Neuropeptides xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Neuropeptides

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

Enhanced radiosensitization of human glioblastoma multiforme cells with phosphorylated peptides derived from Gli2

Lizhang Han[a], Ling Tang[b], Zheng Jiang[a,*], Yuquan Jiang[a,*]

[a] Department of Neurosurgery, Qilu Hospital of Shandong University, Jinan 250012, PR China
[b] Department of Pediatrics, Jinan Central Hospital Affiliated to Shandong University, Jinan 250013, PR China

ARTICLE INFO

Keywords:
Gli2
Hedgehog
Peptides
Radiosensitization
GBM

ABSTRACT

Glioma-Associated Oncogene Family Zinc Finger 2 (Gli2) seems to be the major nuclear effector of Sonic Hedgehog (SHH) signaling to regulate self-renewal and tumorigenic potential of Glioblastoma multiforme (GBM) cells. Three phosphorylated peptides derived from Gli2 were synthesized and combined with cell-penetrating peptide Tat-(47–57) (AYGRKKRRQRRR). Western Blot was applied to detect the phosphorylation level of Gli2 and cell division protein kinase 6 (CDK6) luciferase reporter was utilized to detect the transcriptional activator function of Gli2. Clonogenic survival assay and apoptosis assay were used to testify the radiosensitization effect. The mixed three phosphorylated peptides derived from Gli2 increased the phosphorylation level of Gli2 and decreased Gli2 transcriptional activator activity significantly than the individually used peptide. The mixed three phosphorylated peptides showed greater radiation-sensitizing effects in GBM cells in clonogenic and survival assay compared with control peptide. We present here a novel rational strategy for developing phosphorylated peptides derived from Gli2 to decrease Gli2 transcriptional activator activity and such administration could radiosensitize GBM.

1. Introduction

Glioblastoma multiforme (GBM), also known as grade IV gliomas, is the most common malignant primary brain tumor. Despite aggressive surgery, chemotherapy, radiotherapy, or combination approaches, the survival rate of patients with GBM remains poor and currently, the average time from diagnosis to death is less than one and a half years (Filbin et al., 2013). Cancer stem cells (CSCs) and deregulation of intercellular signaling pathways are major causes of GBM treatment failure, relapse, and drug resistance. Intriguingly, the aberrant activation and constitutive activation of the Sonic hedgehog (SHH) signaling pathway, which is crucial to embryonic development, play critical roles in the developing and maintaining of CSCs and lead to angiogenesis, migration, invasion, and metastasis (Cochrane et al., 2015; Rudin et al., 2009; Xu et al., 2017). Glioma-Associated Oncogene Family Zinc Finger 2 (Gli2), which seems to be the major nuclear effector of Hedgehog signaling (Bai et al., 2002; Lipinski et al., 2006), is correlated with malignancy grade in PDGF-induced gliomas (Hambardzumyan et al., 2008) and involved in oncogenesis through the up-regulation of genes encoding apoptosis inhibitors (Kump et al., 2008; Regl et al., 2004), cell-cycle regulators, and inducers of angiogenesis (Zhang et al., 2011). Furthermore, Hedgehog was also found to be indispensable for tumor formation in glioma xenotransplantation studies (Takezaki et al., 2011). All of these suggest that Gli2 may play a predominant role in the proliferation and apoptosis resistance of GBM cells, and Gli2 target might be a novel anticancer strategy for the treatment of GBM (Bai et al., 2002; Mishra, 2014; Ringuette et al., 2016; Xu et al., 2017).

It is vital to note that Gli2 processing is extremely inefficient and the majority of Gli2 exists in its full-length, Gli2–185 form, while the degradation of Gli2 requires the phosphorylation of a cluster of numerous serine residues in its carboxyl terminus by protein kinase A and subsequently by casein kinase 1 and glycogen synthase kinase 3. The phosphorylated Gli2 interacts directly with SKP1–CUL1–F-box protein ubiquitin ligase (βTrCP), which results in Gli2 ubiquitination and subsequent degradation by the proteasome (Pan et al., 2006). Such regulatory mechanism allows researchers to invent an approach to regulate Gli2 activity through reversible protein phosphorylation regulation.

In this research, phosphorylated peptides derived from Gli2 can promote both Gli2 degradation and radiosensitization in GBM cells, which may have a potential therapeutic value in the treatment of GBM.

* Corresponding authors at: Department of Neurosurgery, Qilu Hospital of Shandong University, No. 107, Wenhua West Road, Lixia District, Jinan 250012, Shandong Province, PR China.
E-mail addresses: doctorjiangzheng@126.com (Z. Jiang), jiangyuquan1964@163.com (Y. Jiang).

https://doi.org/10.1016/j.npep.2018.05.009
Received 25 January 2018; Received in revised form 28 May 2018; Accepted 29 May 2018

2. Methods & materials

2.1. Cell lines and culture

The GBM cell lines U118MG, U251, A172, U87-MG, SW1088, and H4 were obtained from the ATCC (Manassas, VA) and human Astrocyte was ordered from Thermo Fisher Scientific (Rockford, IL, USA). All cell lines were cultured in monolayer and maintained in DMEM (Sigma-Aldrich, Dorset, UK) supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich, Dorset, UK) in a 5% CO_2 incubator at 37 °C. Cells were treated with MG132 (50 μM) plus Cycloheximide (100 uM) (M + C group) as a control or together with Okadaic acid (100 nM) (M + C + O group) for 1 h. All the agents were ordered from Sigma-Aldrich (Dorset, UK) and were dissolved in distilled water or Dimethyl sulfoxide to varied stock concentrations, aliquoted and stored at −20 °C.

2.2. Phosphorylated peptides conjugated with cell-penetrating peptide

Phosphorylated peptides conjugated with a cell-penetrating peptide Tat-(47–57) (AYGRKKRRQRRR) were synthesized by solid-phase techniques and purified by HPLC (98% of purity) (Chinese Peptide Co., Hangzhou, China).

2.3. Phosphoprotein enrichment assay

A commercial Pierce® phosphoprotein enrichment kit (Thermo Fischer Scientific, Rockford, IL, USA) was used to enrich phosphoprotein. In brief, the diluted cell extract (0.5 mg/mL) was added to a pre-equilibrated Phosphoprotein Enrichment Column and incubated for 30 min on a rocking platform at 4 °C. Phosphoproteins were eluted with Elution Buffer (5 × 1 mL) with a 2-to-3 minute incubation between each centrifugation step (1000 × *g*). Elution fractions were centrifuged and pooled. Protein concentrations were determined by Coomassie Plus Bradford Assay (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The relative phosphorylation levels were quantitated by comparing phosphoprotein enrichment elution fractions to the total cell extracts.

2.4. Western blot analysis

Concentrated protein fractions were resolved by SDS-PAGE using 4–10% gradient gels. Antibody recognizing Gli2 (Phospho-Ser136) was purchased from Signalway and antibodies for total Gli2 (ab26056), CDK6 (ab124821), and tubulin (ab18207) were purchased from Abcam. The blots were incubated with HRP-conjugated anti-rabbit IgG and detected with enhanced chemiluminescence (Roche, Mannheim, Germany) and exposed to X-ray film.

2.5. Quantitative real-time RT-PCR (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA according to the manufacturer's instructions. cDNA was reverse-transcribed from 1 μg RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The reaction mixture in 15-μL volume was constructed using 2× FastStart Universal SYBR Green Master Mix (Roche, Mannheim, Germany) in which 0.375 μM of each primer and 0.5 μL cDNA were contained. The reaction procedures were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Expression data were normalized to β-actin mRNA expression. Primer sequences were listed: tubulin, forward primer 5′-GGAGGTCATCGACCTTGTG-3′, reverse primer 5′-AAGTCCA CCACACTGTTCTGC-3′; CDK6, 5′-GCTCTAACCTCAGTGGTCGT-3′, reverse primer 5′-GTAGCTGGACTGGAGCAAGA-3′.

2.6. Luciferase reporter constructs

In order to testify Gli2 transcriptional activator activity, luciferase reporter containing truncated CDK6 promoter sequences as reported (Raleigh et al., 2017) was constructed. In brief, pRL-TK (E2231, Promega, Madison, WI) and firefly luciferase reporters containing either Cdk6 promoter sequences or 8 consecutive Gli2 binding sites in pGL3-Luciferase (E1751, Promega, USA), plus Gli2-CLEG in pCMV-Tag1 (211170, Addgene, Cambridge, MA) were transfected using Lipofectamine LTX. Dual-Luciferase Reporter Assay System (E1910, Promega, USA) and a GloMax 96 Microplate Luminometer (Promega, USA) were utilized to assay luciferase. Firefly luciferase reporter activities were calculated relative to the internal *Renilla* luciferase controls with normalization to the control conditions.

2.7. Clonogenic survival assay

5×10^2 to 1×10^3 cells were plated into 6-well-plates and allowed to attach and proliferate until large clones (> 1 mm, 50 cells or more) were formed, which was further treated with peptide for 12 h. Irradiation was then performed using a 6 MV linear accelerator Siemens MD-2 (20 × 20 cm irradiation field). The irradiation dose varied between 0 and 8 Gy (0 Gy, 2 Gy, 4 Gy, 6 Gy, and 8 Gy). Clones fixation and staining were performed 24 h after radiation exposure with a mixture of 0.5% crystal violet in 50/50 methanol/water for 30 min and the number of clones was counted on the following day. Plating efficiency (PE) and surviving fraction (SF) were calculated as followed:

PE = no. of colonies formed/no. of cells seeded × 100%

SF = no. of colonies formed after treatment/no. of cells seeded × PE

2.8. Apoptosis assay

The Annexin V-FITC Apoptosis Detection kit I (BD Biosciences, USA) was used to detect and quantify apoptosis by flow cytometry. In brief, cells were harvested and collected by centrifugation for 5 min at 800 × *g*, then suspended at a density of 1×10^6 cells/ml, stained with FITC-labeled Annexin V for 5 min, and immediately analyzed by FACScan Flow Cytometer (Becton Dickinson, San Jose, CA, USA). The data obtained were analyzed using CellQuest software.

2.9. Statistical analysis

Quantitative parameters between groups were assessed using *t*-test and one-way ANOVA with post hoc Bonferroni test (significance level was set at a p-value < .05). All statistical analyses were performed using the SPSS 20.0 (SPSS, Inc., Chicago, IL). Image J software was utilized to detect the relative gray value of Western Blot bands.

3. Results

3.1. Decreased phosphorylated Gli2 expression in human GBM cells

After Pierce® phosphoprotein enrichment kit was used to enrich phosphorylated Gli2 (p-Protein elute), the relative expression of phosphorylated Gli2 was further determined by SDS-PAGE and Western Blots (Fig. 1A and B). GBM cell lines had significantly less expression of phosphorylated Gli2 when compared with astrocyte. It is worth noting that the level of phosphorylation might be varied among different GBM cell lines (Fig. 1B). U251 and H4 cell lines, which had the least expression of phosphorylated Gli2, were used to do the further analysis.

It is well reported that phosphorylation and subsequent ubiquitination may induce Gli2 degradation. In this experiment, OKA was used as a phosphatase inhibitor and MG132 plus Cycloheximide were used to prevent new protein synthesis and degradation (Pan et al., 2006). OKA