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Down-regulation of 14-3-3β exerts anti-cancer effects through inducing ER stress in human glioma U87 cells: Involvement of CHOP–Wnt pathway

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ABSTRACT

We previously identified 14-3-3β as a tumor-specific isoform of 14-3-3 protein in astrocytoma, but its functional role in glioma cells and underlying mechanisms are poorly understood. In the present study, we investigated the effects of 14-3-3β inhibition in human glioma U87 cells using specific targeted small interfering RNA (siRNA). The results showed that 14-3-3β is highly expressed in U87 cells but not in normal astrocyte SVGp12 cells. Knockdown of 14-3-3β by Si-14-3-3β transfection significantly decreased the cell viability but increased the LDH release in a time-dependent fashion in U87 cells, and these effects were accompanied with G0/G1 cell cycle arrest and apoptosis. In addition, 14-3-3β knockdown induced ER stress in U87 cells, as evidenced by ER calcium release, increased expression of XBP1S mRNA and induction of ER related pro-apoptotic factors. Down-regulation of 14-3-3β significantly decreased the nuclear localization of β-catenin and inhibited Topflash activity, which was shown to be reversely correlated with CHOP. Furthermore, Si-CHOP and sFRP were used to inhibit CHOP and Wnt, respectively. The results showed that the anti-cancer effects of 14-3-3β knockdown in U87 cells were mediated by increased expression of CHOP and followed inhibition of Wnt/β-catenin pathway. In summary, the remarkable efficiency of 14-3-3β knockdown to induce apoptotic cell death in U87 cells may find therapeutic application for the treatment of glioma patients.

1. Introduction

Approximately 60% of CNS neoplasms are gliomas, and half of these are glioblastomas (GBM), which is classified into grade IV of the WHO grading system [1]. Despite recently achieved advances in surgery, radiotherapy and chemotherapy, including the addition of temozolomide (TMZ), which increased median survival of GBM patients by a mere 2 months, the prognosis for GBM patients remains poor [2,3]. Due to the restricted anatomical location and absence of metastases outside the CNS, which allows delivery of vectors directly to the desired site with little systemic toxicity, GBM is an ideal target for local gene therapy [4]. Targeted molecular drugs that inhibit abnormal cellular signal transduction pathways or the expression of oncogenes have been demonstrated to have potential therapeutic values in experimental models and preclinical trials [5].

The 14-3-3 proteins are phosphor-serine/phosphor-threonine binding proteins, and can be classified into seven isoforms, namely β, γ, ε, δ, θ, η and ζ, all of which adopt a similar horseshoe-like structure capable of binding pS/T residues in a sequence specific context [6]. Through modulating the function of various binding partners, 14-3-3 proteins have become key regulatory components in many vital cellular processes, such as signal transduction protein synthesis, protein folding and degradation, cell cycle, rearrangement of the cytoskeleton, cellular trafficking, DNA replication, apoptosis and survival [7]. Importantly, accumulating evidence has established an association between 14-3-3 proteins and many types of cancers, including lung, breast, neck cancers and brain tumors [8]. However, the role of 14-3-3 proteins in cancer and its interactions with various oncogenic gene and tumor suppressor genes are complex, and specific 14-3-3 isoform has individual expression levels and specific functions [9]. Our previous studies demonstrated that 14-3-3 β, ε, δ, θ, η and ζ isoforms'

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immunoreactivity was seen in the majority of astrocytoma samples and their immunoreactivity scores were increased markedly with an increase in the pathologic grade of human astrocytomas [10]. We also identified 14-3-3 β and η as two tumor-specific isoforms of 14-3-3 in astrocytoma, and they might be useful candidates as targets for gene therapy [11]. More recently, 14-3-3β was shown to regulate proliferation of glioma cells through GSK3β-related signaling pathway [12]. However, the exact role of 14-3-3β in human GBM and possible underlying molecular mechanisms need to be further determined. In the present study, specific targeted siRNA was used to knockdown the expression of 14-3-3β in U87 cells and to investigate the potential anti-cancer effect, as well as possible mechanism with focus on ER stress and CHOP–Wnt pathway.

2. Materials and methods

2.1. Cell culture

Human normal astrocyte SVGp12 and glioma U87 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS), 100 U penicillin and 100 U streptomycin at 37 °C in a humidified incubator of 5% CO_2 and 95% air.

2.2. RNA interference (RNAi)

Small interfering RNA (siRNA) transfection was used to down-regulate 14-3-3β expression, and all the siRNAs were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The 14-3-3β targeted siRNA (Si-14-3-3β) and control siRNA (Si-Control) were dissolved separately in Optimem I (Invitrogen, CA, USA). After 10 min of equilibration at room temperature, each RNA solution was combined with the respective volume of the Lipofectamine 2000 solution, mixed gently and allowed to form siRNA liposomes for 20 min. The U87 cells were transfected with the transfection mixture in antibiotic-free cell culture medium.

2.3. Immunocytochemistry (ICC)

After being fixed with 4% paraformaldehyde for 15 min at room temperature, U87 cells were washed with NaCl/Pi, permeabilized with 0.2% Triton X-100, and incubated with primary anti-14-3-3β antibody overnight at 4 °C. Cells were then incubated with Alexa 488-conjugated secondary antibody for 2 h at 37 °C, and DAPI (10 μg/ml) was used to stain nucleus. Images were captured with an Olympus FV10i Confocal Microscope (Olympus, Tokyo, Japan). All images of one experiment were acquired with the same exposure time.

2.4. Cell viability assay

Cell viability was assessed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions. The absorbance was measured with an automatic microplate reader at a wavelength of 492 nm. Results are presented as a percentage of the control.

2.5. Lactate dehydrogenase (LDH) assay

LDH release into the culture medium was detected with a diagnostic kit (Jiancheng Bioengineering Institute, Nanjing, China) [13]. Briefly, 50 μl of supernatant from each well was collected to assay LDH release. The samples were incubated with NADH and pyruvate for 15 min at 37 °C, and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 440 nm, and background absorbance from culture medium that was not used for any cell cultures was subtracted from all absorbance measurements.

2.6. Flow cytometry

U87 cells were harvested 48 h after siRNA transfection, washed with ice-cold Ca^{2+} free PBS, and re-suspended in binding buffer. Cell suspension was transferred into a tube and double-stained for 15 min with the Alexa Fluor 488-conjugated annexin V (AV) and propidium iodide (PI) at room temperature in the dark. After addition of 400 μl binding buffer, the stained cells were analyzed by an FC500 flow cytometer with the fluorescence emission at 530 nm and >575 nm. The CXP cell quest software (Beckman-Coulter, USA) was used to count the number of AV^+/PI^- and AV^+/PI^+ cells, and analyzed the results.

2.7. Assessment of the cell cycle

U87 cells were treated with Si-14-3-3β or Si-control for 48 h. Thereafter, cells were washed, fixed in citrate buffer, and finally incubated for 1 h at −20 °C. Cells were next incubated in a glycine/NaCl buffer containing 0.1% Nonidet P-40, 10 μg/ml RNase A, and 40 μg/ml of PI for 1 h at 4 °C. Cell distribution across the different phases of the cell cycle was detected with a FACScan and analyzed.

2.8. Measurement of cytochrome c release

Cytochrome c release into the cytoplasm was assessed after subcellular fraction preparation. The levels of cytochrome c in cytosolic and mitochondrial fractions were measured using the Quantikine M Cytochrome C Immunoassay kit obtained from R&D Systems (Minneapolis, MN, USA). Data were expressed as ng/mg protein.

2.9. Electrophysiology and Ca^{2+} imaging

Whole-cell recordings were performed following standard procedures using an EPC9 patch clamp amplifier as described previously [14]. Imaging of transient Ca^{2+} changes in U87 cells was started 25–30 min after establishing the whole-cell configuration. A multipoint confocal microscope using dual spinning disc technology, attached to an upright microscope and equipped with a 403 objective was used to acquire fluorescence images in parallel to the patch clamp recordings.

2.10. Real-time RT-PCR

Total RNA was isolated from U87 cells using Trizol reagent (Invitrogen), and the mRNA levels of XBP1S were quantitated using iQ5 Gradient Real-Time PCR system (Bio-Rad Laboratories). Primers were listed as follow: XBP1S: forward: 5′-GCTTGTGATTGAGAACCAGG-3′, reverse: 5′-GCACCTGCTGCGGACTC-3′; GAPDH: forward: 5′-AAGGTGAAGGTCGGAGT-CAA-3′, reverse: 5′-AATGAAGGGGTCATTGATGG-3′. Samples were tested in triplicates and data from five independent experiments were used for analysis.

2.11. Reporter assay

Topflash and internal control pRL-TK vectors were co-transfected into U87 cells. After transfection for 36 h, U87 cells were lysed using passive lysis buffer. Firefly and Renilla luciferase activities were analyzed using the dual-luciferase reagent assay kit (Promega, USA) according to the manufacturer's instructions.

2.12. Western blot analysis

U87 cells were lysed by RIPA containing protease inhibitor cocktail. Electrophoresis and immunoblotting were done as described previously [15]. For the densitometric analysis, optical density was measured on the inverted digital images using Image J software.

2.13. Statistical analysis

Statistical analysis was performed using SPSS 16.0, a statistical software package. Statistical evaluation of the data was performed by ANOVA followed by Bonferroni's multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Anti-cancer effects of 14-3-3β knockdown in human glioma U87 cells

Expression of 14-3-3β in human normal astrocyte SVGp12 and glioma U87 cells was examined to test their feasibility in studying the biological function of 14-3-3β. As shown in Fig. 1A, the results showed that 14-3-3β protein was highly expressed in U87 cells, whereas there was no 14-3-3β expression in SVGp12 cells. To elucidate the functional role of 14-3-3β in human glioma, U87 cells were transfected with Si-14-3-3β or Si-control, and the results of morphological staining indicated that 14-3-3β expression was significantly reduced after Si-14-3-3β transfection (Fig. 1B). Knockdown of 14-3-3β resulted in a significant decrease in cell viability (Fig. 1C) and increase in LDH release (Fig. 1D) in a time-dependent manner, although it was not effective at 24 h after transfection ($p > 0.05$).

3.2. Down-regulation of 14-3-3β induces apoptotic cells death in U87 cells

To determine whether the anti-cancer effect of 14-3-3β knockdown was due to inducing apoptosis, U87 cells was transfected with Si-14-3-3β or Si-control for 48 h. As shown in Fig. 2A, the results of flow cytometry showed that 14-3-3β knockdown significantly increased the apoptotic rate in U87 cells (Fig. 2B). In addition, the percentage of cells in individual cell-cycle phases was assessed after 14-3-3β knockdown, and representative histograms are shown in Fig. 2C. A marked decrease of the cell fraction with fully replicated DNA (G2/M) occurs after Si-14-3-3β transfection, which corrected with a significant fraction of U87 cells accumulated in the subG1 phase. As expected, we also observed that 14-3-3β knockdown markedly inhibited cytochrome c release as evidenced by an increase in cytosolic cytochrome c (Fig. 2D) and a decreased cytochrome c content in mitochondrial fraction (Fig. 2E).

3.3. Down-regulation of 14-3-3β induces ER stress in U87 cells

To investigate the potential involvement of ER stress in 14-3-3β knockdown induced anti-cancer effect, we detected morphological

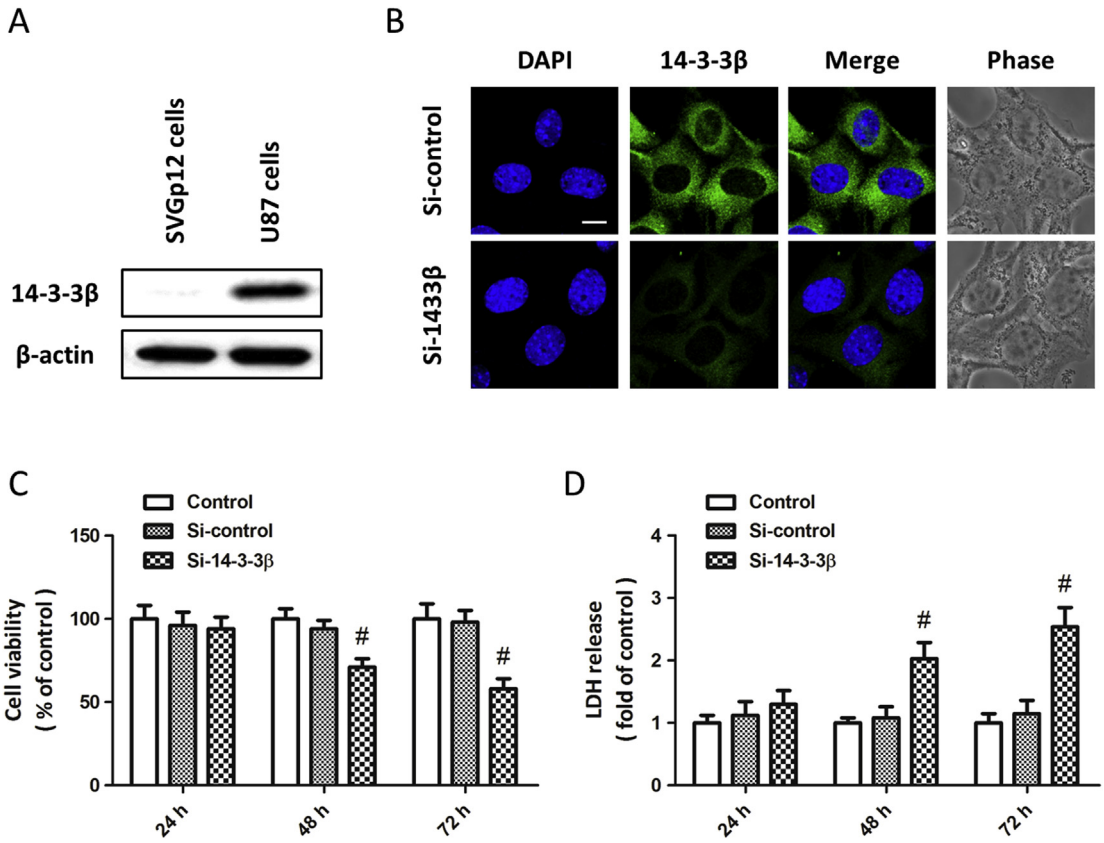

Fig. 1. Anti-cancer effects of 14-3-3β knockdown in human glioma U87 cells. The expression of 14-3-3β in human normal astrocyte cell line SVGp12 and human glioma U87 cells were detected by western blot analysis (A). U87 cells were transfected with 14-3-3β specific siRNA (Si-14-3-3β) or control siRNA (Si-control) for 48 h, and the expression of 14-3-3β was detected by fluorescence staining (B). U87 cells were transfected with 14-3-3β specific siRNA (Si-14-3-3β) or control siRNA (Si-control). The cell viability (C) and LDH release (D) were measured at 24, 48 and 72 h after transfection. Scale bar: 10 μm. Data are either representative of three similar experiments or are shown as mean ± SEM of five experiments. $^{\#}p < 0.05$ vs. Si-control.

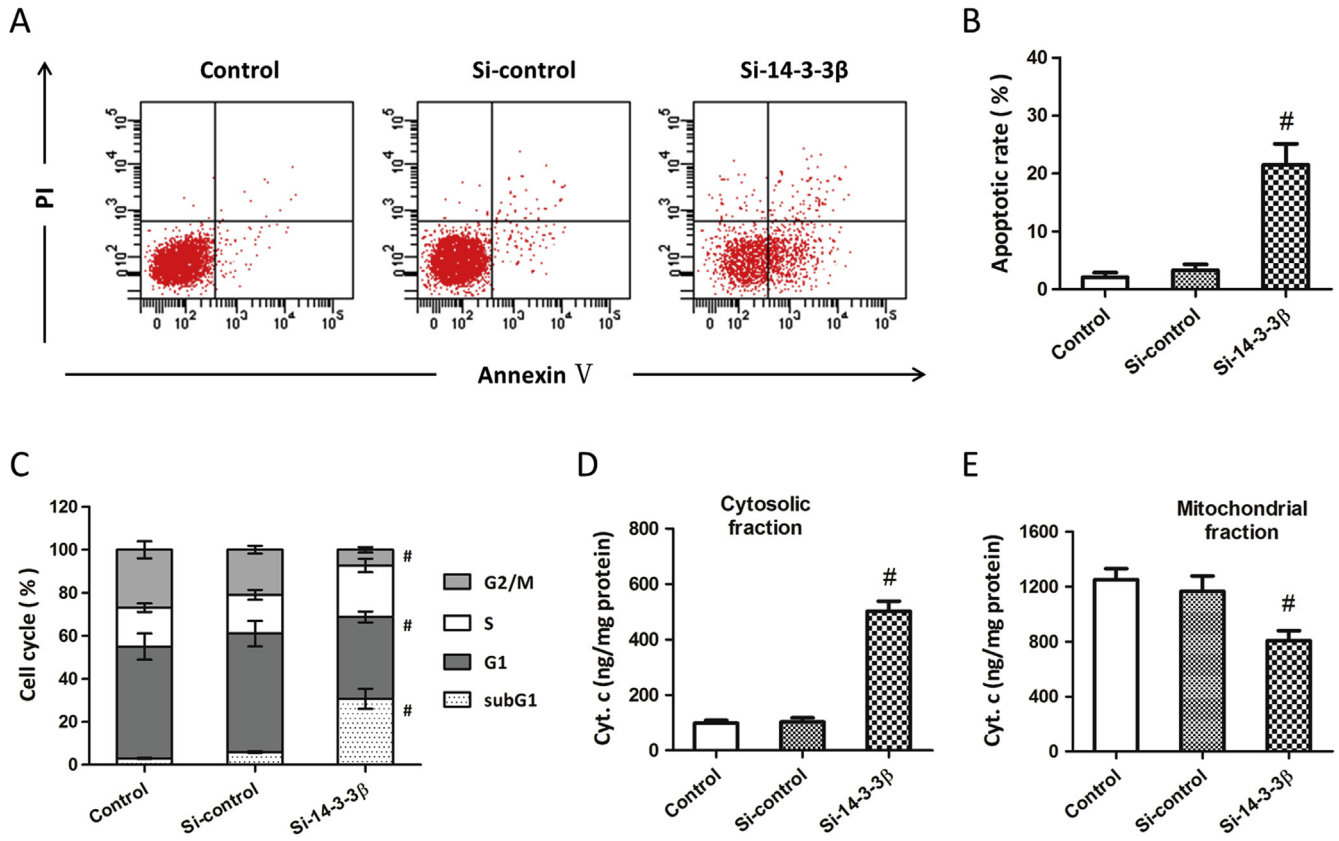

Fig. 2. Down-regulation of 14-3-3β induces apoptotic cells death in U87 cells. U87 cells were transfected with Si-14-3-3β or Si-control. The apoptotic cell death was assayed by flow cytometry (A), and the apoptotic rate was calculated (B). The percentage of cells in individual cell-cycle phases was assessed in each group (C). The mitochondrial cytochrome c release was determined by measuring cytochrome c in cytosolic (D) and mitochondrial fraction (E). Data are shown as mean ± SEM of five experiments. $^{\#}p < 0.05$ vs. Si-control.

changes of ER by ER tracker staining and observed a fluorescence intensity increase in Si-14-3-3β transfected U87 cells (Fig. 3A). We also examined the ER Ca^{2+} metabolism by measuring thapsigargin (Tg) induced changes in Ca^{2+} responses (ΔF/F) (Fig. 3B). The results showed that application of Tg produced a local Ca^{2+} transient in control and Si-control transfected cells, but a smaller Ca^{2+} transient was observed after Si-14-3-3β transfection (Fig. 3C), indicating a Ca^{2+} released from ER after 14-3-3β knockdown. As shown in Fig. 3D, Si-14-3-3β transfection significantly suppressed the expression of XBP1S mRNA. To further confirm the ER stress, we detected the expression of CHOP and caspase-4 by western blot (Fig. 3E). The results showed that the expression of CHOP and activation of caspase-4 were both significantly increased by Si-14-3-3β transfection as compared to Si-control (Fig. 3F).

3.4. CHOP–Wnt pathway contributes to 14-3-3β knockdown-induced anti-cancer effect

To explore the possible underlying molecular mechanism, β-catenin was examined in U87 cells after transfection (Fig. 4A). Our data showed that down-regulation of 14-3-3β significantly decreased the nuclear localization of β-catenin. Si-14-3-3β transfection could also decrease Topflash activity, which was shown to be reversely correlated with CHOP (Fig. 4B). Interestingly, Si-CHOP prevented the decrease of Topflash activity induced by Si-14-3-3β (Fig. 4C), suggesting that Wnt might be a target of CHOP in our in vitro conditions. As shown in Fig. 4F, the Si-14-3-3β-induced decrease in cell viability was full prevented by Si-CHOP transfection. Furthermore, to investigate the role of Wnt in Si-14-3-3β-induced anti-cancer effect, sFRP was used to inhibit Wnt. The results showed that sFRP and Si-14-3-3β had no extra effect on Wnt activation (Fig. 4D) and cell viability (Fig. 4G). To further confirm the involvement of the CHOP–Wnt pathway, Si-CHOP and sFRP were used to inhibit CHOP and Wnt, respectively. In Si-CHOP transfected U87 cells, sFRP and Si-14-3-3β had no extra effect on Wnt inhibition (Fig. 4E) and cell viability (Fig. 4H).

4. Discussion

14-3-3 proteins are highly conserved and have risen to a position of importance in cell biology owing to its involvement in many vital cellular processes [7]. They are highly expressed in most human CNS tumors but not in most human majority of normal brain tissues [16]. In our previous studies, we found that inhibition of 14-3-3 functions with its antagonist, difopein, or siRNA improves sensitivity of U251 and U87 glioma cells to apoptosis and suppresses glioma growth [17]. However, different 14-3-3 isoforms may act as oncogenes or tumor suppressors in different types of cancers. 14-3-3ζ was shown to play important roles in lung cancer cell proliferation and resistance to chemotherapy drugs [18], whereas 14-3-3σ was function as a tumor suppressor due to the frequent gene methylation that occurs in breast cancers [19]. A previous study using mouse mammary epithelial cells also showed that 14-3-3σ and 14-3-3ζ play an opposite role in cell growth inhibition mediated by TGF-β1 [20]. We previously identified 14-3-3β and η as two tumor-specific isoforms in astrocytoma, and they might be involved in astrocytoma tumorigenesis [11]. Here, we found that 14-3-3β is highly expressed in U87 cells but not human normal astrocyte SVGp12 cells. Knockdown of 14-3-3β inhibited cell proliferation through inducing cell cycle arrest and apoptosis,

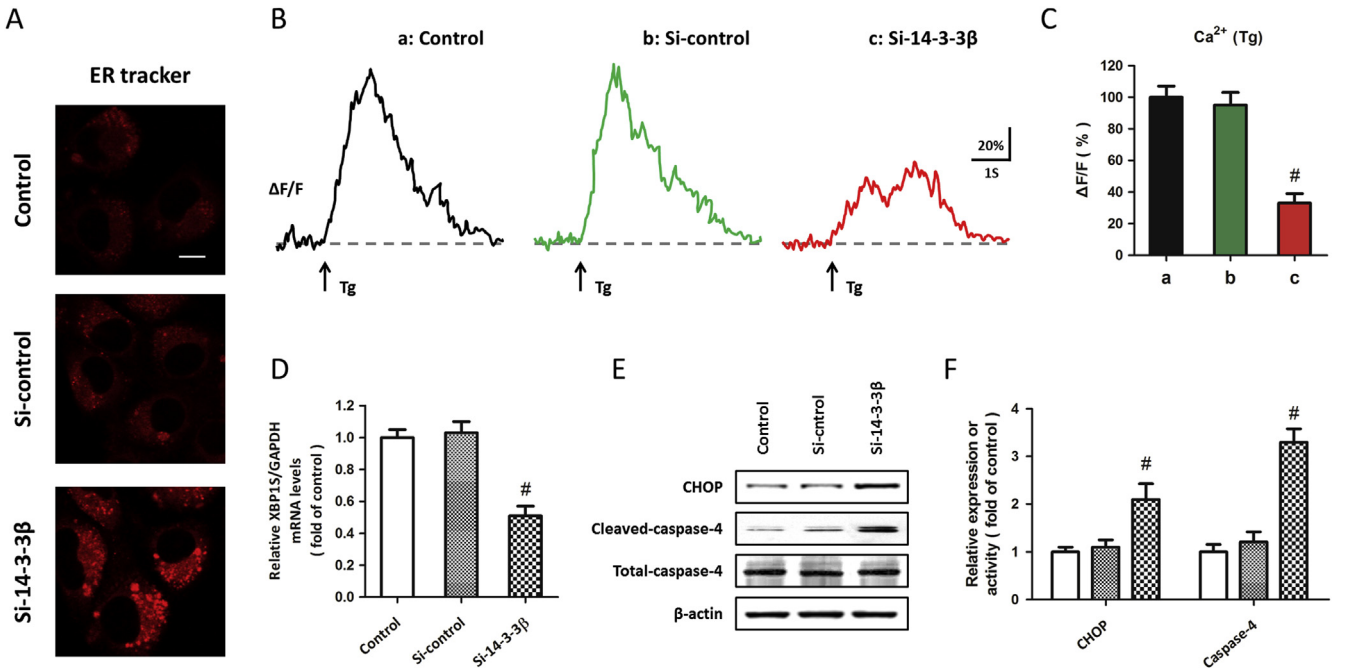

Fig. 3. Down-regulation of 14-3-3β induces ER stress in U87 cells. U87 cells were transfected with Si-14-3-3β or Si-control, and the morphological changes of ER was detected by ER tracker staining (A). Relative changes in Ca^{2+} responses (ΔF/F) to the application of thapsigargin (Tg) were detected (B and C), and the expression of XBP1S was measured by RT-PCR (D). The expression of CHOP and activation of caspase-4 was detected by western blot (E) and calculated (F). Scale bar: 10 μm. Data are shown as mean ± SEM of five experiments. $^{\#}p < 0.05$ vs. Si-control.

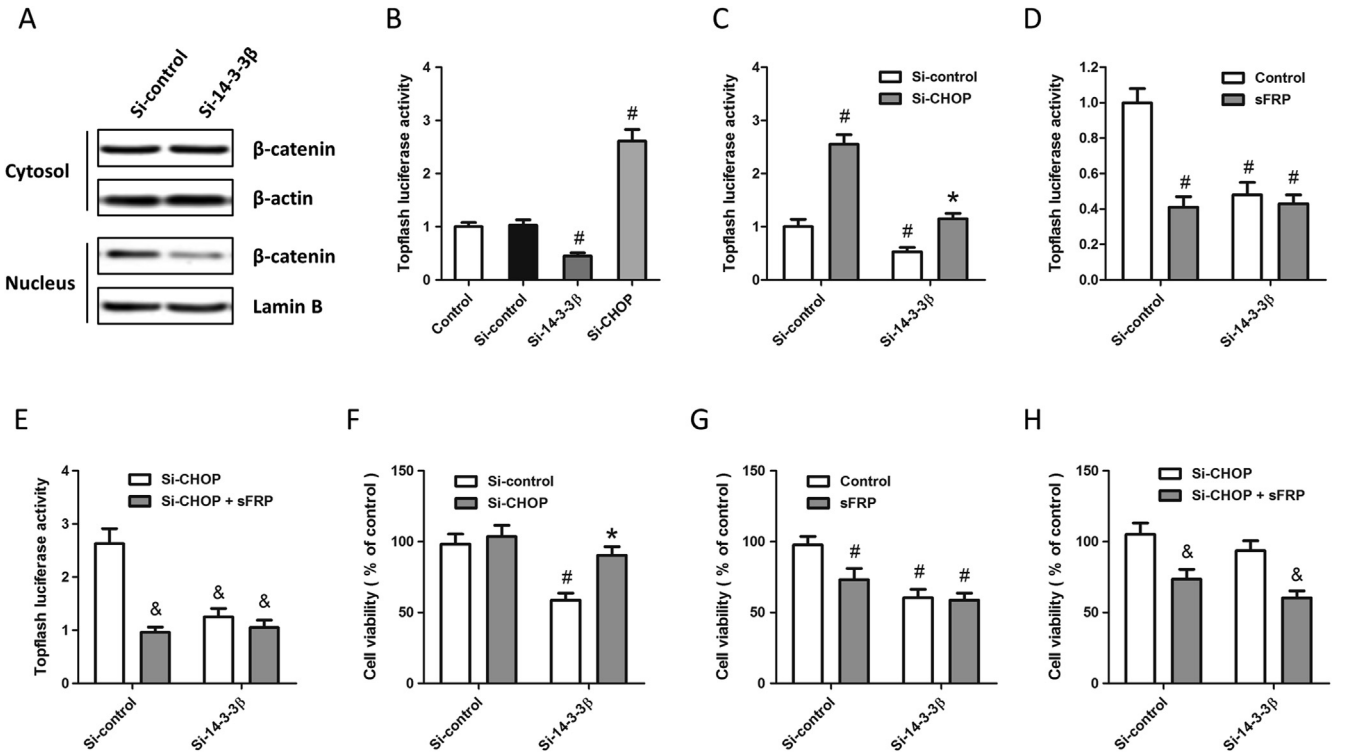

Fig. 4. CHOP–Wnt pathway contributes to 14-3-3β knockdown-induced anti-cancer effect. U87 cells were transfected with Si-14-3-3β or Si-control, and the expression of β-catenin was detected by western blot in cytosolic and nucleus fractions (A). After transfection with Si-14-3-3β or Si-CHOP, Topflash activity was measured (B). PC12 cells were transfected with Topflash and pTK-Rennila luciferase vector with or without Si-CHOP and/or sFRP before Si-14-3-3β transfection, and Topflash activity was measured (C–E). PC12 cells were transfected with Topflash and pTK-Rennila luciferase vector with or without Si-CHOP and/or sFRP before Si-14-3-3β transfection, and cell viability was measured, respectively (F–H). Data are shown as mean ± SEM of five experiments. $^{\#}p < 0.05$ vs. Si-control. $^{*}p < 0.05$ vs. Si-14-3-3β. $^{\&}p < 0.05$ vs. Si-CHOP.

which was consistent with previous reports in several other cancer cells [12,21]. These data, in accordance with previous studies, strongly suggested that targeting 14-3-3β for human astrocytoma therapy may be a promising method.

In previous studies, the investigations of apoptosis in glioma cell lines were commonly focused on cell cycle arrest, mitochondrial pathway or oxidative stress. Recently, a number of anti-cancer therapies have been associated with the induction of ER stress in tumor cells [22]. Under a variety of endogenous and exogenous insults conditions, ER stress is initially shaped to re-establish ER homeostasis through the activation of an intracellular signal transduction pathway termed as unfolded protein response (UPR) [15]. The UPR consists of a complex interplay between three signaling "arms", pancreatic ER kinase (PKR)-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). All these signaling cascades are shown to be differently dys-regulated in cancer patients, and might contribute to anti-apoptotic activity and drug resistance of cancer cells [23]. Indeed, the past few years have witnessed the exciting development and testing of novel anti-cancer agents that take advantage of this, such as proteasome inhibitors, Brefeldin A and Hsp90 inhibitors [24]. In the present study, 14-3-3β knockdown was shown to induce morphological changes in ER lumen and promote ER calcium release. Moreover, Si-14-3-3β transfection also elevated the protein level of CHOP, the cleavage of caspase-4, and XBP1S mRNA expression, which has a diverse range of target genes that associated with apoptotic pathway [25]. When the ER stress is too severe or prolonged to repair, the stressed cell will initiate apoptosis through several signaling cascades, including CHOP and the ER-resident caspase-4, which in turn leads to the activation of caspase-3 [26]. The damage of ER calcium homeostasis and expression of ER stress related factors observed here might contribute to the cell cycle arrest and mitochondrial cytochrome c release in our in vitro model. These findings suggest that 14-3-3β knockdown induces apoptosis via ER stress and activates the UPR branches in U87 glioma cells.

Actually, the molecular mechanisms activated by ER stress in cancer cells are not straightforward and involve signaling pathways with dualistic function in cell survival and death [24,27]. The results of the present study, for the first time, support a model in glioma U87 cells whereby 14-3-3β knockdown increases the expression of CHOP, which is required for counteracting the function of pro-survival Wnt/β-catenin pathway. CHOP, also known as GADD153, is a protein of the C/EBP family of transcriptional regulators, which induces growth arrest and apoptosis after ER stress or DNA damage [28]. Under ER stress conditions, CHOP can promote transcription of Bim while suppressing the induction of Bcl-2 [29]. Our present study extended its pro-apoptotic mechanism to the inhibitory effects on Wnt/β-catenin pathway in glioma cells. Wnt is an essential cell protective mediator that functions as an important effector in the anti-apoptotic signaling [30]. It binds to the cell membrane receptors called the frizzled family via trans-membrane protein LRP5/6, and thereby accumulates β-catenin to translocate to the nucleus to regulate transcription of multiple genes involved in cellular proliferation, differentiation, survival, and apoptosis [30]. Wnt/β-catenin pathway has recently been identified to accumulate oncogenic activities in glioma proliferation, apoptosis inhibition and invasion [31]. In line with this, we found that inhibition of Wnt via sFRP significantly decreased cell proliferation in U87 cells. Canonical Wnt signaling (β-catenin dependent) regulates a wide set of signaling cascades in cancer cells, but its upstream regulation mechanism in glioma cells are not fully elucidated. In the present study, Si-14-3-3β transfection was shown to attenuate the preferential nuclear accumulation of β-catenin, the hallmark of activated Wnt signaling. These effects were also confirmed by Topflash luciferase activity assay, and all these regulations were demonstrated to be mediated by CHOP. In a previous study using *Xenopus laevis* embryos, CHOP was found to function as a specific inhibitor of Wnt/TCF signaling [32]. More recently, 14-3-3β was shown to regulate the proliferation of glioma cells through GSK3β/β-catenin pathway [12]. It is tempting to speculate that the antagonistic effect of CHOP on the Wnt pathway might be part of molecular mechanisms underlying 14-3-3β knockdown induced anti-cancer activity.

In conclusion, this study identifies 14-3-3β knockdown as a growth arrest strategy in human glioma U87 cells, and delineates a novel mechanism for this anti-cancer effect. Down-regulation of 14-3-3β induces ER stress through CHOP activation, and thereby inhibits pro-survival Wnt/β-catenin pathway resulting in apoptotic cell death.

Conflict of interest

None.

Acknowledgments

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