. After 4 weeks, the mice were euthanized, the weight of tumors of the control and shKDELR2 #1 groups were measured. The volume of tumor was calculated by the formula: Volume $(mm^3) = 1/2 \times length \times width^2$.

Statistical Analysis

The IBM SPSS Statistics Program and GraphPad prism version 6.0 were performed for statistical analyses. Student's *t*-test was used to analyze difference between two groups. Kaplan–Meier survival and the log-rank test were used. $P < 0.05$ was considered as statistically significant.

Results

KDELR2 was Over-Expressed in GBM and Suggested Poor Survival

We first analyzed the expression of KDELR2 in 10 pairs of fresh GBM tumor tissues and their paired para-tumor tissues. qRT-PCR (Fig. 1a) and Western blot (Fig. 1b) showed that both mRNA and protein level of KDELR2 were upregulated in GBM tumor tissues. The results were consistent with the significantly increased KDELR2 mRNA expression in GBM tissues from TCGA database (Fig. 1c). Furthermore, analysis of TCGA data showed longer overall survival (OS) in patients with lower KDELR2 expression (Fig. 1e). Similarly, the analysis of glioma patients with different histology in the REMBRANDT database showed high expression of KDELR2 mRNA in any type of glioma, especially in the GBM (Fig. 1d), and also patients with high KDELR2 mRNA expression who had a shorter OS (Fig. 1f). These results implied that elevated KDELR2 expression in glioma may be associated with poor prognosis.

KDELR2 Promoted Proliferation in GBM Cells and Tumor Growth In Vivo

To determine the role of KDELR2 in GBM growth, we established stable cell lines with KDELR2 knockdown (sh#1, sh#2) in LN229 cells and KDELR2 overexpression in T98G cells, and cells were verified by Western blot analysis. The LN229 sh#1 and T98G-KDELR2 were

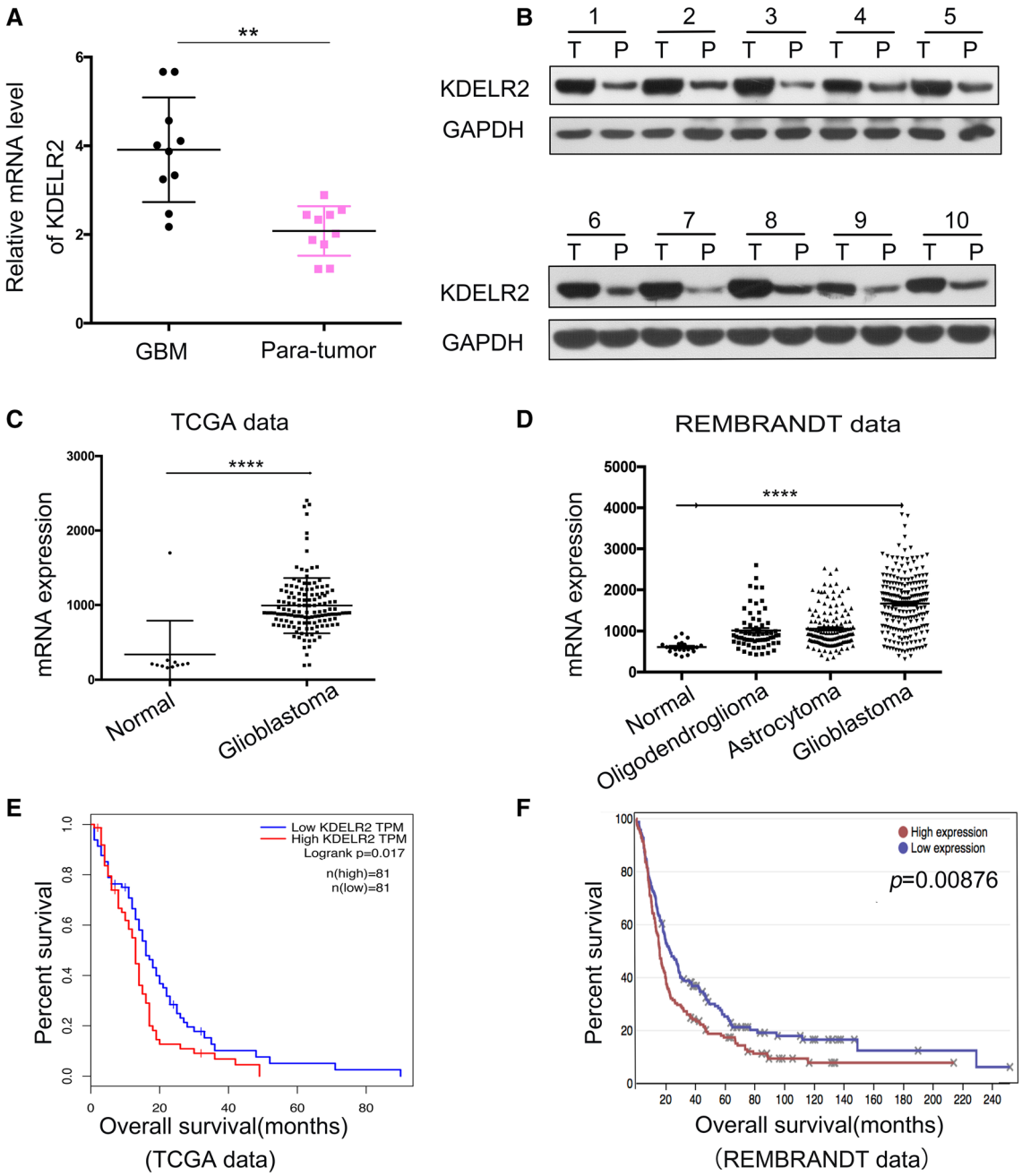

Fig. 1 The expression of KDELR2 was upregulated and suggested poor prognosis in glioma patients. **a**, **b** Ten pairs of fresh GBM tumor and para-tumor tissues were analyzed to compare the KDELR2 expression levels by quantitative real-time RT-PCR (**a**) and Western blotting (**b**). **c** The mRNA expression of KDELR2 in GBM tissues from TCGA database was analyzed, compared to normal tissues. **d** The relative expression of KDELR2 in different pathological subtypes of glioma tissues compared to normal tissues was analyzed on the basis of Rembrandt database. **e**, **f** Kaplan–Meier analysis of survival data of GBM patients from TCGA database(**e**) and REMBRANDT database(**f**) according to the level of KDELR2 expression. The data were showed as mean ± SD (Student's *t*-test, Log-rank tests, $^{****}P < 0.0001$, $^{**}P < 0.001$)

selected for the following studies (Fig. 2a). CCK8 and EdU assays showed that KDELR2 knockdown markedly reduced the cell viability and DNA synthesis of LN229 cells compared to the scramble group (Fig. 2b, d). Additionally, colony formation assays showed that knockdown of KDELR2 had a strong inhibitory effect on colony formation in LN229 cells (Fig. 2e). Conversely, KDELR2 overexpression produced opposite effects in the CCK8, EdU, or colony formation assays (Fig. 2c, d, and f).

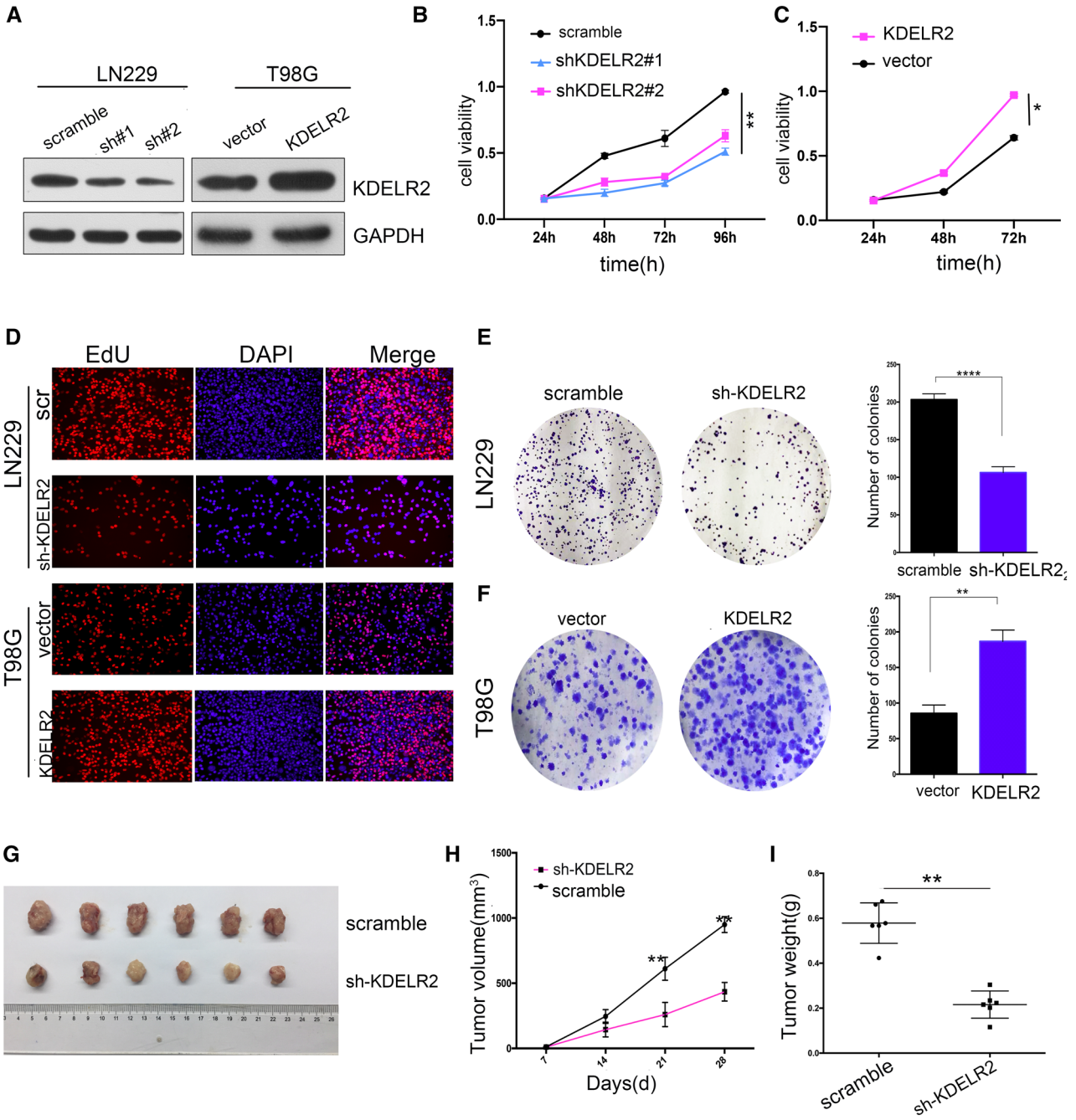

Fig. 2 Overexpression of KDELR2 promotes cell proliferation and tumor growth of GBM in vivo. **a** Stably transfected cells with shKDELR2(LN229) or pLV-KDELR2(T98G) were validated by western blotting. **b–c** The cell proliferation of LN229-shKDELR2 and T98G-KDELR2 cell lines was measured at indicated time by CCK8 assay. **d** Analysis of DNA synthesis by EdU assays of LN229-shKDELR2 and T98G-KDELR2 cell lines. **e** and **f** Colony formation assay of LN229-shKDELR2 and T98G-KDELR2 cell lines. **g–i** 12 BALB/c nude mice were subcutaneously injected with 3×10^6 LN229 shKDELR2 #1 (or LN229 scramble), respectively, ($n=6$ per group) and tumors were obtained from nude mice after 28 days (**g**). Scramble means the control. Tumor volume (**h**) and tumor weights (**i**) were measured at indicated time-point. The data were showed as mean ± SD. Columns indicate three independent experiments (Student's *t*-test, $^{****}P<0.0001$, $^{**}P<0.001$, $^{*}P<0.05$)

In addition, we validated the effect of KDELR2 on glioma tumorigenesis in vivo by subcutaneous implantation of LN229-shKDELR2 #1 cells. Downregulation of KDELR2 induced a significant reduction in tumor weight and tumor volume (Fig. 2g–i), which indicated that KDELR2 affected the growth and survival of GBM cells after subcutaneous implantation. Taken together, the experiments showed that KDELR2 promoted cell proliferation and tumor growth.

Overexpression of KDELR2 Activated the mTORC1 Pathway in GBM Cells

To explore the mechanism underlying the role of KDELR2 in cell proliferation, we performed Gene set enrichment analysis (GSEA) of TCGA data. As shown in Fig. 3a, significant enrichment of components of the mTORC1 pathway was observed, which was confirmed by Western blotting analysis. KDELR2 knockdown decreased the phosphorylation levels of mTOR (Ser2448) and p70s6k1 (Fig. 3b). In contrast, KDELR2 overexpression resulted in the upregulation of these key markers (Fig. 3b). Subsequently, the T98G cells with KDELR2 overexpression (T98G-KDELR2) were treated with rapamycin (RAPA), a mTOR inhibitor (Watson et al. 1999), and presented an obvious decrease in the colony formation ability (Fig. 3c, d). These data suggested that KDELR2 impacted the activity of mTOR pathway in GBM cells.

HIF1α Directly Targeted and Activated KDELR2 Transcription in GBM Cells

Hypoxia, which is a hallmark of malignant solid tumors, induces organ stress responses, including endoplasmic reticulum stress (ERS). KDELR2 is classically retained in the ER and responsible for ER homeostasis. Therefore, we speculated that KDELR2 is closely related to hypoxia. To test this hypothesis, we first analyzed the correlation between KDELR2 and HIF1α expression at the mRNA level in a cohort of 214 GBM patients from the REMBRANDT

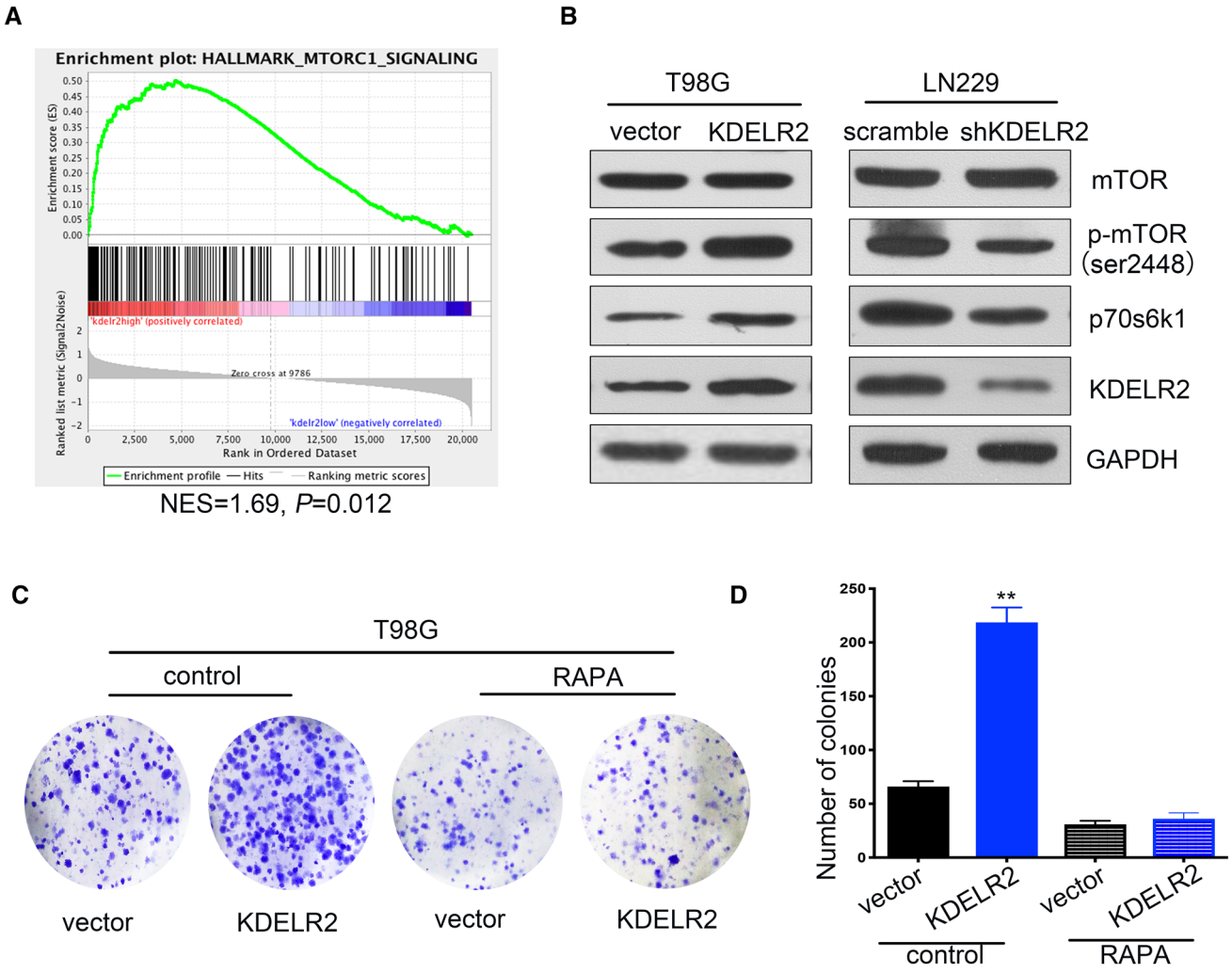

Fig. 3 Over-expressed KDELR2 activates the mTORC1 pathway in GBM cells. **a** GSEA analysis based on the TCGA data of GBM. **b** Western blotting analysis showed the phosphorylation of mTOR (p-mTOR) and its downstream target p70S6K1 in LN229 cells with KDELR2 knockdown and T98G cells with KDELR2 overexpression. **c**, **d** Colony formation assay of T98G cells with KDELR2 overexpression with and without Rapamycin (RAPA 100 nM) treatment for 48 h. The data were showed as mean ± SD. Columns indicate three independent experiments (Student *t*-test, **, vs. group with RAPA, $P < 0.001$)

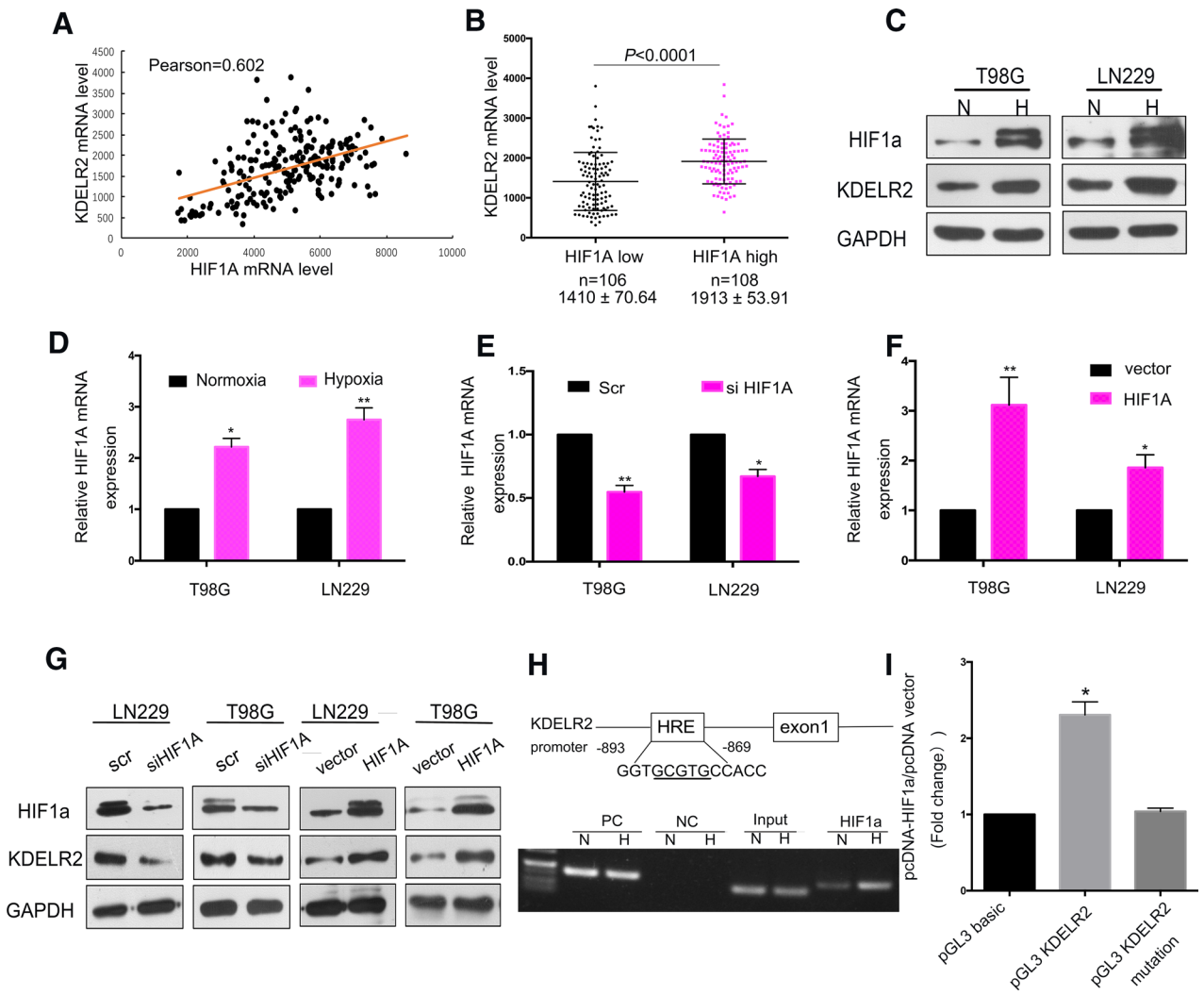

Fig. 4 HIF1-alph expression correlates with KDELR2 expression in GBM cells, and directly drives KDELR2 expression. **a** Correlation analysis of the mRNA expression of KDELR2 and HIF1A in 214 GBM patients from the Rembrandt database using the online Betastasis software. **b** KDELR2 expression of the 214 GBM patients from the Rembrandt database was divided into two groups according to the mRNA levels of HIF1A. **c**, **d** The protein and mRNA levels of HIF1α and KDELR2 determined by Western blotting (**c**) and qRT-PCR analysis (**d**) in the indicated GBM cells incubated under normoxic (20% O_2) and hypoxic (1% O_2) conditions for 24 h. **e**–**g** The indicated cells were transfected with siHIF1a or pcDNA-HIF1a for 48 h, and then assessed by quantitative real-time RT-PCR (**e**–**f**) and Western blotting (**g**) analysis. **h** The DNA sequence of the KDELR2 promoter (upper part). LN229 cells were cultured under normoxia and hypoxia for 12 h, and then subjected to ChIP analysis. The KDELR2 promoter region was analyzed by RT-PCR (lower part). HIF1a overexpression promoted the transcription of KDELR2. PC means positive control; NC means negative control, input means the total DNA products, and the HIF-1a means the DNA products binding with anti-HIF-1a antibody. **i** Luciferase analysis in LN229 cells. The stable cells with pcDNA-vector or pcDNA-HIF1A were transfected with Renila vector (20 ng) and pGL3-basic(1 ug) (or pGL3-KDELR2-promoter or pGL3-KDELR2-promoter mutation), respectively. After 48 h, cells were subjected to luciferase analysis using Dual-Luciferase reporter assay system. Renila vector was an internal control. Results are showed as a fold change relative to the cells transfected with the pcDNA-vector after normalization to Renilla activity. The data were showed as mean ± SD (Student's *t*-tests, $^{**}P<0.001$, $^{*}P<0.05$). Columns indicate three independent experiments. N:normoxia, H:hypoxia

data using the Betastasis software ($R=0.602$, Fig. 4a). Furthermore, we found the mRNA expression of KDELR2 in HIF1A-low patients was significantly lower than that in HIF1A-high patients (Fig. 4b, $P<0.0001$).

RT-PCR and western blot analyses revealed significantly increased KDELR2 expression under hypoxic conditions (Fig. 4c, d). In addition, we established targeting-HIF1α siRNAs and plasmids to reduce or enhance HIF1α expression in GBM cells. HIF1A knockdown decreased KDELR2 expression at the mRNA and protein levels (Fig. 4e, g), while HIF1A overexpression markedly enhanced KDELR2 expression at the mRNA (Fig. 4f, $P<0.05$) and protein (Fig. 4g) levels. These data suggested that KDELR2 expression is associated with HIF1α in GBM cells.

To determine the mechanism by which HIF1 affected KDELR2 expression in GBM cells, the promoter region of human KDELR2 gene was analyzed. The results revealed the presence of one hypoxia response elements (HRE). Then chromatin immunoprecipitation assays were performed in LN229 cells incubated under normoxic (21% O_2) or hypoxic (1% O_2) conditions. The chromatin fragment pulled down by anti-HIF1a antibody was detected and obviously increased under hypoxia (Fig. 4h), which confirmed that hypoxia promoted HIF1α binding to the KDELR2 promoter. Furthermore, Luciferase assays determined that overexpression of HIF1A markedly increased the luciferase activity of the wild-type luciferase vector (2.4 fold) but not the mutated vector (Fig. 4i). The above results demonstrated that HIF1a could activate the KDELR2 transcription.

Discussion

The KDELR is a conserved transmembrane protein and able to bind with the KDEL and HDEL signal sequence for ER-chaperones retrieval (Semenza et al. 1990). The amino terminal of the protein is exposed to the lumen of the organelles, and the carboxyl terminal is in the cytosol (Townsley et al. 1993; Scheel and Pelham 1998). Here, we first investigated the expression and role of KDELR2 in GBM. We found that KDELR2 was abnormally high expressed in GBM tissues compared to the para-tumor tissues, and negatively correlated with survival. It suggested the potential prognostic indicator of KDELR2 in GBM. KDELR2 knockdown could significantly inhibit the proliferation and clonality of GBM cells. Moreover, tumor growth was significantly slowed in the subcutaneous xenograft mouse models, although the tumor microenvironment is not as good as that of orthotopic transplantation tumors. These findings implied that KDELR2 functioned as oncogene in GBM and promoted tumor progression.

KDELRs primarily function as carrier to retrieve several chaperone proteins such as GRP78 from Golgi apparatus to the ER. KDELRs and GRP78 were upregulated response to ER stress. GRP78 is a key regulator in the tumorigenesis. Overexpression of KDELR2 reduced GRP78 expression on the cell surface, but little impacted on the total expression of GRP78 (Tiwarekar et al. 2019). This suggested that higher expression of KDELR2 lead to increased GRP78 in the ER which was favorable for cell survival under stress. In this study, we found that KDELR2 overexpression in GBM cells could significantly upregulate p-mTOR as well as the downstream protein, which was downregulated as KDELR2 knockdown. This suggested that KDELR2 could activate the mTORC1 pathway, which is correlated with cell proliferation and aggressiveness (Ghosh and Kapur 2017). Furthermore, an analysis of mutiple gene expression profile in glioma also identified KDELR2 was a core gene both in GBM and low-grade glioma (LGG), and KDELR2 have the potential ability to differ the patients into different (high/low) risk in survival (Hsu et al. 2019).

Rapidly growing tumors, such as GBMs, are associated with extensive necrosis and hypoxia. The role of hypoxia in promoting tumor growth has attracted increasing attention. Hypoxia-inducible factor 1 (HIF-1) is the representative of the master regulators involved in the responses to hypoxia and constitutively expressed in GBM (Kaur et al. 2005). HIF-1 signaling in GBM is a potent activator of proliferation, invasion, and angiogenesis through the upregulation of target genes critical for these functions. The analysis of REMBRANDT data showed a strong positive correlation between the levels of HIF1α and KDELR2. We also found that HIF1α upregulated KDELR2 mRNA and protein expression in GBM cells. Moreover, HIF1α directly bound to the HRE within KDELR2 promoter region, and enhanced the activity of KDELR2 transcription. These data together indicated that KDELR2 is a direct target gene of HIF1α in GBM.

Antiangiogenesis therapy plays an important role in refractory GBM. Pre-clinical and clinical studies have shown that the growth of tumors was not increased when antiangiogenesis therapy (for example, bevacizumab) was used alone for the normalization of tumor blood vessels (Jain 2005). Any growth of tumors within this short normalization window can be masked by the direct and indirect killing of tumor cells by antiangiogenic drugs. It is a possibility for the indirect role that bevacizumab reversed the hypoxia microenvironment and affected the wide downstream targets of HIF1α including KDELR2 expression, which weakened the cell proliferation.

However, treatment of GBM often fails due to rapid proliferation and enhanced invasiveness. Understanding the potential mechanisms involved in these characteristics may provide new insights into strategies aimed at delaying GBM growth. Therefore, KDELR2 could be as a novel therapeutic target and prognosis factor for the future treatment of GBM.

Author Contributions CS and LM conceived the project and designed the study; ZL and CS performed most of the experiments; ZS and XZ assisted in some in vitro experiments; PL, PW, and WL provided clinical samples; LM provided reagents and conceptual advice; ZL and CS wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Compliance with Ethical Standards

Conflicts of interest The authors declare no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of Ethics Committee of Tianjin Medical University Cancer Institute & Hospital (China) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethics Committee of Tianjin Medical University Cancer Institute & Hospital (China) or practice at which the studies were conducted.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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