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Knockdown of long non-coding RNA LINC00941 suppressed cell proliferation, colony-formation, and migration of human glioblastoma cell lines

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Abstract

Introduction: *Glioblastoma (GBM) represents the most common and lethal type of primary brain tumour in adults, and due to its high invasiveness, treatment of GBM remains challenging. This work is aimed to elucidate the role of LINC00941 in GBM.*
Material and methods: *Expression of LINC00941 in two GBM cell lines U251 and U87-MG was knocked down using siRNA. Cell proliferation and colony-formation ability of LINC00941 knockdown were examined. Apoptosis of the knockdown was evaluated using flow cytometry, with the levels of Bax, Bcl-2, cleaved caspase-3, and phosphorylation of ERK and Akt to be examined using western blotting. Migration and invasion of the knockdown was studied using transwell assays.*
Results: *Expression of LINC00941 was significantly elevated in GBM compared to non-tumour tissues ($p < 0.01$). Statistical analysis on the expression data further revealed the negative correlation between LINC00941 and miR-526b-5p ($r = 0.7494$, $p < 0.001$). LINC00941 was successfully knocked down with RNA interference in U251 and U87-MG. The knockdown significantly suppressed cell proliferation and the ability to form colonies. Percentage of apoptotic cells was elevated by the knockdown in both cell lines as evidenced by flow cytometric analysis, which was accompanied by a significant decrease in Bcl-2 and substantial increases in Bax and cleaved caspase-3. Phosphorylation of ERK and Akt was also enhanced in both cell lines by the knockdown. In addition, knockdown of LINC00941 suppressed migration of both cell lines across transwell membrane and matrigel.*
Conclusions: *LINC00941 is overexpressed in GBM, exhibiting important roles in cell proliferation and survival, migration and invasion.*

Key words: *long non-coding RNA, LINC00941, glioblastoma, miR-526b-5p.*

Introduction

Glioblastoma (GBM) is the most common and the fifth lethal type of primary brain tumour in adults, accounting for 45.2% of all primary malignant brain and central nervous system tumours [13]. It is estimated that the age-adjusted incidence rate is 3.19/100,000 population [13]. GBM has poor prognosis with reported overall survival less than 15 months after diagnosis [10,14]. Currently, the standard therapy of newly diagnosed GBM includes maximal-safe surgical resection followed by radiation therapy and chemotherapy with temozolomide [6]. The surgical resection of GBM remains difficult because of the high degree of invasiveness, and the tumours are frequently located in eloquent regions of the brain which are associated with speech, senses, and motor function [16].

Substantial research effort has been made to identify genetic alterations in GBMs which might help identify new drug targets for treating the disease or define a subgroup of patients with differing responses to therapies [11,12]. Studies revealed a completed genetic

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profile of GBM, and a set of core signalling pathways being commonly impacted, including the receptor tyrosine kinase pathway, retinoblastoma pathway, and p53 pathway [5]. Ongoing efforts have been made to provide detailed analysis of the altered GBM genetic profile, as well as the signalling pathways that are affected.

Long non-coding RNAs, typically with a length of > 200 nucleotides, have emerged as key regulators of gene expression and critical biological functions. Accumulating evidence has shown the abnormal expression of lncRNAs in many cancers and their role in tumour growth and progression [3]. There were several mechanisms suggested for the lncRNA function. lncRNA may interact with chromatic-modifying complexes to direct them to specific genomic location for the regulation of gene expression; lncRNAs can bind to microRNAs to prevent them from binding to the mRNA target, affecting transcription/translation [2]. LINC00941 is a lncRNA that has been reported to have an important role in hepatocellular carcinoma [17], thyroid cancer [8], and lung adenocarcinoma [15]. The loss-of-function assay for LINC009941 showed its role in promoting invasiveness of thyroid cancer cells [8]. To date, the role of LINC009941 in GBM has remained uncertain. In this study, we performed *in vitro* experiments to investigate the biological effects and molecular mechanisms of LINC00941 in GBM.

Material and methods

Tissue samples

A pair of tumour tissue and normal tissue adjacent to the tumour were collected from 63 patients who underwent surgical treatment of GBM in Tangshan Gongren Hospital between 2014 and 2017. Informed written consent forms were collected from all patients prior to tissue collection. All patients were radiotherapy- and chemotherapy-naïve. All samples were frozen and stored at –80°C until use. This study was approved by the ethics committee of the Tangshan Gongren Hospital.

Cell culture

Human glioblastoma cell line U251 and U87-MG cells were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were kept in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin, and 100 µg/ml phytomycin at 37°C in a humidified incubator with 5% CO_2.

Cell transfection

Small interfering RNA (siRNA) targeting LINC00941 (i.e., si-LINC00941) and negative control siRNA (i.e., si-NC) were purchased from GenePharma, China. These siRNAs were transfected into glioblastoma cell lines using Lipofectamine 2000 reagent (Invitrogen, USA) per the manufacturer's instruction. A day before transfection, cells were plated in growth medium without antibiotics with a confluency of 30-50%. On the day of transfection, siRNA was mixed with Lipofectamine reagent and was then added to the cells.

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). One microgram of total RNA was reverse transcribed into the first-strand cDNA. SYBR® Green Master Mix (Takara, Japan) was used for the measurement of relative expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as U6, were used as internal references. The relative gene expression level was analysed using $2^{-\Delta\Delta CT}$ method.

Cell proliferation assay

Cell proliferation was determined by cell counting kit-8 (CCK-8) assay. Briefly, 2×10^3 cells/well were seeded onto 96-well plates. On the next day, 10 µl of CCK-8 reagent was added into each well and then incubated for 2 hours. Absorbance at 450 nm was measured. Cell proliferation was measured on day 1, day 2, and day 3.

Colony formation assay

Cells were seeded onto a 6-well plate at a density of 200 cells/well. The plate was incubated at 37°C for 14 days to allow the formation of colonies. Colonies were fixed, stained with 0.5% crystal violet and counted.

Determination of apoptosis

After transfection for 24 hours, cells were collected and centrifuged. The cell pallet was resuspended with 195 µl annexin V-FITC. A staining solution, 5 µl annexin V-FITC and 10 µl propidium iodide, was added for staining. The cells were incubated in darkness for 10-20 minutes and then placed on ice. The apoptosis was detected using flow cytometry (Jiyuan, Guangzhou, China).

Western blotting

Total protein was extracted from cultured cells. The proteins were electrophoresed onto SDS polyacrylamide gel and then electro-transferred onto a PVDF membrane. After blocking, the membrane was probed with one of the following primary antibodies from Cell Signaling Technology (Danvers, MA, USA): Bax, Bcl-2, Cleaved caspase-3, phosphor-ERK, total ERK, phos-

pho-Akt, and total Akt. GAPDH was used as a reference control. HRP-conjugated antibodies were used as a secondary antibody. The expression of protein was measured using ImageJ software (National Institute of Health).

Invasion assay and migration assay

Invasion chambers coated with Matrigel matrix was used to assess the invasive capacity of the treated cells. Briefly, cells were seeded onto the invasion chamber and incubated overnight at 37°C. Non-invading cells on the apical side of the membrane were removed with a cotton swab. The cells on the lower surface of the membrane were fixed by methanol and stained by 0.1% crystal violet. Five fields were randomly selected for cell counting. Migration assay was performed in the same way as described above, except no Matrigel coating in the chambers.

Statistical analysis

Data were expressed as mean with standard deviation (SD), with comparison between different groups performed using Student's *t* test. Survivals of patients with differential LINC00941 expression were compared using Kaplan-Meier analysis with log-rank test. Statistical significance was indicated with a p value < 0.05.

Results

LINC00941 was elevated in GBM tissues

The level of LINC00941 in 63 pairs of GBM and adjacent non-tumour tissues was studied using real-time PCR (Fig. 1A). Results clearly demonstrated that LINC0094 was significantly elevated in tumour tissues compared to the non-tumour tissues (p < 0.01). Further analysis on the expression of LINC00941 in tumours revealed that high LINC00941 was associated with the dismal survival outcomes of patients with GBM, and the overexpression would increase the risk of death by 2.4 folds (Fig. 1B). LncRNAs regulate negatively the functionality of microRNAs. In the present GBM cohort, a significant negative

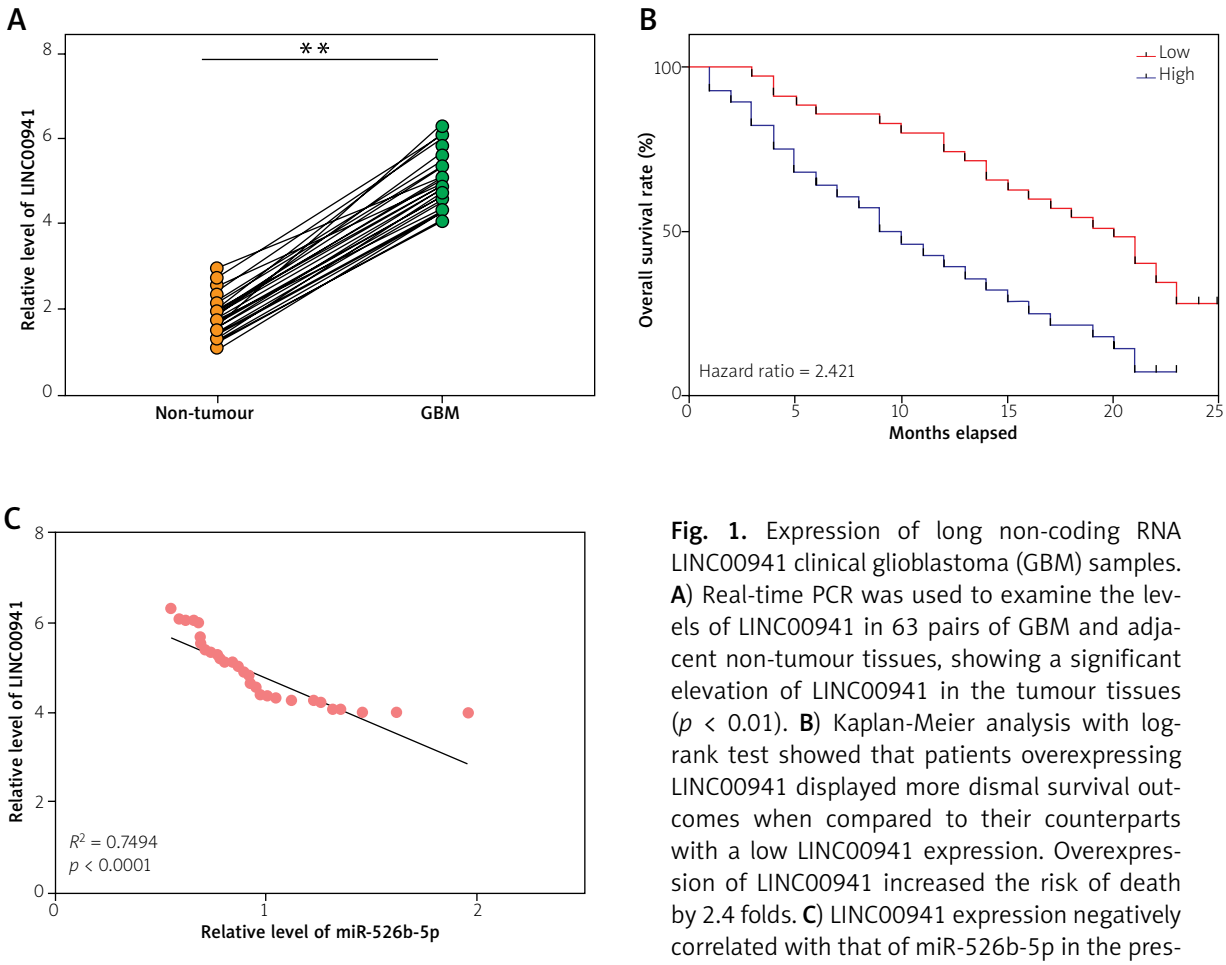

Fig. 1. Expression of long non-coding RNA LINC00941 clinical glioblastoma (GBM) samples. **A**) Real-time PCR was used to examine the levels of LINC00941 in 63 pairs of GBM and adjacent non-tumour tissues, showing a significant elevation of LINC00941 in the tumour tissues (p < 0.01). **B**) Kaplan-Meier analysis with log-rank test showed that patients overexpressing LINC00941 displayed more dismal survival outcomes when compared to their counterparts with a low LINC00941 expression. Overexpression of LINC00941 increased the risk of death by 2.4 folds. **C**) LINC00941 expression negatively correlated with that of miR-526b-5p in the present GBM cohorts.

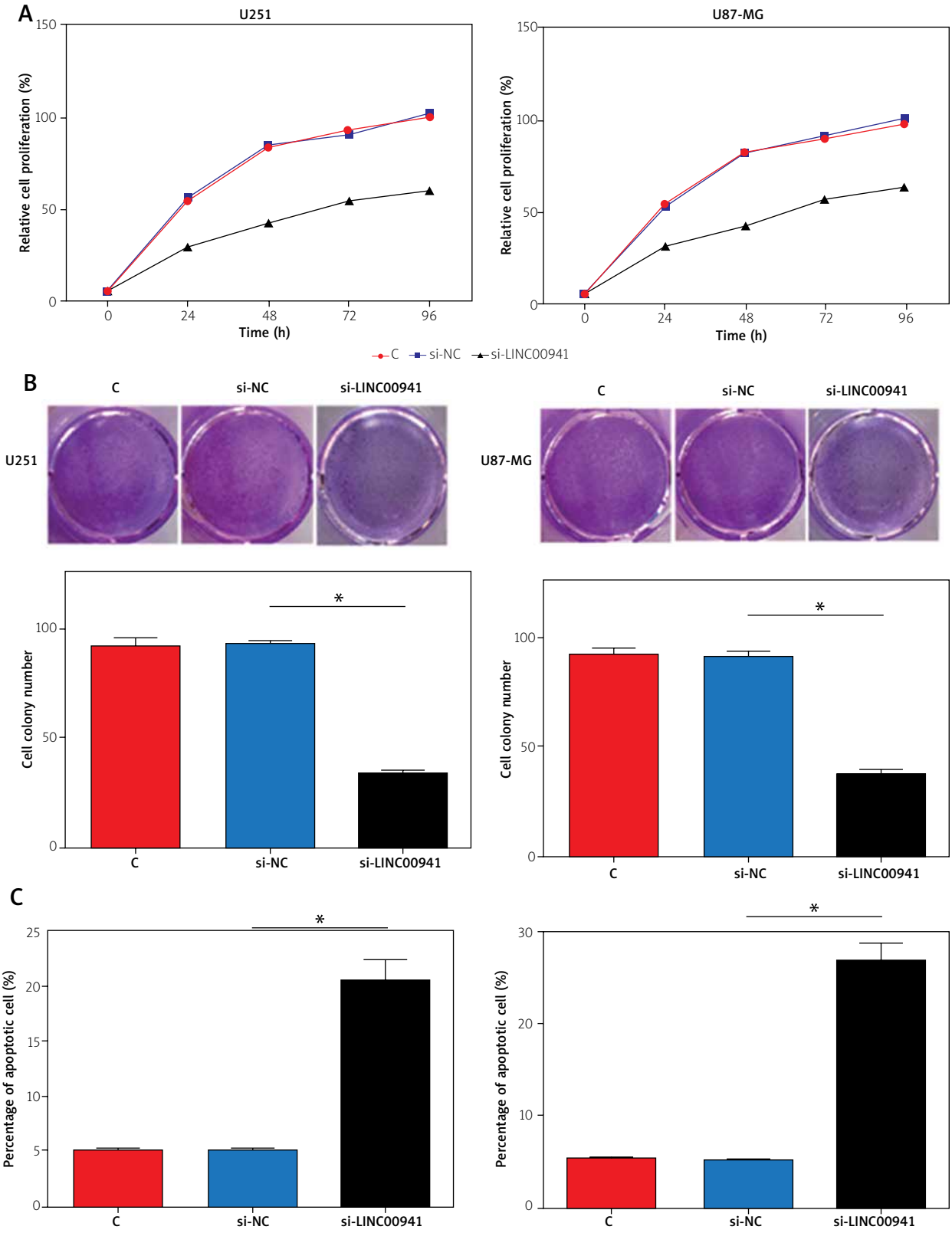

Fig. 2. Effect of LINC00941 knockdown on glioblastoma (GBM) cancer cell proliferation and survival was studied. **A**) Treatment of U251 and U87-MG cells with LINC00941 siRNA significantly reduced proliferation as assessed using CCK-8 assay. **B**) Knockdown of LINC00941 with siRNA suppressed colony formation in U251 and U87-MG cells as shown using colony-formation assay. **C**) Knockdown of LINC00941 resulted in substantial increases in apoptotic cell populations in U251 and U87-MG as examined using flow cytometry.

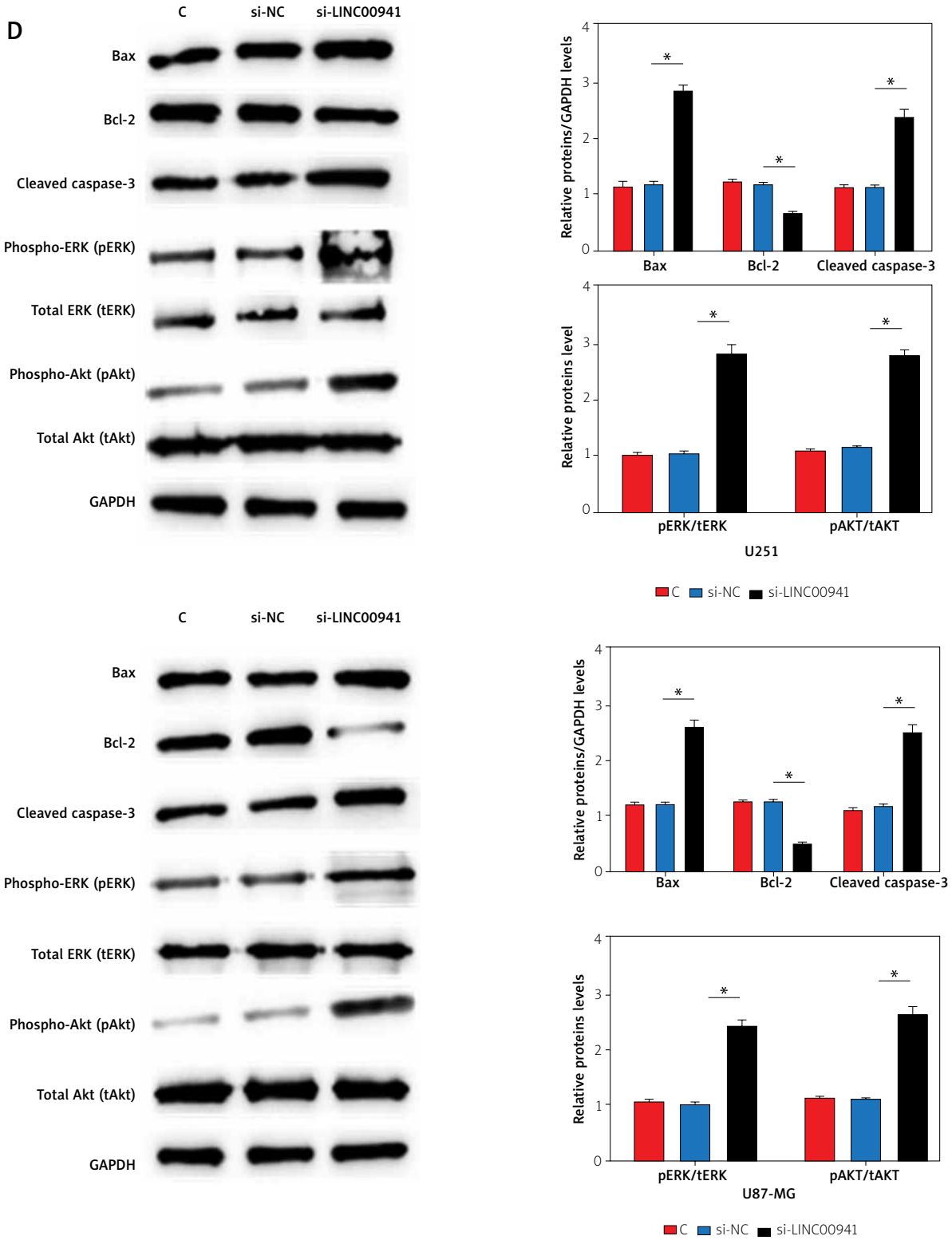

Fig. 2. Cont. **D**) Expression of Bax, Bcl-2, cleaved caspase-3 and phosphorylation of ERK and Akt in the knockdown was assessed using western blotting with band intensity quantified and compared. *$p < 0.05$ when compared to treatment with negative control siRNA. The figure shows the representative set of data of three independent experiments.

correlation between the levels of LINC00941 and miR-526b-5p was found ($r^2 = 0.7494$, $p < 0.0001$) (Fig. 1C).

LINC00941 was essential to GBM cancer cell growth and survival

The functional roles of LINC00941 in GBM were examined by knocking down its expression in two human GBM cell lines U251 and U87-MG with siRNA. Transient knockdown of LINC00941 reduced significantly the proliferation of both GBM cell lines *in vitro* (Fig. 2A). In addition, knockdown of LINC00941 was shown to suppress the ability of U251 and U87-MG to form colonies (Fig. 2B). In both cell lines, the number of colonies formed in the siLINC00941-treated group was substantially lower than that of the scrambled siRNA-treated i.e., si-NC ($p < 0.05$).

Flow cytometric analysis was performed to see whether knockdown of LINC00941 would have any effects on apoptosis (Fig. 2C). Untreated and si-NC-treated U251 and U87-MG cell lines presented basal levels of apoptotic cells of about 5%, while LINC00941 knock-

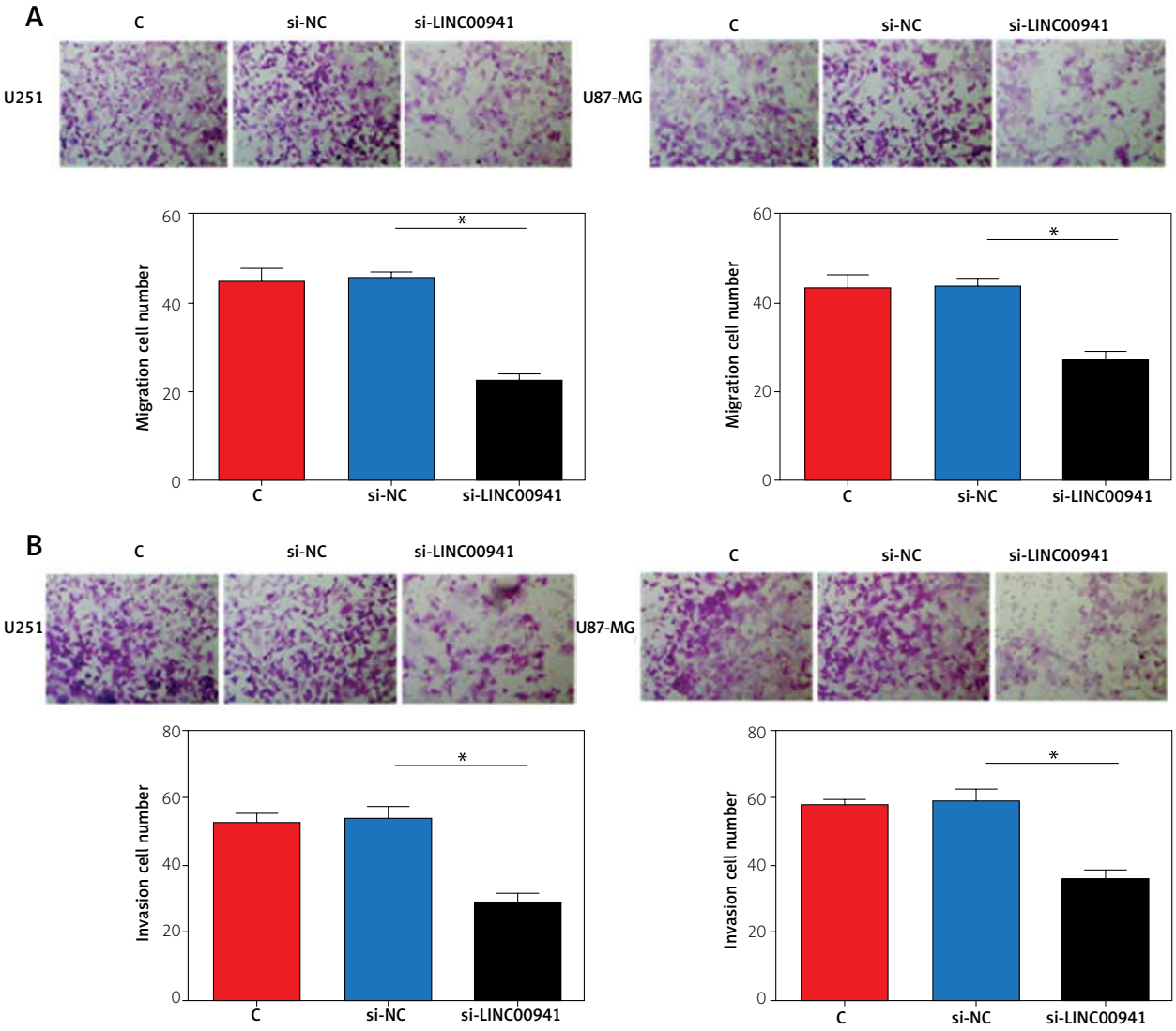

Fig. 3. Effect of LINC00941 knockdown on glioblastoma (GBM) cancer cell migration and invasion was studied using transwell assays. **A**) Migration of U251 and U87-MG cells across transwell membrane was quantified and compared. Knockdown of LINC00941 significantly reduced the number of migrated cells. **B**) Invasive properties of U251 and U87-MG cells after LINC00941 was knocked down were evaluated using matrigel transwell assay. Treatment with LINC00941 siRNA led to substantial decreases in the numbers of invaded cancer cells. *$p < 0.05$ when compared to treatment with negative control siRNA. The figure shows the representative set of data of three independent experiments.

down led to an increase in apoptotic cell populations by 4 and 5 folds in U251 and U87-MG cells, respectively. These increases in apoptotic cell population were all statistically significant ($p < 0.05$).

Western blotting was used to examine whether LINC00941 would affect the expression of pro-apoptotic Bax protein, anti-apoptotic Bcl-2 protein and cleaved caspase-3 in both cell lines (Fig. 2D). In U251 cells, knockdown of LINC00941 resulted in substantial increases in Bax and cleaved caspase-3 proteins, which was accompanied with a significant decrease in Bcl-2 protein. These changes in the protein level in LINC00941 knockdown were found statistically significant when compared with the negative control ($p < 0.05$). Knockdown of LINC00941 in U87-MG cells also led to similar changes in Bax, Bcl-2, and cleaved caspase-3, of which the differences between si-LINC00941 and negative control were statistically significant ($p < 0.05$).

Activation of ERK/Akt pathway in the LINC00941 knockdown was studied by assessing the levels of phosphorylated ERK (pERK) and Akt (pAkt) using western blotting (Fig. 2D). In both cell lines, knockdown of LINC00941 enhanced phosphorylation of ERK and Akt, causing significant increases in pERK/ERK and pAkt/Akt compared to the negative control ($p < 0.05$).

LINC00941 was important to migration and invasion of GBM cell lines

In addition to cell proliferation and survival, the effect of LINC00941 on migration and invasion properties of GBM cell lines was also examined by migration assay with transwell setting and invasion assay using matrix, respectively (Fig. 3). Knockdown of LINC00941 in U251 and U87-MG significantly reduced the number of cells migrating across the transwells (Fig. 3A). In both cell lines, the differences between si-LINC00941 and negative control treatments were statistically significant ($p < 0.05$). Upon invasion assay, LINC00941 knockdown in both U251 and U87-MG cell lines reduced the number of cells that could invade through the matrix. Again, statistically significant differences between si-LINC00941 and negative control treatments were achieved ($p < 0.05$).

Discussion

The role of LINC00941 has been implicated in several types of cancer. The overexpression of LINC00941 promoted tumorigenic properties, such as cell proliferation, migration, and invasion, of colon cancer cells *in vitro*; whereas the silencing of LINC00941 inhibited tumour growth *in vivo* [4]. In oral squamous cell carcinoma, thyroid cancer [1], and pancreatic adenocarcinoma [7], LINC00941 also appeared to have tumour promoting properties. However, a limited study is available elucidating the role of LINC00941 in GMB. In this study, the loss-of-function analysis revealed that LINC00941 promoted cell proliferation and invasiveness in GMB.

The expression of LINC00941 was higher in tumour tissues than that of the adjacent non-tumour tissues; and the high expression was associated with a poor survival rate of the patients. The high expression of LINC00941 in cancer may be attributed to the genomic amplification, at least in the context of lung adenocarcinoma [15]. The association of LINC00941 expression level with survival outcome suggested the potential use of this lncRNA as a prognostic marker.

The present study provided findings to support the important roles of LINC00941 in driving the proliferation and survival of GBM cancer cells. Knockdown of LINC00941 reduced proliferation and suppressed colony-formation of human GBM cell lines, which could be due to the ability of LINC00941 to prevent apoptosis. Resisting cell death has been characterized as one of the cancer hallmarks [9]. Studies have identified multiple mechanisms rendering cancer cells the ability to resist cell death. These mechanisms include disruption on the balance of pro-apoptotic and anti-apoptotic proteins, reduction in caspase function, and impairment in death receptor signalling. Echoed with these studies, findings of this work implicated the modulating effects of LINC00941 on pro-apoptotic Bax protein, anti-apoptotic Bcl-2 protein and cleaved caspase-3 in human GBM cell lines. Whether LINC00941 would have any effects on death receptor signalling has remained to be investigated.

Knockdown of LINC00941 was shown to be suppressive on the migration and invasion of GBM cell lines, implicating its important roles in initiating metastasis of GBM. A high rate of metastasis is a key feature of GBM, making the management of patients with GBM very challenging. Suppression of the metastatic spread of GBM cancer cells is therefore believed to benefit the survival outcome of patients, and this study has provided insights of targeting LINC00941 as a potential approach to suppress metastasis. Degradation of the extracellular matrix (ECM) at the primary tumour site is a prerequisite to the transmigration of cancer cells into the bloodstream and subsequent homing to the secondary site. Matrigel transwell assay is a widely-used functional assay to examine the invasiveness of cancer cells, in which the matrigel mimics the heavily-deposited ECM in tumour sites. Knockdown of LINC00941 significantly reduced the number of invaded cells in the transwell assay, clearly implicating that LINC00941 would provide GBM cancer cells with the ability to degrade and migrate across ECM. Cancer cells utilize a panel of metalloproteinases (MMPs) to degrade ECM.

It has remained imperative to investigate whether LINC00941 would regulate MMP function in GBM cell lines.

The present study has provided evidence to support the oncogenic roles of LINC00941 in GBM. Targeting LINC00941 with RNA interference would hold promise as a novel modality to suppress tumour growth and metastasis of GBM, a clinical feature that adversely affects the prognosis of patients. The efficacy of such an approach however needs to be assessed in relevant animal models of GBM. Long non-coding RNA modulates gene expression at both epigenetic and microRNA levels. Expression of LINC00941 and miR-526b-5p negatively correlated with each other in the present GBM cohort. The detailed mechanistic link between the two would require further elucidation.

Disclosure

The authors report no conflict of interest.

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