Kavain ablates the radio-resistance of glioblastoma multiforma by targeting LITAF/NF-κB pathway

Jianzhong Yu
Children's Hospital of Fudan University, Fudan University
Fang Yuan
Shanghai Jiao Tong University School of Medicine
Jun Shi
Tongji University School of Medicine
Liyuan Zhuang
Tongji University School of Medicine
Hui Zeng
Tongji University School of Medicine
Lin Ge
Tongji University School of Medicine
Chuantao Fang
Tenth People's Hospital of Tongji University, Tongji University School of Medicine
Xinyu Mei
Tongji University School of Medicine
Hao Li (lihao7272@163.com)
Children's Hospital of Fudan University, Fudan University
Xiongwei Wang
Tongji University School of Medicine

Research Article

Keywords: Glioblastoma multiforma, NF-κB pathway, LITAF, kavain

Posted Date: June 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1715223/v1

Abstract

Glioblastoma multiforma (GBM) is the most malignant intrinsic tumor of the central nervous system (CNS), with high morbidity of 3.19/100,000 per year and a poor 5-year survival rate (< 5%) in worldwide. Numerous studies have indicated that GBM shows remarkable radioresistance and aggressive recurrence. However, the mechanisms to endow GBM cells with radioresistance are complex and unclear. Cell growth curve and Colony formation assay were used to analyzed radio-resistance of GBM. Immunoprecipitation and immunoblotting experiments were carried out to analyze protein expression and interaction. In the present study, we found that LITAF, lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α factor, is up-regulated both in mRNA and protein in GBM tumors. Meanwhile, we observed that high LITAF expression contributes to radioresistance of GBM cell lines, corroborated by LITAF$^{-/-}$ U87 and LITAF-low DK are more sensitive to radiation than wild type U87 (LITAF-high) both *in vitro* and *in vivo*. Furthermore, we demonstrated that kavain, an active constituent of *Piper methysticum Forst.*, effectively ablates U87 cells' radioresistance in a LITAF dependent manner. In mechanism, our results indicated that 1) the elevation of LITAF in GBM cells activates the NF-κB pathway to promote mesenchymal transition, and 2) kavain disturbs STAT6/LITAF protein interaction and then expels LITAF from the nucleus. Therefore, we consider that kavain may be a potential candidate to develop irradiation therapy adjuvant for GBM.

Introduction

Glioblastoma multiforma (GBM) is the most malignant intrinsic tumor of the central nervous system (CNS) with a World Health Organization (WHO) classification of Grade-IV, belonging to high-grade gliomas [1, 2]. In the clinic, GBM frequently manifests as symptoms including headaches, motor weakness, nausea and vomiting, cognitive impairment and seizures, with high morbidity of 3.19/100,000 per year and a poor 5-year survival rate less than 5% in the United States [1, 3]. In treatment, patients with GBM are generally subjected to a maximum surgical resection followed by radiation therapy (RT) as well as oral temozolomide because the tumors frequently infiltrate the surrounding normal tissues [4]. Mostly, patients who received a standard treatment will recur within 6.9 months of initial diagnosis, and no strong evidence supposes re-radiation therapy is effective for recurrent patients [1, 3, 5]. In fact, a great number of studies have indicated that GBM shows prominent radioresistance and generating aggressive recurrent tumors [6–8]. However, the mechanisms to endow GBM cells with radioresistance are complex and unclear.

Radiation therapy (or radiotherapy, RT), a therapy using ionizing radiation delivered by a linear accelerator, has become a curative treatment of many epithelioid cancers such as head and neck gynecologic and gastrointestinal cancers, especially with the development of image guidance and computer control system [8, 9]. Generally, three critical factors have been widely accepted to contribute to mechanisms of how radiotherapy induces cancer cell death: 1) direct DNA damage, 2) indirect DNA damage caused by reactive oxygen species, and 3) secondary intracellular ion buffer dysregulation [10]. Glioma stem cells (GSCs), specific subpopulations in GBM, have been frequently demonstrated to

contribute to this malignant tumor recurrence and aggressive development in patients who received standard therapy [8]. Researchers from independent groups reported that GSCs derived from human tumors (CD133-positive tumor cells) show more distinguishable radioresistance compared with non-GSC-matched populations [11, 12]. Increasing clinical observations show that tumors received chemo- and radiotherapies contain a higher GSCs proportion [6, 13], supporting a notion that GSCs which survived from radiation treatment rebuild more malignant tumors with aggressive irradiation-resistant recurrences [14]. In mechanisms, on one hand, GSCs possess enhanced instincts to respond to DNA damage (such as elevated ATM, CHK1/2 activity) and activate the DNA repair pathway (highly expressed RAD51) [11, 15]. On the other hand, elevated HIF1a expression in GSCs benefits them survival in hypoxic niches within GBM tumors, which protect DNA from ROS attack [16, 17]. Therefore, blockade of GSCs' repopulation may be an available strategy to GBM's radioresistance.

Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), as a transcription factor family containing RelA/B, c-Rel, and NF-κB1/2, displays versatility in embryonic development, host immunity and tumorigenesis [18, 19]. In canonical NF-κB pathway, transcription factors are cytoplasmically sequestered by the inhibitor of NF-κB (IκB) when cells are in resting conditions, and on activation, degradation of IκB after phosphorylation leads to NF-κB release and further nuclearly translocalize to promote target genes expression [20, 21]. Previously, Bhat and co-workers observed that the NF-κB pathway involves the differentiation from proneural (PN) to mesenchymal (MES) GSCs subtypes, and the later ones exhibit insensitivity to radiation [22]. Therefore, the NF-κB signaling pathway may be the potential for developing drugs to promote the curative effects of irradiation therapy on GBM.

Lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α factor (LITAF, also named as PIG7) was initially found to be induced in p53-overexpressed apoptotic DLD-1 cells [23]. In 1999, Myokai and co-workers reported that LITAF responses LPS stimulation to translocate into the nucleus to promote TNF-α expression [24]. In fact, LITAF, as a transcription factor, also directly induces the expression of other cytokines including CCL2, CCL5, CXCL1, IL1A, and IL10 in response to LPS stimulation [25, 26]. Kavain (Kava), an active constituent of *Piper methysticum Forst.*, is widely used as a dietary supplement for the treatment of anxiety, stress, and insomnia in the western world [27, 28]. In 2009, Kava was initially identified as a potent NF-κB inhibitor in lung adenoma tissues [29]. Currently, increasing evidence indicates that kavain inhibits LITAF's transcriptional activity although the underlying mechanisms are still controversial [30–32]. In this study, we aimed to fix two concerns: 1) whether kavain affects the radioresistance of GBM, and 2) if so, what is the underlying mechanism.

Methods And Materials

Human specimens

In the present study, tumor and adjuvant normal brain tissues of 10 GBM surgical specimens (male=4, female=6, median age=11.8±3.7 years) were collected from Children`s Hospital of Fudan University. In detail, these GBMs consist of 5 primary (male=2, female=3, median age=12.1±3.9 years) and 5 recurrent

(male=2, female=3, median age=11.7±3.1 years) tumors. All the samples were immersed in RNAlater Stabilization Solution (ThermoFisher Scientific, Cat:AM7020) and stored in -80 ^{o}C freezer. Pathological examination of all participants was independently confirmed by 3 board-certified neuropathologists according to the 2016 World Health Organization (WHO) grading classification [33]. All the protocols involved in this study have been approved by the ethics committee of the Children`s Hospital of Fudan University. All families of involved participants have signed the written informed consent.

Reagents

Antibodies of anti-LITAF(sc-166719) and anti-γH2AX (sc-517336) were purchased from Santa Cruz Biotechnology (Shanghai, China), antibodies of anti-ACTIN (#4970), anti-phos-γH2AX (Ser139, #80312), anti-STAT6 (#5397), anti-STAT3 (#9139), anti-TLR1 (#2209), anti-STAT5A (#4807), anti-IFNAR1 (sc-7391), anti-Flag (#14793), anti-phos-ERK1/2 (#8544), anti-ERK1/2 (#4695), anti-p38 (#8690), anti-phos-p38 (#4511), anti-AKT (#4691), anti-IκB-α (#4812), anti-phos-IκB-α (#2859) and HRP-linked secondary mouse (#7076)/rabbit(#7074) antibody were from Cell Signaling Technology (Danvers, USA). Florescence secondary antibodies Alexa Fluor Plus 488(#A32723)/555(#A32727) were purchased from ThermoFisher Scientific (Waltham, USA). Kavain (sc-201077) was purchased from Santa Cruz Biotechnology (Dallas, USA). DAPI (4',6-Diamidino-2-phenylindole Hydrochloride) dye (sc-3598) and EdU (2'-Deoxy-5-ethynyluridine-d1) were purchased from Santa Cruz Biotechnology (Dallas, USA). PI (Propidium Iodide, #4087) was from Cell Signaling Technology (Danvers, USA).

Cell culture

U87 (also U-87 MG, from American Type Culture Collection) cells were maintained in ATCC-formulated Eagle's Minimum Essential Medium (Cat: No. 30-2003) containing 10% FBS (Gibco, Cat: 10091148) and 50 mg/mL penicillin/streptomycin (P/S, Cat: 10378016). DK (also DK-MG, from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) cells were cultured in RPMI-1640 containing 10%FBS, 50 mg/mL P/S and 2 mM L-glutamine. T98G cells also were purchased from ATCC and cultured in the same condition with U87 cells. HEK293t cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Cat: 11965092) containing 10% FBS and 50 mg/mL P/S. All cells were maintained in an incubator with 37°C and 5% CO_2 conditions.

Construct of stable cell lines

For lentivirus packaging in constructing stable cell lines, HEK293A cells were co-transfected with viral vectors (expressing shLITAF or LITAF) and packaging plasmids (psPAX.2 and pMD2.G) using polyjet reagent from Signagen (Frederick, USA) following manufacturer’s instructions. 48 hours later of transfection, lentivirus supernatant was filtered using a 0.45-mm filter for the further infection of target cells with 5 mg/ml polybrene (GM-040901B) from Genomeditech (Shanghai, China). 1 day later of

infection, the medium was refreshed using a culture medium containing 1 mg/ml puromycin (A1113802) from ThermoFisher Scientific (Waltham, USA).

Quantitative Real-Time PCR (qPCR)

Total RNA was purified using the Trizol reagent (Cat: 15596026, ThermoFisher Scientific). Subsequently, 1 μg total RNA was reversely transcribed into cDNA using the PrimeScript^{TM} RT reagent Kit (Cat: RR036A, TaKaRa). Finally, qPCR was performed using TB Green Fast qPCR Mix (Cat: RR430A, TaKaRa) on 7500 Real-Time PCR systems (Applied Biosystems, Waltham, USA). The ΔΔCt method was used for relative quantification of target genes with the internal control of GAPDH mRNA.

Oligos for qPCR were listed as follows. LITAF: Forward, 5'-ATG TCG GTT CCA GGA CCT TAC-3', Reverse, 5'-TAC GAA GGA GGA TTC ATG CCC-3', TNF-α: Forward, 5'-CCT CTC TCT AAT CAG CCC TCTG-3', Reverse, 5'-GAG GAC CTG GGA GTA GAT GAG-3', CCL-2: Forward, 5'-CAG CCA GAT GCA ATC AAT GCC-3', Reverse, 5'-TGG AAT CCT GAA CCC ACT TCT-3', IL-1: Forward, 5'-TGG TAG TAG CAA CCA ACG GGA-3', Reverse, 5'-ACT TTG ATT GAG GGC GTC ATTC-3', CXCL-1: Forward, 5'-GCT GCT CCT GCT CCT GGT AG-3', Reverse, 5'-ACA GCC ACC AGT GAG CTT CC-3', STAT3: Forward, 5'-CAG CAG CTT GAC ACA CGG TA-3', Reverse, 5'-AAA CAC CAA AGT GGC ATG TGA-3', STAT5A: Forward, 5'-CAG TGG TTT GAC GGG GTG AT-3', Reverse, 5'-GTC GTG GGC CTG TTG CTT AT-3', GAPDH: Forward, 5'-GGA GCG AGA TCC CTC CAA AAT -3', Reverse, 5'-GGC TGT TGT CAT ACT TCT CAT GG-3'.

Western blot (WB)

Tumor tissues were lysed using tissue total protein lysis buffer (Cat: C500028-0010) from Sangon Biotech (Shanghai, China), followed by quantifying using BCA Protein Assay Kit (Cat: PC0020) from Solarbio company (Beijing, China). Cells were lysed in a home-made 1×SDS loading buffer containing 50mM Tris pH6.8, 2% SDS, 0.025% Bromophenol blue, 10% glycerol, and 5% BME. Firstly, protein samples were separated using a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose (Cat:10600002, Whatman) filter membrane. Subsequently, membranes were blocked using 5% skim milk/TBST buffer for 1 hr at room temperature. After washed with TBST, membranes were probed using primary antibodies (4^{o}C, overnight). The next day, membranes were probed using HRP-conjugated secondary antibodies at room temperature for 1 hr. Finally, protein bands were quantified using ECL substrates and imaged using Tanon-5200Multi equipment (Shanghai, China).

Immunofluorescence (IF)

Cells were seeded in a 6-well plate with condition medium with/without Kavain (50 μg/ml, for 6 hr). After briefly washed using cold 1×PBS, cells were fixed with 4% paraformaldehyde fix solution (Cat: E672002, Sangon Biotech) for 15 min, followed by being permeabilized with 0.2% Triton X-100 (Cat: T8200,

Solarbio) at room temperature for 15 min. Subsequently, cells were blocked using 5% BSA/PBS in room temperature for 30 min and then incubated with first antibodies (in 5% BSA/PBS) at 4°C overnight. The next day, cells were briefly washed using 1×PBS, and then incubated with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (in 1×PBS) at room temperature for 1.5 hr. Finally, cells were washed using 1×PBS for 3×5 min at room temperature and then subjected to microscopy.

Immunoprecipitation (IP)

HEK293t cells transfected with indicated plasmids were lysed using cold mild lysis buffer (MLB) containing 2 mM EDTA, 20 mM Tris, 1% NP-40, 100 mM NaCl, 1 mM Na3VO4, 50 mM NaF plus protease inhibitor cocktail (Cat: HY-K0010, MedChemExpress). After quantified using BCA Protein Assay Kit (Cat: PC0020), equal cell lysates were incubated with anti-Flag antibody at 4°C for 2 hours, and subsequently with Anti-FLAG M2 magnetic beads (Cat: M8823, Merck) at 4°C for 3-5 hours. Finally, the beads were washed with cold MLB 3×10 min, eluted by 1×SDS loading buffer (~1/10 input), boiled at 100°C for 10 min, and then separated by SDS-PAGE to WB detection.

Cell growth curve

Cells were cultured in growth medium (with/without 50 μg/ml) in 96-well microplate (Cat: nos. 3798, Corning) overnight. The next day, cells were subjected to X-ray irradiation treatments (1 Gy/min) using X-RAD 320 device (Precision X-ray). After irradiation, cells were maintained in a CO_2 incubator (at 37°C), and cell viability was determined at indicated time points using MTT Assay Kit (Cat: ab211091, Abcam) following manufacturer's instruction. Each treatment was replicated at least three wells, and the relative MTT optical density (OD) by normalized by the value of 0 day.

Colony formation assay

Melted agarose (1.0% in ddH_2O) was fully mixed with an equal volume of 2× DMEM medium at a temperature of 42-45°C in a sterile condition and then placed onto 3.5-cm dishes to form base agarose gel. U87 and DK cells (1000 cells/well) suspended in DMEM medium with 10% fetal bovine serum and 0.3-0.4% agarose were plated onto the prepared plates with base agarose gel. If required, cells were treated with PBS or Kavain (200 mg/ml) every 2 days. As to irradiation, cells were irradiated (4Gy, with a rate of 1.8 Gy/min) at 3, 8, 13 and 18 day post-implantation. Finally, the colonies were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet and counted.

Tumor xenograft assay

BALB/C nude mice (4-5 weeks) were purchased from Shanghai SLAC laboratory animal Co. Ltd (Shanghai, China). DK and U87 cell lines were trypsinized and then resuspended with a mixture of equal

volume PBS and Matrigel (10^7 cells/ml). The prepared cell suspensions were injected subcutaneously into both shoulders of a nude mouse. In case of Fig2, mice injected with wt and shLITAF (or oeLITAF) cells were divided into two subgroups (n=4): (1), Control (Con) group without irradiation and (2), Irradiation (IR) group receiving 5-Gy localized radiation at 5, 10 and 15 dpi (dpi, day post-injection). In case of Fig3, mice injected with U87(wt and LITAF$^{-/-}$) or DK cells lines were divided into four subgroups (n=4): (1), Control group, only receiving equal volume PBS treatment, (2), IR group, receiving 5-Gy localized radiation at 5, 10 and 15 dpi, (3), Kava group, receiving an intratumoral kavain treatment (100 μl, 4 mg/kg) every 3 days, (4), IR+Kava group, receiving irradiation and kavain treatment as (2) and (3). The tumor sizes were measured every 5 days to draw a tumor growth curve. One month later, mice were sacrificed with sodium pentobarbital (50 mg/kg) via intraperitoneal (i.p.) injection. The wet weight of each tumor was measured.

Public data analysis

LITAF gene expression in gliomas tissues and LITAF-based overall survival analysis (Illumina Hiseq platform) were analyzed using a web-based tool in the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/index.jsp).LITAF protein level in (low and high) gliomas tissues are quantified using the public data from The Human Protein Atlas database.

Statistical analysis

In the present study, all the data were presented as mean ± SD with at least 3 duplications. Statistical analyses were conducted on the software of GraphPad Prism7 (San Diego, USA). For comparisons between two groups, student's *t*-test was used, for comparisons between three or more groups, one- or two- way ANOVA test was used. A P value < 0.05 was considered as statistical significance.

Data availability

The data that support the findings of this study are available from the corresponding authors upon request.

Results

LITAF expression is elevated in tumor tissues of glioblastoma patients

To determine the clinical significance of LITAF in glioblastoma patients, we analyzed its expression and promoter DNA methylation level in gliomas tissues using public data from the Chinese Glioma Genome Atlas database. We found that LITAF mRNA level in tumor tissues was significantly increased along with its WHO grade, which is exactly correlated to its promoter DNA methylation level (Fig. 1A). While, overall survival analysis showed that, only in WHO III (but not WHO IV) gliomas, high LITAF exhibited

unfavorable role in patients' prognosis outcomes (Fig. 1B). In protein level, immunohistochemistry data from human protein atlas also indicated that LITAF expression was dramatically elevated in high-grade gliomas (Fig. 1C). All the evidence impelled us to collect glioblastoma multiforme (GBM, WHO IV) specimen to confirm these public data. Consistently, our qPCR results demonstrated that LITAF mRNA was obviously increased in GBM patients compared with that of normal controls (Fig1. 1D). Furthermore, we observed that LITAF protein in GBM was significantly higher than adjacent normal controls, determined by Western blot (Fig. 1E). More interestingly, the protein level of LITAF in recurrent GBM tumors was further higher than that in primary GBM tumor (Fig. 1F), implying it may contributes GBM recurrence. Altogether, these findings demonstrated that LITAF expression is up-regulated in tumor tissues of GBM patients.

LITAF expression involves cytokines produce of NF-κB pathway

Previously, Bhat et al. reported that patient-derived glioma sphere cultures undergo differentiation from proneural (PN) to mesenchymal (MES) transcriptomic subtype in a TNF-α/NF-κB-dependent manner [22] (Bhat et al. 2013). Moreover, NF-κB activation can efficiently induce the up-regulation of the master transcriptional factors including STAT3, CEBPB, and TAZ [22] (Bhat et al. 2013). In the public database of Gene Expression Profiling Interactive Analysis, we also observed that STAT3 and TAZ (but not CEBPB) mRNA levels were up-regulated in GBM patient tumor specimens and correlated with LITAF mRNA (Fig. S1A and S1E). Consistently, our qPCR and WB results for GBM clinical samples also confirmed these findings (Fig. S1B and C). All the evidence demonstrates NF-κB signaling pathway exhibits crucial roles in the development of GBM. So, whether LITAF involves in the regulation of the NF-κB signaling pathway in the context of GBM or not? Firstly, we determined LITAF expression in several common GBM cell lines by WB and observed that, in fact, LITAF is expressed quite differentially (Fig. 2A). We also noticed that LITAF subcellularly localized both in the nucleus (active form) and cytoplasm (inactive form) (Fig. S1D). Our immunofluorescence (IF) data showed that in U87 and DK cells, LITAF mainly localized in the nucleus, suggesting the protein constitutively activated. Therefore, these two cell lines were chosen for further experiments due to LITAF's significantly different expression. Subsequently, we constructed several LITAF-knockdown (by shRNA) cell lines in U87 and LITAF-overexpressed cell lines in DK (Fig. 2C). Intriguingly, we found depletion (in U87) and overexpression (in DK) of LITAF markedly decreased and increased the target genes expression of the NF-κB signaling pathway including TNF-α, CCL-2, IL-1 and CXCL-1 (Fig. 2D), respectively. More importantly, the expression of mesenchymal markers [34] including TIMP1, OSMR, SERPINE1 and EHD1 were accordingly changed after manipulating LITAF expression in U87 and DK cells (Fig. 2E). Collectively, these findings indicate that LITAF is sufficient to affect NF-κB activity in GBM cells.

LITAF elevation contributes to radioresistance of GBM cells

As demonstrated in Bhat et al study, the activation of the NF-κB signaling pathway correlates with GBM patients' poor radiation response and unfavorable survival [22]. In our experiments, we found that LITAF expression also correlated with IR-induced G2M phase arrest in GBM cell lines (Fig. 3B and S2A), suggesting LITAF elevation may contribute to cells' radioresistance. Indeed, the cell growth curve showed

that the depletion of LITAF in U87 cells efficiently released IR-induced cell growth arrest (Fig. 3A). Meanwhile, ectopic LITAF significantly enhanced cell radioresistance in DK cells (Fig. S2B). IR treatment on U87 and DK cells induced G2M arrest (Fig. 3B and S2C) as well as γ-H2AX phosphorylation (DNA damage) (Fig. 3C and S2D), and intriguingly, these phenotypes were obviously affected by LITAF expression level (Fig. 3B, 3C, S2C and S2D). In addition, LITAF also obviously influenced IR-induced cell proliferation arrest in U87 and DK cells, determined by EdU staining (Fig. 3D and S2E). In radiation-insensitive cells (U87), depletion of LITAF significantly boosted the increase in cells' tumorigenicity induced by IR-treatment both *in vitro* and *in vivo* (Fig. 3E-G), evaluated by soft agar and xenograft assay. In contrast, ectopic LITAF in DK cells weakened the effects of IR treatment on tumorigenicity both *in vitro* and *in vivo* (Fig. S2F and G). Altogether, our results display that LITAF contributes to the radioresistance of GBM cells.

Kavain weakens the radiation-resistance of LITAF-high GBM cells

Kavain has been demonstrated to be an effective LITAF inhibitor and influences TNF-α induced NF-κB activation in the context of human leukemia cells [30-32]. Therefore, we wondered whether kavain improves the clinical effect of irradiation on GBM. Intriguingly, our results displayed that kavain administration dramatically augmented cell growth arrest induced by IR treatment in U87 cells (Fig. 4A). Also, we constructed several LITAF knockout cell lines using CRISPR/Cas9 technology (Fig. S3A and B). Inhibitory effects of kavain on U87 cells' radioresistance were substantially blocked by depletion of LITAF protein (Fig. 4B), indicating kavain executes its functions in a LITAF dependent manner. Meanwhile, kavain administration in U87 cells significantly enhanced IR-induced G2M arrest (Fig. 4C) and proliferation (Fig. 4D), and these effects were stronger than those of $LITAF^{-/-}$ U87 and DK (LITAF-low expression) cells. Furthermore, kavain administration in U87 cells efficiently facilitated the decrease of tumorigenicity after IR treatment (Fig. 4E), determined by colony size change in soft agar assay. However, in $LITAF^{-/-}$ U87 and DK cells, the effect of kavain treatment was distinguishably worse compared with that of wild type U87 cells *in vitro* (Fig. 4E). In the U87 xenograft assay, kavain administration substantially improved the clinical effects (both in tumor growth and final tumor weight) of IR treatment, which was largely blocked by depletion of LITAF (Fig. 4F). Taken together, our findings indicate that kavain weakens the radioresistance of GBM cells by targeting LITAF.

Kavain suppresses NF-κB signaling by targeting LITAF

In molecular mechanism, activated LITAF (such as by LPS stimulation) has been well established to interact with signal transducer and activator of transcription (STAT) 6A and thereby translocate to the nucleus to mediate target genes expression including TNF-α, CCL-2, IL-1 and CXCL-1 [30, 31, 35]. Indeed, our ectopic Co-IP results displayed that the protein interaction between LITAF and STAT6 was weakened by kavain stimulation in HEK293t cells (Fig. 5A). Moreover, we observed that LITAF protein was mainly located in the nucleus in wild type U87 cells, and was expelled to the cytoplasm after kavain stimulation (Fig. 5B). Therefore, we supposed that: 1) elevation of LITAF induces cytokines such as TNF-α product

(primer signal) thereby to primer NF-κB signaling, and 2) kavain inhibits LITAF activity to resolve the radioresistance of GBM cells (Fig. 5E). In our experiment, we found that kavain treatment in U87 cells significantly inhibited the phosphorylation level of IκB-α in a dose-dependent manner, and this effect was blocked by the depletion of LITAF (Fig. 5C). Moreover, our observation showed that kavain pre-treatment in U87 cells obviously inhibited LPS-induced NF-κB targets expression in including TNF-α, CCL-2, IL-1, and CXCL-1, and these phenotypes were substantially blocked in LITAF$^{-/-}$ U87 cells (Fig. 5D). Taken together, all these findings indicated that kavain restrained the activation of the NF-κB signaling pathway in a LITAF dependent manner.

Discussion & Conclusion

LITAF, as a transcription factor, responds to LPS stimulation to promote the target genes expression (including TNF-α, IL-6, CCL-2, etc), and thereby further activates the NF-κB signaling pathway [35]. In quiescent conditions, LITAF protein mainly is located on late endosome and lysosome membranes under the control of E3 ligase NEDD4, and somehow functions with endosomal sorting complex [36, 37]. However, in our experiment, we observed that LITAF protein mainly takes the nuclear form in GBM cells such as U87 and U251 cells (but not A431) without LPS stimulation, suggesting LITAF is constitutively activated in GBM context. Meanwhile, our results displayed that the expression both in mRNA and protein of LITAF are significantly elevated in Grade-IV glioma (GBM) when compared with Grade-II/III ones. These findings also implied that the activated LITAF may be a primer cue to ignite canonical NF-κB signaling, which further mediates the differentiation of GSCs from proneural (PN) to mesenchymal (MES) subtypes. Indeed, we found that the depletion of LITAF in U87 cells reversed GSC subtype from MES to PN, indicated by the alteration in OLIG2 (PN marker) and CD44 (MES marker) mRNA level. Nonetheless, no distinguishable difference was observed in the overall survival of GBM analyzed by a cutoff of the LITAF expression medium. A probable explanation is that GBM is a malignant tumor with less than 500 days of the half-survival period. Moreover, we observed that depletion of LITAF in U87 cells sensitized cells to irradiation treatment, and LITAF-low DK cells exhibited higher sensitivity to irradiation than that of LITAF-high U87 cells. Collectively, these data demonstrate that LITAF contributes to GBM's radioresistance by activating the NF-κB pathway.

Over the past century, radiation therapy (RT) has gradually become a standard oncologic treatment especially in solid malignancies such as head and neck, gastrointestinal, and gynecologic cancers due to its increasing effectiveness with the development of image guidance and computer control system [8]. In the case of GBM, the clinical performance of RT is currently unsatisfactory because the tumor cells naturally resist IR-induced cell death and frequently recover to aggressive recurrent tumors after treatment [1]. In the present study, we first reported that kavain significantly improves the effects of IR on GBM cell lines both *in vitro* and *in vivo*, indicated by that Kava pre-treatment remarkably exaggerated the irradiation-induced cell growth slowdown (MMT assay), G2M phase arrest (PI staining), cell proliferation inhibition (EdU staining) and tumorigenicity attenuation (soft agar and xenograft assay). Kavain is widely used in the treatment of inflammatory diseases in ethnopharmacology [27, 38], and was reported to

involve the transcriptional regulation of TNF-α by independent groups [30, 31, 39]. Therefore, the activity of kavain resolving the radioresistance of GBM examples of this drug's novel function, which needs further investigation in other tumor contexts.

In 2016, Tang et al. established that kavain inhibits LITAF activity by inactivating Akt in RAW264.7 cells [30, 31]. In collagen antibody-induced arthritis (CAIA) mice, kavain also suppresses the activation of LITAF in response to LPS stimulation [30, 31]. All these observations collectively suggest a potential mechanism of kavain/LITAF/NF-κB in kavain resolving the radioresistance of GBM cells. Indeed, we observed that, in LITAF$^{-/-}$ U87 cells, all the alteration in cell growth, proliferation, and tumorigenicity induced by kavain pre-treatment were largely reduced although not completely eliminated. Meanwhile, the effects of kavain on radioresistance in LITAF-low DK cells were incomparable with that of LITAF-high (U87) cells. These results definitely demonstrate that kavain achieves its bioactivity mainly in a LITAF mediated manner. To further explain the mechanism of how kavain inhibits the transcriptional activity of LITAF, we determined the phosphorylation level of ERK1/2, p38 and AKT in wild type and LITAF$^{-/-}$ U87 cells [30, 31]. Unfortunately, our results indicated that kavain pre-treatment failed to alter these proteins' phosphorylation induced by LPS. Intriguingly, we found kavain treatment weakened the protein interaction between LITAF and STAT6 in HEK293 cells. In 2005, Tang et al. reported that, in LPS-stimulated U2OS cells, LITAF and STAT6 synergistically translocate into the nucleus to regulate the expression of inflammatory cytokines [26]. Consistently, we observed that kavain treatment expelled LITAF protein from the nucleus in U87 cells. Besides, kavain also restrained the LPS-induced expression of TNF-α, CCL2, IL-1, and CXCL-1 (NF-κB targets) in a LITAF mediated manner. Overall, our findings indicate that kavain inhibits NF-κB pathway activity by targeting LITAF.

In summary, we highlight two conclusions: 1) the elevated LITAF expression ignites the NF-κB pathway and thereby contributes to GBM's radioresistance, and 2) kavain resolves GBM's radioresistance by targeting LITAF. Therefore, kavain may be a potential candidate to develop irradiation therapy adjuvant for GBM.

Declarations

Ethical Approval and Consent to participate

All the protocols involved in this study have been approved by the ethics committee of the Children`s Hospital of Fudan University. All families of involved participants have signed the written informed consent.

Consent for publication

Not applicable.

Acknowledgements

This work was supported by Cyrus Tang Foundation.

Author contributions

Investigation and validation: Jianzhong Yu, Fang Yuan ,Jun Shi, Liyuan Zhuang, Hui Zeng, Lin Ge, Chuantao Fang, Xinyu Mei.

Data curation: Jianzhong Yu, Fang Yuan, Jun Shi, Liyuan Zhuang, Hui Zeng, Lin Ge, Chuantao Fang, Xinyu Mei.

Writing—original draft preparation: Jianzhong Yu, Fang Yuan and Jun Shi.

Writing—review and editing: Hao Li and Xiongwei Wang.

Supervision: Hao Li and Xiongwei Wang

Funding acquisition: Hao Li

Competing interests

The authors declare no competing interests

Funding

This study was supported by Cyrus Tang Foundation.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files.

Authors' information

Authors

Jianzhong Yu[2,a], Fang Yuan[4,a], Jun Shi[1,a], Liyuan Zhuang[1], Hui Zeng[1], Lin Ge[1], Chuantao Fang[3], Xinyu Mei[1],Hao Li[2,*], Xiongwei Wang[1,*],

Affiliations

[1]Center for Clinical Research and Translational Medicine, Department of Neurosurgery，Yangpu Hospital, Tongji University School of Medicine, Shanghai 200090, China.

[2]Department of Neurosurgery, Institute of Pediatrics, Children's Hospital of Fudan University, Fudan University, Shanghai, 201102, China.

[3]Tenth People's Hospital of Tongji University, Tongji University School of Medicine, Shanghai, China.

[4]Department of Neurosurgery, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200072, China.

[a]These authors contributed equally to this work

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Figures

Figure 1

LITAF expression is elevated in tumor tissues of glioblastoma patients

A. LITAF expression (mRNA) and promoter DNA methylation levels in gliomas tissues. Data were collected from the CGGA database. WHO II/III/IV, World Health Organization classification grade, GBM belongs to WHO IV or high-grade gliomas. One-way ANOVA was used for statistical analysis

B. LITAF-based overall survival analysis of gliomas. Data were collected from the CGGA database. The medium of LITAF expression level was used as a cutoff value.

C. LITAF protein level in gliomas tissues. Data were collected from the human protein atlas database. Relative optical density (normalized by low-grade) was calculated for each sample.

D. qPCR determined the mRNA level of LITAF in human specimens. NC, adjacent normal controls, Tumor, GBM tumors (both primary and recurrent GBM). n=10. ***P<0.001 by student's *t*-test.

E. Western blot showed the protein level of LITFA in human specimens. Samples are same as B. N, adjacent normal controls, T, GBM tumors (both primary and recurrent GBM).

F. Western blot showed the protein level of LITFA in GBM tumors. #, a patient.

Figure 2

LITAF expression involves cytokines produce of NF-KB pathway

A. Western blot showed the protein level of LITFA GBM cells. N/T, pooled protein samples of normal (N)/tumor (T) tissues.

B. IF determined the subcellular localization of LITAF in U87 and DK cells. The left panel shows the quantification of IF. shLITAF, knockdown of LITAF by shRNA, oeLITAF, over-expression of LITAF. Scale bar, 50 μm.

C. Western blot showed the knockdown (U87) and overexpression (DK) efficiency of GBM cell lines. Scr, scramble shRNA control, #1/2/3, three independent shRNA targets, Vec, vector control.

D. qPCR determined target genes expression of the NF-κB pathway and mesenchymal markers in U87 and DK cell lines. GAPDH serves as the internal control. *P<0.05, **P<0.01 and ***P<0.001 by student's *t*-test.

Figure 3

LITAF elevation contributes to radioresistance of GBM cells

A. Cell growth curve determined by MTT assay. U87 cell lines received IR treatment with a dose of 4 Gy, and the cell growth was determined at the indicated time points. IR, irradiation treatment. N.S, no statistical significance and ***P<0.001 by two-way ANOVA.

B. The cell cycle phase was determined by PI staining. U87 cell lines received IR treatment with a dose of 4Gy, and 24 hr later, PI staining was performed. .2N indicates the G1 phase, 4N indicates the G2M phase. The percentage of cells in G2M phase was analyzed (Right). *P<0.05 and ***P<0.001 by student's *t*-test.

C. WB showed the indicated protein levels in U87 cells. Cells were treated as B. **P<0.01 and ***P<0.001 by the student's *t*-test.

D. EdU staining determined the proliferation of indicated cells. Cells were treated as B. EdU positive cells were counted (Right). **P<0.01 and ***P<0.001 by student's *t*-test.

E. Soft agar assay determined tumorigenicity of indicated cells *in vitro*. Colony formation (Left) and size (Right) were analyzed. **P<0.01 and ***P<0.001 by student's *t*-test.

F. Tumor growth curve of U87 xenograft. Statistical analysis by two-way ANOVA followed by posthoc Bonferroni test.

Final tumor weight of U87 xenograft. *P<0.05 and ***P<0.001 by student's *t*-test.

Figure 4

Kavain weakens the radiation-resistance of LITAF-high GBM cells

A-B. Cell growth curve determined by MTT assay. Wild type (A)/LITAF$^{-/-}$(B)U87 cell lines received IR treatment with a dose of 4 Gy, and the cell growth was determined at the indicated time points. N.S, no statistical significance, *P<0.05 and ***P<0.001 by two-way ANOVA followed by posthoc Bonferroni test.

C. The cell cycle phase was determined by PI staining. Indicated cell lines received IR treatment with a dose of 4Gy, and 24 hr later, PI staining was performed. The relative G2M arrest changes with/without kavain treatment in each cell line were used for statistical analysis (Up). *P<0.05 and **P<0.01 by student's *t*-test.

D. EdU staining determined the proliferation of indicated cells. Cells were treated as C. The relative proliferation changes with/without kavain treatment in each cell line were used for statistical analysis (Up). *P<0.05 by student's *t*-test. Scale bar, 100 μm.

E. Soft agar assay determined tumorigenicity of indicated cells *in vitro*. Relative colony size changes with/without kavain treatment in each cell line were used for statistical analysis. **P<0.01 by student's *t*-test.

Tumor growth curve and final tumor weight of U87 xenograft. Two-way ANOVA followed by posthoc Bonferroni test for tumor growth curve and student's *t*-test for tumor weight.

Figure 5

Kavain suppresses NF-KB signaling by targeting LITAF

A. Protein interaction between ectopic LITAF and STAT6 was determined by Co-IP. HEK293t cells were transfected with Flag-tagged LITAF plasmid. 36 hr after transfection, cells were treated with kavain (50 μg/ml) for 8 hr, and then were subjected to Co-IP.

B. The subcellular localization of LITAF was determined by IF. U87 cells were pre-treated with kavain (50 μg/ml) for 8 hr. LITAF protein distribution was quantified (Right). ***P<0.001 by student's *t*-test.

C. WB showed the indicated protein levels in U87 cells. Cells were treated with kavain (50, 100 μg/ml) for 24 hr. N.S no significance, *P<0.05 and ***P<0.001 by student's *t*-test.

D. qPCR determined the mRNA level of indicated the NF-κB target genes. U87 cells were pre-treated with kavain (50 μg/ml) for 8 hr and then stimulated with LPS (0.1μg/ml) for 6 hr. N.S no significance, *P<0.05 and ***P<0.001 by student's *t*-test.

Supplementary Files

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