LncRNA HULC promotes the proliferation, migration, and invasion of glioblastoma cells through microRNA-128- and tenascin-R-mediated regulation of PI3K/AKT pathway

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Article

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Abstract

Glioblastoma multiforme (GBM) is an extremely aggressive and malignant tumor of the central nervous system in adults. Therefore, understanding its pathogenesis is urgently needed. This study aimed to investigate the role of long noncoding RNA (lncRNA) highly upregulated in liver cancer (HULC) in GBM. The levels of lncRNA HULC and proteins in PI3K/AKT pathway in GBM tissues were measured. Kaplan–Meier method was used to analyze the survival. An in-vivo tumor xenograft model in mice was constructed and monitored. In GBM SGH44 and U87 cells, the proliferation, migration, invasion, cell cycle and apoptosis, cellular expression of proteins in PI3K/AKT pathway, and that of lncRNA HULC and microRNA (miR)-128 were determined. The interactions between lncRNA HULC, miR-128, and tenascin-R (TNR) were verified. The result showed lncRNA HULC was an oncogene that can promote cell proliferation, migration, and invasion in human GBM tissue, orthotopic transplantation in mice, and GBM cell lines. LncRNA HULC was further confirmed to affect TNR expression via sponging miR-128 and activating the PI3K/AKT pathway to promote cell proliferation in GBM. LncRNA HULC can affect the expression of TNR protein, activate the PI3K/AKT pathway, promote GBM cell proliferation, migration, and invasion, and inhibit apoptosis mediated by miR-128.

Introduction

Glioblastoma multiforme (GBM) is the most common primary tumor in the adult brain. According to the pathological classification by the World Health Organization (WHO), GBM belongs to grade IV glioma, whose malignancy is the highest among tumors. GBM mostly originates from abnormal astrocytic cells, and then it infiltrates and invades into the nearby regions in the brain. Due to the destructive growth and invasion of the tumor, the mortality of those with GBM is high, and the prognosis is not good, as demonstrated by a 1-year survival rate of < 10% and a median survival time of only 12–14 months [1]. To date, effective treatments for GBM include cytoreductive surgery, temozolomide chemotherapy, external beam radiation therapy, and combinations of these methods. However, it is still difficult to completely remove tumor cells, which makes relapse common in GBM patients [2]. Both genetic (e.g., epidermal growth factor receptor, p53, and isocitrate dehydrogenase 1 mutations) and environmental (e.g., smoking, pesticides, and viral infections) risk factors contribute to the pathogenesis of GBM. Many investigations have focused on the mechanism of tumorigenesis and progression of GBM, including complicated interactions and signaling pathways, such as the chemokine (C-X-C motif) ligand 2–chemokine (C-X-C motif) receptor 2 and c-myc pathways [3, 4]. Cancer stem cells [5], cellular metabolism [6], ion channels [7], microRNA [8], etc. also have been shown to be involved in the pathogenesis of GBM. Nevertheless, until now, the detailed mechanism of the pathogenesis of GBM is still not fully understand. Therefore, more research on the molecular mechanisms of GBM development and progression is needed in order to discover new treatments for GBM.

Long noncoding RNA (lncRNA) is a type of RNA that can exert biological functions via interacting with other RNAs, DNAs, and proteins at the mRNA level without being translated into proteins. The functions of lncRNAs include epigenetic chromosome modifications, transcription, and post-transcriptional regulation.
as well as effects on the biological activity of tumor and immune cells [10], epithelial–mesenchymal transition (EMT), and cytoskeleton integrity [11, 12]. Besides, lncRNAs can regulate the expression of downstream genes by sponging microRNAs (miRs) and participating in various signaling pathways in tumorigenesis [13]. In 2007, the lncRNA highly upregulated in liver cancer (HULC), as demonstrated by its name, was first recognized as an extremely upregulated lncRNA in hepatocellular carcinoma [14]. Since then, lncRNA HULC has been found to participate in the occurrence and development of various cancers besides liver cancer [15, 16]. However, the specific mechanism remains unclear due to the complexity. In 2016, Yan et al [17] reported that IncRNA HULC is highly expressed in human glioma tissues and can increase cell proliferation and colony formation of glioma cell lines, indicating its important role in glioma progression. In addition, silencing IncRNA HULC can inhibit the angiogenesis and growth of human glioma cells by regulating endothelial cell-specific molecule 1 via the phosphoinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway [18]. Moreover, Yin et al [19] have found that IncRNA HULC can promote EMT and vasculogenic mimicry in GBM. IncRNA HULC also has been reported to play a positive role in maintaining the stemness of glioma stem cells to enhance their tumorigenicity through the forkhead box M/anterior gradient 2/hypoxia-inducible factor 1-alpha pathway [20]. However, until now, only a few studies have been carried out on the interconnection and mechanism of lncRNAs and GBM. More investigations should be performed to elucidate them, which may provide novel strategies for GBM treatment.

To clarify the elaborate regulatory pathways in which IncRNA HULC is involved in GBM, the specific target of IncRNA HULC was determined. As reported, in human dermal microvascular endothelial cells, IncRNA HULC has been shown to enhance the lipopolysaccharide-induced inflammatory response via downregulating miR-128-3p [21]. The correlation of IncRNA HULC and miR-128-3p also has been observed in coronary heart disease [22]. Furthermore, many reports have demonstrated that miR-128 plays a role in GBM. For example, Sun et al have suggested that miR-128 may be a specific biomarker of GBM [23]. Additionally, Shan et al [24] have reported that miR128-1 inhibits the growth of GBM cells by targeting BMI1 and E2F3. Similarly, miR-128 can inhibit the proliferation and invasion of glioma cells via cyclooxygenase-2 [25]. Moreover, miR-128-3p can enhance the chemosensitivity of temozolomide in GBM by targeting c-Met and EMT [26]. miR-128-3p also inhibits glioma cell proliferation and differentiation by targeting neuronal pentraxin 1 through the insulin receptor substrate 1/PI3K/AKT signaling pathway [27]. Thus, we hypothesized that IncRNA HULC targets miR-128 during tumor cell proliferation in GBM; however, further investigation is necessary.

Tenascin-R (TNR), which is one of the four members of the tenascin family, is a glycoprotein in the extracellular matrix [28]. It is primarily expressed in the central nervous system. The primary components of the extracellular matrix include collagen, proteoglycan, laminin, and fibronectin, which are involved in tumor angiogenesis and metastasis, especially in tumor cell adhesion and migration [29]. TNR can combine with integrins to activate focal adhesion kinase (FAK), phosphorylate PI3K, and activate the PI3K/AKT signaling pathway [30]. TNR has been hypothesized to affect cell migration, adhesion, and differentiation; however, there is no remarkable connection of its dysfunction and clinical features [31]. Although its exclusive expression in the central nervous system suggests that it may have a primary or
secondary role in neurological diseases, such as cancer and neurodegenerative disorders, scant evidence has emerged to prove it. Therefore, more investigations regarding TNR in human diseases, such as cancer, are needed.

In the present study, the role and interconnection of IncRNA HULC, miR-128, and TNR in GBM, especially in tumor cell proliferation, migration, and invasion, were investigated *in vitro* and *in vivo*. The downstream regulatory pathway was also analyzed. Our study may provide new evidence and clues for the mechanism and therapeutic target of GBM.

**Results**

**LncRNA HULC was highly expressed in GBM and correlated with the patients’ overall survival (OS)**

By the BaseScope *in situ* hybridization assay, among the 263 GBM patients, IncRNA HULC was shown to be highly expressed in the normal adjacent brain tissue of tumors in 60 patients (22.8%) and in tumor tissue in 173 patients (65.8%), indicating a significant difference in the expression pattern in the normal adjacent brain tissue and tumor tissue (*P* < 0.01) (Fig. 1A). The OS of all the patients ranged from 1 to 74 months, the mean survival time was about 15 months, and the median survival time was 12 months. The expression of IncRNA HULC was significantly different between the patients aged ≥ 45 years old and those aged < 45 years old (*P* = 0.009), and between patients with relapse ≤ 6 months and those with relapse > 6 months (*P* = 0.001). However, no differences were observed in the expression of IncRNA HULC between females and males or between those with different tumor sizes (tumor diameter ≥ 4.5 cm or < 4.5 cm) (Fig. 1A). All of the patients were divided into two groups based on the IncRNA HULC level. The OS was significantly less in the GBM patients with high expression of IncRNA HULC compared to those with low expression of IncRNA HULC (*P* = 0.002) (Fig. 1B). Besides, the OS in patients aged < 45 years old was more than that in those aged ≥ 45 years old (*P* < 0.001). According to the Cox proportional hazards regression model, it was revealed that age and the IncRNA HULC expression level were two independent risk factors affecting the prognosis of the patients (*P* < 0.05) (Fig. 1C).

The expression levels of TNR, p-AKT/AKT, p-FAK/FAK, p-PI3K/PI3K, and p-PTEN/PETN in the GBM brain slices were also determined by tissue microarray immunohistochemistry. The protein expression levels of TNR, p-AKT/AKT, p-FAK/FAK, and p-PI3K/PI3K in the tumor tissues were significantly increased compared with those in the corresponding normal adjacent brain tissues, while the expression levels of p-PTEN in the tumor tissues were significantly decreased (Fig. 1D). Of these 263 cases, 62 (23.6%) and 215 (81.7%) patients were found to have positive expression of TNR in the normal adjacent brain tissue of the tumor and the tumor tissue, respectively (Supplementary Table 1). Spearman correlation analysis showed that TNR expression was negatively correlated with the expression of IncRNA HULC and p-FAK in human GBM (*P* < 0.05). In addition, P-FAK expression was positively correlated with PI3K and p-AKT expression (*P* < 0.05) (Supplementary Table 2).
The expression of Ki67, which is used for the identification of GBM immunophenotypes during diagnosis, was significantly increased in GBM tissue with high lncRNA HULC expression (Fig. 1D). Altogether, these data suggest that lncRNA HULC plays an important role in GBM and may be involved in tumor cell proliferation.

**Tumor-promoting effect of lncRNA HULC in mice with GBM transplantation**

To confirm the tumor promotion function of lncRNA HULC in vivo, we constructed a xenograft tumor model in nude mice with different lncRNA HULC expression levels. By hematoxylin and eosin staining and microscopy, the tumor tissue showed an unclear boundary and invasive growth, and the GBM cells showed obvious atypia and mitosis, myxoid degeneration, map necrosis, and vascular formation (Fig. 2A). The results showed that the GBM xenograft transplanted tumor model was successfully constructed and was in line with its malignant growth characteristics. The expression of lncRNA HULC was also checked; the expression level of lncRNA HULC was significantly increased in the lncRNA HULC-overexpression group and significantly decreased in the lncRNA HULC-silenced group compared with the NC group (Fig. 2B). The levels of Ki67 were also assessed; Ki67 expression was significantly increased in the lncRNA HULC-overexpression group and significantly decreased in the lncRNA HULC-silenced group compared with the NC group (Fig. 2C). The level of lncRNA HULC was positively correlated with that of Ki67 and exerted a tumorigenic effect in vivo.

Furthermore, the survival times of the mice in all groups were calculated. The results showed that the survival time was significantly shorter in the lncRNA HULC-overexpression group but longer in the lncRNA HULC-silenced group compared with the NC group (Fig. 2D). Besides, the tumors in the lncRNA HULC-overexpression group were larger in size and had a heavier weight compared with the VEC control, while the lncRNA HULC-silenced group showed the opposite results compared with the NC group (Fig. 2E). The magnetic resonance imaging results demonstrated that lncRNA HULC led to a larger amount of blood fluid and average pass time but a shorter maximum time (Fig. 2F). Altogether, these results confirm that lncRNA HULC can promote GBM growth in mice and shorten the survival time.

LncRNA HULC promoted proliferation, migration, and invasion as well as inhibited apoptosis in cultured GBM cells

To further investigate the mechanism of lncRNA HULC promoting GBM cell proliferation, we established an in vitro cell model by using the human GBM cell lines SHG44 and U87. LncRNA HULC was stably overexpressed in the two GBM cell lines. The expression of lncRNA HULC was confirmed by RT-qPCR (Fig. 3A). In both cell lines, the CCK8 assay (Fig. 3B) and colony formation assay (Fig. 3C) showed that overexpression of lncRNA HULC markedly promoted cell proliferation, while silencing of lncRNA HULC inhibited cell viability. Moreover, the wound healing assay (Fig. 3D) and the transwell assay (Fig. 3E), respectively, confirmed that lncRNA HULC overexpression facilitated the wound-healing and invasion abilities, while silencing of lncRNA HULC weakened the wound-healing and invasion abilities. Furthermore, cell cycle analysis demonstrated that GBM cells with high lncRNA HULC expression had
greater proportions of cells in the S and G2/M phases, compared to lncRNA HULC-silenced cells and the NC cells (Fig. 3F). Cell apoptosis was also assessed by flow cytometry, and the results showed that GBM cells with high lncRNA HULC expression had fewer apoptotic cells (Fig. 3G), indicating the inhibitory effect of lncRNA HULC on cell apoptosis. The expression patterns of apoptosis-related proteins confirmed this result. The levels of Bax, caspase-3, and caspase-8 were significantly inhibited in GBM cells with high lncRNA HULC expression (Fig. 3H). Altogether, these results demonstrate that lncRNA HULC promotes cell proliferation, migration, and invasion but inhibits cell apoptosis in GBM cells.

We also checked the expression of p-AKT/AKT, FAK, PI3K, and PETN in lncRNA HULC-overexpressed GBM cells. The western blot results showed that the levels of FAK, PI3K, and p-AKT were increased and that the level of PTEN was decreased in the lncRNA HULC-overexpression group compared with the control group; while the levels of FAK, PI3K, and p-AKT were decreased and the level of PTEN was increased in the lncRNA HULC-silenced group compared with the control group (Fig. 3I), indicating activation of the PI3K/AKT pathway. Therefore, the PI3K/AKT pathway is involved in the effect of lncRNA HULC on GBM cells.

The effect of lncRNA HULC on GBM cells was mediated by miR-128

To search for the specific target of lncRNA HULC in promoting cell proliferation of GBM, published data and The Cancer Genome Atlas database were referenced. In particular, miR-128 stood out. To investigate the role of miR-128 in the effect of lncRNA HULC on GBM cells, we first checked the function of miR-128 in GBM proliferation. miR-128 was overexpressed in the two GBM cell lines by transfection of the miR-128 mimic into SHG44 and U87 cells, respectively (Supplementary Fig. 1). In both cell lines, the CCK8 assay and colony formation assay showed that miR-128 markedly inhibited cell proliferation, while inhibition of miR-128 promoted cell proliferation. Besides, the wound-healing assay and transwell assay, respectively, confirmed that miR-128 weakened the wound-healing and invasion abilities, while silencing of lncRNA HULC facilitated the healing and invasion abilities. Furthermore, cell apoptosis was also assessed by flow cytometry, and the results showed that U87 cells treated with the miR-128 mimic caused more apoptosis, indicating the promotion effect of lncRNA HULC on cell apoptosis. The expression patterns of apoptosis-related proteins confirmed this result. The expression levels of Bax, caspase-3, and caspase-8 were significantly increased in GBM cells bearing the miR-128 mimic.

Altogether, these results demonstrate that miR-128 inhibits cell proliferation, migration, and invasion but promotes cell apoptosis in GBM cells. We also determined the expression of p-AKT/AKT, FAK, PI3K, and PETN in GBM cells bearing the miR-128 mimic. The western blot results showed that the levels of FAK, PI3K, and p-AKT were decreased and the level of PTEN was increased in the miR-128 mimic group compared with the control group; while the levels of FAK, PI3K, and p-AKT were increased and the level of PTEN was decreased in the miR-128 inhibition group compared with the control group, indicating the inactivation of the PI3K/AKT signaling pathway with miR-128 treatment. The PI3K/AKT pathway was shown to be involved in the effect of miR-128 on GBM cells.
Next, we investigated the influence of lncRNA HULC on miR-128. When SHG44 and U87 cells were transfected with lncRNA HULC, respectively, the expression level of miR-128 was shown to be decreased; meanwhile, silencing of lncRNA HULC significantly increased the level of miR-128 (Fig. 4A). When the miR-128 mimic was transfected into lncRNA HULC-overexpressed GBM cells, cell proliferation, migration, and invasion were detected by the CCK8 assay (Fig. 4B), colony formation assay (Fig. 4C), wound healing assay (Fig. 4D), and transwell assay (Fig. 4E), respectively. These results suggest that the proliferation, migration, and invasion abilities of the cells were downregulated with miR-128 mimic transfection; however, the promoting effect on cell proliferation, migration, and invasion of lncRNA HULC could not be reversed. Nevertheless, the proliferation, migration, and invasion abilities of the cells were upregulated after miR-128 was inhibited, which coordinated the promoting effect of lncRNA HULC. Furthermore, the interaction of lncRNA HULC and miR-128 was verified by the ChIRP assay (Fig. 4F).

**TNR was involved in the effects of lncRNA HULC and miR-128 on GBM cells**

Next, we chose the stable lncRNA HULC-transfected cell line U87 for proteomics analysis. Among all 4630 proteins analyzed, 232 upregulated proteins and 319 downregulated proteins were detected in the lncRNA HULC-overexpression group, while 112 upregulated proteins and 24 downregulated proteins were observed in the lncRNA HULC-silenced group, compared to their controls, respectively (Fig. 5A, Supplementary Table 3).

GO analysis showed that miR-128 was associated with activities of the PI3K/AKT pathway, Wnt pathway, MAPK pathway, and epidermal growth factor pathway and affected cell signaling, protein phosphorylation, the cell cycle, apoptosis, angiogenesis, and cell growth (Fig. 5B).

By informatic analysis using the Targetscan database, miR-128 and TNR protein were shown to be possible targets of lncRNA HULC (Fig. 5C). A dual-luciferase reporter assay was adopted to verify the correlation between miR-128 and TNR. The results showed that miR-128 can influence the expression of wild-type TNR, indicating that TNR might be the target of miR-128 (Fig. 5D). Altogether, these data indicate that TNR might be involved in the effects of lncRNA HULC and miR-128 on GBM cells.

**Discussion**

GBM is a huge threat to human life and leads to a poor prognosis. Despite diverse methods of treatment, the cure rate remains unsatisfactory with inevitable recurrence. To improve the cure rate and ameliorate clinical outcomes, more in-depth studies are currently being prioritized. Apart from the basic methods of treatment, it has been proposed that affiliated local medical treatment after local resection surgery can bring about better therapeutic effects, alleviate body reactions, and improve the life quality of patients [32]. Tumor occurrence and progression are influenced by multiple factors, such as regulation of immune cells and cytokines in the tumor microenvironment [33], interactions between IncRNA and signaling pathways [34, 35], etc. All of these factors can affect GBM progression and affect the prognosis.
Therefore, research regarding GBM tumorigenesis and effective therapeutic approaches show promise to make breakthroughs in clinical treatment. The present study focused on the effect of IncRNA HULC on the pathogenesis of GBM and indicated that IncRNA HULC promotes the proliferation, migration, and invasion of GBM cells through miR-128- and TNR-mediated regulation of the PI3K/AKT pathway.

Noncoding RNAs can be divided into small noncoding RNAs and IncRNAs; while IncRNAs consist of long intergenic noncoding RNAs, long intronic noncoding RNAs, antisense RNAs, and enhancer RNAs in light of the location related to protein-coding genes and enhancer regulatory elements [9]. IncRNAs can execute various complex biological functions at the RNA level via interactions between other DNAs, RNAs, and proteins [36]. For instance, IncRNAs can affect glycolysis and metabolism in tumors [37] as well as regulate downstream target proteins by sponging miRNAs [38]. Thus, they play a crucial role in tumorigenesis and immune response. In GBM, IncRNAs do not only affect treatment, but they also indicate the prognosis to some extent [39, 40]. A previous study has found that IncRNA PRADX recruits the PRC2/DDX5 complex to promote the trimethylation of H3K27 in the promoter region of UBXN1, thus inhibiting the expression of UBXN1 and enhancing the activity of nuclear factor-κB to promote tumorigenesis in GBM [41]. Besides, IncRNA OXCT1-AS1 has been shown to be a potential therapeutic target with a tumorigenic ability to promote the expression CDC25A by sponging miR-195-5p as an endogenous competitive RNA [42]. In addition, IncRNA HULC was first found to be remarkably highly expressed in liver cancer [43] and has been verified to act as an oncogene in pancreatic cancer, osteosarcoma, etc [44]. Moreover, other studies have discovered that IncRNA HULC can facilitate aerobic glycolysis via combining the glycolysis enzyme lactate dehydrogenase and pyruvate kinase isozyme M2 [45]. The regulatory functions of IncRNA HULC still remain ambiguous and unclear, although the sponge absorption with downstream miRNAs accounts for a huge part. To date, it has been validated that IncRNA HULC can modulate the expression of MYH9 by sponging miR-9-5p to promote tumor progression in gastric cancer [46]. In addition, IncRNA HULC can activate the PI3K/AKT pathway by reducing miR-15a expression to promote cell proliferation, migration, and invasion in pancreatic cancer [47]. Likewise, IncRNA HULC can regulate the expression of rhotekin by sponging miR-613 to affect tumor growth and metastasis in colon cancer [48]. Until now, it has been reported that IncRNA HULC can facilitate GBM progression [49], EMT, and vasculogenic mimicry [19]. However, research on IncRNA HULC in GBM is still incomplete. Here, we first verified a markedly high expression level of IncRNA HULC in GBM tissues compared with adjacent normal tissues, and it was correlated with recurrence and a poor prognosis. Then, we confirmed that IncRNA HULC promoted GBM cell proliferation, migration, and invasion in vivo and in vitro. To further explore the specific mechanism of IncRNA HULC in GBM, we took its downstream target miR-128 into consideration with bioinformatic analysis using Targetscan. Cell experiments manifested the reverse regulatory effects of IncRNA HULC and miR-128 in GBM cell proliferation, migration, and invasion. At the same time, a ChIRP assay confirmed the correlation between IncRNA HULC and miR-128, illustrating that miR-128 may serve as the target RNA of IncRNA HULC in GBM. In addition, by proteomics analysis on the stably transfected GBM cell line U87, we found that TNR was obviously highly upregulated in the IncRNA HULC-overexpression group, indicating that TNR may be a downstream target protein of IncRNA HULC. A dual-luciferase reporter assay further confirmed the
relationship between miR-128 and TNR. Accordingly, IncRNA HULC can modulate TNR by sponging miR-128 to promote cell proliferation, migration, and invasion. Based on this study, we proposed the role of IncRNA HULC in GBM. LncRNA HULC can be used as a new molecular marker of GBM, and it is expected to provide a new therapeutic strategy for GBM as well as a new avenues for the prevention and treatment of GBM. Nevertheless, additional investigations on the interplay of IncRNA HULC, miR-128, and TNR protein should be carried out to confirm their roles in GBM.

GBM is a heterogeneous disease, and its occurrence and development involve complex signaling pathways such as PI3K/AKT, JAK/STAT, Notch, MAPK/ERK, nuclear factor-κB, Wnt, and TGF-β as well as abnormal gene expression. Currently, it is believed that the core signaling pathways and gene variations are as follows: (1) the growth factor signaling pathway is involved through amplification and mutation of receptor tyrosine kinase genes; (2) the PI3K signaling pathway is abnormally activated; and (3) inactivation of p53 and Rb tumor suppressors [1]. Activated AKT directly phosphorylates the key kinase mTOR downstream of PI3K/AKT to inhibit apoptotic factors or activate antiapoptotic factors to promote tumor growth. PI3K can also regulate serum/glucocorticoid-regulated kinase 1, Rac family small GTPase 1/cell division control protein 42 homolog, and protein kinase C activities, leading to survival (antiapoptosis), cell scaffold rearrangement and transformation, and regulation of tumor cell proliferation, growth, and angiogenesis. The tumor suppressor PTEN can dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate at D3 to generate PIP2, thereby inhibiting the PI3K/AKT signaling pathway, inhibiting cell proliferation, and promoting cell apoptosis. The PI3K/AKT/mTOR signaling pathway plays an important role in cancer stem cell self-renewal and resistance to chemotherapy or radiotherapy, and it is considered to be the root of treatment failure as well as cancer recurrence and metastasis. In-vitro and in-vivo glioma experiments have confirmed that the isocitrate dehydrogenase 1 mutation inhibits the PI3K/AKT pathway [50]. Thus, the PI3K/AKT signaling pathway plays a key role in promoting tumor proliferation, apoptosis, invasion, metastasis, tumor angiogenesis, EMT, and extracellular matrix degradation. Therefore, it is of great translational medical value to analyze the molecular mechanism that regulates the PI3K/AKT signaling pathway in the development of GBM.

In conclusion, we verified a high expression level of IncRNA HULC in GBM and that it can be used to indicate clinical outcomes. We also confirmed that IncRNA HULC promotes the proliferation, migration, and invasion of GBM cells in vivo and in vitro. Furthermore, the specific mechanism by which IncRNA HULC regulates GBM cells was proposed to be mediated by miR-128 and TNR protein through the PI3K/AKT pathway.

**Methods**

**Clinical samples and animals**

Paraffin-embedded tumor tissue samples from 263 GBM patients were obtained from the Department of Pathology, The First Affiliated Hospital of the University of Science and Technology of China from September 2010 to January 2017. Informed consent was obtained from all subjects or their legal
guardians. All experiments were carried out in accordance with ARRIVE guidelines. All procedures were approved by the Ethics Committee of The First Affiliated Hospital of the University of Science and Technology of China ( Permit No. USTCACUC1801040). All methods were carried out in accordance with relevant guidelines and regulations.

The tumor xenograft model was designed by the Animal Experiments Ethics Committee of the University of Science and Technology of China (No: USTCACUC1801033). All operations and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. Female nude mice (BALB/c-Foxn1nu/Nju, 5–7 weeks old) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China) and raised in a specific pathogen-free animal laboratory. After 5 days of acclimation, the mice were randomly divided into four groups (n = 10): HULC, vector (VEC), HULC-siRNA, and negative control (NC), to build the tumor xenograft model. The movement and nervous system signs of the mice were observed by a Signa HDX 3.0 T Magnetic Resonance scanner (GE, USA) every 3 days.

Details of material and methods were shown in Supplementary Material and Methods.

**Tissue microarray RNAscope in situ hybridization**

A total of 263 tumor tissues and 263 corresponding adjacent normal tissues were used for tissue microarray analysis. The BA-Hs-HULC probes were from the Advanced Cell Diagnostics Company (USA). The procedure was performed according to the manufacturer's instructions.

**Immunohistochemistry**

Paraffin-embedded GBM tissues were sliced into 3-µm-thick sections. Immunohistochemistry experiments were performed by using an autostainer (Bench Maker XT, Switzerland). The following primary antibodies were used: TNR (Sigma, USA), p-AKT (Ser473, D9E, CST, USA), AKT (11E7, CST), p-phosphatase and tensin homolog (PTEN) (Thr366, ab109454, Abcam, USA), PTEN (D4.3, CST, USA), p-FAK (Tyr397, Sigma, USA), FAK (CST), p-PI3K (Tyr607, ab182651, Abcam), PI3K (19H8, Sigma), and Ki67 (Zsbio, China).

**Cell culture and transfection**

The human GBM cell lines U87 and SHG44 were purchased from the China Center for Type Culture Collection (Wuhan, China) and were cultured in complete cell culture medium, which consisted of Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, USA) with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% penicillin/streptomycin solution (Beyotime biotechnology, China) under 5% CO₂ and 37°C. All experiments were performed with mycoplasma-free cells.

Stable HULC-overexpression and HULC-silenced cells were constructed by lentiviral packaging, transfection with Lipofectamine 2000 (GeneParma, Shanghai, China), and purinomycin screening. The HULC-related sequences were synthesized by GeneParma. Transient transfection of miR-128 mimic and
inhibitor was also performed with Lipofectamine 2000 (GeneParma), and fluorescence microscopy was used to observe the transfection efficiency.

**Cell counting kit-8 (CCK8) assay**

Approximately 1500 cells were cultured in each well of a 96-well plate overnight (n = 3). After incubation for 24 h, 48 h, 72 h, and 96 h, CCK8 solution was added. Then the plates were incubated for 2 h at 37°C and 5% CO₂, and the optical density at 450 nm was measured by a microplate reader.

**Colony formation**

The cells were divided into a 6-well plate at 300 cells per well (n = 3) and cultured for another 2 weeks until an obvious cell mass appeared. The culture medium was replaced every 3 days. Afterwards, the cells were fixed with 4% paraformaldehyde for 30 min, and then they were dyed with 0.1% crystal violet for 10 min. The colony formation rate was calculated with the following formula: colony formation rate (%) = colony number/cultivated cell number×100%.

**Cell cycle and apoptosis**

Cell cycle and apoptosis were assayed by a flow cytometer with propidium iodide and FITC staining. Data were analyzed with FlowJo software.

**Wound healing assay**

Cells were seeded into 6-well plates at 5×10⁵ cells per well (n = 3). When the cells reached about 80% confluency, straight scratches were made by a 10-µL pipette tip. Cell migration was observed by microscopy and photographed at 0 h, 24 h, and 48 h after scratching. The migration rate was calculated according to the following formula: migration rate (%)=(original width − closure width)/original width×100%.

**Transwell assay**

Transwell assay was performed using 24-well transwell chamber plate (Corning, USA) according to the manual. ImageJ was used to analyze the results and calculate the relative invasion rate.

**Western blot**

The performance of Western blot was according to the standard procedure. Antibodies against Bax, caspase-3, caspase-8, TNR, FAK, p-FAK (Tyr397), PI3K p85α, AKT (pan), p-AKT (Ser473), PTEN, and p-PTEN (Ser385) were used.

**Reverse transcription–quantitative polymerase chain reaction (RT-qPCR)**

RT-PCR was performed according to the manual using SYBR Green. The 2⁻ΔΔ Ct value was used to analyze the results.
Dual luciferase reporting assay

Fragments of the 3′-untranslated region of TNR were amplified and cloned into the dual-luciferase reporter vector to construct reporter plasmids containing h-TNR-WT and h-TNR-MUT. Next, 293T cells were seeded in 96-well plates. Then, the reporter plasmids h-TNR-WT and h-TNR-MUT were respectively cotransfected with miR-128 mimic or miR-NC by the transfection reagent Lipo 6000™. After 48 h, the cells were harvested and measured with a Dual-Luciferase Reporter Assay System. Firefly luciferase (experimental group) activity was normalized to Renilla luciferase (control group) activity for each sample.

Chromatin isolation by RNA purification (ChIRP)

ChIRP was performed using probes (Ribo Biology, China) and C-1 magnetic beads (Thermo Fisher Scientific, USA). RNA was separated from the samples and tested by qPCR to analyze the enrichment function of miR-128 towards lncRNA HULC.

Data analysis

The results were analyzed by SPSS 16.0, and graphs were completed by GraphPad Prism 7. All data were described as the mean ± standard deviation. The $t$ test was used when data were in accordance with normal distribution and equal variance. Chi-squared test and Fisher’s exact method were used to analyze differences in the same group. Relative analysis was performed by Spearman correlation. Survival curves were completed by the Kaplan–Meier method, while the diversity was compared by the Log-rank test. The Cox proportional hazard model was applied in single-factor and multiple-factor analysis for prognosis. $P < 0.05$ was considered significant.

Declarations

Acknowledgments

None.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions
Jie He designed the research and drafted the manuscript. Yiran Wang, Yuchen Hu, Qian Li, Tiantian Yin, Shan Ye, Jing Wu conducted the research. Yiran Wang, Shan Ye analyzed the data. All authors contributed to the review and editing of the manuscript.

**Ethics statement**

The animal experiments in this study have been reviewed and approved by Laboratory Animal Management Committee of University of Science and Technology of China, and the clinical trials have been approved by Institutional Review Board of University of Science and Technology of China.

**Funding**

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**References**


**Figures**

A. Correlation analysis between IncRNA HULC and pathological characteristics of GBM

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>sum (n)</th>
<th>HULC high expression</th>
<th>HULC low expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (years old)</td>
<td></td>
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<tr>
<td>&gt; 45</td>
<td>213</td>
<td>148</td>
<td>65</td>
<td>0.009</td>
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<tr>
<td>≤ 45</td>
<td>50</td>
<td>25</td>
<td>25</td>
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<tr>
<td>gender</td>
<td></td>
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<tr>
<td>male</td>
<td>190</td>
<td>108</td>
<td>51</td>
<td>0.387</td>
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<tr>
<td>female</td>
<td>104</td>
<td>65</td>
<td>39</td>
<td></td>
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<tr>
<td>diameter of tumor (cm)</td>
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<tr>
<td>&gt; 4.5</td>
<td>136</td>
<td>96</td>
<td>40</td>
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<tr>
<td>≤ 4.5</td>
<td>127</td>
<td>77</td>
<td>50</td>
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<tr>
<td>time of recurrence</td>
<td></td>
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<tr>
<td>≤ 6 months</td>
<td>90</td>
<td>70</td>
<td>20</td>
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<tr>
<td>&gt; 6 months</td>
<td>85</td>
<td>47</td>
<td>38</td>
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B. Overall survival

- HULC low expression
- HULC high expression

P = 0.002

C. Analysis of OS by the Cox proportional hazards regression

<table>
<thead>
<tr>
<th>Clinical pathological factors</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
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<td>gender (male/female)</td>
<td>1.146 (0.671-1.508)</td>
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<tr>
<td>age (&lt;45/≥45)</td>
<td>1.955 (1.381-2.770)</td>
<td>&lt;0.001</td>
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<td>tumor size (&lt;5 cm or ≥ 5 cm)</td>
<td>0.906 (0.693-1.163)</td>
<td>0.466</td>
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<tr>
<td>HULC expression (high/low)</td>
<td>1.837 (1.377-2.451)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
**Figure 1**

Analysis of the correlation of IncRNA HULC and the occurrence of GBM.

The tumor samples of 263 GBM patients were obtained in this study, and the expression level of IncRNA HULC was assessed by a BaseScope *in situ* hybridization assay. All the patients were divided into two groups based on the level of IncRNA HULC. Correlation analysis between IncRNA HULC and pathological characteristics of GBM (A). Analysis of the overall survival (OS) of patients with different IncRNA HULC expression levels or at different ages was performed (B). The Cox proportional hazards regression model was used to analyze the contribution of sex, age, tumor size, and IncRNA HULC expression to OS (C). Besides, the expression of TNR, p-AKT/AKT, p-FAK/FAK, p-PI3K/PI3K, p-PTEN/PETN, and Ki67 in the GBM brain slices was checked by tissue microarray immunohistochemistry (D).
Figure 2

The effect of IncRNA HULC in mice with GBM transplantation.

A xenograft tumor model was constructed in nude mice by inoculation of GBM cells that were transfected with IncRNA HULC-overexpression plasmid, HULC siRNA, and their controls, respectively. Hematoxylin and eosin staining of the brain slices was performed, and microscopy was used to observe the tumor cells
(A). The expression of IncRNA HULC (B) and Ki67 (C) was assessed by immunohistochemistry. The survival time of each group was calculated (D), the tumor sizes were measured (E), and magnetic resonance imaging was used to assess the blood in the brain (E). *P<0.05, **P<0.01.
LncRNA HULC promoted the proliferation, migration, and invasion as well as inhibited apoptosis in cultured GBM cells.

The human GBM cell lines SHG44 and U87 were transfected with IncRNA HULC-overexpression plasmid, IncRNA HULC siRNA, and their controls, respectively. The expression of IncRNA HULC was confirmed by RT-qPCR (A). The CCK8 assay (B), colony formation assay (C), wound-healing assay (D), transwell assay (E), cell cycle analysis (F), cell apoptosis analysis (G) by flow cytometry, western blot with Bax, caspase-3, and caspase-8 antibodies, respectively (H), and western blot with p-AKT/AKT, FAK, PI3K, and PETN antibodies, respectively (I), were performed. *P<0.05, **P<0.01, ***P<0.001.
Figure 4

The co-effect of lncRNA HULC and miR-128 on the proliferation, migration, and invasion of GBM cells.

The human GBM cell lines SHG44 and U87 were transfected with lncRNA HULC-overexpression plasmid, lncRNA HULC siRNA, and their controls, respectively. The expression of miR-128 was confirmed by RT-qPCR (A). miR-128 mimic was transfected into ncRNA HULC-overexpressed GBM cells, and the CCK8
assay (B), colony formation assay (C), wound-healing assay (D), and transwell assay (E) were performed. The ChIRP assay was also performed in GBM cells by using miR-128 sense and antisense DNA probes (F). *P<0.05, **P<0.01, ***P<0.001.

Figure 5

TNR was predicted to be the downstream target of lncRNA HULC and miR-128.

U87 cells were transfected with plasmids bearing lncRNA HULC, lncRNA HULC siRNA, and their controls, respectively. Cell lysates were collected for proteomics analysis. The differentially expressed proteins are shown (A). GO analysis on the biological function of miR-128 (B). The target network figure of lncRNA HULC was predicted by RNAplex and the Targetscan database (C). The terms in the dotted circles are the GO terms to analyze the function of each group of target genes. The blue hexagonal nodes represent lncRNA HULC, the diamond nodes represent miRNAs, and the yellow oval nodes represent the target genes of miR-128. A dual-luciferase reporter assay was performed by using miR-128 mimic and the TNR 3′-untranslated region (D). *P<0.05.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial2022.12.07docx.pdf
- Supplementarymaterial.docx
- supplymentarymaterial.zip