LABORATORY INVESTIGATION

Heat shock protein 47 regulated by miR-29a to enhance glioma tumor growth and invasion

Dan Zhao · Xiaochun Jiang · Chengyun Yao · Li Zhang · Huixiang Liu · Hongping Xia · Yongsheng Wang

Abstract Heat shock protein 47 (HSP47), also known as SERPINH1, is a product of CBP2 gene located at chromosome 11q13.5, a region frequently amplified in human cancers. HSP47 has been demonstrated to effect on limiting tumor invasion and motility. The previous studies showed that HSP47 is overexpressed in many human cancers, including stomach cancer, lung cancer, pancreatic ductal adenocarcinoma, and ulcerative colitis-associated carcinomas. However, the role of HSP47 in human glioma is still unknown. Here, we examined the expression of HSP47 in a group of glioma tumors and matched non-tumor brain tissues using qRT-PCR. We found that HSP47 is significantly overexpressed in glioma tissues and cell lines and associated with glioma tumor grade. Next, we knockdown the expression of HSP47 in the glioma cells using small interfering RNAs. The result showed that knockdown of HSP47 inhibits glioma cell growth, migration and invasion in vitro. We further investigated the posttranscriptional regulation of HSP47 by microRNAs using bioinformatics analysis and experimental validation. The results suggested that the expression of HSP47 is regulated by miR-29a. Finally, stable knockdown of HSP47 using shRNA inhibits glioma tumor growth and induces apoptosis in mice models in vivo. Therefore, our data suggested that HSP47 regulated by miR-29a to enhance glioma tumor growth and invasion. Taken together, HSP47 plays important role in tumor growth and invasion and thus could be a therapeutic target for treating glioma in the future.

Keywords Heat shock protein 47 · MicroRNA · Glioma · Cell growth · Invasion

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Introduction

Glioma is the most common primary malignant brain tumor and glioblastoma multiforme (GBM) is the most common glioma arising in adults and accounts for approximately 75 % of all newly diagnosed glioma cases. Gliomas display histological similarities to glial cells, including astrocytes and oligodendrocytes. Malignant gliomas can be classified as astrocytomas, oligodendrogliomas, or oligoastrocytomas based on the cell types. According to the 2007 World Health Organization (WHO) classification, gliomas can be categorized to low-grade gliomas (WHO grade I and II) and high-grade (WHO grade III and IV). More than half of gliomas are GBM (WHO grade IV astrocytoma) [1]. Recent genetic studies have

identified a number of recurrent chromosomal abnormalities and genetic alterations in malignant gliomas, particularly in GBM [2, 3]. Currently, maximum safe optimal surgical resection followed by adjuvant partial brain radiotherapy with concurrent temozolomide and subsequent continuation of temozolomide for 6 cycles is considered as standard treatment approach for patients with GBM [4]. However, the overall survival in GBM is usually only around 12 months and the overall 5 years survival rate of GBM remains <5 % [5]. This dismal clinical outcome makes glioma an urgent subject of cancer research for identification of novel factors associated with glioma development.

Heat shock protein 47 (HSP47), also known as SERPINH1, is a member of the serpin superfamily of serine proteinase inhibitors which serve as a human chaperone protein for collagen. HSP47 has been shown to specifically and transiently bind to procollagen, and considered to play important role in collagens maturation and function [6]. Expression of HSP47 has been demonstrated to be up-regulated in lesions of fibrotic diseases such as systemic sclerosis, renal scaring and idiopathic lung fibrosis [7]. However, except the role in fibrosis, few studies have showed the role of HSP47 in liver cancer invasion and metastasis which may be associated with collagens formation and stromal fibrosis. HSP47 is a product of CBP2 gene located at chromosome 11q13.5, a region frequently amplified in human cancers [8]. HSP47 has been demonstrated to effect on limiting tumor invasion and motility. The previous studies showed that HSP47 is overexpressed in many human cancers, including stomach cancer [7], lung cancer [9], pancreatic ductal adenocarcinoma [10], and ulcerative colitis-associated carcinomas [11]. However, the role of HSP47 in human glioma is still unknown. Here, we examined the expression of HSP47 in a group of glioma tumor and non-tumor brain tissues and glioma cell lines. We further investigated the role of HSP47 in glioma cell growth and invasion in vitro and in vivo. Our data suggested that HSP47 plays important role in tumor growth and invasion and thus could be a therapeutic target of treating glioma in the future.

Materials and methods

Cell lines and samples

Human glioma or glial cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in recommended medium with 10 % FBS and 100 units/mL of penicillin and 100 μg/mL of streptomycin (GIBCO, Grand Island, NY, USA). The glioma samples used in this study were collected and proved by the Committees for Ethical Review of Research Involving Human Subjects at the Nanjing University and Wannan Medical College. None of these patients received preoperative chemotherapy or radiotherapy.

Real-time quantitative reverse transcription PCR (RT-qPCR)

Total RNA from glioma tissues and cell lines was extracted using TRIzol reagent (Invitrogen, CA). The concentration of isolated total RNA was measured by NanoDrop ND-1000 Spectrophotometer (Agilent, CA). For mRNA detection, the total RNA was reversely transcribed by using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, CA). The qPCR were performed by using SsoFast^{TM} EvaGreenH Supermix (Bio-Rad). Amplification was done on a Bio-Rad CFX96 system in 20 uL. The HPRT1 internal control was used as an endogenous control, and fold changes were calculated via relative quantification ($2^{-\Delta Ct}$) [12].

MTS assays

The glioma cells were transfected with MISSION$^{®}$ pre-designed HSP47 siRNA (siHSP47) or MISSION$^{®}$ siRNA Universal Negative Control (siControl) (Sigma) using Lipofectamine RNAiMAX (Invitrogen, life Technologies, Carlsbad, CA, USA). 24 h after transfection, transfected cells were seeded into 96-well plates at a density of 5×10^3 per well (100 μL). For the MTS assay, the CellTiter 96$^{®}$ AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) was used following the manufacturer's instruction. Briefly, at 2 h before each of the desired time points (24, 48, 72 h), 20 μL of the MTS reagent was added into each well and cells were incubated at 37 °C for around 2 h. The absorbance was detected at 490 nm using a Wallac Victor 1420 Multilabel plate reader. All the experiment was repeated three times.

Wound healing assay

One day before transfection, equal numbers of glioma cells (5.0×10^4) were seeded into 24-well tissue culture plates without antibiotics. Cells were then transfected with 50 nM siHSP47 or siControl using Lipofectamine RNAiMAX transfection reagent (Invitrogen), respectively. When the cell confluence reached about 90 % at 48 h post-transfection, an artificial homogenous wound was created onto the monolayer with a sterile plastic 100 μL micropipette tip. After wounding, the debris was removed by washing the cells with serum-free medium. Migration of cells into the wound was observed at different time points. Cells that

migrated into the wounded area or cells with extended protrusion from the border of the wound were visualized and photographed under an inverted microscope (40× objective) (Leica, Solms, Germany). A total of three areas were selected randomly from each well and the cells in three wells of each group were quantified in each experiment.

In vitro Matrigel invasion assay

The cell invasiveness was assessed with the use of BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA). Medium with 10 % FBS was added to the lower chamber as chemoattracctant. Then equal numbers of transfected glioma cells were resuspended in 500 μL serum-free medium and seeded into the rehydrated insert. After 24 h of incubation at 37 °C, non-invading cells on the upper surface of the Matrigel membrane were gently removed with a cotton-tipped swab. The cells were then fixed with 100 % methanol and stained with 1 % toluidine blue (Sigma). The stained invasive cells on the lower surface of the membrane were photographed under an inverted light microscope (40× objective) and quantified by manual counting in three randomly selected areas. This experiment was performed in duplicate in three independent experiments.

Luciferase reporter assay

The potential microRNAs targeting HSP47 were selected by bioinformatics analysis. The 3′-UTR sequence of HSP47 predicted to interact with the microRNAs or a mutated sequence within the predicted target sites were synthesized and inserted into the XbaI and FseI sites of pGL3 control vector (Promega, Madison, WI). For reporter assay, U87 cells were plated onto 24-well plates and transfected with the above constructs and pLL3.7-miR-29a overexpression vector (p-miR-29) or the pLL3.7-control vector (p-miR-control) using GenJet™ Plus DNA in vitro tranfection reagent (SignaGen, MD). A Renilla luciferase vector pRL-SV50 (Promega, Madison, WI) was also co-transfected to normalize the differences in transfection efficiency. After transfection for 48 h, cells were harvested and assayed with Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. This experiment was performed in duplicate in three independent experiments.

Western blotting

The U87 cells were transfected with p-miR-29a or p-miR-control and solubilized in radioimmunoprecipitation assay lysis buffer. The supernatants, which contained the whole-cell protein extracts, were obtained after centrifugation of the cell lysates at 12,000×g for 10 min at 4 °C. Heat-denatured protein samples (20 μg per lane) were resolved by SDS–poly-acrylamide gel electrophoresis (PAGE) and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated for 60 min in PBS containing 0.1 % Tween 20 and 5 % skim milk to block nonspecific binding, followed by incubation for 1 h at room temperature with anti-HSP47 primary antibody (Abcam). The membrane was washed three times for 10 min in PBS with 0.1 % Tween 20 and then incubated for 1 h with a secondary antibody. The membrane was washed thoroughly in PBS containing 0.1 % Tween 20, and the bound antibody was detected with the use of enhanced chemiluminescence detection reagents (Amersham Biosciences) according to the manufacturer's instructions.

Animal studies

Stable knockdown cells of HSP47 in U87 cells by shRNA-HSP47 or shRNA-control vector were implanted into the right and left flanks (1.5×10^6 cells per flank) of BALB/c athymic mice by subcutaneous injection. Tumor volumes were determined by measuring the length (a) and the width (b). The tumor volume (V) was calculated according to the formula $V = ab^2/2$. The tumor tissues were collected and the protein were isolated for western blot with primary antibody of anti-intact/cleaved caspase 3 and anti- intact/cleaved PARP (Abcam) as the protocol mentioned above. Statistical significance between shRNA-HSP47 or shRNA-control vector transfected group animals was evaluated using Student's t test.

TUNEL assay

Glioma tissues were fixed in 4 % paraformaldehyde in PBS embedded in paraffin and cut into around 5 mm sections through the center of the tissue. The diameter of the tumor slices examined varied according to the tumor volume. The presence of apoptotic cells within the tumor sections was evaluated using the DeadEnd Fluorometric TUNEL System following the manufacturer's protocol (GenScript USA Inc.). Percent apoptosis was determined by counting the number of apoptotic cells divided by the total number of cells in the field [13].

Statistical analysis

Data are presented as the mean ± standard deviation. The data were analyzed using the SPSS 12.0 Windows version software. Statistical analyses were done by analysis of

variance (ANOVA) or Student's t test. P value <0.05 was considered statistically significant.

Results

HSP47 is overexpressed in glioma tissues and cell lines and associated with tumor grade

To investigate the clinical significance of HSP47 in glioma development, the expression of HSP47 mRNA in 40 samples of gliomas (20 high grade and 20 low grade) and 20 samples of non-tumor brain tissues was detected by qRT-PCR. The relative expression level of HSP47 was significantly higher in glioma tumor tissues compared with their non-tumor counterparts ($P < 0.0001$; paired Student's t test; Fig. 1a). Next, we further analyzed the expression of HSP47 in the high grade and low grade glioma. Interestingly, we found that the expression of HSP47 is associated with the grade of glioma. The high grade glioma has significantly higher expression of HSP47 than the low grade glioma ($P = 0.026$; Fig. 1a), which implies that HSP47

may play a role in glioma development. Next, we further detected the expression of HSP47 in a panel of established glioma cell lines. The results also showed that the expression of HSP47 is significantly higher in glioma cells than in non-tumor brain tissues (Fig. 1b). Therefore, HSP47 is overexpressed in glioma tissues and cell lines and associated with tumor grade.

Knockdown of HSP47 inhibits glioma cell growth

To explore the functional role of HSP47 in tumorigenicity and development of glioma, a validated siRNA targeting HSP47 was selected and transfected into the glioma cell lines A172, U-87 MG and U-251 MG or glial cell line SVGp12. The expression of HSP47 in siHSP47-transfected cells was confirmed by qRT-PCR and western blot analysis. The significantly silencing of HSP47 expression was observed after 48 h after transfection in glioma cells A172, U-87 MG and U-251 MG, but not in glial cells SVGp12 (Fig. 2a, b). Subsequently, the effect of HSP47 knockdown on glioma or glial cell growth was assessed for 3 days by MTS assay. Compared with siControl-transfected cells, siHSP47-transfected cells exhibited significantly decreased growth rates in glioma cells A172, U-87 MG and U-251 MG, but not in glial cells SVGp12 after 48 and 72 h after transfection (Fig. 2b, c), indicating that knockdown of HSP47 inhibits glioma cell growth in vitro.

Knockdown HSP47 inhibits glioma cell migration and invasion

To further investigate the potential effects of HSP47 inhibition on glioma development, the wound healing and in vitro Matrigel invasion assay were performed to investigate the effects of HSP47 silencing on glioma cell migration and invasion. Both wound healing and in vitro Matrigel invasion assay demonstrated that the migration and invasion capability of glioma cells with silencing of HSP47 expression was significantly decreased compared with siControl-transfected glioma cells (Fig. 3a, b). Compared with siRNA control transfected cells, wound repair is significantly decelerated in glioma cells with knockdown of HSP47 (Fig. 3a). Consistently, knockdown of HSP47 also significantly decreased the invaded cell number (Fig. 3b). These results indicate that knockdown HSP47 inhibits glioma cell migration and invasion in vitro.

The expression of HSP47 is regulated by miR-29a

Since the important role of HSP47 in glioma cell growth, migration and invasion, it is interesting to investigate the expression regulation of HSP47. Previous studies suggested that at least one-third of human genes are estimated
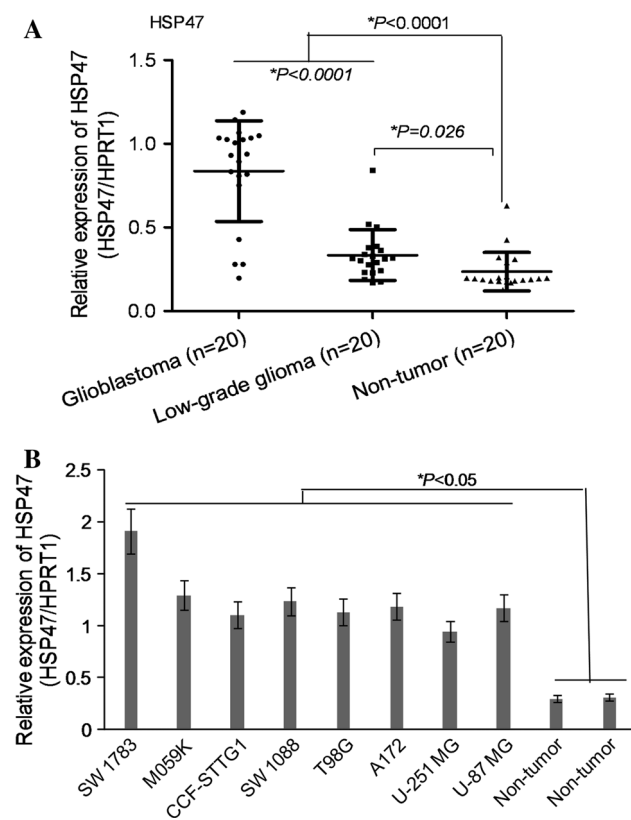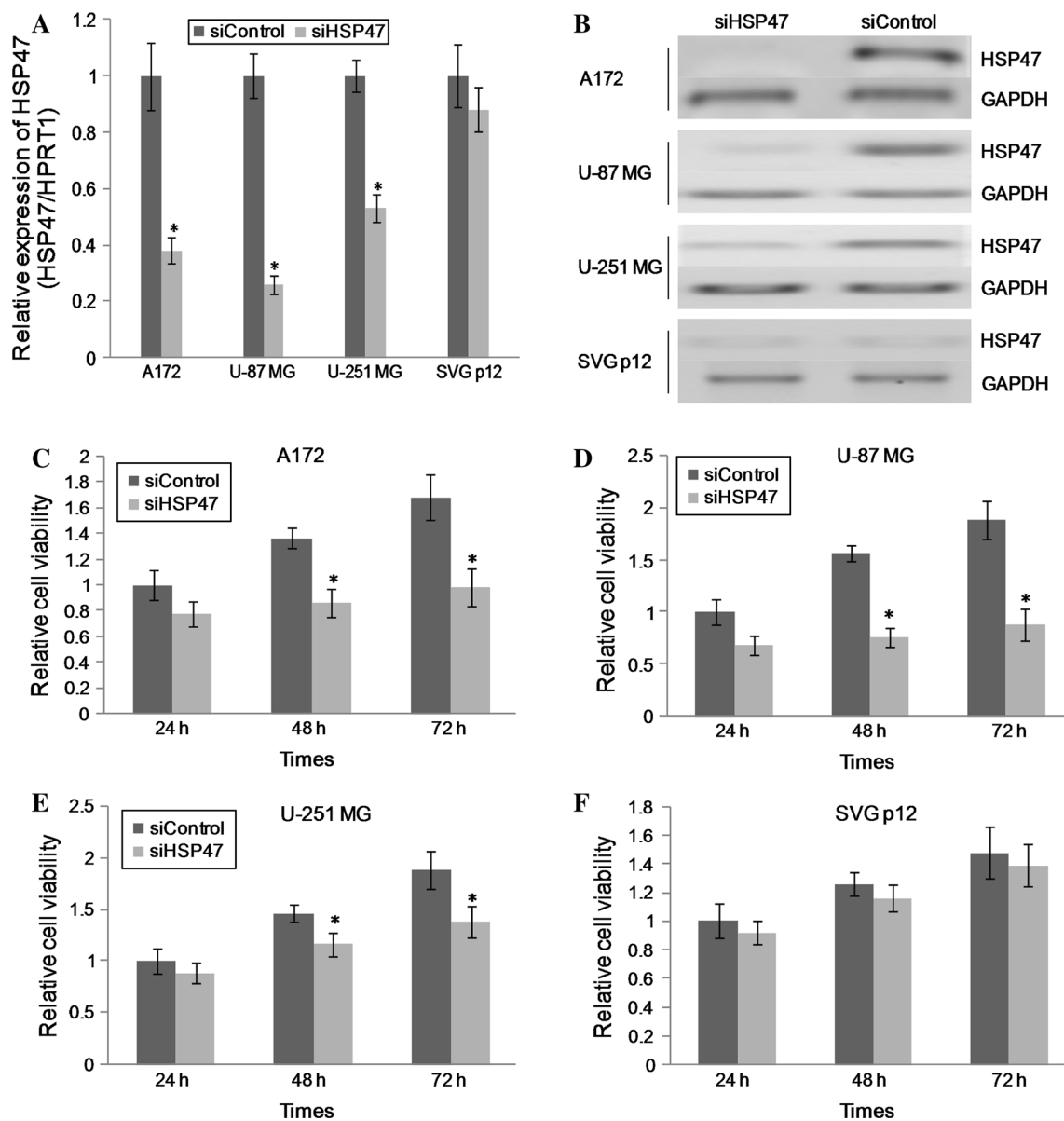

Fig. 1 HSP47 is overexpressed in glioma tissues and cell lines and associated with tumor grade. **a** The expression of HSP47 in 40 cases of glioma tumor and 20 non-tumor brain tissues was analyzed by qRT-PCR. **b** The expression of HSP47 in a panel of glioma cell lines and 2 non-tumor brain tissue controls was analyzed by qRT-PCR

Fig. 2 Silencing of HSP47 inhibits glioma cell growth. **a** The mRNA expression of HSP47 in glioma or glia cells transfected with si-control or siHSP47 by qRT-PCR analysis. **b** The protein expression of HSP47 in glioma or glia cells transfected with si-control or siHSP47 by western blot analysis. **c–f** The cell growth rate was detected in A172, U-87 MG, U-251 MG and SVGp12 cells transfected with siHSP47 or siControl by MTS assay at different time points (24, 48 and 72 h). *$P < 0.05$

to be miRNA targets, so the regulation mediated by miRNA at the post-transcriptional level is pervasive in animals [14]. To idenfiy the potential posttranscriptional regulation of HSP47 by miRNAs, we used the TargetScan for prediction. The prediction indicates that HSP47 is potentially regulated by miR-29 family. To validate whether miR-29a directly recognizes the 3′-UTRs of HSP47 mRNA, we cloned a sequence containing the predicted target sites or a mutated sequence with the predicted target sites downstream of the pGL3 luciferase reporter gene to generate pGL3-HSP47-3′UTR-wt or pGL3-HSP47-3′UTR-mut vector (Fig. 4a). The vectors were then co-transfected with the

p-miR-29a or p-miR-control into U87 cells. A renilla luciferase vector (pRL-TK) was used to normalize differences in transfection efficiency. Luciferase activity in U87 cells co-transfected with p-miR-29a and pGL3-HSP47-3′UTR-wt vector was decreased when compared with the control (Fig. 4b). Next, we further detected the protein expression of HSP47 in U87 cell after transfection with p-miR-29a and p-miR-control, or the protein expression of HSP47 in SVGp12 cell after transfection with miR-29 inhibitor and inhibitor-control. The result also showed that overexpression of miR-29a decreased the expression of HSP47 in glioma U87 cells, while knockdown of endogenous miR-29a
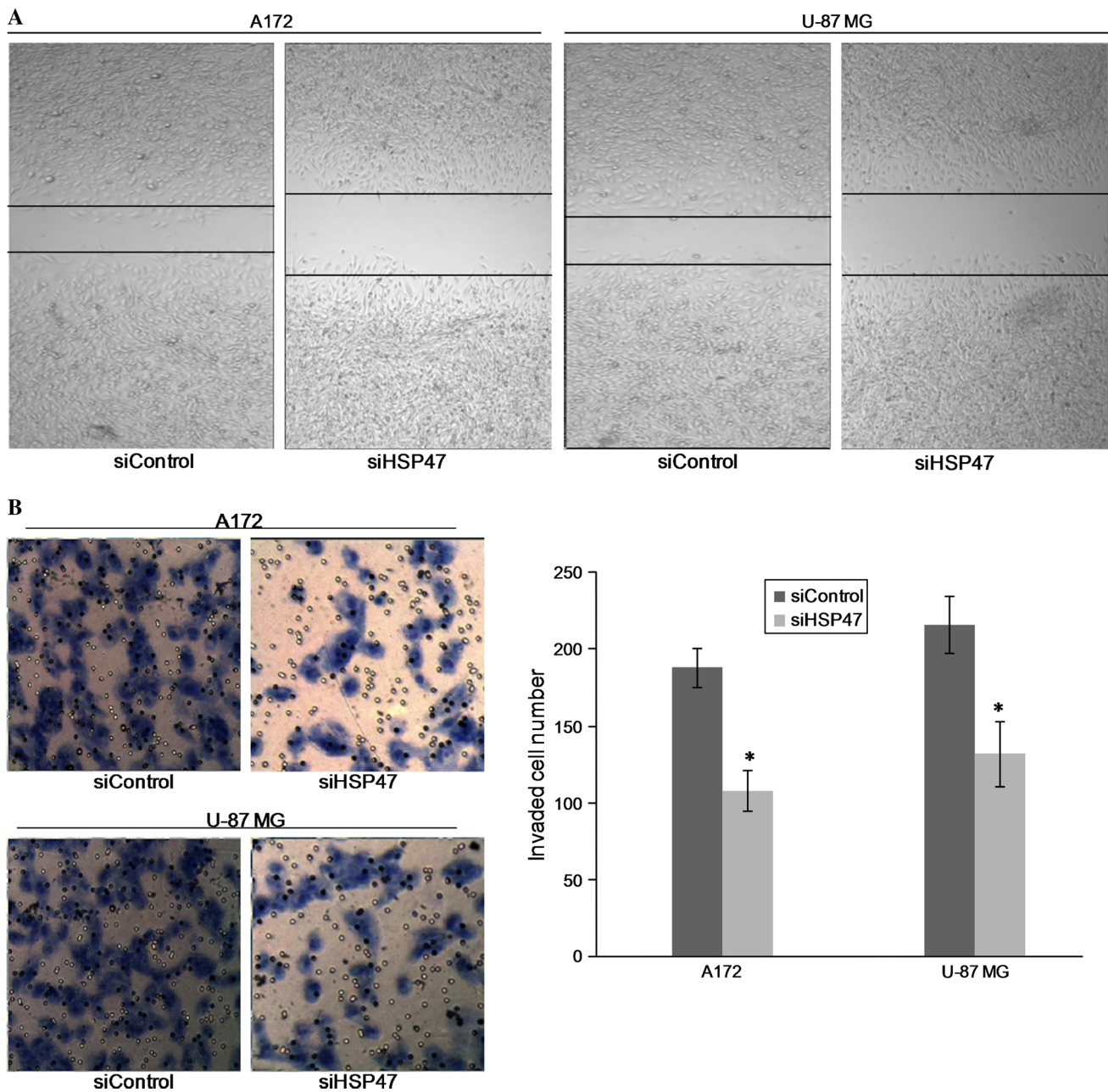
A

B

Fig. 3 Silencing of HSP47 inhibits glioma cell migration and invasion in vitro. **a** Image of cell migration in glioma cells transfected with si-control or siHSP47 by wound healing assay analysis. **b** Image of cell invasion in glioma cells transfected with si-control or siHSP47 by transwell Matrige invasion assay. The average invasive cells was counted from three independent repeated wells and shown in the graph. *$P < 0.05$

increased the expression of HSP47 (Fig. 4c). These data suggested that the expression of HSP47 is regulated by miR-29a.

Stable knockdown of HSP47 inhibits glioma tumor growth in vivo and induces apoptosis

To substantiate the roles of HSP47 in glioma carcinogenesis, we further assessed the effects of HSP47 knockdown on tumorigenicity and apoptosis of glioma cells in vivo. At 42 days post-injection of stable transfected U87 cells with HSP47 shRNA vector, the mean volumes of tumors generated from U87-shHSP47 were significantly smaller than those originated from U87-shRNA-control cells (Fig. 5a). The tissue sections from each group were collected and assayed by TUNEL analysis to detect apoptotic cells. The results showed that the apoptotic percentage (TUNEL + cells) was significantly increased in tumor tissues
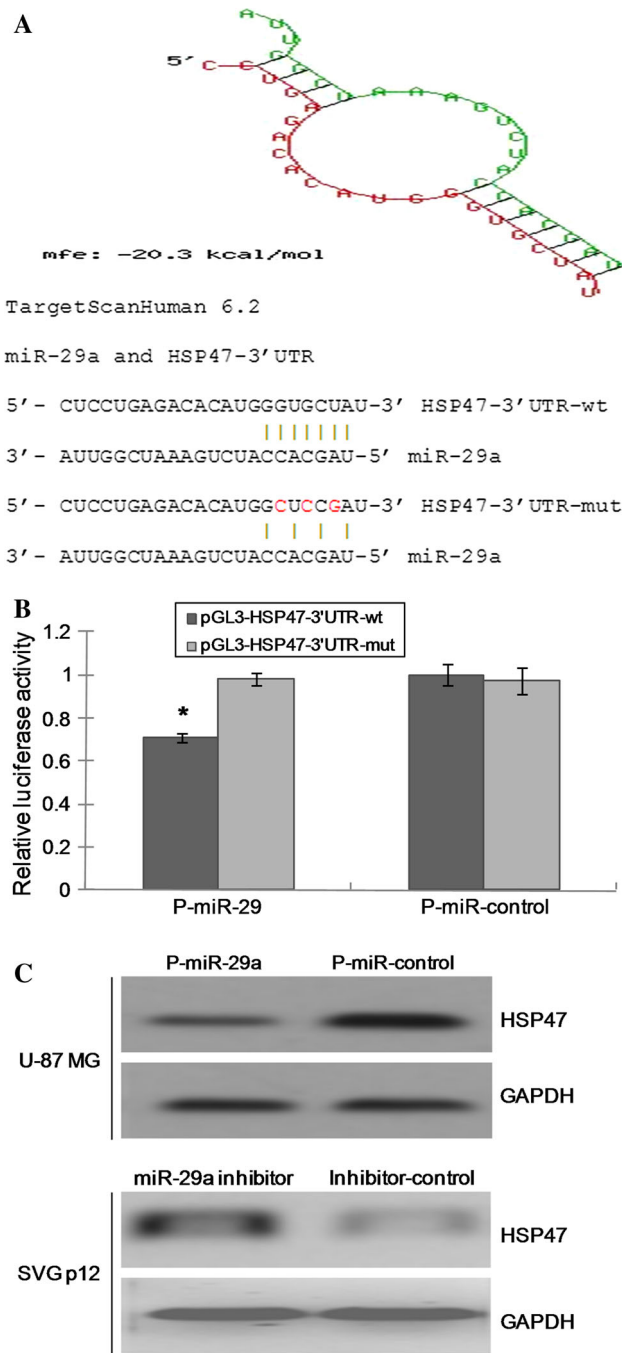
A

mfe: −20.3 kcal/mol

TargetScanHuman 6.2

miR-29a and HSP47-3'UTR

5′- CUCCUGAGACACAUGGGUGCUAU-3′ HSP47-3′UTR-wt
                     | | | | | | |
3′- AUUGGCUAAAGUCUACCACGAU-5′ miR-29a

5′- CUCCUGAGACACAUGGCUCCGAU-3′ HSP47-3′UTR-mut
                   | | | |
3′- AUUGGCUAAAGUCUACCACGAU-5′ miR-29a

B

C

Fig. 4 The expression of HSP47 is regulated by miR-29a. **a** HSP47 mRNA 3′-UTR putative sites or mutated sites targeted by miR-29a (TargetScan). **b** U87 cells were transfected with HSP47-3′-UTR-wt reporter vector or 3′-UTR mutant reporter vector and with P-miR-29a or P-miR-control. **c** Western blot analysis of total cell lysates extracted from P-miR-29a and P-miR-control transfected U87 cells or miR-29 inhibitor and Inhibitor-control transfected SVGp12 using HSP47 antibody. Data were mean ± SD of three independent experiments. *$P < 0.05$

derived from U87-shHSP47 cells compared with tumor tissues from U87-shControl cells (Fig. 5b). Meanwhile, the intact/cleaved caspase 3 and intact/cleaved PARP was also

analyzed and further showed that shRNA knockdown HSP47 induces increased expression of cleaved caspase 3 and PARP, indicating shRNA knockdown HSP47 induces apoptosis in vivo. These results indicate that knockdown of HSP47 expression restrains glioma tumorigenicity and induces apoptosis in vivo.

Discussions

Overexpression of HSP47 has been demonstrated in various cancers such as lung cancer, pancreatic carcinoma, gastric carcinoma, ulcerative colitis-associated carcinomas and head and neck squamous cell carcinoma [8, 11]. This study firstly demonstrated the increased expression of HSP47 in human gliomas both in patients' tissues and cell lines. The expression of HSP47 is strongly associated with the grade of glioma and also overexpressed in most of glioma cell lines. The high grade glioma has significantly higher expression of HSP47 than the low grade glioma. Low-grade gliomas are slow-growing gliomas, while high-grade gliomas are more aggressive gliomas. Prognosis for gliomas, particularly high-grade gliomas, remains poor [15]. The association of HSP47 with the grade of glioma supports it to be further validated as a prognostic factor of glioma. Therefore, HSP47 clearly has the potential to be a prognostic biomarker and therapeutic target of glioma.

The expression of HSP47 on cell surface following alkalization of the endosomal compartments under conditions of stress indicated that HSP47 is a serpin family protein that may modulate cell migration during development and invasion and metastasis in cancer. To investigate the role of increased HSP47 expression in glioma, we selected a validated siRNA targeting HSP47 to infect glioma cells with high HSP47 expression. We demonstrated that knockdown of HSP47 in glioma cells can inhibit the growth of cancer cells, more than that, the knockdown can also prevent invasion and metastasis of cancer cells. These data indicated that HSP47 plays an important role glioma invasion and could be used as a prognostic marker and therapeutic target for the invasive activity of human glioma cells.

The expression of oncogenes can be regulated by miR-NAs, small RNAs 21–25 nucleotides in length that control most of gene expression regulation by downregulating them [16]. Dysregulation of such miRNAs could lead to activation of oncogenes. So we next investigated the posttranscriptional regulation of HSP47 by miRNAs. The results suggested that the expression of HSP47 is regulated by miR-29a using bioinformatics analysis and experimental validation, such as luciferase reporter assay and western blotting. Previous study indicated that miR-29a was significantly downregulated in all of the glioma samples and continuously decreased as the malignant grade of the
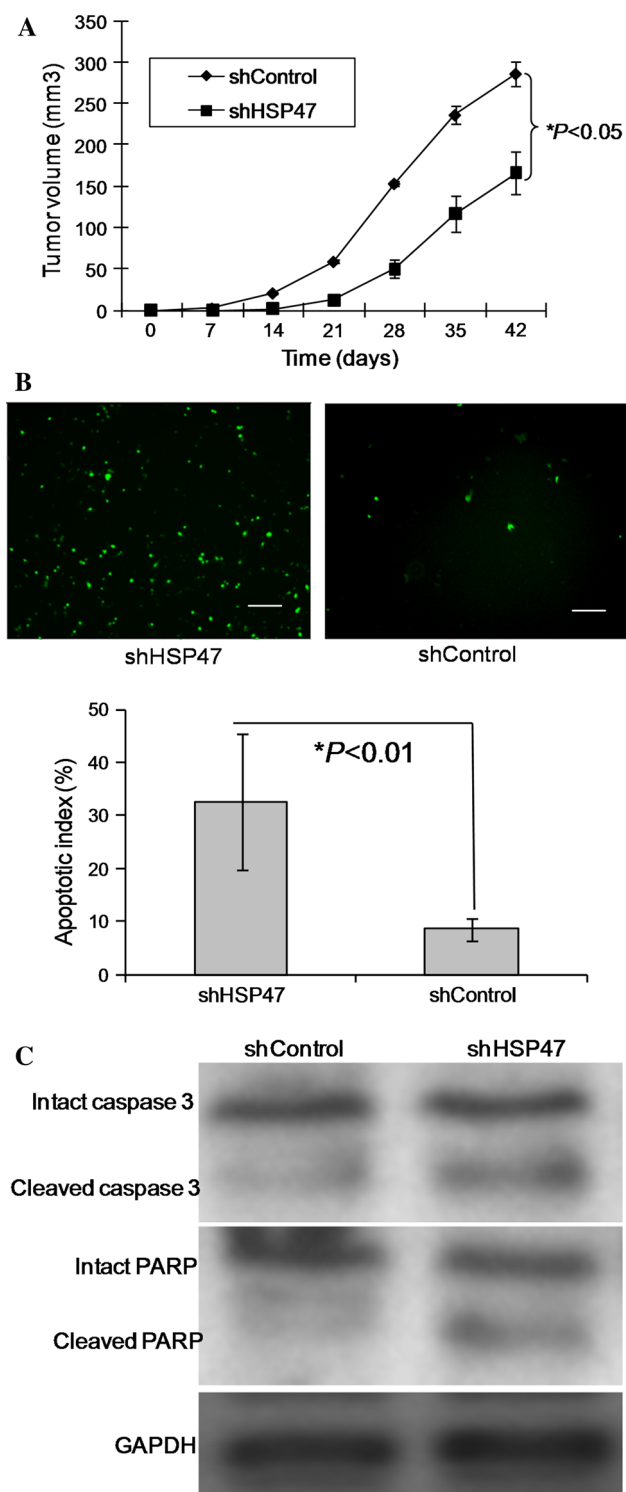
◀ **Fig. 5** Stable knockdown of HSP47 inhibits glioma tumor growth in vivo and induces apoptosis. **a** Determination of the tumor growth. Tumor volume was calculated every week after injection. Data were mean ± SD of three independent experiments ($n = 15$), *$P < 0.05$. **b** TUNEL staining of tumor tissues. The staining showed the representative fields from tumor tissues in the shHSP47 or shControl stable transfected U87 cells group. *Scale bar*, 100 μm. The *graph* showed the percent apoptosis in each group. Values were expressed as mean ± SD (*$P < 0.05$). **c** The expression of intact/cleaved caspase 3 and intact/cleaved PARP was assessed by western blot in the tumor tissues from the shHSP47 or shControl stable transfected U87 cells group

Silencing HSP47 clearly has the potential to inhibit cell growth, migration and invasion in glioma cells. Further studies are required to elucidate in vivo role of HSP47 on tumorigenicity of glioma cells. Stable knockdown of HSP47 using shRNA showed suppression of glioma cell tumorigenicity in vivo and HSP47 expression in tumor tissue by qRT-PCR, suggesting that silencing HSP47 expression restrains glioma tumorigenicity. Although the mechanism of inhibition of glioma cell remains to be clarified, apoptosis could be involved. The tissue sections from each group were collected and assayed by TUNEL analysis to detect apoptotic cells. The results showed that the apoptotic percentage was significantly increased in tumor tissues derived from U87-shHSP47 cells compared with tumor tissues from U87-shControl cells. These results indicate that knockdown of HSP47 expression restrains glioma tumorigenicity and induces apoptosis in vivo.

In conclusion, HSP47 is significantly overexpressed in glioma tissues and cell lines and associated glioma tumor grade. Knockdown of HSP47 inhibits glioma cell growth, migration and invasion in vitro. The expression of HSP47 is regulated by miR-29a. Stable knockdown of HSP47 using shRNA inhibits glioma tumor growth and induce apoptosis in mice models in vivo. Therefore, HSP47 plays important role in glioma cell growth and invasion and thus could be a therapeutic target of treating glioma in the future.

Conflict of interests None.

tumors increased. The decrease of miR-29a in glioma facilitated the migration and invasion of glioma cells [17]. These data suggested that HSP47 may be a potential downstream target for modulating miR-29a regulated migration and invasion of glioma cells.

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