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Carboxyl terminus of Hsp70-interacting protein (CHIP) contributes to human glioma oncogenesis

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Malignant glioma is the most common adult primary brain tumor, and the mechanism of its oncogenesis is poorly understood. Growing evidence has shown that E3 ubiquitin ligases can promote tumorgenesis of glioma. CHIP is an E3 ubiquitin ligase that can induce ubiquitylation and degradation of many tumor-related proteins, and it has been reported to act as an upstream regulator in breast cancer; however, its role in human gliomas has not been evaluated yet. In this study, the expression of CHIP in glioma tissues was studied using immunohistochemistry. CHIP expression in glioma cells was studied by real-time RT-PCR, western blot and double immunofluorescence staining. The role of CHIP in glioma oncogenesis was investigated by lentivirus-mediated RNA interference (RNAi) and overexpression *in vitro* and *in vivo*. We showed CHIP expression in glioma samples was related to tumor grades, with stronger staining in high-grade gliomas than in low-grade gliomas. Knocking down of CHIP suppressed proliferation, colony formation of U251 and U87 glioma cells, while overexpression of CHIP resulted in enhanced proliferation and colony formation *in vitro*. In a nude mouse xenograft model, intratumoral injection of CHIP RNAi lentivirus significantly delayed tumor growth. In contrast, overexpression of CHIP resulted in enhanced tumor growth *in vivo*. After CHIP RNAi, both survivin mRNA and protein were decreased, while CHIP overexpression induced increased mRNA and protein levels of survivin. This is the first study demonstrating CHIP contributes to oncogenesis of glioma. (*Cancer Sci* 2011; 102: 959–966)

Malignant gliomas are the most common and aggressive primary brain tumors. They are highly invasive, with a median survival time of 2–5 years for patients with anaplastic glioma and 12–15 months for patients with glioblastoma.[1] Despite recent advantages in surgery, chemotherapy and radiotherapy, the survival time of glioma patients has not improved much.

The etiology of glioma is not clearly defined. However, several signal transduction pathways have been implicated. Large-scale multi-dimensional analysis of genomic data helped reveal molecular characteristics in human cancer. According to the Cancer Genome Atlas Research Network, three critical signaling pathways (phosphatidylinositol-3-OH kinase [PI3K] pathway, p53 pathway and RB signaling pathway), with their frequent genetic alterations, were involved in oncogenesis of glioblastoma.[2] They found 74% glioblastoma harbored aberrations in all three pathways, a pattern suggesting that deregulation of the three pathways is a core requirement for glioblastoma pathogenesis. Many important molecules like epidermal growth factor receptor (EGFR), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ErbB-2), Forkhead transcription factors (FOXO) and v-akt murine thymoma viral oncogene homolog (Akt) were involved in these pathways, with point mutation or amplification in glioma samples. Targeted therapy against these pathways and core molecules provided new insights for neurosurgeons.

The ubiquitin-proteasome system played an important role in glioma oncogenesis; our team reported that ubiquitin-conjugating enzyme E2C/UbcH10 may serve as one important molecular mechanism that underlies the astrocytic carcinogenesis.[3] In this paper, we studied the role of an E3 ubiquitin ligase, carboxyl terminus of Hsp70-interacting protein (CHIP), in glioma oncogenesis. CHIP interacts with heat shock proteins and negatively regulates chaperone functions.[4] The U-box domain at the carboxyl terminus of CHIP contains its E3 ubiquitin ligase activity, and could induce ubiquitylation and degradation of many tumor-related proteins.[5,6] P53 is a common tumor suppressor protein, which is frequently mutated in cancers, that can be targeted for degradation by the CHIP ubiquitin ligase with the chaperones Hsp70 and Hsp90 in both wild-type and R175H mutated form.[7] By abrogating p53-dependent apoptotic pathways and by ubiquitylation competitive with Daxx (death domain-associated protein) sumoylation, CHIP integrates the proteotoxic stress response of the cell with cell cycle pathways that influence cell survival.[8] FOXO1 a main member of the forkhead transcription factor family, has been shown to play an important role in cell cycle arrest and apoptosis of various cell lines.[9] FOXO1 can be phosphorylated by activated Akt, resulting in cytoplasmic retention and proteasomal degradation by CHIP, thus promoting cell survival status.[10] PTEN is a major negative regulator of the PI3K/Akt pathway, once PTEN is mutated or deleted FOXO1 will be inactivated through ubiquitylation and degradation by CHIP. It is a possible mechanism for cancer initiation and progression, which indicates CHIP might contribute to oncogenesis of some PTEN-deficient cancers like glioma. Other tumor-related proteins like C-ErbB2/neu[11] and hypoxia-inducible factor (HIF)-1alpha[12] can also be regulated by CHIP.

Because CHIP can regulate these tumor-related proteins through ubiquitylation and degradation, it might play an important role in cancers. A recent study indicated that CHIP acts as an upstream regulator of oncogenic pathways and suppresses tumor progression in human breast cancer by degrading oncogenic proteins including SRC-3.[13] However, whether it contributes to tumorgenesis or tumor suppression in human glioma remains unclear. In the current study, we tested the hypothesis that CHIP might also play an important role in human gliomas. Our results revealed a positive correlation of CHIP expression with tumor grade. To explore the function of CHIP in glioma cell proliferation, colony formation and invasion, we used lentivirus-based RNA interference (RNAi) and an overexpression system to change the expression of CHIP in U251 and U87

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glioma cell lines. Decrease in CHIP levels induced by RNAi resulted in a delay of cell proliferation, and inhibition of soft agar clonogenic growth. An increase in CHIP levels by overexpression resulted in enhancement of cell proliferation and colony formation. Tumor growth in the xenografts was decreased after intratumoral injection of CHIP shRNA, while enhancement of tumor growth was detected after intratumoral injections of the CHIP overexpression system. Our data suggested that CHIP might contribute to the tumorigenesis of human glioma both *in vitro* and *in vivo*.

Materials and Methods

Tumor samples. The specimens from the glioma patients were provided by the Department of Neurosurgery, Changzheng Hospital, Shanghai Institute of Neurosurgery, China. All patients gave informed consent according to a study protocol that was approved by the Tissue Committee and Research Ethics Board. The tissues from 20 low-grade glioma (LGG; WHO grade I–II) patients and 20 high-grade glioma (HGG; WHO grade III–IV) patients were obtained during operations. Normal brain tissues were obtained from surgical resections of three trauma patients, for whom a partial resection of normal brain tissue was required as decompression treatment for their severe head injuries to reduce increased intracranial pressure, under the permission of each of the patient's family. All samples used in this research were under the National Regulation of Clinical Sampling in China. The pathological diagnoses of all enrolled patients were confirmed by two different pathologists.

Immunohistochemistry. Formalin-fixed, paraffin-embedded, 3-μm tissue sections were cut for immunohistochemistry. Two adjacent sections in one sample were used for staining by CHIP or Ki-67 primary antibody, respectively. Immunostaining was performed following standard methods. Sections were deparaffined in xylol and rehydrated in a graded ethanol series. Antigen retrieval was performed using a microwave heating method for 20 min with 1 mM EDTA buffer (pH 8.0). Thereafter, endogenous peroxidise activity was eliminated by 3% methanolic hydrogen peroxide solution for 30 min. The slides were incubated in nonimmune serum for 30 min. CHIP primary antibody (Abgent, San Diego, CA, USA) with 1:100 dilution and Ki-67 primary antibody (Maxin Biotechnology Inc, Fujian, China) with 1:75 dilution were added afterwards and the sections were incubated overnight at 4°C, followed by staining with an ABC kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The sections were counterstained with hematoxylin. To ensure specificity of the immunostaining, sections in which the primary antibody was replaced by non-immune serum served as a negative control.

For evaluating CHIP and Ki-67 immunoreactivity, representative visual fields (×400 magnifications) were chosen to calculate the percentage of positively stained cells over the total number of tumor cells by two independent pathologists. The results were evaluated quantitatively and divided into four groups: −, negative; +, <30% of tumor cells were immunopositive; ++, 30–60% of tumor cells were immunopositive; and +++, >60% of tumor cells were immunopositive. Data sets were combined after the completion of scoring and analyzed by Fisher' s exact test.

Cell culture. The human glioma cell lines U251 and U87 were purchased from American Type Culture Collection (ATCC). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) accompanied by 10% fetal bovine serum supplemented with 100 U/mL penicillin and 100 ug/mL streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Indirect double immunofluorescence staining. For immunocytochemistry, U87 cells were seeded onto precoated glass coverslips for 2 days. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS for 5 min, and then incubated with rabbit anti-CHIP polyclonal antibody (Abgent) and mouse anti-glial fibrillary acidic protein (GFAP) (Abcam Inc., Cambridge, MA, USA) at a dilution of 1:200 in PBS containing 5% normal goat serum overnight at 4°C. The final detection was made by incubating with FITC-conjugated anti-rabbit IgG antibody and TRITC-conjugated anti-mouse IgG antibody (Kangcheng Biotechnology Inc., Shanghai, China; 1:400 dilution in PBS containing 5% normal goat serum) for 1 h at 37°C. Hochest was used to stain the nuclear. An Olympus microscope (IX70) (Olympus, Tokyo, Japan) fitted for fluorescence detection connected to a personal computer via a charge-coupled device (CCD) camera was used to visualize the slides, and the photographs were analyzed by image analysis software (Molecular Devices, Inc. Sunnyvale, CA, USA).

siRNA sequences and constructs. CHIP protein was encoded by *STUB1* (NM_005861). Two candidate sequences (5′-GGAGGTTATTGACGCATTC-3′, 5′-GAAGAGGAAGAAGC-GAGACAT-3′) in the *STUB1* cDNA sequence were selected for small interfering RNA (RNAi). For stable RNAi, we designed a short hairpin RNA (shRNA) sequence that contains both sense and antisense siRNA sequences against *STUB1*, flanking with the *Hpa I* and *Xho I* sites. A loop sequence was used to separate the complementary domains.

CHIP siRNA and overexpression lentivirus constructs and preparations. The recombinant lentiviruses were packaged using the pMagic4.0 lentivirus RNAi system and the pLV expression system (Sunbio Medical Biotechnology Co., Ltd, Shanghai, China). Briefly, recombinant was produced by co-transfecting human embryonic kidney (HEK) 293T cells with the lentivirus RNAi plasmid (pMagic4.0-shRNA) or overexpression plasmid (pLV-CHIP) and packaging plasmids (pLV1.0, pLV2.0 and pLV3.0) using lipofectamine2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. U251 and U87 glioma cells were then infected with the lentivirus producing shRNA directed against CHIP ($CHIP^{KD1}$, $CHIP^{KD2}$), lentivirus overexpressing CHIP ($CHIP^{OE}$) or lentivirus with negative-control sequences ($CHIP^{Scr}$). Total RNA and proteins were extracted at 24 and 48 h after transfection, respectively.

Quantitative real-time reverse transcription-PCR. Quantitative real-time reverse transcription–PCR (RT-PCR) was used to validate *STUB1* gene knockdown and overexpression effect. Total RNA was extracted from $CHIP^{KD1}$-, $CHIP^{KD2}$-, $CHIP^{OE}$- or $CHIP^{Scr}$-infected U251 and U87 cells with TRIzol (Invitrogen). Reverse transcription (RT) was performed using a Reverse Transcriptase kit (Promega, San Luis Obispo, CA, USA) following the manufacturer's instructions. *Beta-actin* was used as an endogenous control. Quantitative real-time PCR was performed in triplicate using SYBR Green Mastermix (TaKaRa, Kyoto, Japan) on the TaKaRa TP800 System. The PCR conditions were 95°C for 15 s, 40 cycles of 95°C for 5 s and 60°C for 30 s. Values were normalized to the expression of the *GAPDH* gene using the Delta–Delta CT ($2^{-\Delta\Delta Ct}$) method.

Western blot. Protein was isolated from $CHIP^{KD1}$-, $CHIP^{KD2}$-, $CHIP^{OE}$- or $CHIP^{Scr}$-transfected U251 and U87 cells, suspended in Tris-buffered SDS lysis buffer (50 mM Tris, pH 6.8/2% SDS/10% glycerol/1% β-Mercaptoethanol) and sonicated. Samples were spun at 4°C to pellet insolubles, and the supernatant was measured with a BCA Protein Assay Kit (HyClone-Pierce, South Logan, UT, USA). A total of 30 μg of protein per lane was electrophoresed in 10% sodium dodecyl sulfate (SDS)-PAGE gels, transferred to the polyvinylidene difluoride (PVDF) membrane, blocked for 1 h with 5% powdered skim milk–0.5% Tween 20 in TBS, and incubated with rabbit anti-CHIP (1:50 dilution; Abgent) or mouse anti-GAPDH

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(1:5000 dilution; Santa Cruz Biotechnology) at 4°C overnight. After several washes, the membranes were incubated in peroxidase-coupled secondary antibodies against rabbit or mouse (1:5000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature, washed, and revealed using ECL Plus (Amersham Biosciences, Piscataway, NJ, USA) and exposed to X-OMAT film (Kodak Japan, Tokyo, Japan). The downstream signaling factors were detected by the antibodies against bcl-2, HSP70, HSP90, Akt and Survivin, respectively (1:1000 dilution; Cell Signaling Technology Inc., Danvers, MA, USA).

MTT assay. After the gene knockdown and overexpression effect of CHIP expression was confirmed by real-time PCR and western blot, U251 and U87 cells successfully transfected with $CHIP^{KD1}$, $CHIP^{KD2}$, $CHIP^{OE}$ or $CHIP^{Scr}$ were subjected to MTT assay. Cells (1×10^4 cells/well) were seeded in triplicate in 96-well plates, and an assay was performed at 24, 48, 72 and 96 h. The cells were stained and cultured with 10 μL 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL; Sigma, St Louis, MO, USA) for 4 h. The culture medium was then removed and 100 μL dimethylsulfoxide (DMSO, Sigma) was added to each well and thoroughly mixed for 10 min. The absorbance values at 570 nm (A570) were measured on a Spectra Microplate Reader 1420 multilabel counter (Perkin Elmer, Foster City, CA, USA).

Colony formation assay. Soft agar assays were preformed as follows. Briefly, 0.5 mL under layers consisting of 0.8% agar medium was prepared in six-well plates. U251 and U87 cells transfected with $CHIP^{KD1}$, $CHIP^{KD2}$, $CHIP^{OE}$ or $CHIP^{Scr}$ were trypsinized, centrifuged, resuspended in 0.4% agar medium (equal volumes of 0.8% Noble agar and culture medium), and plated onto the top agar at 200 per well. The cells were kept for growth for 14 days at 37°C. Colonies were visualized using cell staining GIEMSA solution (Chemicon, Millipore Corporation, Billerica, Danvers, MA, USA) and counted under the microscope.

Nude mouse xenografts. Sixteen female BALB/c-nu mice with 7–8 weeks of age were obtained from the Institute of Neuroscience Lectures, Shanghai, China. Each mouse was inoculated with 3×10^7 U251 tumor cells in 0.2 mL of medium subcutaneously in the forelimb. After 5 days, when the tumors were palpable, these mice were randomly divided into four groups. Group 1 mice received intratumoral injections (30 uL/mouse in 3–5 sites) of $CHIP^{KD1}$ (5×10^9 TU/mL) twice weekly at various times, group 2 received intratumoral injections of $CHIP^{KD2}$ (5×10^9 TU/mL), group 3 received intratumoral injections of $CHIP^{OE}$ (5×10^9 TU/mL) and group 4 received intratumoral injections of $CHIP^{Scr}$ (5×10^9 TU/mL). Tumor sizes were measured everyday in two dimensions using a caliper, and the volume (mm^3) was calculated using the formula $V = 0.5 \times \text{larger diameter} \times (\text{smaller diameter})^2$. The tumor volume was calculated and normalized to the initial volume before intratumoral injection to form the curve of glioma cell growth rate. These mice were killed and the tumors were excised after treatment for 12 days. All procedures involving animals were approved by the Animal Care and Use Committee of Second Military Medical University.

Statistical analysis. Fisher's exact test was used to compare the immunolabelling results of CHIP and Ki-67 between high-grade and low-grade gliomas. For other experiments, differences between groups were measured by the Student *t* test, and for comparing means >2 groups, one-way ANOVA was used. SPSS 15.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis and a significance level of $P < 0.05$ was used to evaluate the difference between groups.

Results

Immunohistochemistry and double immunofluorescence analysis. To test whether CHIP was involved in the development of human brain tumorigenesis, we first examined the expression of CHIP in glioma cells using a double immunofluorescence labelling assay and demonstrated a close co-localization of CHIP and GFAP in the cytoplasm of U87 glioma cells (Fig. 1). Because GFAP was an important marker for both normal glial cells and glioma cells, its expression could be detected in the cytoplasm.

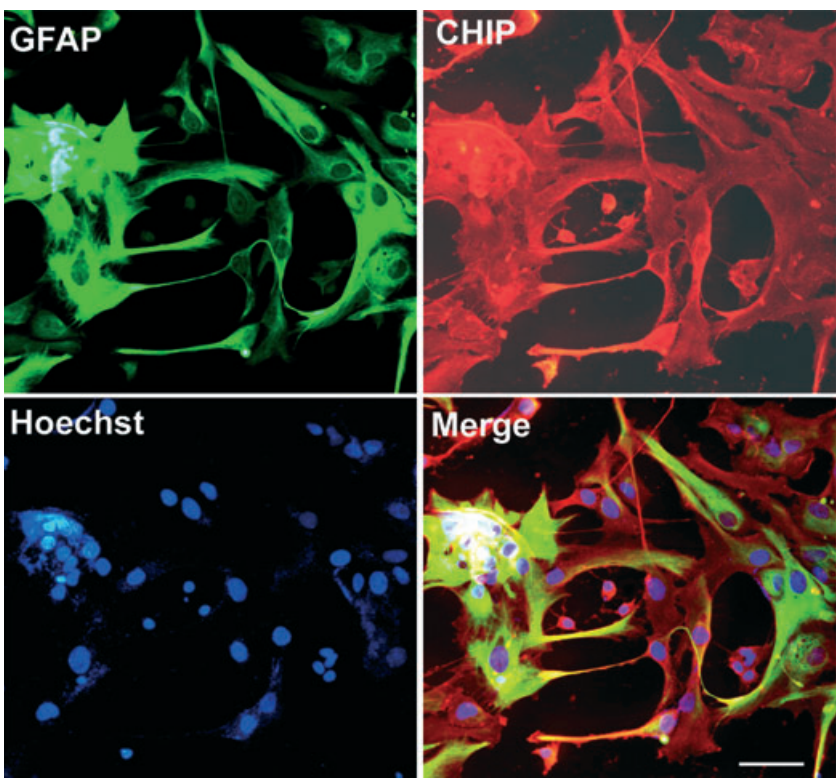

Fig. 1. Expression of CHIP in glioma cells. Double immunofluorescence labelling assay demonstrated a close co-localization of CHIP and glial fibrillary acidic protein (GFAP) in the cytoplasm of U87 glioma cells.

Our results confirmed the expression of CHIP in the cytoplasm of glioma cells. We then examined the expression of CHIP in different grades of gliomas and normal brain samples by immunohistochemistry. As shown in Figure 2, in normal brain, CHIP expression was present in the neurons. In glioma samples, CHIP was mainly expressed in the cytoplasm while Ki-67 was mainly expressed in the nucleus of glioma cells. The frequencies of intensity scores for CHIP and Ki-67 in 40 samples are listed in Table 1. Statistical analysis showed both increased CHIP and Ki-67 staining intensity in high-grade gliomas versus low-grade gliomas ($P = 0.000$ and $P = 0.000$, respectively).

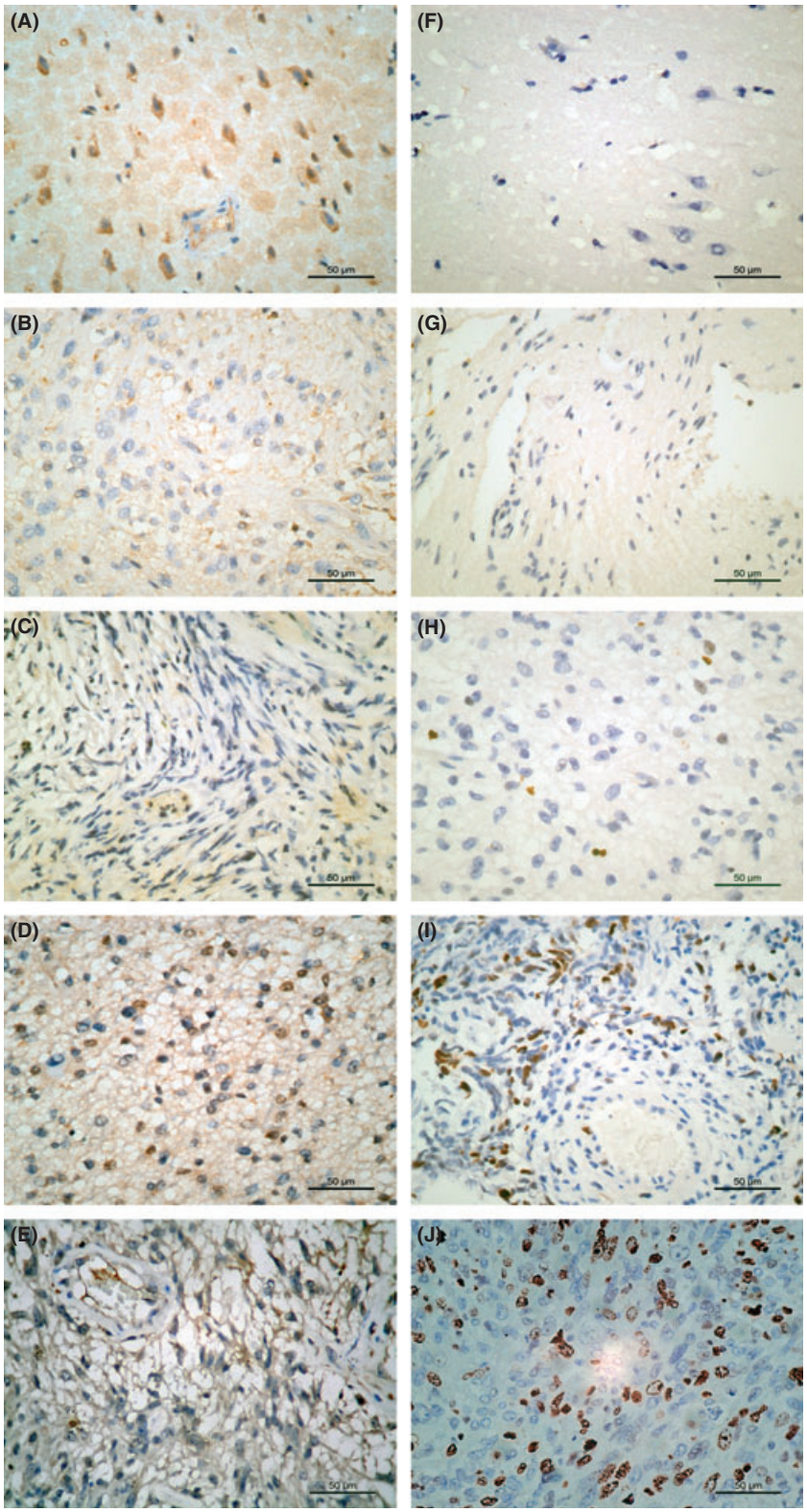

Fig. 2. Expression of CHIP and Ki-67 in normal brain and glioma samples. Immunohistochemistry in normal brain showed CHIP mainly expressed in the cytoplasm of neurons (A), while Ki-67 showed no staining (F). Stronger staining of CHIP was detected in high-grade gliomas (D,E) rather than low-grade gliomas (B,C). Ki-67 was also upregulated in high-grade gliomas (I,J) rather than low-grade gliomas (G,H) (original magnification, ×400).

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Table 1. CHIP and Ki-67 protein expression in glioma tissue samples

	Frequencies of intensity scores				*P* value
	–	+	++	+++	
CHIP					
Low-grade glioma (*n* = 20)	0	11	7	2	0.000
High-grade glioma (*n* = 20)	0	0	8	12	
Ki-67					
Low-grade glioma (*n* = 20)	0	16	4	0	0.000
High-grade glioma (*n* = 20)	0	0	7	13	

Lentivirus-based RNAi and overexpression system successfully changed CHIP expression. To investigate the role of CHIP in the development of human glioma cells, the level of CHIP expression was decreased in human glioma cell lines U251 and U87 by the lentivirus-based small RNA interference, as well as increased by CHIP overexpression. As shown in Figure 3A–C, both CHIP mRNA and protein expression were significantly inhibited by $CHIP^{KD1}$ and $CHIP^{KD2}$, but increased by $CHIP^{OE}$ in the U251 cell line. Similar results were obtained in the U87 cell line (Fig. 3D–F).

Effects of lentivirus-based RNAi and overexpression of CHIP on glioma cell growth and colony formation *in vitro*. To assess the role of CHIP in the growth of human glioma cells, we performed MTT assays to examine the cell proliferation of U251 and U87 after CHIP RNAi or overexpression. As shown in Figure 4A,B, decreasing of CHIP by $CHIP^{KD1}$ and $CHIP^{KD2}$ caused a significant delay of both U251 and U87 cell growth compared with the negative control ($P < 0.05$), while overexpression of CHIP enhanced cell growth in both cell lines ($P < 0.05$).

To determine a role of CHIP in human glioma tumorigenesis *in vitro*, we assayed the colony formation of two human glioma cells U251 and U87 in soft agar. Reduction of CHIP in U251 and U87 cell lines caused a substantial reduction in colony formation in soft agar compared with the $CHIP^{Scr}$ cells ($P < 0.01$). Overexpression of CHIP increased colony formation in soft agar compared with $CHIP^{NC}$ ($P < 0.01$; Fig. 4C,D). The results of the MTT assay and colony formation indicated that CHIP might be involved in human glioma cell growth *in vitro*.

Effects of lentivirus-based RNAi and overexpression of CHIP on glioma cell growth *in vivo*. Given that there is a role for CHIP in proliferation and colony formation, we examined the effect of CHIP RNAi and overexpression on glioma cell growth *in vivo*. U251 cells were injected subcutaneously into nude mice. Five days later, the palpable tumors were injected with lentivirus-based $CHIP^{KD1}$ and $CHIP^{KD2}$, as well as $CHIP^{Scr}$ and $CHIP^{OE}$. The relative proliferation rates were calculated. As shown in Figure 5, shCHIP significantly inhibited tumor growth speed in mice compared with $CHIP^{Scr}$, and $CHIP^{OE}$ significantly enhanced tumor growth. These results indicated that using CHIP RNAi could efficiently inhibit U251 tumor growth in a nude mouse model.

CHIP regulate survivin expression. To investigate the effects of CHIP on important components in signal transduction pathways, we detected the expression of survivin, MMP-2, AKT,

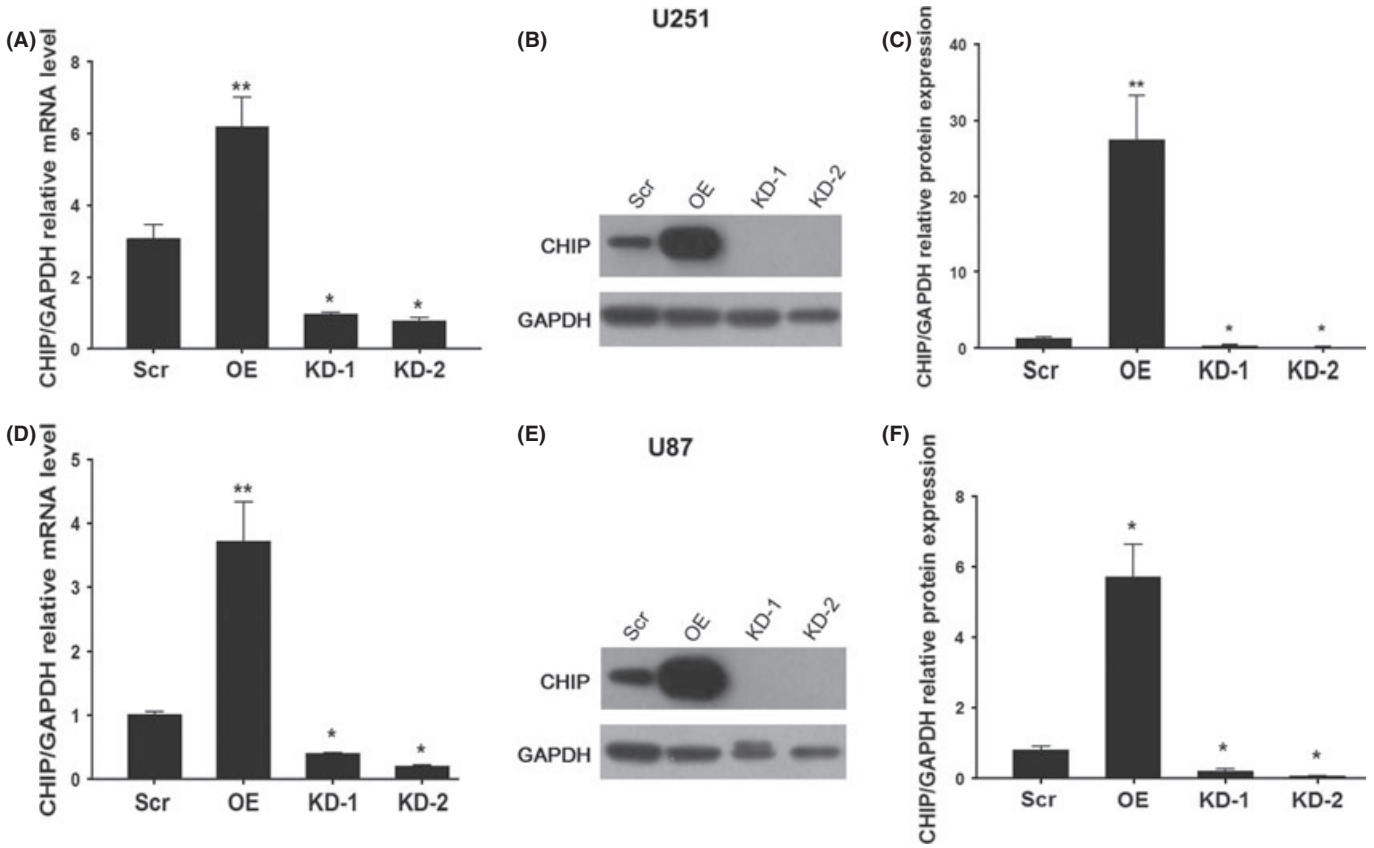

Fig. 3. CHIP siRNA and overexpression validation were determined by western blot and real-time PCR. U251 or U87 cells were transfected with lentivirus with negative-control sequences ($CHIP^{Scr}$; line 1) or lentivirus overexpressing CHIP ($CHIP^{OE}$; line 2) or lentivirus producing shRNA directed against CHIP ($CHIP^{KD1}$, $CHIP^{KD2}$; lines 3 and 4). The expression of CHIP mRNA and protein following siRNA or overexpression transfection were determined by real-time PCR (A,D) and western blot (B,C for U251, E,F for U87). Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences between transfected cells and mock-infected cells.

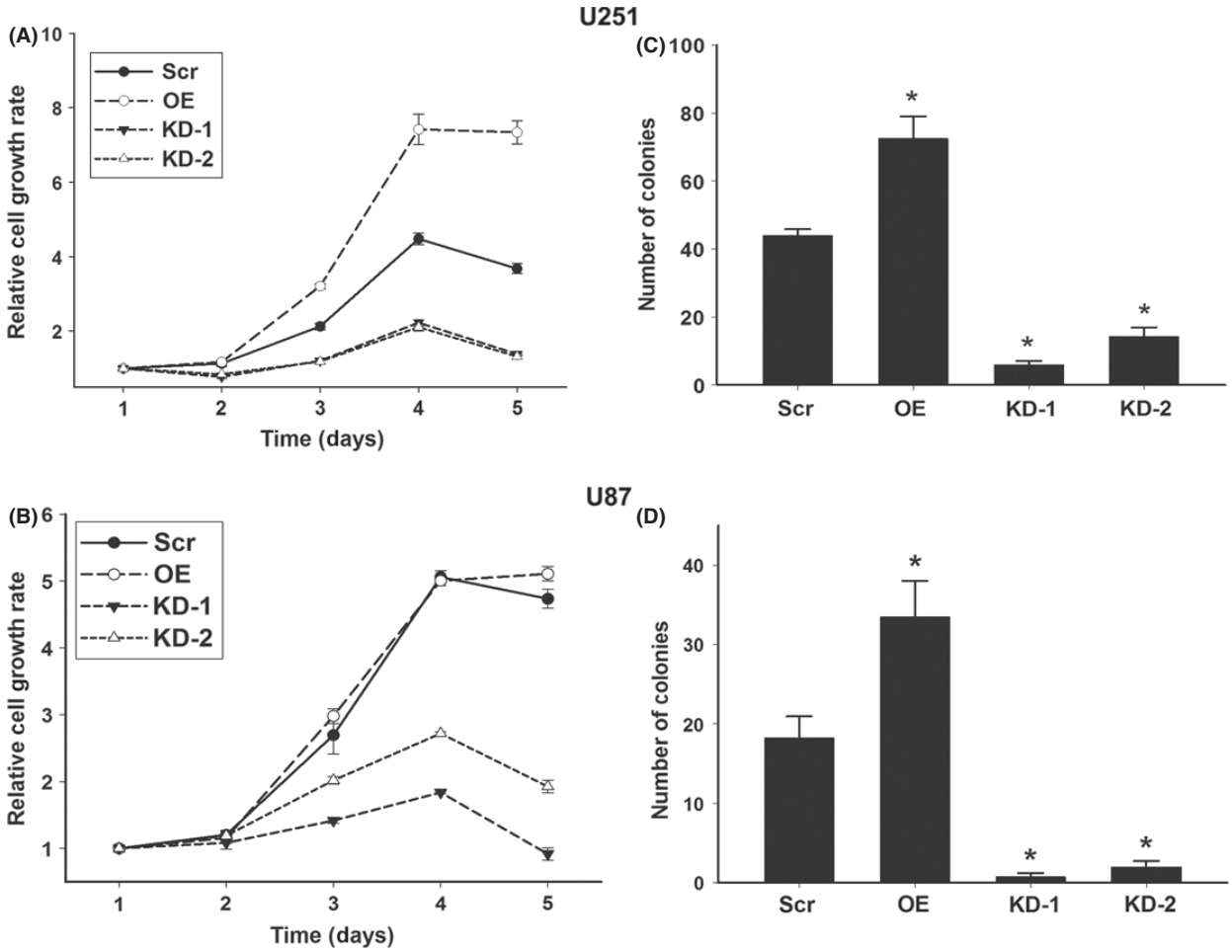

Fig. 4. Cell proliferation and tumorigenesis were examined in human glioma cells treated with CHIP siRNA or overexpression. MTT assay (A,B) and soft agar assays (C,D) were performed on U251 cells (A,C) and U87 cells (B,D) transfected with $CHIP^{Scr}$, $CHIP^{OE}$, $CHIP^{KD1}$ or $CHIP^{KD2}$. Data were presented as mean ± SD. *$P < 0.01$, significant differences between transfected cells and mock-infected cells.

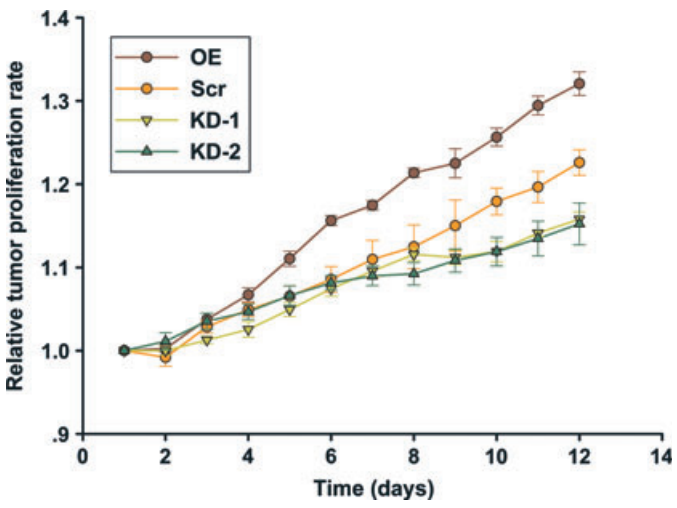

Fig. 5. Glioma cell growth rate after CHIP siRNA or overexpression was examined *in vivo*. Xenografts assays were performed to examine the effect of CHIP on tumorigenesis *in vivo*. Four groups of nude mice received intratumoral injection of $CHIP^{Scr}$, $CHIP^{OE}$, $CHIP^{KD1}$ or $CHIP^{KD2}$ after tumors were palpable in the forelimbs. The tumor volume was calculated and normalized to the initial volume before intratumoral injection to form the curve of glioma cell growth rate. Data are presented as mean ± SE.

Hsp90, Bcl-2 and Hsp70 using quantitative RT-PCR and western blot analysis. No significant changes of mRNA or protein were observed in the tested molecules except survivin (Fig. 6).

As shown in Figure 7, in both U251 and U87 glioma cells with downregulated CHIP, survivin mRNA and protein were decreased. In glioma cells with upregulated CHIP, both survivin mRNA and protein were increased.

Discussion

In the current study, we demonstrated increased CHIP expression associated with histological grade of glioma. This was accompanied with an increase of Ki-67 expression in high-grade glial tumors. In normal brain, CHIP is mainly expressed in the cytoplasm of neurons. Modulating CHIP levels may be a critical determinant in the oncogenesis of glioma. Overexpression of CHIP can enhance proliferation and colony formation of U251 and U87 glioma cell lines *in vitro*, while CHIP RNAi can get opposite results. Moreover, tumor growth in the xenografts was significantly slowed after intratumoral injection of lentivirus containing CHIP shRNA, while enhancement of tumor growth was detected after intratumoral injections of CHIP overexpressed lentivirus. These results indicated that CHIP is required for tumorigenesis *in vitro* and *in vivo* and could induce oncogenesis of human gliomas. Our results can be supported by some published datasets in Oncomine (http://www.oncomine.org). Two of

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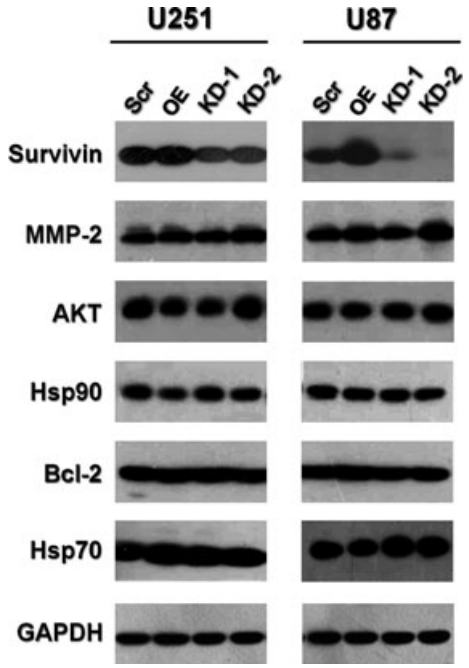

Fig. 6. Total levels of proteins potentially involved in the malignant transformation of CHIP RNAi or expression cells were analyzed by western blot. The protein levels of survivin changed significantly after CHIP RNAi or overexpression, while the expression patterns of other proteins did not change significantly.

four datasets that containing gene chip profiles classified by glial tumor grade (Yamanaka Brain and Kotliarov Brain) showed a correlation between CHIP expression and tumor grade, consistent with our results.

Interestingly, the roles of CHIP that we found in gliomas were totally opposite to those in breast cancer. Kajiro *et al.*[13] showed that CHIP suppressed tumor progression in human breast cancer by inhibiting oncogenic pathways. Tumor growth and invasiveness of shCHIP cells was significantly elevated due to increased expression of Bcl2, Akt1, Smad and Twist. Transcriptional co-activator SRC-3 was found to be a direct target for ubiquitylation and degradation by CHIP.[13] These results indicate that CHIP might play different roles in different human cancers; whether it contributes to tumorgenesis or tumor suppression in other human cancers remains unclear.

E3 ubiquitin ligases were studied a lot in the field of cancer, and their role in cancer proliferation and tumorigenesis is well established.[14] Overexpression of several E3 ubiquitin ligases has been proven to be associated with poor prognosis, such as inhibitor of apoptosis protein (IAP)-family genes,[15] murine double minute 2 (MDM2),[16] Casitas B-lineage lymphoma (Cbl) family[17] and Anaphase promoting complex (APC).[18] Some of them have emerged as therapeutic targets for cancer.[14] As a member of the E3 ubiquitin ligases, CHIP has also been proven in this paper to be involved in proliferation and tumorigenesis of glioma cells. To the best of our knowledge, these results have not been reported before, which might support the idea that targeting of E3 ligases might be a therapeutic approach for glioma.

In the central nervous system, CHIP has been studied in some neurodegenerative diseases. Leucine-rich repeat kinase-2 (LRRK2) is the most common cause of late-onset Parkinson's disease (PD). CHIP showed protective effects by preventing the deleterious effects of LRRK2, and offered potential treatment options for LRRK2-associated PD.[19] In tauopathies, CHIP can ubiquitinate phosphorylated tau, and rescue phosphorylated tau-induced cell death. Therefore, the CHIP-Hsp70 complex may provide a new therapeutic target for tauopathies.[20]

The protective role of CHIP in neurodegenerative disease and its oncogenic role in glioma remind us of an interesting hypothesis: are PD patients protected from some kinds of cancer?[21] It is reported in several epidemiological studies that patients with Parkinson's disease will have a relatively lower risk for some cancers, but will have a relatively higher risk for breast cancer and malignant melanoma.[22,23] The mechanism is still unknown. Kim and Mak[24] proposed that cancer and PD are two pathological processes signaling by one of two sets of opposing forces. We guess that CHIP might also be involved in

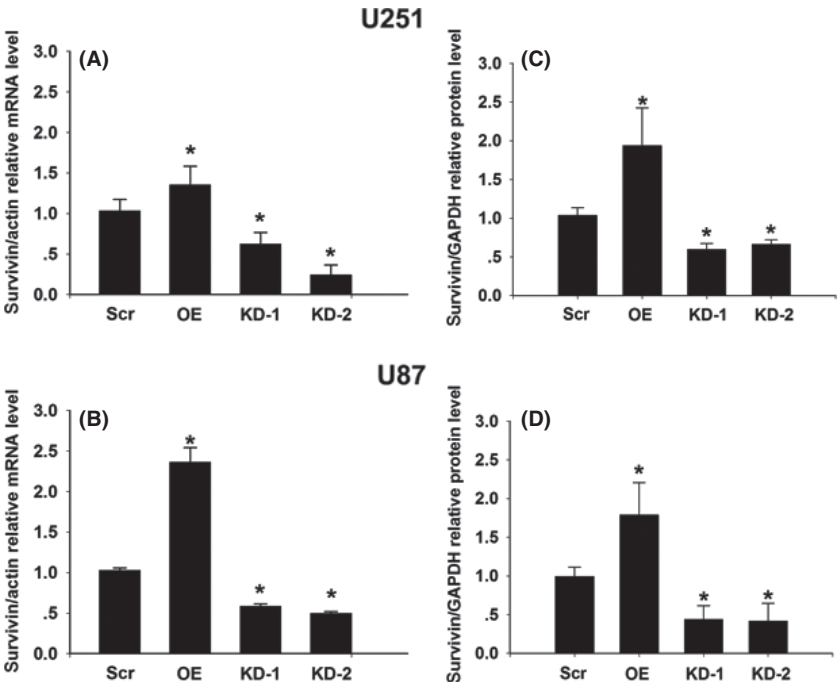

Fig. 7. Expression of survivin mRNA and protein after CHIP RNAi or overexpression in U251 and U87 cells. Both survivin mRNA (A,B) and protein increased (C,D) after CHIP overexpression, but decreased after CHIP RNAi. Data are presented as mean ± SD. $*P < 0.05$, significant differences between transfected cells and mock-infected cells.

these processes. This hypothesis can be supported by the existing evidence of CHIP in cancers and PD. In PD patients, the ubiquitin system of CHIP might be impaired, which could induce protein accumulation and neurotoxicity.[25] The impaired ubiquitin system might also induce breast cancer, since CHIP acts as a tumor suppressor with a relatively low expression in breast cancer. That might be, at least partly, the reason why PD patients have a relatively higher risk of breast cancer. The role of CHIP in glioma and PD might be just the opposite. Parkinson's disease patients might have a relatively lower risk for glioma. Further study about the role of CHIP in other cancers could provide more evidence for this hypothesis.

Survivin was found to be regulated by CHIP in the present study. A lot has been learned previously in human gliomas. Overexpression of survivin was seen frequently in glioma samples and was related to poor prognosis.[26] Our study revealed the same results, that both mRNA and protein levels of survivin were decreased in cells with impaired proliferation and colony formation ability, suggesting that CHIP might regulate tumorigenesis of glioma cells through survivin. Moreover, decreased survivin can effectively sensitize multiple malignant glioma cell lines to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis,[27,28] indicating a potential therapeutic effect on combined CHIP-RNAi and TRAIL on human glioma cells.

In conclusion, we demonstrated increased CHIP expression associated with histological grade for the first time. Knocking down of CHIP can suppress the proliferation and colony formation of U251 and U87 glioma cell lines both *in vitro* and *in vivo*, while overexpression of CHIP results in enhanced proliferation and colony formation. The E3 ubiquitin ligase CHIP might contribute to tumorigenesis of malignant glioma.

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Disclosure Statement

The authors declare no conflict of interests.

References

1 Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med* 2008; **359**: 492–507.
2 The Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; **455**: 1061–8.
3 Jiang L, Huang CG, Lu YC *et al.* Expression of ubiquitin-conjugating enzyme E2C/UbcH10 in astrocytic tumors. *Brain Res* 2008; **1201**: 161–6.
4 Ballinger CA, Connell P, Wu Y *et al.* Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. *Mol Cell Biol* 1999; **19**: 4535–45.
5 Connell P, Ballinger CA, Jiang J *et al.* The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol* 2001; **3**: 93–6.
6 Meacham GC, Patterson C, Zhang W, Younger JM, Cyr DM. The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol* 2001; **3**: 100–5.
7 Esser C, Scheffner M, Hohfeld J. The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation. *J Biol Chem* 2005; **280**: 27443–8.
8 McDonough H, Charles PC, Hilliard EG *et al.* Stress-dependent Daxx-CHIP interaction suppresses the p53 apoptotic program. *J Biol Chem* 2009; **284**: 20649–59.
9 Huang H, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci* 2007; **120**: 2479–87.
10 Li F, Xie P, Fan Y *et al.* C terminus of Hsc70-interacting protein promotes smooth muscle cell proliferation and survival through ubiquitin-mediated degradation of FoxO1. *J Biol Chem* 2009; **284**: 20090–8.
11 Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C, Neckers L. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc Natl Acad Sci U S A* 2002; **99**: 12847–52.
12 Luo W, Zhong J, Chang R, Hu H, Pandey A, Semenza GL. Hsp70 and CHIP selectively mediate ubiquitination and degradation of hypoxia-inducible factor (HIF)-1alpha but Not HIF-2alpha. *J Biol Chem* 2010; **285**: 3651–63.
13 Kajiro M, Hirota R, Nakajima Y *et al.* The ubiquitin ligase CHIP acts as an upstream regulator of oncogenic pathways. *Nat Cell Biol* 2009; **11**: 312–9.
14 Lakshmanan M, Bughani U, Duraisamy S, Diwan M, Dastidar S, Ray A. Molecular targeting of E3 ligases–a therapeutic approach for cancer. *Expert Opin Ther Targets* 2008; **12**: 855–70.
15 Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* 2002; **3**: 401–10.
16 Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997; **387**: 296–9.
17 Duan L, Miura Y, Dimri M *et al.* Cbl-mediated ubiquitinylation is required for lysosomal sorting of epidermal growth factor receptor but is dispensable for endocytosis. *J Biol Chem* 2003; **278**: 28950–60.
18 Harper JW, Burton JL, Solomon MJ. The anaphase-promoting complex: it's not just for mitosis any more. *Genes Dev* 2002; **16**: 2179–206.
19 Ko HS, Bailey R, Smith WW *et al.* CHIP regulates leucine-rich repeat kinase-2 ubiquitination, degradation, and toxicity. *Proc Natl Acad Sci U S A* 2009; **106**: 2897–902.
20 Shimura H, Schwartz D, Gygi SP, Kosik KS. CHIP-Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. *J Biol Chem* 2004; **279**: 4869–76.
21 Inzelberg R, Jankovic J. Are Parkinson disease patients protected from some but not all cancers. *Neurology* 2007; **69**: 1542–50.
22 Olsen JH, Friis S, Frederiksen K, McLaughlin JK, Mellemkjaer L, Moller H. Atypical cancer pattern in patients with Parkinson's disease. *Br J Cancer* 2005; **92**: 201–5.
23 Minami Y, Yamamoto R, Nishikouri M, Fukao A, Hisamichi S. Mortality and cancer incidence in patients with Parkinson's disease. *J Neurol* 2000; **247**: 429–34.
24 Kim RH, Mak TW. Tumours and tremors: how PTEN regulation underlies both. *Br J Cancer* 2006; **94**: 620–4.
25 Dickey CA, Patterson C, Dickson D, Petrucelli L. Brain CHIP: removing the culprits in neurodegenerative disease. *Trends Mol Med* 2007; **13**: 32–8.
26 Chakravarti A, Noll E, Black PM *et al.* Quantitatively determined survivin expression levels are of prognostic value in human gliomas. *J Clin Oncol* 2002; **20**: 1063–8.
27 Kim JY, Kim EH, Kim SU, Kwon TK, Choi KS. Capsaicin sensitizes malignant glioma cells to TRAIL-mediated apoptosis via DR5 upregulation and survivin downregulation. *Carcinogenesis* 2010; **31**: 367–75.
28 Son YG, Kim EH, Kim JY *et al.* Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. *Cancer Res* 2007; **67**: 8274–84.

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