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Corticotropin-releasing factor suppresses glioma progression by upregulation of long non-coding RNA-p21

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ABSTRACT

Corticotropin-releasing factor (CRF) plays a key role in neuroendocrine regulation of hypothalamo-pituitary-adrenal axis under normal condition and stress by binding to CRF receptor1 (CRFR1). CRF and its receptors have been reported in many types of tumors. Little is known about the role of CRF in the development of glioma. And lincRNA-p21 was reported to act as a role in progression of some cancers. The aim of the present study was to investigate the levels of CRF in glioma, and explore the link between CRF and lincRNA-p21 in this disease. In this study, we found CRF mRNA expression was significantly down-regulated in glioma mice. Moreover, CRF could suppress the proliferation of glioma cells and promote the expression of lincRNA-p21. Afterwards, lincRNA-p21 repressed the proliferation and invasion of glioma cells, which was reversed by miR-34c targeted with 3′-UTR. Furthermore, miR-34c decreased the expression of CRFR1 by binding with the 3′-UTR, which interact with CRF to inhibit the proliferation of glioma cells. Together, these results CRF plays as an important role in glioma progression and metastasis through activation of lincRNA-p21, providing a novel insight for the pathogenesis and underlying therapeutic target for glioma.

1. Introduction

A glioma is one of the most common primary tumors that arise in the glial cells of the brain or the spine [1]. Gliomas account for about 30% of all brain tumors and central nervous system tumors, and 80% of all malignant brain tumors [2]. The surgical operation treatment is still a first-selected way for the patients with glioma at present. Despite advances in surgical techniques and other adjuvant therapies, the prognosis of glioma patients remains poor [3]. Therefore, further investigation of glioma development at a molecular level is necessary.

Corticotropin-releasing factor (CRF) is a 41-amino acid peptide which was first isolated in hypothalamus of sheep [4]. CRF plays a key role in neuroendocrine regulation of hypothalamo-pituitary-adrenal (HPA) axis under normal condition and stress by binding to CRF receptor (CRFR) [5,6]. CRF and its receptors have been reported in many types of tumors. Graziani et al. revealed that CRF inhibited the proliferation of human breast cancer [7]. Conversely, Yang et al. observed that CRF promoted the migration of melanoma cells via the ERK pathway [8]. These findings indicate that CRF has both pro- and anti-cancer activity. However, the biological roles of CRF in gliomas are still poorly understood.

As a member of non-coding RNAs, long non-coding RNA (lncRNA), > 200 nucleotides in length, plays an important role in diverse cellular processes, such as regulation of gene expression, post-translational processing and tumorigenesis [9,10]. Long intergenic non-coding RNA p21 (lincRNA-p21) was first reported as a direct transcriptional target of p53, it mainly regulates the cell cycle and apoptosis [11].Several studies have manifested that lincRNA-p21 could inhibit the progression of colorectal cancer and hepatocellular carcinoma [12,13]. Nevertheless, the role of lincRNA-p21 in glioma remains elusive.

MicroRNAs are a class of highly conservative non-coding small RNAs (about 19–23 nucleotides) that regulate gene expression in signal pathways, which play an important role in maintaining the homeostasis [14]. It regulates post-transcriptional gene silencing, induces the mRNA degradation or inhibits the transcription by endogenously binding to the 3′-untranslated region (3′UTR) of target genes [15]. Currently, it is widely accepted that miRNAs may act as oncogenes or suppressor genes during tumor development [16]. Previous studies have confirmed the function of miR-34c as a tumor regulator in various human cancers, like osteosarcoma, hepatocellular carcinoma and endometrial carcinoma [17–19]. Whether miR-34c acts on glioma cells is still not very clear.

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Thus, the aim of the present study was to investigate the level of CRF expression in the progression of glioma, and explore the link between CRF and lincRNA-p21 in this disease.

2. Material and methods

2.1. Experimental animals, glioma model construction

6–8 week old female BALB/c nude mice (body weight 20.0 ± 2 g) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The mice were anesthetized with 0.75% pentobarbital sodium (50 mg/kg intraperitoneally) and placed in ALC-H type mouse brain sterotactic instrument. After incising the scalp longitudinally, 1×10^5 U87 or U251 cells (purchased from Tongpi Biological Technology Co., Ltd.) were implanted 3 mm deep in the right cerebral hemisphere (1 mm posterior and 2 mm lateral to the bregma) of BALB/c mice as previously described [20]. All animal experiments were performed according to institutional, local and national guidelines on animal research and ethics.

2.2. ACTH and cortisol measurement

For determination of plasma ACTH and cortisol levels, mice were euthanized by decapitation between 8:00 and 9:00 AM. Blood from angulus oculi vessels was collected in heparinized tubes and allowed to clot for 30 min at room temperature before centrifugation for 10 min at 3000 ×*g* at 4 °C. Mice ACTH and cortisol concentration determination was performed using ELISA kits according to the manufacturer's instructions.

2.3. Cell culture

The human glioma cell lines, U87 and U251, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA) and 1% penicillin/streptomycin at 37 °C in a humidified, 5% CO_2 atmosphere randomly. The U87 and U251 cells were passaged four times and divided into 4 groups (3 sample sizes in each group) randomly. DMEM containing 10^{-7} mol/L, 10^{-8} mol/L, 10^{-9} mol/L and 0 mol/L CRF was added in 50 mL culture flasks respectively.

2.4. Cell transfection

For transfection, cells were seeded into plates and transfected with Lv-lincRNA-p21, si-lincRNA-p21 or their parental negative controls (NCs) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA) according to the manufacturer's protocols. Knockdown of lincRNA-p21 expression was achieved using small interfering RNA (siRNA) against lincRNA-p21. To establish a stably overexpressed or silenced SNHG7 cell line, lentivirus particles derived from the plasmid GV113 containing the Lv- or si-lincRNA-p21 RNA were used to infect U87 or U251 cells.

2.5. Real time quantitative PCR analysis

The whole hypothalamus were dissected between 8:00 and 9:00 AM and homogenized in 200 ml physiological saline (mixed liquor). The hypothalamus CRF mRNA and the cells total RNA were extracted using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a reverse transcription kit (Takara Biotechnology, Dalian, China). Relative quantity of cDNA was assessed by quantitative PCR. Relative levels of gene expression was expressed relative to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Cell proliferation assay

Cells (3×10^3) were cultured in 96-well plates and incubated for 24 h and stained with 0.5 mg/ml MTT for 4 h. Supernatant was discarded and 200 μl of dimethylsulfoxide (DMSO) was added to dissolve precipitates. Samples were measured at 490 nm using an ELISA reader.

2.7. Cell migration assay

U87 and U251 cells at density of 1×10^5 per well were respectively seeded into the upper chamber containing with serum-free DMEM. The lower chamber was filled with DMEM supplemented with 10% FBS medium. After 24 h incubation, cells adhered on the upper surface of membrane were removed, while the cells migrated into the lower chamber were stained with 0.1% crystal violet.

2.8. Apoptosis analysis by flow cytometry

U87 and U251 cells were examined for apoptotic status by double-staining with Annexin V and PI using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen, Nanjing, China), followed by flow cytometric analysis.

2.9. Luciferase reporter assay

The3′-UTR of lincRNA-p21 and CRFR1 containing miR-34c binding sites was amplified and cloned into the pGL3-basic luciferase vector (Promega, USA), respectively. Cells were seeded into plates and transfected with involved oligonucleotides (the lincRNA-p21 wild-type or mutant reporter vector, miR-34c mimics or negative control) mixed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA) according to the manufacturer's protocols. Luciferase activity was detected after transfection 24 h using Dual-Luciferase Reporter System (Promega, Madison, WI, USA). Renilla luciferase was used as an internal reference.

2.10. Western blotting analysis

The cultured cells were washed with ice-cold PBS and lysed in RIPA buffer supplemented with protease inhibitor mixture. After electrophoresis, the protein samples were incubated with the primary antibody (anti-CRFR1; 1:1000dilution). Samples were incubated with secondary antibodies conjugated by HRP. Bands were quantified using ImageJ software.

2.11. Statistical analysis

Results are expressed as the mean ± standard error. The Student's *t*-test and ANOVA were performed among different groups. All calculations were performed using SPSS 17.0 software (IBM Software, Chicago, IL, USA) and GraphPad (vision 6.0, USA).The correlation analysis of continuous variables was based on Spearman test correlation method. A value of $P < 0.05$ indicated a statistically significant difference.

3. Results

3.1. Hormonal changes in the HPA axis of mice with glioma

In order to investigate the hormonal profiles of the HPA axis in mice model of glioma, we measure CRF mRNA expression using qRT-PCR and the plasma ACTH and Cortisol levels using ELISA. Fig. 1A and B showed the level of CRF mRNA expression was significantly down-regulated in U87 and U251 mice with glioma. However, no obvious change was found in the concentration of plasma ACTH and Cortisol in mice injected with U87 or U251 cells (Fig. 1C, 1D, 1E and 1F).

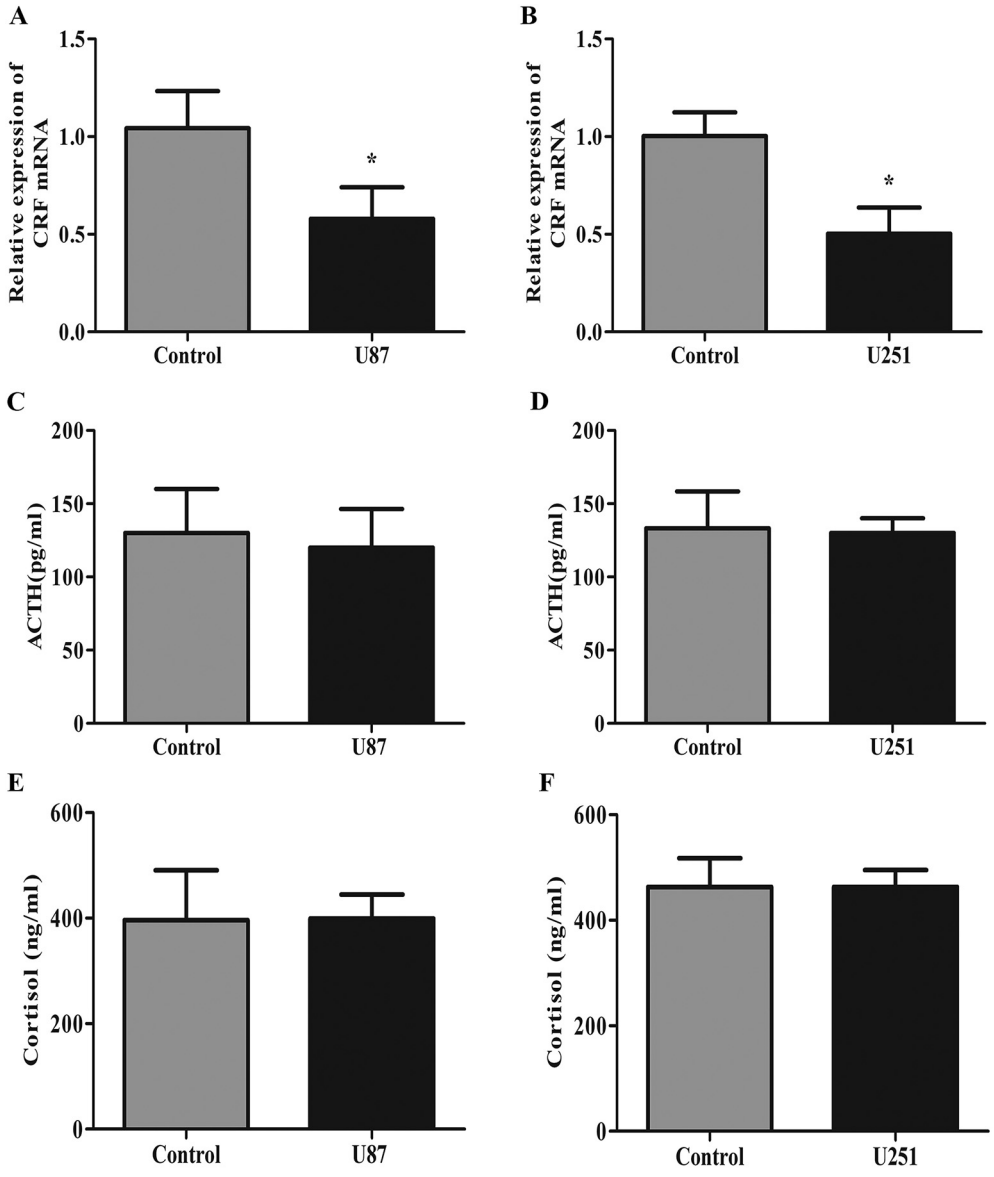

Fig. 1. Hormonal changes in the HPA axis of mice with glioma. A and B showed the level of CRF mRNA expression was significantly down-regulated in U87 and U251 mice with glioma (*$P < 0.05$). C and D demonstrated no obvious change in the concentration of plasma ACTH in U87 and U251 mice with glioma. E and F demonstrated no obvious change in the concentration of plasma Cortisol in U87 and U251 mice with glioma.

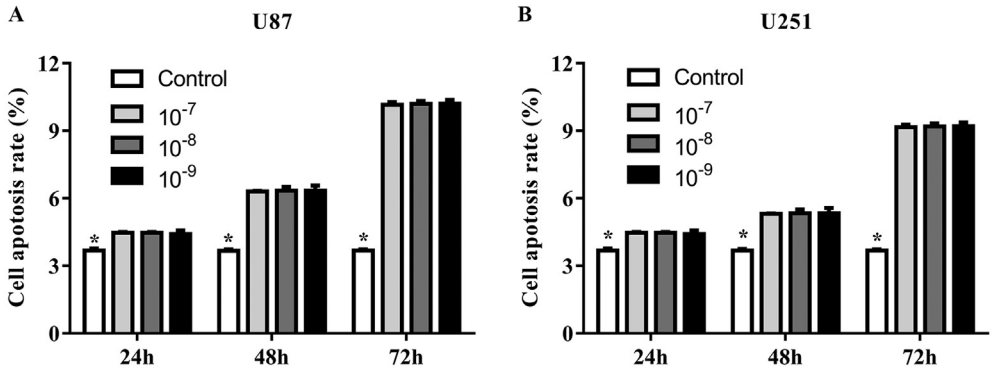

Fig. 2. Glioma cell lines U87 and U251 were treated by different concentrations of CRF. U87 and U251 cells was divided into four groups, which were dealt with by different concentrations of CRF(0, 10^{-7}, 10^{-8} and 10^{-9} mol/L).In the test groups, CRF showed no statistical differences on U87 and U251 proliferation among each time point ($P > 0.05$). Compared with control group, cell apoptosis rate in test groups increased with CRF concentration, and presented a positive correlation (*$P < 0.05$) (A and B).

3.2. Glioma cell lines U87 and U251 were treated by different concentrations of CRF

U87 and U251 cells was divided into four groups, which were deal with by different concentrations of CRF(0, 10^{-7}, 10^{-8} and 10^{-9} mol/L).In the test groups, CRF showed no statistical differences on U87 and U251 proliferation at indicated time point. Compared with control group, cell apoptosis rate in test groups increased with CRF

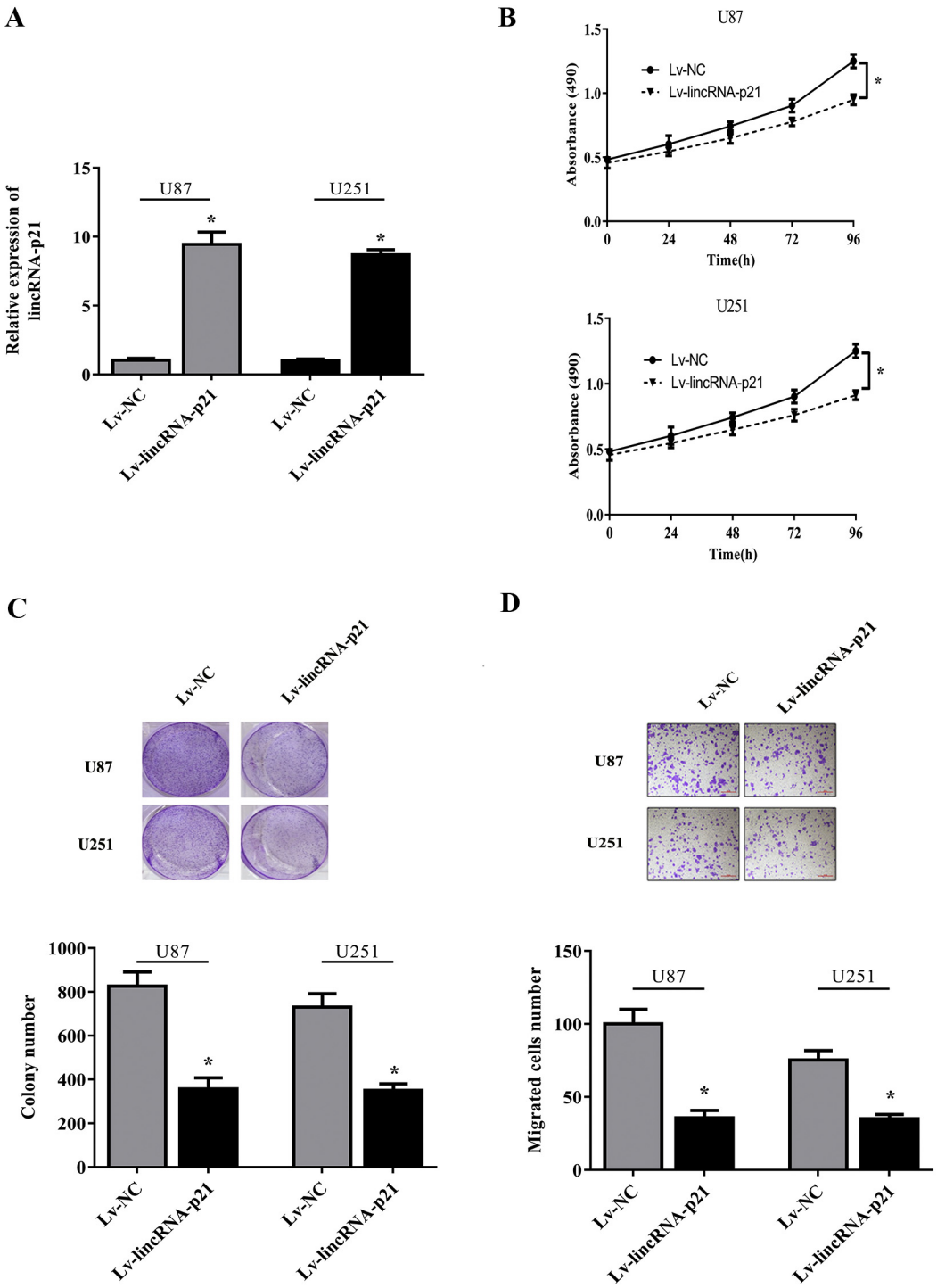

Fig. 3. LincRNA-p21 suppressed the proliferation and invasion of glioma cells in vitro. A, LincRNA-p21 level was significantly elevated in two cell lines transduced with Lv-lincRNA-p21 compared with Lv-NC (*$P < 0.05$). B, the growth rate of cells transfected with Lv-lincRNA-p21 was significantly down-regulated compared to that with Lv-NC in two cell lines (*$P < 0.05$). C, Colony formation assay revealed that lincRNA-p21 transfected U87 and U251 cells formed fewer colonies than negatively control group (*$P < 0.05$). D, Enhanced expression of lincRNA-p21 significantly decreased cell migration in U87 and U251 cells (*$P < 0.05$).

concentration, and presented a positive correlation (Fig. 2A, B).

3.3. LincRNA-p21 suppressed the proliferation and invasion of glioma cells in vitro

To characterize the role of lincRNA-p21 in the progression of glioma, the lincRNA-p21-overexpression in two cell lines (U87 and U251) was constructed via retrovirus infection. Results of qRT-PCR manifested that lincRNA-p21 level was significantly elevated in two cell lines transduced with Lv-lincRNA-p21 compared with Lv-NC (Fig. 3A). To explore the role of lincRNA-p21 in cell proliferation, a MMT assay was performed to assess the viability of glioma cell lines. Results revealed that the growth rate of cells transfected with Lv-lincRNA-p21 was significantly down-regulated compared to that with Lv-NC in two cell lines (Fig. 3B). Colony formation assay revealed that lincRNA-p21 transfected U87 and U251 cells formed fewer colonies than negatively

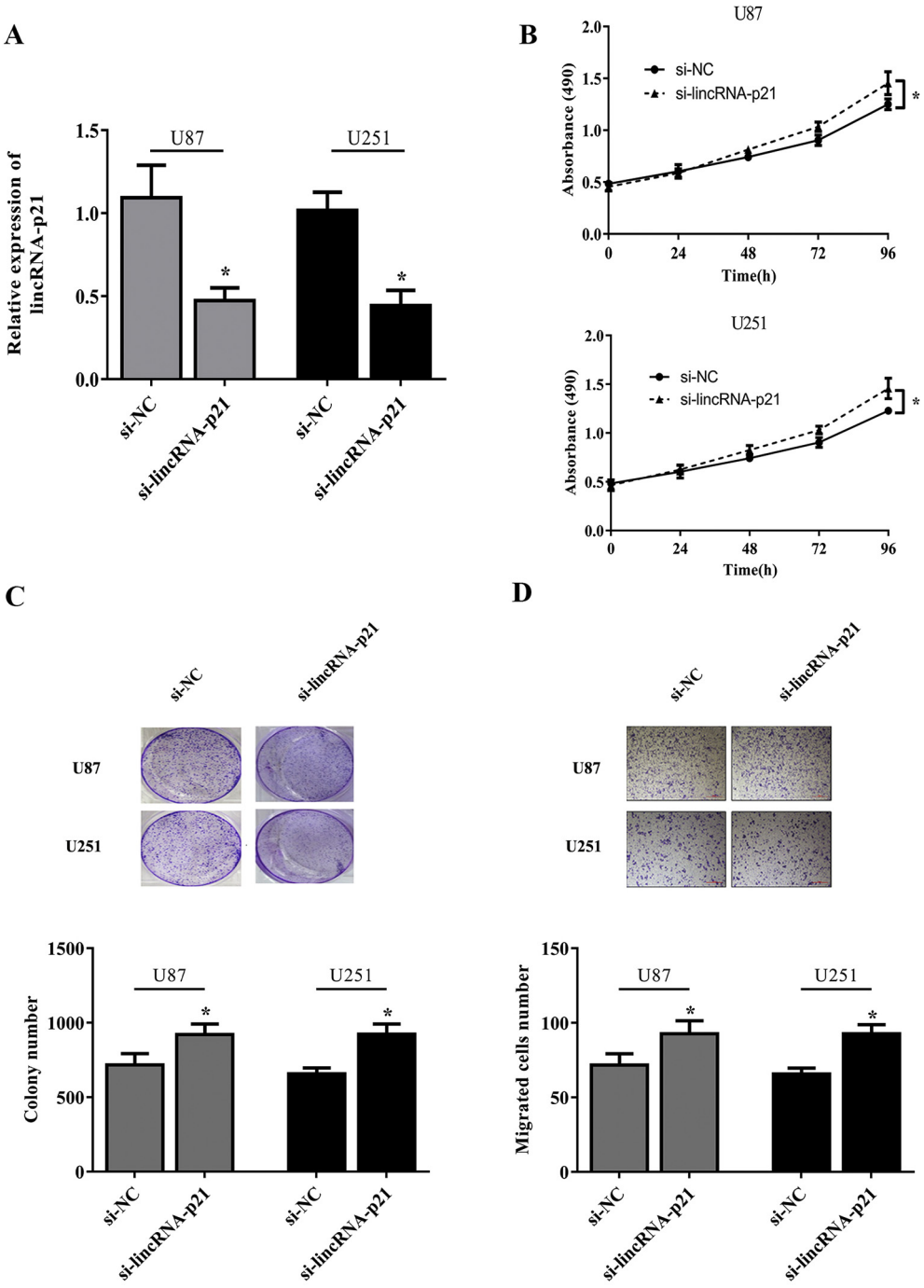

Fig. 4. LincRNA-p21 silencing promoted the proliferation and migration of glioma cells in vitro. LincRNA-p21 silencing was established via siRNA transfection in these two cell lines (Fig. 4A, $P < 0.05$). MTT assay showed that lincRNA-p21 silencing inhibited the proliferation of U87 and U251 cells (Fig. 4B, $P < 0.05$). Colony formation assay revealed that lincRNA-p21 silencing could promote U87 and U251 cells formed significantly more colonies than control these two cell lines (Fig. 4C, $P < 0.05$). Transwell assays showed lincRNA-p21 silencing aggravated the migratory ability of glioma cells (Fig. 4D, $P < 0.05$).

control group (Fig. 3C). As presented in Fig. 3D, enhanced expression of lincRNA-p21 significantly decreased cell migration in U87 and U251 cells.

3.4. LincRNA-p21 silencing promoted the proliferation and invasion of glioma cells in vitro

To further verify the role of lincRNA-p21 in the development of glioma, we stably established lincRNA-p21 silencing via siRNA transfection in these two cell lines in vitro (Fig. 4A). MTT assay showed that lincRNA-p21 silencing promoted the proliferation of U87 and U251 cells (Fig. 4B). Colony formation assay revealed that lincRNA-p21 silencing could promote U87 and U251 cells formed significantly more colonies than control these two cell lines (Fig. 4C). As demonstrated by transwell assays, lincRNA-p21 silencing aggravated the migratory ability of glioma cells (Fig. 4D).

3.5. LincRNA-p21 acts as a molecular sponge for miR-34c

Increasing evidence had illustrated that lncRNAs function as sponges to bind specific miRNAs. Bioinformatics analysis was used to predict the candidate targets of miRNAs binding with lincRNA-p21.

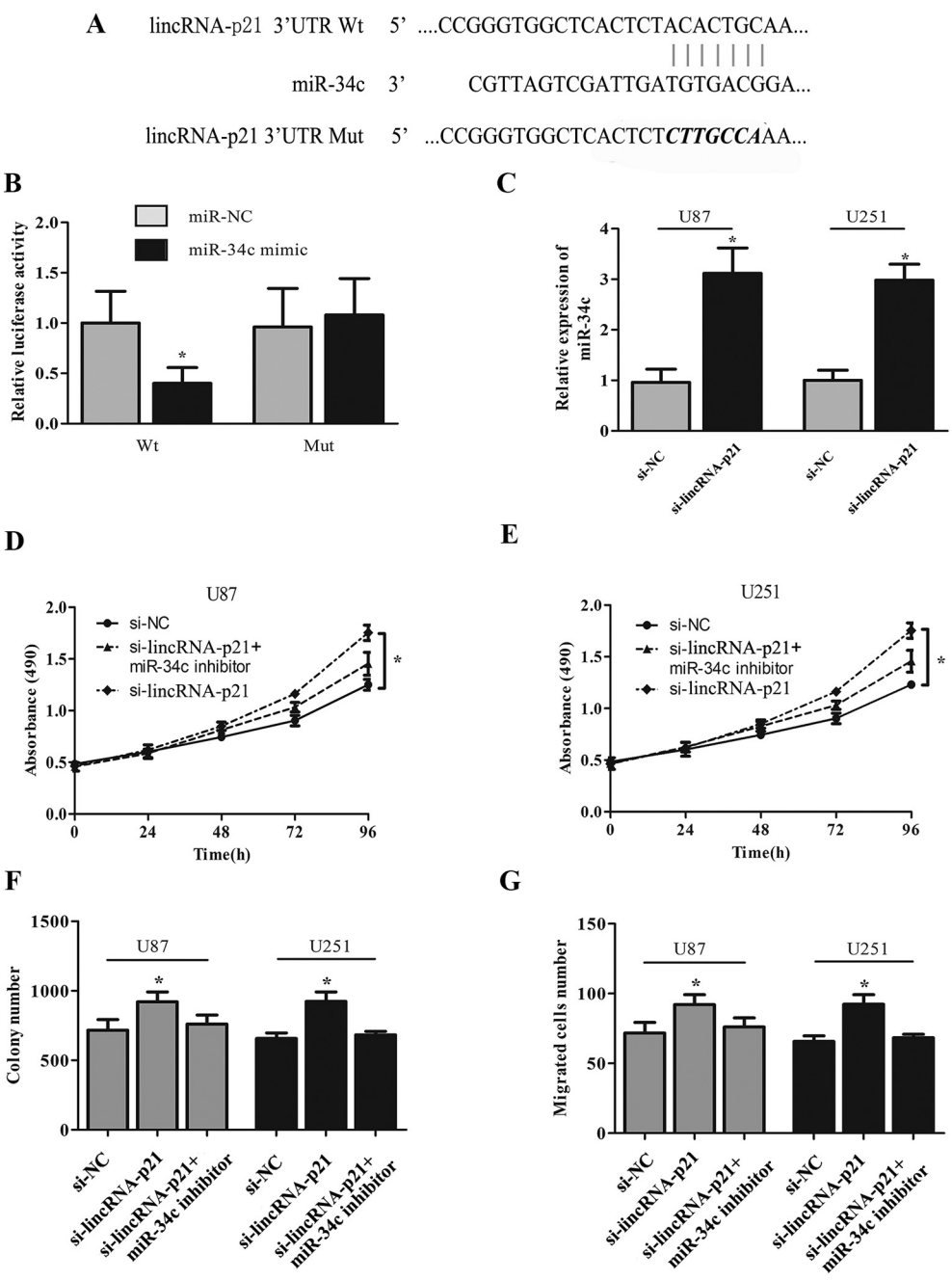

Fig. 5. LincRNA-p21 acts as a molecular sponge for miR-34c. The 3′-UTR binding sites can be seen in Fig. 5A. Luciferase reporter assay confirmed the binding with the decreasing fluorescence within miR-34c mimic and lincRNA-p21 wild type (Fig. 5B, $^*P < 0.05$). After glioma cells (U87 and U251) were transfected with si-lincRNA-p21, miR-34c expression levels were significantly up-regulated (Fig. 5C, $^*P < 0.05$). MTT assay revealed that miR-34c inhibitor could rescue the suppression by si-lincRNA-p21 in the proliferation of glioma cells (Fig. 5D and E; $^*P < 0.05$). Colony formation assay revealed that miR-34c inhibitor could rescue the suppression by si-lincRNA-p21 in the proliferation of glioma cells (Fig. 5F, $^*P < 0.05$). Transwell assay showed that miR-34c inhibitor repressed the migration of glioma cells induced by si-lincRNA-p21 (Fig. 5G, $^*P < 0.05$).

Results found 3′UTR of lincRNA-p21 was highly conserved to bind with miR-34c. The 3′-UTR binding sites can be seen in Fig. 5A. Luciferase reporter assay confirmed the binding with the decreasing fluorescence within miR-34c mimic and lincRNA-p21 wild type (Fig. 5B). Moreover, after glioma cells (U87 and U251) were transfected with si-lincRNA-p21, miR-34c expression levels were significantly up-regulated (Fig. 5C). Colony formation assay and MTT assay revealed that miR-34c inhibitor could rescue the suppression by si-lincRNA-p21 in the proliferation of glioma cells (Fig. 5D, E and F). Besides, transwell assay showed that miR-34c inhibitor repressed the migration of glioma cells induced by si-lincRNA-p21 (Fig. 5G).

3.6. miR-34c directly targets with 3′-UTR of CRFR1

We examined computationally predicted targets of miR-34c using bioinformatics analysis. Results revealed 3′UTR of CRFR1 was highly conserved to bind with miR-34c. The 3′-UTR binding sites can be seen in Fig. 6A. Luciferase reporter assay showed transfection of miR-34c could significantly restrict the relative luciferase activity in cells, suggesting miR-34c has inhibitory effects on CRFR1 expression via interaction with the 3′-UTR of CRFR1 (Fig. 6B). Moreover, as shown in Fig. 6C and D, overexpression of miR-34c obviously downregulated CRFR1 expression in U87 and U251 cells at the mRNAand protein levels. Overall, our study discovered that miR-34c suppressed the expression of CRFR1 by binding with the 3′-UTR.

3.7. The level of lincRNA-p21, miR-34c and CRFR1 expression in glioma cells treated by CRF

Next, we detected the lincRNA-p21, miR-34c and CRFR1 expression in glioma cells following treatment with CRF. Results showed the expression of lincRNA-p21 was significantly up-regulated in cells treated by CRF compared to that in cells without treatment (Fig. 7A). The levels of miR-34c expression were obviously decreased in glioma cells

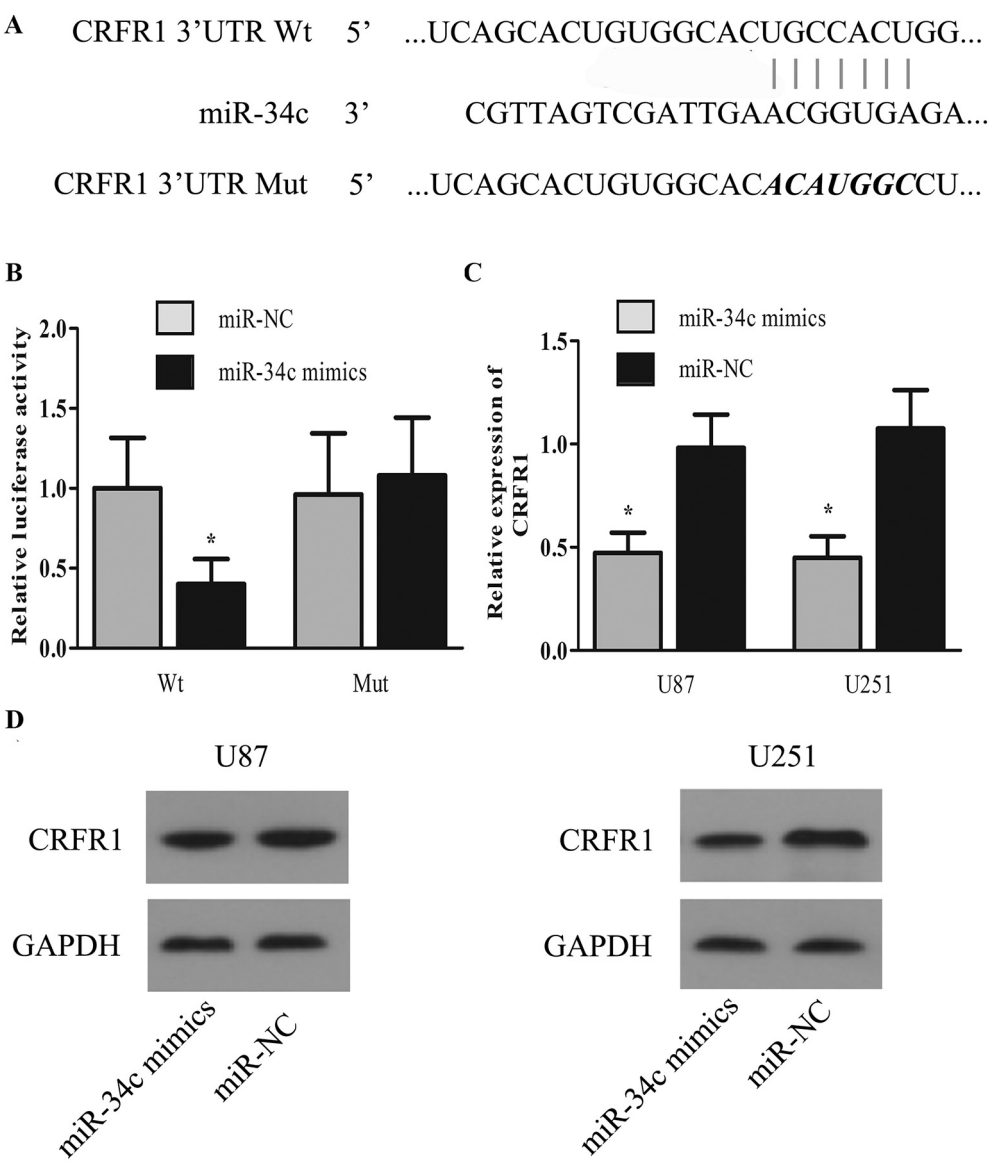

Fig. 6. MiR-34c directly targets with 3′-UTR of CRFR1. The 3′-UTR binding sites can be seen in Fig. 6A. Luciferase reporter assay showed transfection of miR-34c could significantly restrict the relative luciferase activity in cells (Fig. 6B, $*P < 0.05$). As shown in Fig. 6C and D, overexpression of miR-34c obviously down-regulated CRFR1 expression in U87 and U251 cells at the mRNA and protein levels.

following treatment with CRF (Fig. 7B). As shown in Fig. 7C, there was a significant increase of CRFR1 in glioma cells treated by CRF at the mRNA and protein levels.

4. Discussion

Corticotrophin-releasing factor (CRF) was originally identified as a regulator of the stress response in hypothalamic-pituitary-adrenal axis [21]. The CRF system, consisting of CRF and CRFR, has been detected in the cardiovascular, reproductive and gastrointestinal systems [22]. The former researches have confirmed that CRF and its receptors exert their function as pro- or anti-factor in different types of tumors [8,23]. The present study showed the level of CRF mRNA expression was significantly in glioma cells. However, no obvious change was found in the concentration of plasma ACTH and cortisol. Furthermore, CRF could suppress the proliferation of glioma cells.

Long non-coding RNAs are involved in various biological processes and diseases. LincRNA-p21 was first found by Huarte M et al. as a tumor suppressive noncoding RNA with a length of ~3 kb [11]. The present study focused on the role of lincRNA-p21 in glioma cells. Results showed that, in line with previous studies, lincRNA-p21 act as tumor-inhibiting factor which could suppress the proliferation and invasion of glioma cells. We also found that knocking down lincRNA-p21 expression promoted cell migration and invasion of glioma cell lines.

lncRNAs function as sponges for common miRNAs and abolished the endogenous suppressive effect of these miRNAs on their targeted transcripts [24]. Utilizing bioinformatics prediction program, we found 3′UTR of lincRNA-p21 was highly conserved to bind with miR-34c. After glioma cells were transfected with si-lincRNA-p21, miR-34c expression levels were significantly up-regulated. And miR-34c inhibitor could rescue the suppression by si-lincRNA-p21 in the proliferation of glioma cells. These data illustrate lincRNA-p21 exerted the anticancer role by targeting miR-34c.

Haramati, S. et al. have reported that stress-related CRFR1 mRNA is one of the miR-34c targets [25]. Consistent with previous research, bioinformatics analysis showed that miR-34c closely target with 3′-UTR of CRFR1 in our study. Moreover, over-expression of miR-34c could lower the CRFR1 mRNA and related protein production.

CRF could suppress the proliferation of melanoma cells through activation of endogenous CRFR1 [23]. Similarly, the results showed a

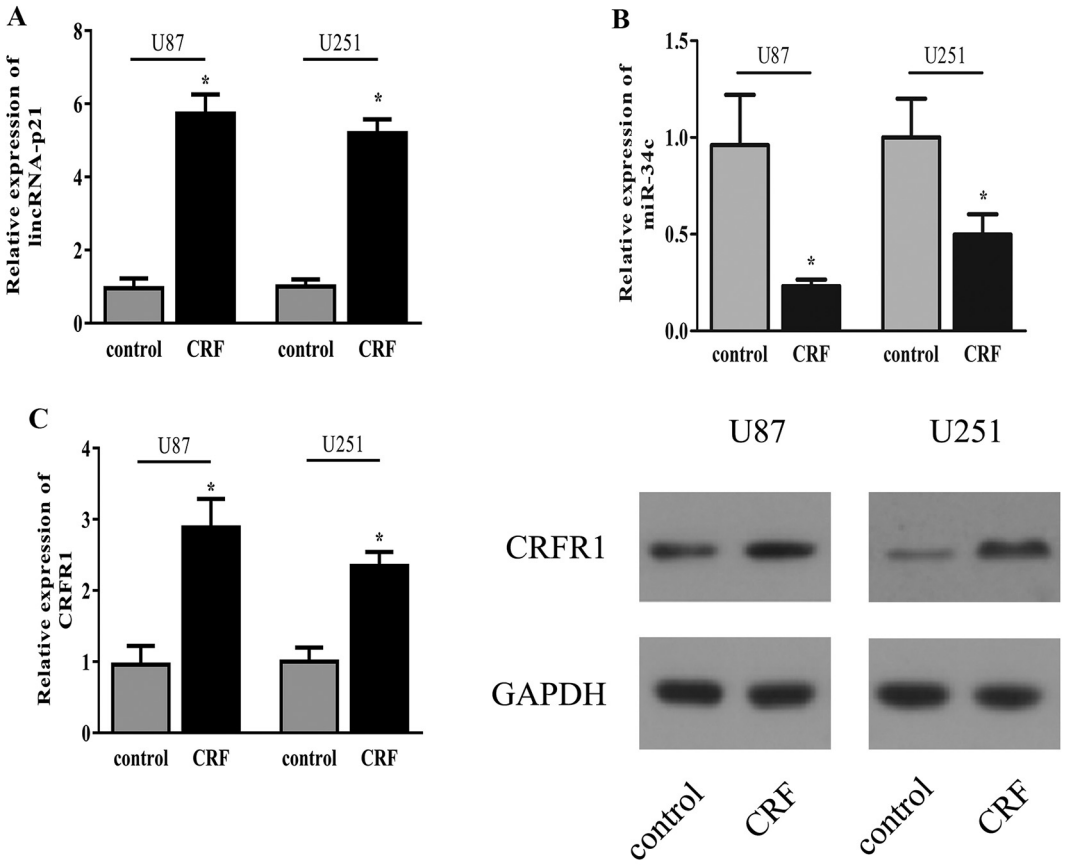

Fig. 7. The level of lincRNA-p21, miR-34c and CRFR1 expression in glioma cells treated by CRF. The expression of lincRNA-p21 was significantly up-regulated in cells treated by CRF compared to that in cells without treatment (Fig. 7A, $*P < 0.05$). The levels of miR-34c expression were obviously decreased in glioma cells following treatment with CRF (Fig. 7B, $*P < 0.05$). As shown in Fig. 7C, there was a significant increase of CRFR1 in glioma cells treated by CRF at the mRNA and protein levels.

significant increase of CRFR1 in glioma cells following treatment with CRF at the mRNA and protein levels. Besides, CRF could promote the expression of lincRNA-p21, which was targeted by miR-34c at 3′UTR.

5. Conclusion

The current study suggests CRF plays as an important role in glioma progression through activation of lincRNA-p21, providing a novel insight for the pathogenesis and underlying therapeutic target for glioma.

Conflict of interest

All authors declare no conflicts of interest.

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