Pathology - Research and Practice 215 (2019) 600–606

Contents lists available at ScienceDirect

Pathology - Research and Practice

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

Molecular mechanism of SSFA2 deletion inhibiting cell proliferation and promoting cell apoptosis in glioma

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ARTICLE INFO

Keywords:
SSFA2
Glioma
Cell proliferation
Cell cycle
Apoptosis

ABSTRACT

Gliomas are the most common primary brain malignant tumors in humans. Glioblastoma multiforme(GBM) is the most malignant intracranial tumor with a relatively poor prognosis. There promote us to find effective anti-cancer therapies to reduce cancer mortality. By using bioinformatic analysis, we found SSFA2 as a gene with elevated expression in the glioma tissues. We detected the expression of SSFA2 in glioma tissues and in the glioma cell lines, as well as in normal brain tissues. SSFA2 expression was higher in glioma tissues, especially in glioblastoma multiforme than normal brain tissues. Subsequently, we found that down-regulate SSFA2 in glioma cell lines can regulate the cell cycle to reduce the proliferation ability and induce the early apoptosis rate in shSSFA2 cells relative to control cells. Moreover, we found that down-regulate SSFA2 in glioma cell line U87(shSSFA2-U87) inhibited the growth effectiveness compared to the control cell line U87. These result reveals us that SSFA2 may act as oncogene to promote the progression of glioma. For further research specific mechanisms of SSFA2 in gliomas, we used the gene chip to detect the downstream gene in U87. We found that 30 genes also may be as target gene of SSFA2, and we testify the protein expression by western-blot. The result reveal that IL1A, IL1B and CDK6 as target gene of SSFA2 to regulate the progression of glioma. These finding suggest that SSFA2 could be a new therapeutic target for gliomas.

1. Introduction

Gliomas are the most common primary brain malignant tumors in humans, accounted for 40%–50% of brain tumors. Now, the treatment of glioma has always been a clinical problem [1]. Glioblastoma multiforme (GBM) are diffuse, highly invasive tumors with poor prognosis [2]. Despite take comprehensive treatment measures, including surgery, radiotherapy and chemotherapy, the prognosis of patients has not significantly improved and the median survival time is only 12–15 months [3,4]. In recent years, many genetic changes related to glioma progression have been discovered. However, the molecular mechanisms underlying glioma progression remain largely unknown.

SSFA2, also name as KRAP, (Ki-ras-induced actin-interacting protein). The human KRAP gene was originally identified as one of the genes whose expression level was up-regulated by activated KRAS in human colon cancer HCT116 cells [5,6]. In recent years, more and more research reveal that SSFA2 may as a potential target for cancer. For example, SSFA2 is associated with liver-preferential metastasis of small cell lung cancer [7]; p38 MAPK inhibition can down-regulated the expression of SSFA2 to promote the progression of lymphoma;and SSFA2 can affect the proliferative phenotype in chronic lymphocytic leukemia [8]. In addition, the other data suggest that SSFA2 is associated with the development of malignancy in cancers [9,10]. However, the regulatory mechanisms and the significance of the changes in the expression of SSFA2 are important in determining glioma phenotypes are not clear.

Recently, we previously check The Cancer Genome Atlas (TCGA), found that SSFA2 was higher expression in Glioblastoma multiforme (GBM) than normal brain tissues. We explored the mechanisms of SSFA2 dysregulation in GBM and compared its prognostic value in glioma patients. In addition, we decipher the molecular mechanism of SSFA2 in vitro and in vivo.

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https://doi.org/10.1016/j.prp.2018.12.035
Received 3 September 2018; Received in revised form 9 December 2018; Accepted 30 December 2018

2. Materials and methods

2.1. Bioinformatic analysis using GEPIA

ECT2 expression in some solid tumors and in corresponding normal tissues was analyzed by using data from the Cancer Genome Atlas (TCGA). Data analysis was performed by using GEPIA (http://gepia.cancer-pku.cn/detail.php), which provides access to analyze data generated by TCGA [11].

2.2. Patient and sample collection

Normal brain tissues (6 patients) were harvested from patients with craniocerebral injuries. These brain tissues were partially resected to reduce intracranial pressure based on decompression treatment guidelines. Human brain glioma tissue specimens 30 glioma patients (LGG:18; GBM:12), there were 16 male patients and 14 female, mean age at the time of surgery was 50.6 years. Tissue samples were obtained from January 2015 through July 2018 from the Department of Neurosurgery of the First Affiliated Hospital of Soochow University. All tissue samples were immediately collected and stored in liquid nitrogen after resection from patients and then used for analysis. Informed consent was obtained from the patient or patient family members and the study was approved by the local ethics committee of our hospital.

2.3. Cell culture

Glioma cell lines U87 and U251 was purchased from the Cell Bank of Chinese Academy of Science (shanghai). U87 and 251 was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (FBS, Gibco) at 37℃ and humidified atmosphere of 5% CO2.

2.4. Real time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted using the Trizol reagent method. Reverse transcription in 20 μL was preformed following the Applied Bio-systems protocol. Primers were synthesized by Sangon Biotech (shanghai). SYBR Green I qRT-PCR kit (Applied LightCycler480) was used to analyze the mRNA expression levels of SSFA2. The expression of GAPDH was used as the endogenous control. Primer sequences are as follows, SSFA2:F-GACGTGATGAACCAGATATTGCT,R-TTGACGAAAACGGCTTGTTAAAG;GAPDH:F-CAGGAGGCATTGCTGATGAT,R-GAAGGCTGGGGCTCATTT. The relative expression of mRNA was calculated using the comparative threshold cycle (Ct) ($2^{-\Delta\Delta Ct}$) method. Samples were analyzed in triplicate.

2.5. Lentiviral vector construction and transfection

The lentiviral vector(HIV-1) with down-regulate SSFA was designed by Genechem Co.,Ltd (Shanghai China). Transfection was performed following the manufacturer's protocol.

2.6. Cell proliferation, cell cycle, and cell apoptosis

The cell proliferation was measured with the MTT assay. Briefly, U87 and U251 cells were seeded into 96-well plates (3000 cells/well) and incubated overnight at 37 ℃, then treated with 0, 6, 12, 25, 50 and 100 μmol/L compounds for 24, 48 and 72 h. Next, 20 μL MTT solution (5 mg/mL) was added into each well and incubated for another 4 h, followed by media removal and solubilization in 200 μL DMSO. The absorbance value was determined at 570 nm using a microplate reader (Bio-Tek, Winooski, VT, USA). Three independent experiments were carried out.

Absorbance values were measured at a wavelength of 450 nm. Cell cycle and cell apoptosis were analyzed by Flow cytometer (CY TOMICS, FC, 500 Beckman-coulter, CA, USA). Transfected cells were fixed in 75% ethanol at 4 ℃ and stained with propidium iodide (PI), and analyzed by Flow cytometer for Cell cycle. Cells were collected and washed with ice-cold PBS. To analyze the cell apoptosis, cells were resuspended in 100 μl binding buffer containing 5 μl of 7-AAD (7-amino-actinomycin D) and 5 μl of PI at room temperature in the dark for 10–15 min, and detected using the Annexin V-PE Apoptosis Detection Kit PE (eBioscience, San Diego, CA, USA) and flow cytometry. All experiments were performed in triplicate.

2.7. Western blotting

Total protein was extracted from cells using RIPA buffer and protein was quantified using the BCA (bicinchoninic acid) assay kit (Beyotime, Shanghai, China). Protein samples were separated using 10% SDS-PAGE gel and then transferred onto PVDF membranes. PVDF membranes were incubated with the relevant primary antibodies overnight at 4 ℃. Membranes were then washed and incubated for 2 h with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (Prosci Inc.1:3,000; Poway, CA, USA), followed by detection and visualization using ECL Western blotting detection reagents (Pierce antibodies, Thermo Fisher, USA).

2.8. Xenograft tumor models and hematoxylin-eosin staining

All animal experiments were carried out in accordance with institutional guidelines and regulations of the institute. Female BALB/c nude mice were purchased from the China Academy of Sciences (Shanghai) and randomly divided into two groups(shSSFA group and shCtrl group) and 10 mice per group. Glioma cell line U87 were counted and then re-suspended to 1×10^5 cells/μL using PBS. The cells were then subcutaneously implanted into right forelimb armpit(the injection volume is 10ul per mice). Mice were observed after injection to monitor the size and the weight of subcutaneous xenograft tumor. Hematoxylin-eosin staining was accorded to the description of the HE staining kit(BBI Life Sciences;Number：E607318).

2.9. GeneChip

Total RNA was isolated from 3 independent scrambled siRNA and shSSFA2 transfected U87MG cells and hybridized to Affymetrix U133Plus 2.0 gene chip. The results were analyzed initially using GeneChip operating software and the data were subsequently processed using ArrayAssist (Agilent) to statistically analyze changes in gene expression.

2.10. Statistical analysis

Statistical analyses were carried out by using SPSS version 18.0 statistical software program (SPSS, Chicago, IL, USA). All results were expressed as the mean ± standard deviation (SD). The data were analyzed using two-tailed Student's *t*-test and ANOVA. Data were considered statistically significant at an alpha value of $P < 0.05$.

3. Results

3.1. Expression of SSFA2 is higher in GBM compared to normal brain tissues

To testify the expression of SSFA2 in GBM patients, we checked the expression of SSFA2 from TCGA. The result showed that SSFA2 expression was higher in GBM tissues than normal brain tissues (Fig. 1a). For further research whether expression of SSFA2 could be implicated as a predictor in glioma patients prognosis. We found that patients with high levels of SSFA2 were associated with a significantly shorter overall

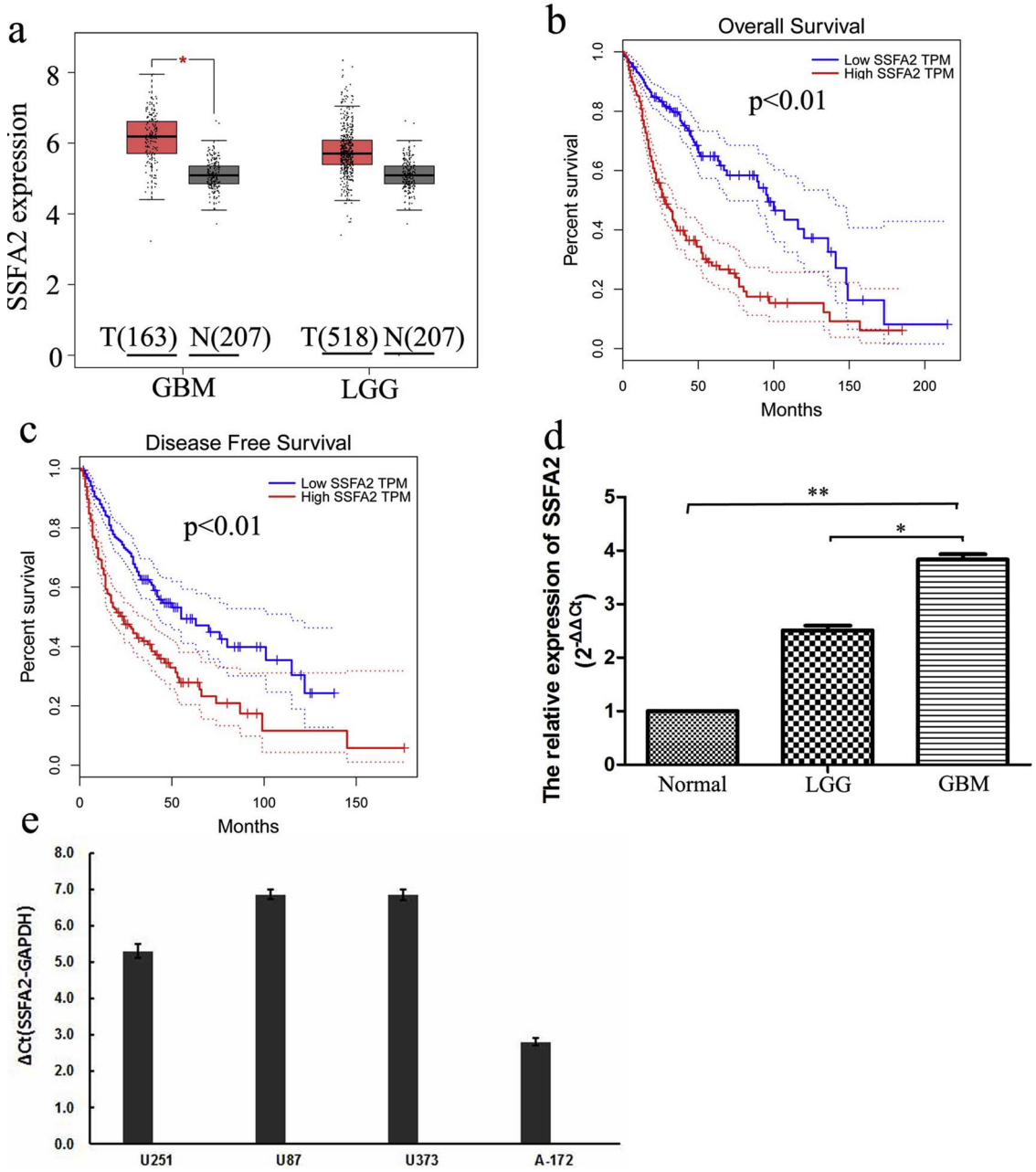

Fig. 1. Expression of SSFA2 in GBM. a:SSFA2 expression was higher in GBM tissues than normal brain tissues (*p < 0.05). b,c:SSFA2 were associated with overall survival time and disease free survival(p < 0.01). d:SSFA2 expression was higher in glioma tissues than normal brain tissues by RT-qPCR (*p < 0.05;**p < 0.01). e:SSFA2 was relatively high abundance in glioma cell lines compared to GAPDH.

survival time and disease free survival (Fig. 1b,c). In addition, real time quantitative polymerase chain reaction (RT-qPCR) was used to measure the expression of SSFA2 in glioma patients, glioma cell lines (U87,U251,U373,A172) and normal brain tissues. SSFA2 expression in glioma tissues was higher compared to normal brain tissues (p < 0.01; Fig. 1d) and relatively high abundance in glioma cell lines (Fig. 1e). This suggested that SSFA2 may act as oncogene to promote the progression of GBM.

3.2. Transfection efficiency and RNA interference of SSFA2

Here, we constructed a stable down-expression of SSFA2 in glioma cell lines U251 and U87 by lentiviral vector with siRNA(The siRNA sequence:ATAATTCCAGTATTACACAGC). We detected transfection efficiency using fluorescence microscopy and used RT-qPCR to determine the expression level of SSFA2 in si-SSFA2 U251 and si-SSFA2 U87. The expression level of SSFA2 were significantly lower than in negative group and untreated glioma cell lines (p < 0.01; Fig. 2a). Down-regulation of SSFA2 was also observed at the protein level by western-blot (Fig. 2b).

3.3. SSFA2 regulated cell proliferation and cell cycle

Next, we assessed the effect of SSFA2 expression on the regulation of glioma cell viability. MTT assay showed that relative proliferative capacity of shSSFA2 cells grew significantly slower at 96 and 120 h relative to shCtrl cells (P < 0.05; Fig. 2c,d). The cell cycle of glioma cells to measured by flow cytometer, the result showed that shSSFA2 induced more cells to rest on G1/G2 phase and fewer cells in the S-phase than the negative control groups and blank groups without treatment

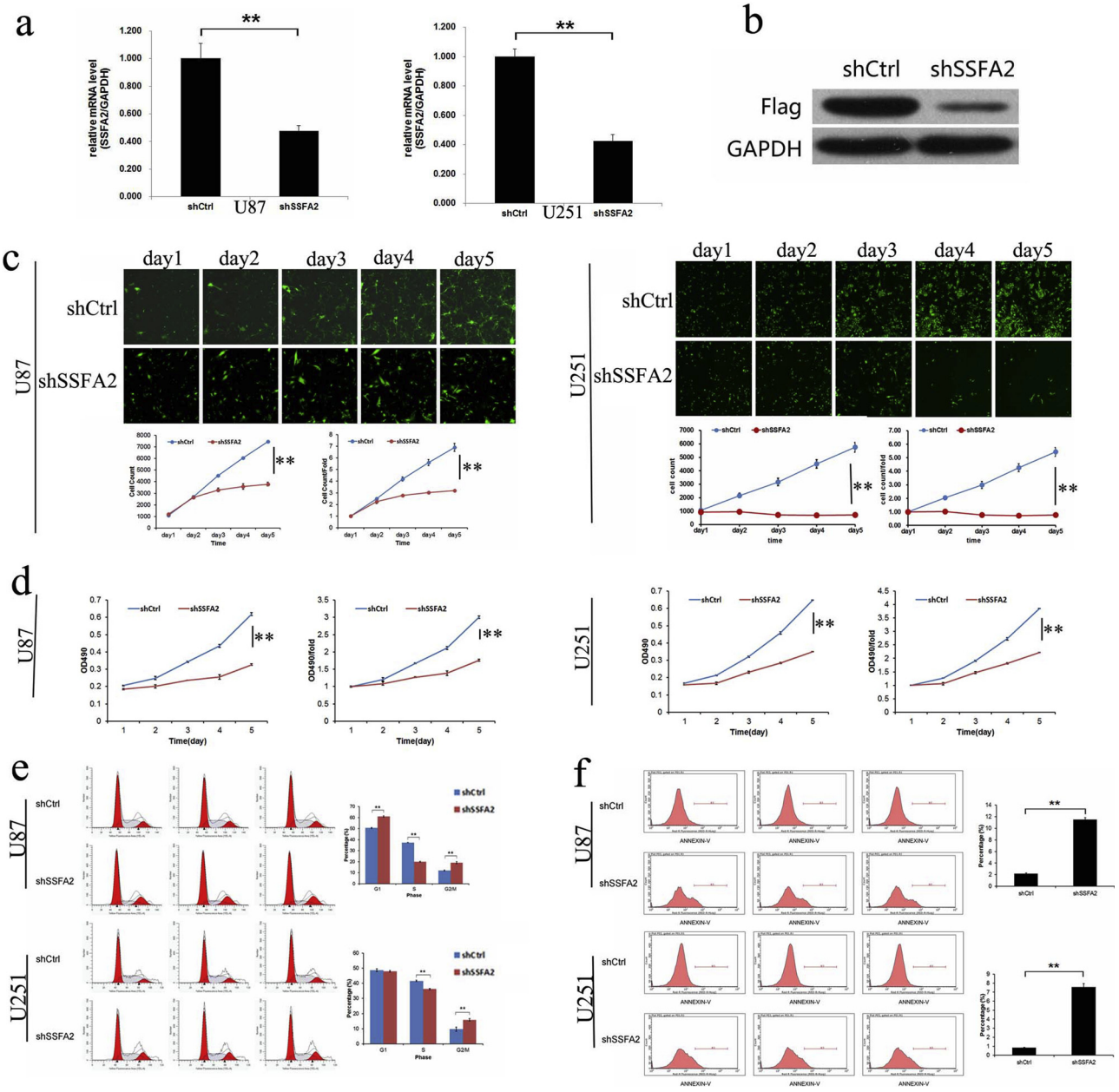

Fig. 2. Transfection efficiency and SSFA2 regulated cell proliferation,cell cycle, cells apoptosis. a,b:The expression level of SSFA2 were significantly lower than in negative group and untreated glioma cell lines. c,d:relative proliferative capacity of shSSFA2 cells grew significantly slower at 96 and 120 h relative to shCtrl cells (**p < 0.01). e:shSSFA2 induced more cells to rest on G1/G2 phase and fewer cells in the S-phase than the negative control groups and blank groups without treatment. f:shSSFA2 can induce the early apoptosis in glioma cell lines (*p < 0.05;**p < 0.01).

(Fig. 2e).

3.4. ShSSFA2 induced cells apoptosis

We used the flow cytometer system to assess the effect of shSSFA2 on the apoptosis of glioma cell lines. The result suggest that there was a significant increase in the early apoptosis rate in shSSFA2 cells relative to control cells (Fig. 2f).

3.5. ShSSFA2 reduces glioma growth in subcutaneous xenograft tumor models

To investigate the effect of shSSFA2 in tumorigenesis, we established a subcutaneous xenograft tumor model (Fig. 3a). There show that shSSFA2 reduced the size and the weight of tumors compared with the control groups ($p < 0.05$; Fig. 3c,d). In addition, we used the hematoxylin-eosin staining to observe the shape of the subcutaneous xenograft tumor cells (Fig. 3b).

3.6. RNA interference of SSFA2 about gene expression profiling

Global gene expression profile of U87MG cells after mock transfection or transient silencing of SSFA2 was determined by using human U133 plus 2 array from Affymetrix (Fig. 4a,b,c). We used the qPCR to measured the gene expression, fortunately, we obtained many variant gene in shSSFA2 U87 compared to negative control. Subsequently, IPA(Ingenuity Pathway Analysis) was used to analysis the result of gene expression profile, there showed that NLRP12 is predicted to be strongly activated, there are 10 genes that are uniformly activated in this gene, and IL2 is predicted to be strongly inhibited, and there are 29

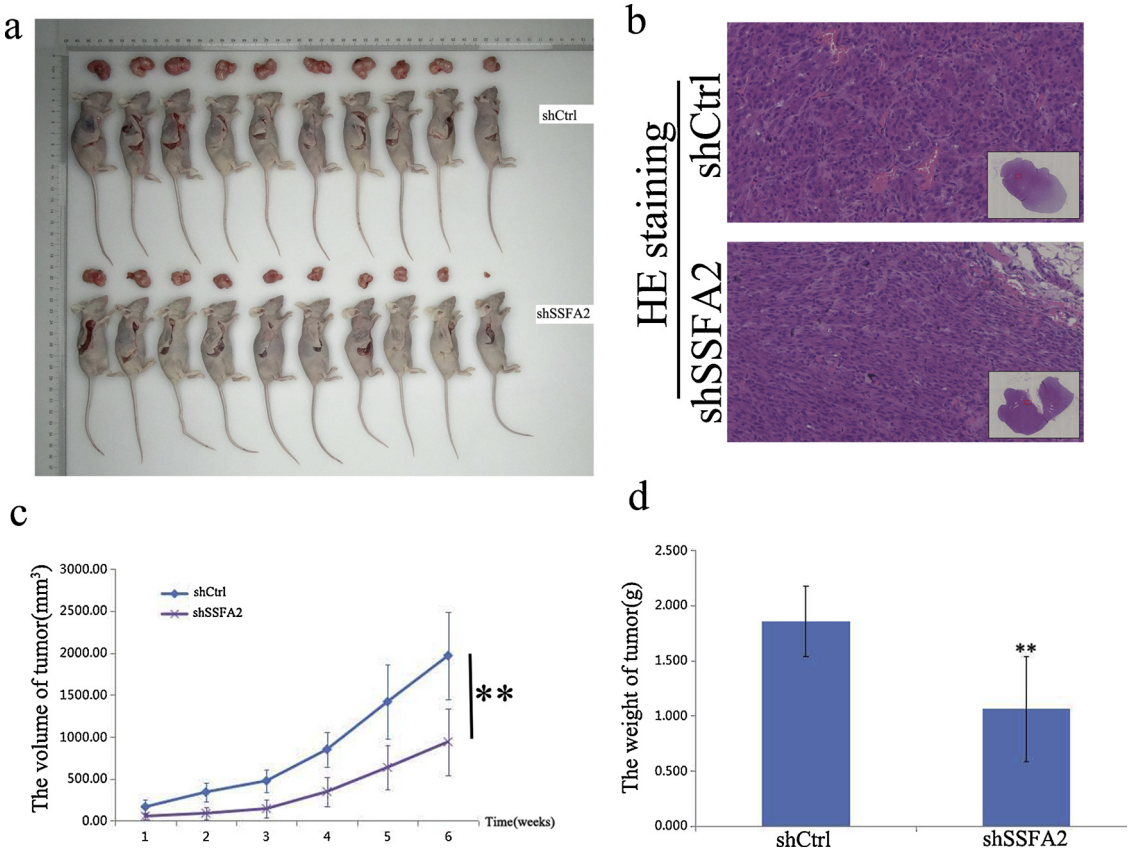

Fig. 3. ShSSFA2 reduces glioma growth in subcutaneous xenograft tumor models. a:This showed subcutaneous xenograft tumor model. b:The the hematoxylin-eosin staining showed the shape of the subcutaneous xenograft tumor cells. c,d:shSSFA2 reduced the size and the weight of tumors compared with the control groups (**$p < 0.05$).

genes that are uniformly inhibited by this gene (Fig. 4d). In addition, the gene interaction network map shows a network of interactions between molecules within a defined functional region (Fig. 4e,f). Finally, we detected the expression of related proteins by western-blot, the result showed that IL1A protein was down-regulated by 76%, IL1B protein was down-regulated by 54%, and CDK6 protein was down-regulated significantly (Fig. 4g). It is speculated that the target gene SSFA2 regulates the progression of glioma by regulating genes such as IL1A, IL1B and CDK6.

4. Discussion

Glioblastoma(GBM) is the most malignant glioma in astrocytic tumors. Although tremendous improvements have been made to treat this cancer, such as surgical resection, radiotherapy and chemotherapy, the overall outcome of patients with glioma is disappointing. Due to the insensitivity of glioblastoma to radiotherapy and chemotherapy, gene therapy has received an important role in malignant tumors in recent years. However, the choice of the ideal target is still the focus of researchers. Several studies have found that some effective target in glioblastoma, such as EGFR, BRAF, KPNB1 [12,13,14].

SSFA2 also name as KRAP, have been founded that play an important role in malignant tumors [15–17]. SSFA2 might be a cytoskeleton-associated protein involving the structural integrity and/or signal transductions in human cancers [18]. Inokuchi et al. found that KRAP might be involved in the regulation of filamentous actin and signals from the outside of the cells [6].

Previous studies have shown that the deletion of SSFA2 can lead to a decrease in mouse IGF1 protein levels [19]. IGF (insulin-like growth factors) is an active protein polypeptide substance necessary for the physiological action of growth hormone, and it is known to include both IGF1 and IGF2. IGF can exert its biological effects as an extracellular ligand by binding to receptors on the cell membrane through various secretory modes, including IGF-1R, IGF-2R and the like. Among them, IGF-1R mediates many biological reactions of IGF-1 and IGF-2. IGF-1R belongs to the tyrosine protein kinase family and is widely expressed on many types of cell surfaces. Free IGF binds to IGF-1R, resulting in autophosphorylation of the tyrosine of the receptor beta subunit, triggering multiple downstream signaling activation reactions. The current research includes the PI3K/AKT pathway and the Ras/MAPK pathway, which can stimulate cell proliferation and inhibit apoptosis. Among them, the PI3K/AKT pathway is particularly important in this process [20,21]. Combined with the experimental data of this project, the deletion of SSFA2 may inhibit the proliferation and metastasis of tumor cells by down-regulating the expression level of IGF1 and inhibiting the signal transduction of a series of downstream signaling molecules such as PI3K/AKT and Ras/MAPK.

In our study, we found that SSFA2 was an upregulation in primary GBM. We analysis the data from TCGA and used the qPCR measured the SSFA2 expression, the result suggest that SSFA2 may act as oncogene in glioma. There prompted us to undertake approach to identify specific impact on glioma in vitro. Subsequently, we examined its effect on the proliferation of glioma cells. The result reveal that down-regulation of SSFA2 inhibits proliferation of glioma cells. In addition, the result from flow cytometer on apoptosis assay suggested that the cells group with shSSFA2 had higher apoptosis rates as compared with empty vector or untreated cells, which suggested that apoptosis was obviously influenced by SSFA2 expression. Moreover, shSSFA2 can inhibited the growth efficiency in vivo. All in all, our result demonstrated that SSFA2 may be used as a potential therapeutic target gene in the treatment of glioma.

All the result promote us to undertake an approach to understand

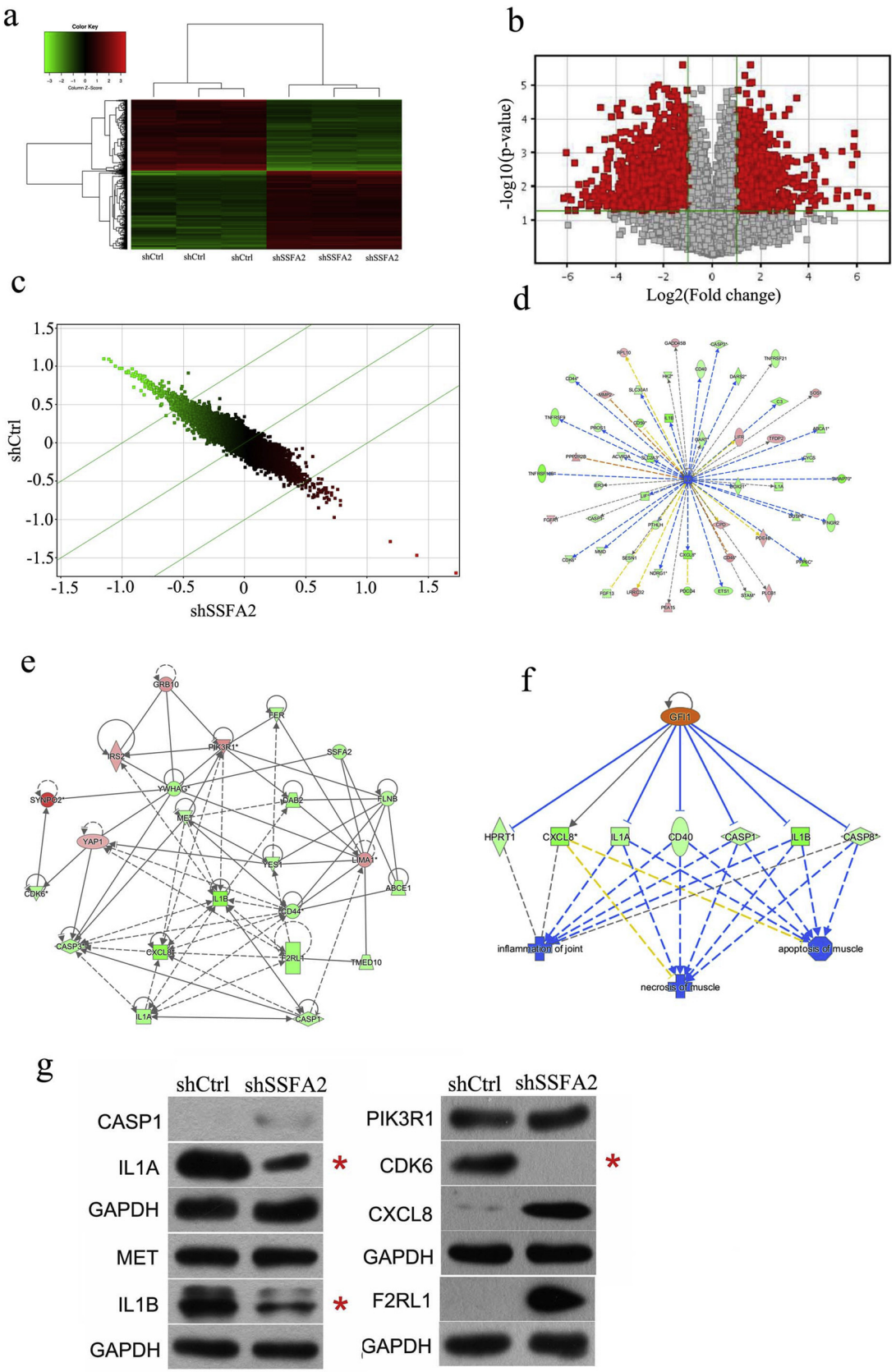

Fig. 4. Gene chip and the gene interaction network map. a:The heat map shows the aggregation of all samples and differential genes at the expression level. b:The volcano map shows the differential gene distribution between the experimental and control groups. c:The scatter plot shows the distribution of signal values between the experimental and control groups on the Cartesian coordinate plane. d:The upstream regulatory subnetwork map shows the interaction between upstream regulatory factors and downstream molecules that are directly related to and present in the data set. e:The regulatory effect network map shows the interaction between genes and regulators and functions in the dataset. f:The gene interaction network map shows a network of interactions between molecules within a defined functional region. g:The protein expression of SSFA2 in glioma cell line.

the variant gene that are intimately influenced by SSFA2. For further research the molecular mechanism of SSFA2 in glioma, we used the gene chip to measure the target gene about SSFA2 and used the qPCR to verify the gene expression. We found that 30 genes also may be as target gene of SSFA2, and we testify the protein expression by western-blot. The result reveal that IL1A, IL1B and CDK6 as taget gene of SSFA2 to regulate the progression of glioma.

Down-regulation of SSFA2 can reduce the ability of proliferation and induce the cell early apoptosis by regulating IL1A, IL1B and CDK6 in glioma cell lines. All the experiments promote us that SSFA2 was a oncogene in the malignant processes and carcinogenesis of gliomas and may be used to develop a potential therapeutic target in glioma therapy.

Conflict of interest

All authors have no conflict of interest.

Acknowledgments

We thank our central lab for providing technical instruction and assistance. We thank Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) for offering us glioma cells line. We thank The First Affiliated Hospital of Soochow University for providing us with glioma samples.

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