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LINC00470 represses cell autophagy and cisplatin sensitivity of glioma *via* suppressing PTEN expression

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Abstract

Introduction: *Glioma is one of primary brain tumours which has the worst clinical prognoses of patients. As an alternative chemotherapeutic drug for malignant glioma, the therapeutic effect of cisplatin (CDDP) is devastatingly affected due to resistance in patients. In this study, we investigated the effect of LINC00470/PTEN on the CDDP sensitivity of glioma cells.*

Material and methods: *Differentially expressed lncRNAs and the downstream regulators in glioma tissue were obtained via bioinformatics analysis. LINC00470 and PTEN mRNA expression levels were detected using qRT-PCR. IC50 values of glioma cells were examined using Cell Counting Kit-8 (CCK-8). Cell apoptosis was revealed by flow cytometry. The expression level of autophagy-related protein was detected by western blot. Intracellular autophagosome formation was detected by immunofluorescence staining, and the methylation level of PTEN promoter was detected via methylation-specific PCR (MSP).*

Results: *Through the above steps, we found that LINC00470 was highly expressed in glioma cells, and the survival rate of patients was reduced in the presence of high expression of LINC00470. Silenced LINC00470 promoted LC3 II expression and autophagosome formation, and facilitated cell apoptosis to inhibit resistance to CDDP. While silenced PTEN could successfully reverse the previous effects on glioma cells.*

Conclusions: *Based on the above, LINC00470 repressed cell autophagy by constraining PTEN, thereby enhancing CDDP resistance of glioma cells.*

Key words: *LINC00470, PTEN, autophagy, glioma, cisplatin resistance.*

Introduction

The most frequently developed brain tumour is glioma, and patients with this tumour have the poorest clinical prognosis among those with other primary brain tumours [17]. The median survival for grade IV glioma patients ranges from 12 to 15 months. The current standard of care consists of maximal surgical resection followed by concomitant chemotherapy and radiotherapy [8]. The standard chemotherapy accepted by the international community for glioma is a combination of radiotherapy and temozolomide [9,32]. However, due to the side effects of these therapies, the alternative therapy is desired. As one of the most effective anti-tumour drugs, cisplatin (CDDP) is often chosen as an alternative chemotherapeutic drug for malignant glioma [9]. CDDP induces apoptosis-mediated cell death by interacting with and damaging DNA to target tumour cells [19]. A report revealed that tumour cells developed chemotherapeutic resistance to CDDP due to the repeated usage of this drug in clinical practice. Accordingly, dissection of the mechanism of CDDP resistance and exploration of effective combination therapy for eliminating such resistance could offer unique molecular targets for glioma treatment.

Recent research has revealed many long non-coding RNAs (lncRNAs) that regulate biological and pathological processes [12]. LINC00470 was proven as an oncogene

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in human cancers. For instance, LINC00470 facilitated phenotypes of gastric cancer cells *via* the promotion of PTEN mRNA degradation [28]. LINC00470 stimulated methylation of PTEN to facilitate the progression of endometrial cancer by recruiting DNMT3a *via* MYC [30]. LINC00470 directly interacted with FUS and repressed autophagy as an AKT activator to facilitate glioma cell phenotypes. Besides, LINC00470 could affect tumour cell resistance. For example, LINC00470 reduced PTEN stability through RNA methyltransferase METTL3, thereby inhibiting autophagy and promoting CML chemoresistance [10]. LINC00470 promoted expression of MYC and ABCC1 *via* inhibiting miR-134, which facilitated glioma cell proliferation and invasion, and reduced the chemosensitivity of glioma cells to temozolomide. Taken together, LINC00470 played a crucial part in tumour progression and chemoresistance. However, the underlying mechanism by which LINC00470 affected glioma CDDP resistance was elusive.

PTEN encodes phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that includes a typical catalytic domain of the dual-specificity protein tyrosine phosphatases, and a tensin-like domain [24]. PTEN plays a tumour suppressor role [3]. The publication also revealed that PTEN/PI3K/AKT is a pivotal signalling pathway regulating various biological processes such as apoptosis, metabolism, proliferation, and growth [3]. Besides, PTEN is a mutated gene that is widely found in human cancers, and the AKT pathway can activate the loss or mutation of PTEN, which develops resistance to therapies with tyrosine kinase inhibitors (TKIs) [20,24]. Silenced miR-152 facilitates progression of bladder cancer *via* DNMT regulating PTEN expression [14]. miR-92 facilitates prostate cancer cell proliferation and suppresses apoptosis by constraining PTEN/Akt signalling pathway activity [29].

Herein, we found that LINC00470 was upregulated in glioma, while suppressing LINC00470 facilitated cell apoptosis and autophagy, and enhanced CDDP sensitivity of glioma cells. We discovered that LINC00470 was able to inhibit autophagy and enhance CDDP resistance of glioma cells *via* constraining PTEN expression. The findings may shed new light on glioma patients' treatment.

Material and methods

Bioinformatics analysis

Analysis of PTEN expression in tissue (normal: 5, tumour: 698) was acquired from the Cancer Genome Atlas-Glioblastoma Multiforme (TCGA-GBM) dataset. *Via* package "survival" [25], we also conducted a survival analysis of LICN00470 and PTEN expression in glioma patients.

Cell cultivation

Normal human glial cells HEB (BNCC358606) and three human glioma cell lines H251 (BNCC337874), LN299 (BNCC342090), and U87 (BNCC337885) were produced by BeNa Culture Collection (China). The H251, HEB and LN299 cell lines were placed in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, USA) containing 10% foetal bovine serum (FBS) (Gibco, USA). U87 cells were maintained in Minimal Essential Medium (MEM) (HyClone, USA) containing 10% FBS. The above cell lines were placed under 5% CO_2 at 37°C. Cells were collected for subsequent assays after resuscitation and 2 or 3 passages.

Cell transfection

sh-LINC00470, oe-LINC00470, sh-PTEN, and their negative controls were produced by RiboBio (China). The above plasmids were transfected into GBM cells using Lipofectamine 2000 kit (Thermo Fisher, USA) complying with the kit instructions. This process lasted 24 h and cells were collected thereafter.

qRT-PCR

We extracted total RNA by the Trizol method, and reversely transcribed RNA into cDNA by PrimeScript RT reagent Kit (Takara, Japan) and Oligo (dt) 18 primers. The relative expression of LINC00470 was detected by qRT-PCR kit (Takara, Japan), with β-actin as an internal reference. All primer sequences involved are manifested in Table I.

Western blot

The detailed steps were undertaken as described previously [4], and the experiment was repeated three times. Primary rabbit anti-human LC3 I/II (LC3B) antibody (ab192890), β-actin (ab8227), and secondary goat anti-rabbit IgG antibody (ab96899) were produced by Abcam (UK).

Cell Counting Kit-8 detection of the inhibition rate of cell proliferation and half-maximal inhibitory concentration

Cell Counting Kit-8 (CCK-8) kit (Solarbio, China) was the tool for examining viability of H251 and U87

Table I. List of primers for qRT-PCR

Gene		Sequence
LINC00470	Forward primer	5'-AGACACAGCCTCTACTGTACT-3'
	Reverse primer	5'-CCTCGTCACCTTACGTCAATAC-3'
PTEN	Forward primer	5'-CCCACCACAGCTAGAACTTATC-3'
	Reverse primer	5'-TCGTCCCTTTCCAGCTTTAC-3'
β-*actin*	Forward primer	5'-CCCTTCATTGACCTCAACTACA-3'
	Reverse primer	5'-ATGACAAGCTTCCCGTTCTC-3'

cells. To explore CDDP sensitivity, cells in 96-well plates (2×10^4 cells/well) were transfected with sh-NC and sh-LINC00470, followed by cultivation with different concentrations of CDDP (Sigma, USA) (0, 0.5, 1, 1.5, 2 μg/ml) for 48 h [13]. CCK-8 reagent was then introduced into each well for another 4 h of cell cultivation. The optical density was measured at 450 nm by a microplate reader. The half-maximal inhibitory concentration (IC50) value of CDDP was calculated by plotting the survival curve.

Flow cytometry for cell apoptosis examination

Annexin V-FITC/propidium iodide [24] kit (Solarbio, China) was utilized to test the cell apoptosis rate. H251 and U87 cells (1×10^6) were resuspended in the binding buffer, and exposed to Annexin V-FITC for 10 min and to PI for 5 min in the dark. A flow cytometer (BD Bioscience, USA) was utilized for assessing the cell apoptosis rate, whose results were analysed by Cell Quest software (BD Biosciences, USA).

Immunofluorescence analysis

The specific steps were conducted as listed in a previous publication [27]. The included antibody LC3 (ab192890) was produced by Abcam (UK).

Methylation-specific PCR detection

We followed the specific steps as described in a previous publication [30]. Whole genomic DNA was extracted, and unmethylated cytosines in which were converted to uracil. PCR products were subject to agarose gel electrophoresis and were imaged by a gel imaging system. Primer sequences are manifested in Table II.

Statistical analysis

Data were obtained from at least three independent experiments. Data were processed by GraphPad Prism 8.0 software, and presented in the form of mean ± standard deviation. Analysis of variance was adopted beforehand, and *t*-test was introduced for inter-group analysis. $P < 0.05$ indicated statistically significant difference.

Results

LINC00470 is upregulated in glioma cells

Given that LINC00470 is upregulated in gastric cancer [28] and hepatocellular carcinoma [7], we posited that LINC00470 level was also remarkably high in glioma cells. Detection results revealed a higher level of LINC00470 in glioma cells H251, LN299, and U87 over the normal human glial cell HEB (Fig. 1A). In addition, Kaplan-Meier

Table II. List of primers for MSP

Gene		Sequence
PTEN	U-Forward	5'-AGACACAGCCTCTACTGTACT-3'
	U-Reverse	5'-CCTCGTCACCTTACGTCAATAC-3'
PTEN	M-Forward	5'-AGACACAGCCTCTACTGTAACT-3'
	M-Reverse	5'-CCTCGTCACCTTACGTCAATAC-3'

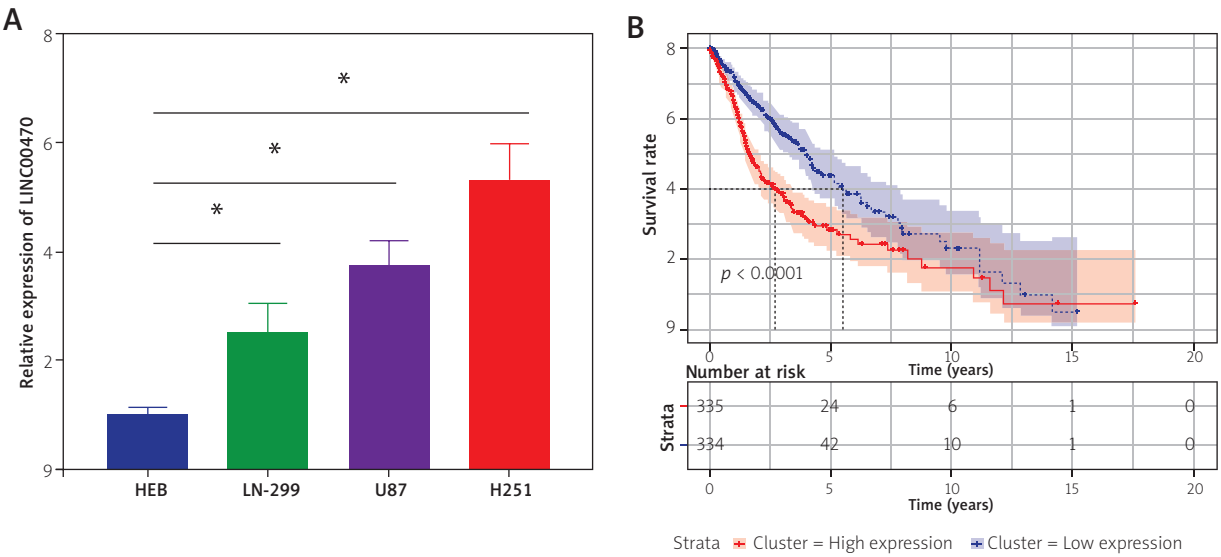

Fig. 1. LINC00470 is upregulated in glioma cells. **A**) LINC00470 level in three human glioma cell lines (LN299, U87, H251) and one human normal glial cell line HEB; **B**) Curves of LINC00470-related survival analysis in glioma patients; *$p < 0.05$.

survival analysis revealed that patients with high-level LINC00470 had a relatively low survival rate (Fig. 1B). To conclude, LINC00470 was upregulated in glioma cells.

The way LINC00470 affects glioma cell apoptosis, autophagy, and CDDP chemotherapeutic sensitivity

H251 and U87 cells with the highest expression of LINC00470 were selected for subsequent experiments. First, sh-NC and sh-LINC00470 were transfected into glioma cell lines, and the efficiency of transfection was examined *via* qRT-PCR. We observed that compared to the control group, the transfection of sh-LINC00470 significantly lowered the LINC00470 level (Fig. 2A). Then, the apoptosis rate of H251 and U87 was detected *via* flow cytometry. In contrast to the control group, the cell apoptosis rate was prominently increased in the sh-LINC00470 group, indicating that suppressing LINC00470 enhanced cell apoptosis (Fig. 2B). Informed by publications, cell apoptosis and autophagy are two main pathways that lead to tumour cell death, and cell autophagy can be induced by plenty of anti-tumour drugs that induce cell apoptosis [16,26]. Further, we explored whether LINC00470 would affect the cell autophagy level. We first examined the levels of autophagy-related protein in H251 and U87 cells *via* western blot. Then, sh-LINC00470 was indicated to facilitate LC3 II expression in H251 and U87 cells (Fig. 2C). The aggregation of LC3 examined *via* immunofluorescence assay was significantly enhanced in sh-LINC00470-treated H251 and U87 cell lines (Fig. 2D). To explore the way sh-LINC00470 affected CDDP resistance of glioma cells, we examined the IC50 values of H251 and U87 against CDDP. IC50 value of sh-LINC00470-treated cells was revealed to be significantly reduced in contrast to cells treated with sh-NC (Fig. 2E). Ultimately, LINC00470 knockdown facilitated autophagy, apoptosis of glioma cells, and enhanced chemotherapeutic sensitivity of glioma cells to CDDP.

LINC00470 suppresses PTEN expression *via* facilitating PTEN methylation

PTEN is downstream of LINC00470, and LINC00470 may regulate PTEN expression by affecting the driver methylation level of PTEN [30]. We revealed *via* bioinformatics analysis that PTEN level in tumour tissue was notably lower than in normal tissue (Fig. 3A). The Kaplan-Meier survival analysis suggested that the survival rate of patients with upregulated PTEN expression was significantly increased (Fig. 3B). We proved the above result at the cellular level that PTEN was notably downregulated in glioma cell lines in contrast to the normal human glial cell line (Fig. 3C). The correlation analysis suggested a negative interplay between LINC00470 and PTEN expression (Fig. 3D). Through qRT-PCR analysis, PTEN mRNA level was prominently upregulated upon silencing LINC00470 (Fig. 3E). *Via* MethPrimer database (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), we predicted the CpG island where PTEN promoter was enriched (Fig. 3F). DNA methylation is the covalent transfer of methyl groups to the 5'position of the cytosine ring, mainly at the dinucleotide CpG [21], so we next explored the methylation level of PTEN. Then, we discovered *via* methylation-specific PCR (MSP) that PTEN was highly methylated in glioma (Fig. 3G). U87 cells, treated with oe-LINC00470 or 5-Aza-CdR, were subjected to qRT-PCR to analyse PTEN expression and MSP to evaluate PTEN methylation. It turned out that LINC00470 overexpression significantly reduced PTEN level and PTEN was highly methylated at the same time, while 5-Aza-CdR treatment reversed this effect (Fig. 3H, I). The above indicated that LINC00470 repressed PTEN level by promoting PTEN methylation.

Regulation of PTEN level by LINC00470 impacts on autophagy, apoptosis, and CDDP chemoresistance of glioma cells

We carried out a rescue assay to validate that LINC00470 regulated PTEN expression to affect autophagy, apoptosis, and CDDP chemoresistance of glioma cells. Due to the significance shown in apoptotic capacity, autophagy, as well as drug resistance, H251 cells were selected for subsequent analyses. Treatment groups were set up as follows: sh-NC, sh-LINC00470 + sh-NC, and sh-LINC00470 + sh-PTEN. First, the mRNA expression levels of LINC00470 and PTEN were measured *via* qRT-PCR. It was found that PTEN expression was increased by sh-LINC00470, which was further restored by sh-PTEN (Fig. 4A). Flow cytometry revealed that sh-LINC00470 facilitated glioma cell apoptosis, while sh-PTEN was able to reverse such effect (Fig. 4B). The expression level of autophagy-related protein LC3 in H251 cells was analysed by western blot. We found that the ratio of LC3 II/LC3 I increased in the sh-LINC00470 + sh-NC group compared to the control group, while the ratio of LC3II/LC3II was restored to the level in the sh-NC group after transfecting sh-LINC00470 plus sh-PTEN simultaneously, which suggested that LINC00470 could inhibit the level of autophagy, while PTEN played a role in promoting cell autophagy (Fig. 4C). Immunofluorescence suggested that sh-LINC00470 significantly enhanced LC3 aggregation, while further introduction of sh-PTEN constrained such effect (Fig. 4D). Besides, CCK-8 results suggested that sh-LINC00470 could reduce the IC50 value of H251

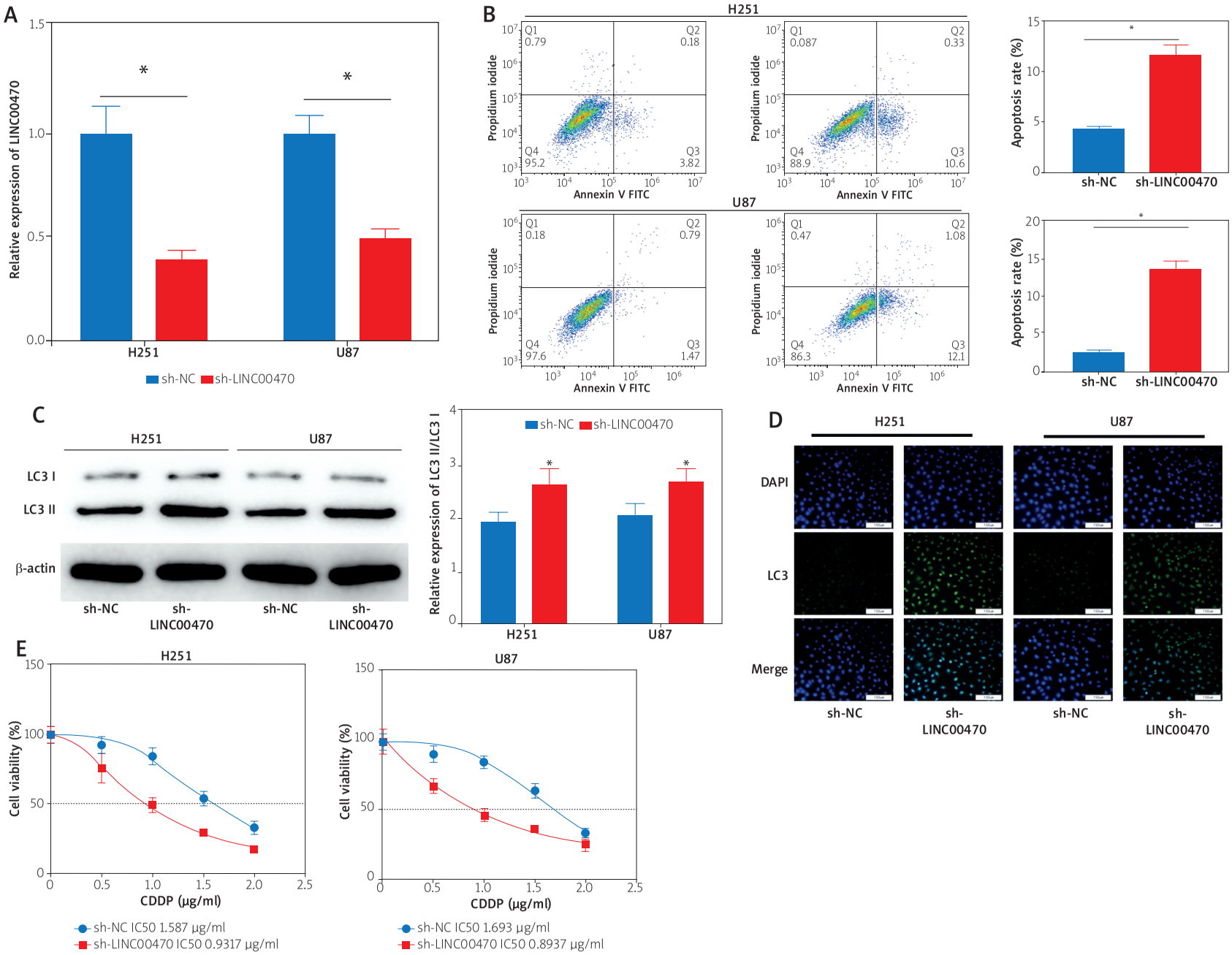

Fig. 2. LINC00470 facilitates cell apoptosis, autophagy, and CDDP chemotherapeutic sensitivity of glioma cells. **A**) Transfection efficiency of LINC00470 in H251 and U87 cells; **B**) Apoptosis of H251 and U87 cells; **C, D**) LC3 I, LC3 II, and LC3 protein levels in H251 and U87 cells; **E**) Cell viability of H251 and U87 cells after 48 h of treatment with different concentrations of CDDP (0, 0.5, 1, 1.5, 2 μg/ml); *$p < 0.05$.

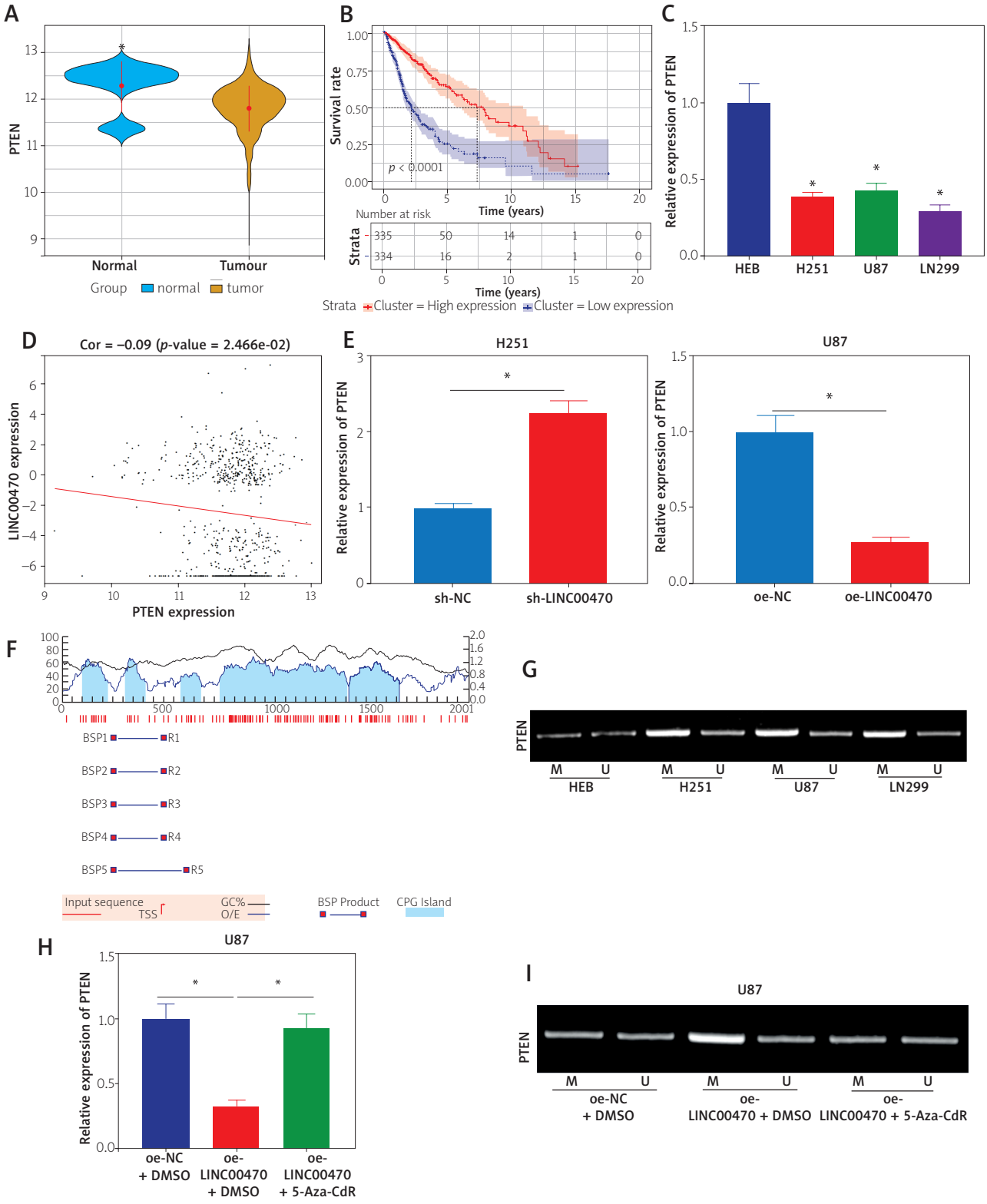

Fig. 3. LINC00470 represses PTEN expression *via* PTEN methylation. **A**) TCGA-GBM database was the base for analysis of PTEN level, of which yellow indicates tumour and blue indicates normal tissue; **B**) The PTEN-related survival curve of glioma patients; **C**) PTEN level in 3 human glioma cell lines (LN-299, U87, H251) and 1 normal human glial cell line HEB; **D**) Interplay between LINC00470 and PTEN levels in glioma cells; **E**) PTEN level in U87 and H251 cells treated by oe-LINC00470 or sh-LINC00470; **F**) Abundant CpG islands in gene promoters predicted *via* MethPrimer (http//www.urogene.org/cgi-bin/methprimer/methprimer.cgi); **G**) The methylation status of PTEN in glioma cells, where U indicates unmethylated cells and M indicates methylated ones; **H**) PTEN expression in U87 cells treated by oe-LINC00470 or 5-Aza-CdR; **I**) PTEN methylation status after oe-LINC00470 or 5-Aza-CdR treatment, where U represents unmethylated and M indicates methylated; *$p < 0.05$.

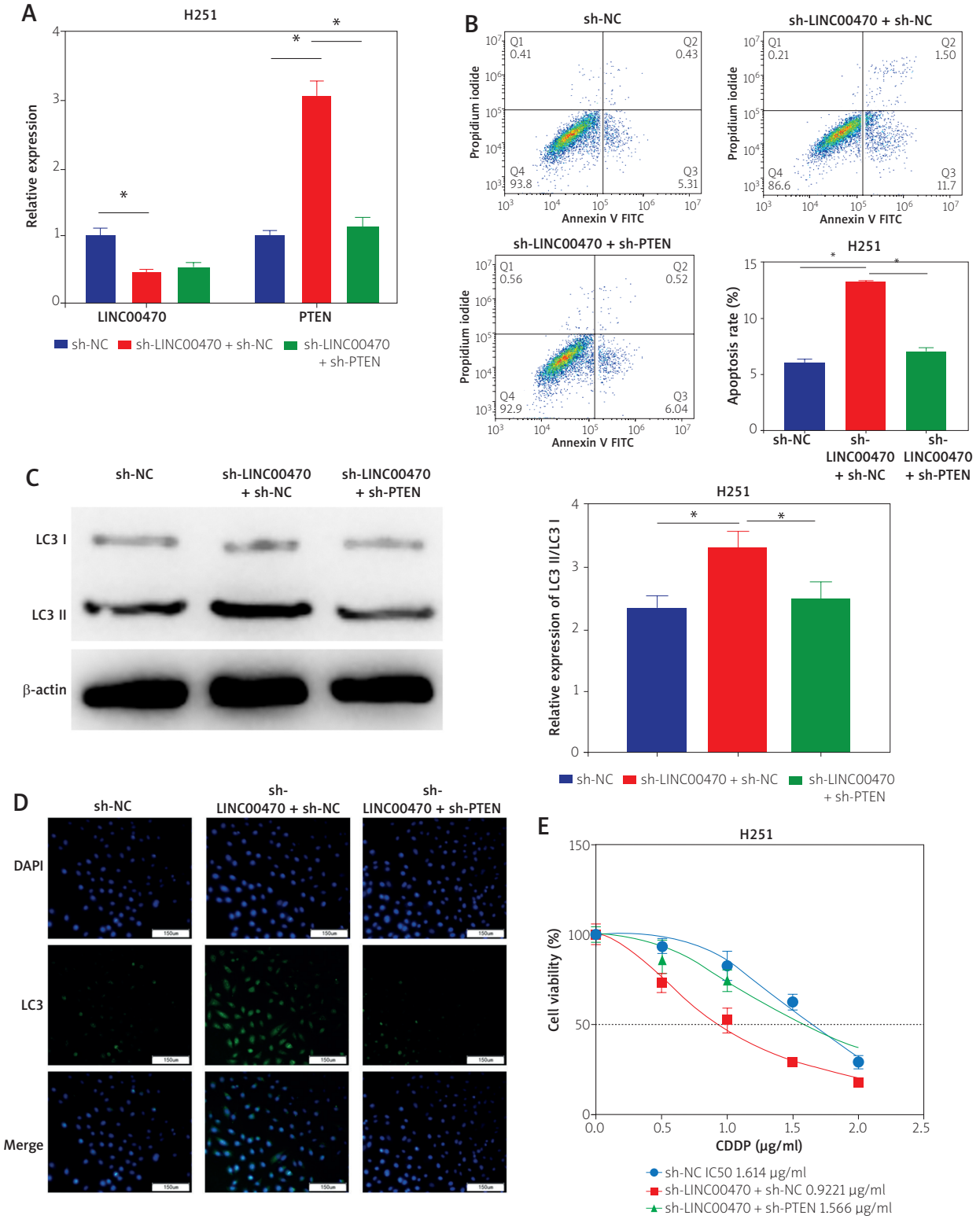

Fig. 4. Regulation of PTEN level by LINC00470 impacts on autophagy, apoptosis, and CDDP chemoresistance of glioma cells. **A**) Expression levels of LINC00470 and PTEN in each transfection group; **B**) The apoptosis of each transfection group; **C, D**) LC3 I, LC3 II and LC3 protein levels in each transfection group; **E**) Cell viability of each transfection group treated with different concentrations of CDDP (0, 0.5, 1, 1.5, 2 μg/ml); *$p < 0.05$.

against CDDP, while sh-PTEN reversed this phenomenon (Fig. 4E). To sum up, silencing LINC00470 could promote autophagy and reduce CDDP resistance of glioma cells by promoting PTEN expression.

Discussion

As a common primary brain tumour, gliomas are linked to high morbidity and mortality. Clinical cases have proven that effective chemotherapy after successful surgery is one of the most effective ways to treat malignant glioma, and chemotherapy itself can notably enhance the survival and survival time of the patients [17]. However, CDDP chemoresistance largely curbs the therapeutic efficacy on glioma patients [15]. Given that, elucidating the mechanism of CDDP resistance at the molecular level could provide novel targets to be applied for glioma treatment. LncRNAs were studied copiously in various cancers. High expression of LINC00470 can facilitate progression of gastric cancer [28], endometrial cancer [30], and glioma [12]. In this study, we discovered *via* bioinformatics approaches that LINC00470 was upregulated in glioma tissue and such upregulation was related to low survival of patients. Cell functional assays suggested that LINC00470 was up-regulated in glioma cells, while silencing this gene facilitated cell apoptosis, which is consistent with conclusions of previous reports. Further, the interplay between LINC00470 and chemoresistance was reported, such as LINC00470 overexpression facilitating chemoresistance of leukaemia cells [10]. Nevertheless, few reports focused on the mechanism of LINC00470-mediated CDDP resistance. We discovered in this work that silencing LINC00470 enhanced the CDDP sensitivity of glioma cells, suggesting LINC00470 as a key target for glioma therapy.

PTEN is widely recognized as a tumour suppressor that usually inactivates in human cancers, including gliomas [2]. Aberrant PTEN levels develop in many cancers and the mutation or loss of PTEN always affects tumour progression [22]. Moreover, PTEN knockdown is able to facilitate endometrial cancer cell proliferation and repress apoptosis [31]. miR-29a was reported to repress colon cancer cell proliferation and reverse p-glycoprotein-mediated drug resistance in colon cancer cells by upregulating PTEN [23]. We discovered *via* bioinformatics approaches that LINC00470 was negatively correlated with PTEN expression. Cell experiments revealed that knockdown of LINC00470 was able to facilitate PTEN expression, thus promoting glioma cell apoptosis, and reducing CDDP resistance of glioma cells, consistent with previous findings.

Autophagy is a self-degrading process involved in maintaining cellular homeostasis and controlling cellular components *via* impelling the clearance or turnover of long-lived or misfolded proteins, protein aggregates, and damaged organelles [6]. In recent years, autophagy in therapeutic response has been studied from various perspectives, making us recognize its role as both the oncogene and tumour suppressor in cancers [1]. To illustrate, paclitaxel can provoke cytoprotective autophagy and repress osteosarcoma cell apoptosis [5]. On the other hand, uric acid can entice autophagy in oral cancer cells and it is conducive to apoptosis [11]. Besides, TOPK suppresses autophagy by phosphorylating ULK1, which in turn promotes temozolomide resistance of glioma cells [18]. LINC00470 facilitates chemoresistance in leukaemia cells via repressing cell autophagy [10]. We found that knockdown of LINC00470 facilitated glioma cell autophagy, which in turn enhanced CDDP sensitivity of glioma cells.

To conclude, this study elucidated the mechanism of LINC00470 promoting CDDP resistance of glioma cells at the molecular level. We concluded that LINC00470 repressed PTEN expression, thus inhibiting autophagy and enhancing CDDP resistance of glioma cells. However, we only studied this at the cellular level. Further validation was needed at the animal and clinical levels. Above all, LINC00470 is an important marker for predicting CDDP resistance of glioma cells and is also a potential target for glioma therapy.

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Disclosure

The authors report no conflict of interest.

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