Long non-coding RNA AB209630 suppresses cell proliferation and metastasis in human hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide and the second most common cause of cancer-related mortalities. With a high potential for metastasis and recurrence, HCC is refractory to cure. The present study aimed to explore the role of a recent-discovered LncRNA, AB209630, in human HCC, in order to provide new insights useful for clinical HCC diagnosis and treatment. Reverse transcription-quantitative polymerase chain reaction was performed to examine the expression of AB209630 in clinical HCC samples and the adjacent non-cancerous tissues. The reduced expression of AB209630 observed in HCC tissues and cultured HCC cells compared with normal hepatic tissues and cells prompted the construction of an AB209630-expressing plasmid with a CBP tag on the plasmid backbone. Cell proliferation and colony formation assays were conducted to detect the effects of AB209630 on HCC cell proliferation. In addition, Transwell assay and wound-healing assays were performed, the results of which further indicated that the overexpression of AB209630 inhibited the migration and invasion of HCC cells. These results revealed the inhibitory effects of AB209630 on HCC progression, and suggest the potential of AB209630 as an inhibitor of HCC for clinical use.

Introduction

Liver cancer is a common disease, particularly in Asia and parts of sub-Saharan Africa, and may occur as the result of an infection several decades earlier (1). It is the sixth most frequently occurring cancer worldwide; in 2012, there was an estimated morbidity of 782,000 cases, and the number of mortalities due to liver cancer was estimated to be ~746,000 (2). Notably, liver cancer is the second most common cause of cancer-related mortalities worldwide (3,4). Liver cancer can be divided into three categories, namely hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma and mixed hepatocarcinoma, according to the origin of the cancer, among which HCC is the most common (5). Despite the great progress made in understanding the tumorigenesis of the disease and therapeutic options in previous decades, HCC continues to respond poorly to traditional therapies and relapses shortly following clinical treatment (6,7). Moreover, its potential for metastasis and intrahepatic transfer makes it challenging to cure (8). Therefore, the identification of novel approaches to diagnose and treat this disease is urgently necessary.

Long non-coding RNA (LncRNA) is a class of non-protein coding transcripts of >200 nucleotides and <100 kb (9). LncRNAs are reported to regulate the expression of genes in the biological processes of differentiation, proliferation, metastasis, reprogramming of induced pluripotent stem cells and apoptosis (10,11) by interacting with chromatin-modifying enzymes (12), RNA processing (13) and structural scaffolds (14). LncRNAs have also been demonstrated to play significant roles in the tumorigenesis of HCC. For example, Zhou and Gao found that BRAF-activated non-coding RNA contributed to HCC initiation and progression by upregulating cell proliferation and metastasis, in addition to promoting cell apoptosis (15). Esposti *et al* detected 5,525 LncRNAs across various tissue types and found 57 differentially expressed LncRNAs in HCC, all of which were co-expressed with genes involved in liver metabolism, cell cycle regulation and the transforming growth factor β1 signaling pathway (16).

AB209630 is a recently-discovered LncRNA, which was demonstrated to have a notable role in hypopharyngeal squamous cell carcinoma (HSCC) in a previous study (17). In that study, the expression of LncRNA AB209630 was found to be markedly decreased in HSCC tissues in comparison with the adjacent non-cancerous tissue. Overexpression of AB209630 was observed to inhibit cell proliferation and colony formation, and also to suppress cell metastasis. Furthermore, AB209630 promoted cell apoptosis and was associated with the prognosis of patients with HSCC. However, the detailed role of AB209630 in HSCC and the potential effects of AB209630 on other types of tumor remain unknown.

The present study explored the role of AB209630 in HCC *in vivo* and *in vitro*. Firstly, the expression of AB209630 in clinical HCC tissues and cultured HCC cells was

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evaluated. Subsequently, an AB209630-expressing plasmid was constructed using a V51 pIRESpuro-GLUE empty vector (pGLUE) containing a CBP-tag (N terminal on the plasmid backbone) and the effects of AB209630 overexpression were explored. The results of this study may provide new information useful in the diagnosis and treatment of patients with HCC.

Materials and methods

Human tissues. In this study, 40 patients (45±5 years old, male:female=22:18) with HCC were enrolled by the Department of Interventional Radiology of the People's Hospital of Weifang (Weifang, China). Prior to the surgery, none of the patients had received chemotherapy or radiotherapy and after the surgery, the HCC tissues and their adjacent non-cancerous tissues were frozen in liquid nitrogen immediately. Written informed consent was obtained from each patient and the study was approved by the Ethics Committee of the People's Hospital of Weifang.

Cell culture. THLE-3 human normal liver cells and the HCC cell lines MHCC97-L, MHCC97-H and HCCLM3 were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). During the study, cells were cultured at 37˚C in a humidified incubator containing 5% CO_2. The culture medium was replaced every other day, or as stated.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from clinical tissues and cultured HCC cells was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) and quantified using a Nanodrop 2000 instrument (Thermo Fisher Scientific, Inc.) according to the optical density values at 260 and 280 nm in each sample. Complementary DNA (cDNA) was synthesized using PrimeScript Reverse Transcriptase (Takara Biotechnology Co., Ltd. Otsu, Japan) and 10 ng total RNA with the following protocol: 37˚C for 15 min and 85˚C for 5 sec. qPCR was performed with a SYBRgreen kit (Takara Biotechnology Co., Ltd.) using an ABI7900 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a volume of 10 μl according to the manufacturer's instructions. The thermocycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. The primers used were as follows (17): AB209630 forward: 5'-GGGCTATTGTCCCTA AGTTGAT-3' and reverse, 5'-TGTCTTGTAGAGCATAAG GAAACC-3'; GAPDH forward, 5'-GGGAAACTGTGGCGT GAT-3' and reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'. All quantitative data were quantified using the $2^{-\Delta\Delta Cq}$ method (18).

Plasmid construction and transfection. pGLUE empty vector was purchased from Addgene, Inc. (Cambridge, MA, USA). Restriction enzyme sites for *Eco*RV and *Bam*H1 were used to clone AB209630. The full-length DNA sequence of human AB209630 was cloned from cultured 293T cells with PCR. The AB209630 sequence was connected to the pGLUE vector with DNA ligase for 2 h at room temperature. Afterwards, the combined plasmid was confirmed by sequencing (Jieli Co., Shanghai, China). Prior to the experiments, AB209630-expressing plasmid was transfected into HCCLM3 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. HCCLM3 cells transfected with empty vector and untransfected cells served as the vector and control groups.

Western blot analysis. Protein expression was evaluated by western blot analysis. Briefly, total protein from HCCLM3 cells was collected using lysis buffer (NP-40, Beyotime Institute of Biotechnology, Nantong, China) on ice according to the manufacturer's protocol and quantified using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein (40 μg) were loaded onto 10% SDS-PAGE for electrophoresis and then transferred to a nitrocellulose membrane (EMD Millipore, Bedford, MA, USA). The membrane was blocked for 2 h with 5% skimmed milk at room temperature and then incubated with primary antibodies overnight at 4˚C. The primary antibodies against CBP-tag (sc-1211, 1:1,000) and GAPDH (sc-32233, 1:1,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The membrane was then incubated with secondary goat-anti-rabbit (sc-2004) and goat-anti-mouse (sc-2005) antibodies (Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C with a dilution of 1:1,000. Finally, the proteins were quantified using ECL Prime Western Blotting Detection reagent (GE Healthcare, Parsippany, NJ, USA) and an ImageQuant LAS 4000 Mini Biomolecular Imager (GE Healthcare).

Cell proliferation. HCCLM3 cells were seeded in a 96-well plate at a concentration of $1x10^3$/well. After 24 h, cells were transfected with AB209630-expressing plasmid or control (empty) plasmid. Cell proliferation was examined every day in the consecutive 5 days using a CellTiter 96 AQueous Non-Radioactive Cell Proliferation kit (Promega Corporation, Madison, WI, USA) following the manufacturer's protocol. The cell proliferation rate was determined by measuring the absorbance at 490 nm using a microplate reader (Tecan, Männedorf, Switzerland).

Colony formation assay. HCCLM3 cells were transfected with AB209630-expressing plasmid or control (empty) plasmid and cultivated in six-well plates at a density of 200 cells/well. The culture medium was changed every 2 days. After 2 weeks, the cell colonies that contained >50 cells were counted by staining with 0.5% crystal violet and observation under a light microscope with a magnification of x200.

Transwell assays. For cell migration assays, HCCLM3 cells were trypsinized and collected by low-speed centrifugation (840 x g, 4˚C) with serum-free DMEM. A total of $1x10^4$ cells (~150 μl) were transferred into the upper chamber. The lower chamber was filled with 600 μl medium containing 10% FBS. Afterwards, the plate was put back into the 37˚C incubator and the cells are allowed to grow freely. At 24 h post-seeding, the membrane was fixed with pre-cooled methanol and stained with crystal violet (0.5%) for 5 min. Cell migration ability was assessed by counting the cells that had migrated through the membrane. Five random fields of view were selected and images captured under a Nikon light microscope (Nikon Corporation, Tokyo, Japan) at a magnification of x100. For cell invasion

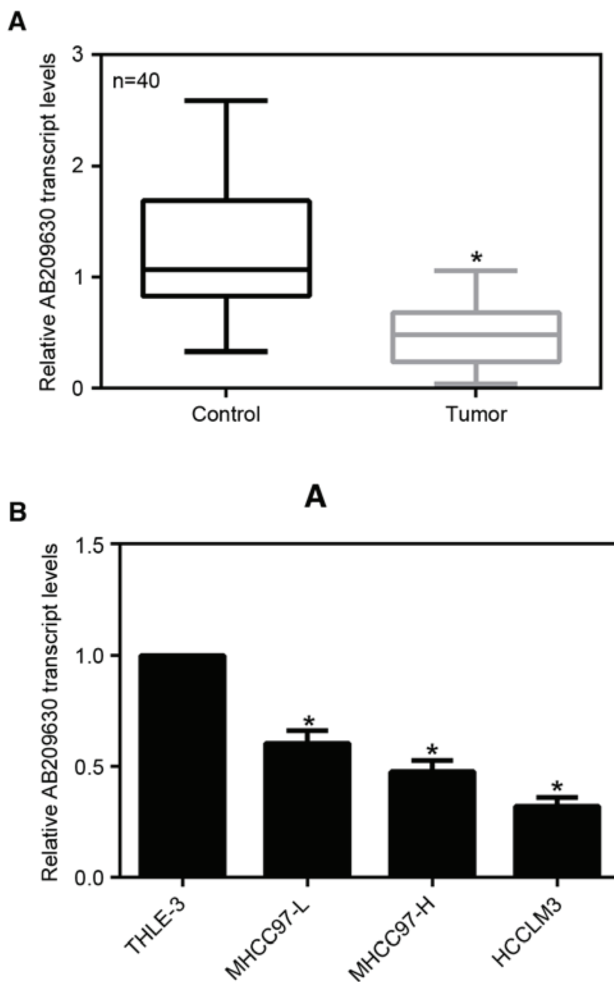

Figure 1. Expression of LncRNA AB209630 was downregulated in clinical HCC tissues and cultured HCC cells. (A) In 40 patients with HCC, the expression of AB209630 was significantly decreased in HCC tissues compared with the adjacent non-cancerous counterparts. *P<0.05 vs. control. (B) Reverse transcription-quantitative polymerase chain reaction analysis revealed the relative transcript level of AB209630 was decreased in HCC cells compared with THLE-3 cells, a normal liver cell line. *P<0.05 vs. THLE-3. LncRNA, long non-coding RNA; HCC, hepatocellular carcinoma.

assays, the membrane was pre-coated with Matrigel (Corning Incorporated, Corning, NY, USA) for 6 h at 37˚C incubator.

Wound-healing assay. HCCLM3 cells were transfected with AB209630-expressing plasmid or control (empty) plasmid, cultured in DMEM in a six-well culture plate at a density of $5x10^5$ cells/well and allowed to grow to a confluence of 90% overnight. The culture medium was replaced with serum-free DMEM. A line was scratched in the single cell layer using a 10-μl pipette tip and the cells were then washed with PBS three times. Following incubation for 24 h, images of the migrating cells were observed and images captured using a light microscope.

Statistical analysis. *In vitro* experiments were repeated three times in triplicate, giving reproducible results. Results are presented as the mean ± standard deviation following Student's t-test analysis. Data were analyzed using Prism 6 (GraphPad Software Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of LncRNA AB209630 is downregulated in clinical HCC tissues and cultured HCC cells. To examine the transcript levels of AB209630, 40 HCC patients from the People's Hospital of Weifang were included in the study. A tumor section and adjacent non-cancerous tissues were obtained from each patient. RT-qPCR analysis revealed a markedly lower expression of AB209630 in HCC tissues compared with the adjacent non-cancerous tissues (Fig. 1A).

MHCC97-L, MHCC97-H and HCCLM3 are three HCC cell lines, listed in order of increasing metastatic potential (19). The results shown in Fig. 1B indicate that the AB209630 transcript levels were decreased in the three HCC cell lines compared with those in THLE-3 normal liver cells, and were the lowest in HCCLM3 cells, which have the highest ability to transfer to other organs in patients. These data suggest that the transcript level of AB209630 was notably downregulated in HCC tissues and cultured HCC cells.

Construction of AB209630 plasmid successfully increases the expression of AB209630 at the mRNA and protein levels. To explore the role of decreased AB209630 expression in HCC cells, an expression plasmid containing the whole sequence of human AB209630 was prepared (17). Fig. 2A shows the pGLUE empty vector, in which the restriction enzyme *Eco*RV was used to insert the AB209630 sequence. The ability of the constructed plasmid to upregulate the expression of AB209630 was examined. In comparison with the untransfected control group, the transcript level of AB209630 was highly increased upon AB209630-expressing plasmid transfection but was not changed when the empty vector was transfected (Fig. 2B). Afterwards, western blot analysis was performed to detect the transfection efficiency of the AB209630-expressing plasmid. As shown in Fig. 2C, the protein level of the CBP tag was highly increased upon transfection with the AB209630-expressing plasmid compared with the control. These results verified that the AB209630-expressing plasmid was effective in promoting the transcription of AB209630.

Overexpression of AB209630 inhibits cell proliferation and colony formation in HCC cells. The effect of AB209630 on the proliferation of HCC cells was examined. After HCCLM3 cells were transfected with AB209630 plasmid, the proliferation rate remained unchanged in the following 3 days among the three experimental groups; however, on day 4, the proliferation rate of the HCCLM3 cells was inhibited by 42% in the AB209630-overexpressing group compared with the control group, and the inhibitory effect was even stronger on day 5 after transfection (Fig. 3A). A similar phenomenon was observed in the colony formation assays. In the control group, >230 colonies were formed 2 weeks post-seeding and there were 222 colonies in the vector group (P>0.05); however, only 76 colonies were observed when AB209630 was overexpressed in HCCLM3 cells (Fig. 3B). These data reveal that the overexpression of AB209630 in HCCLM3 cells significantly suppressed cell proliferation.

Overexpression of AB209630 suppresses the migration and invasion of HCC cells. Transwell assays and wound-healing

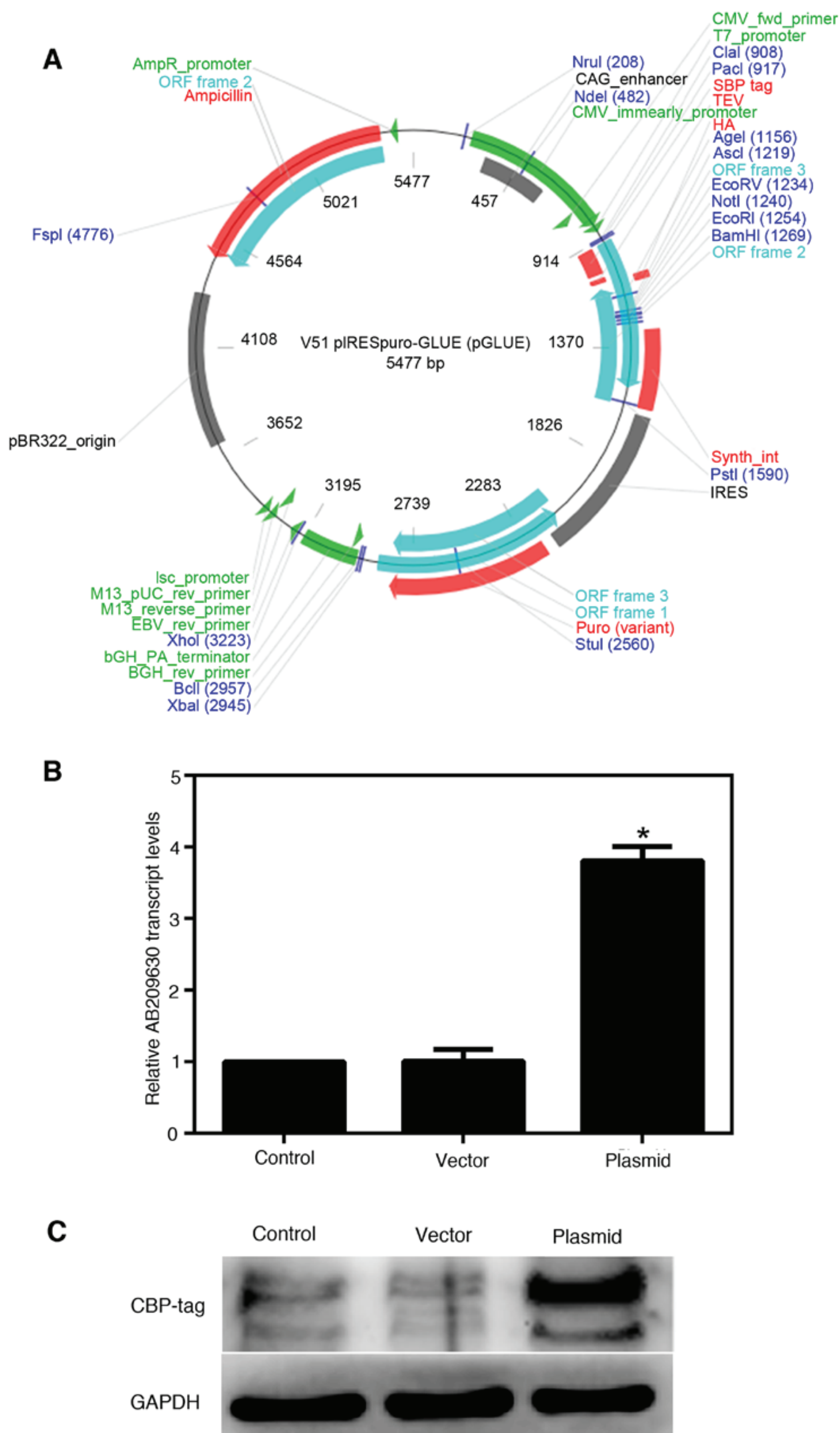

Figure 2. Construction of AB209630 plasmid successfully increased the expression of AB209630 at the mRNA and protein levels. (A) V51 pIRESpuro-GLUE (pGLUE) empty vector, used to clone the AB209630-expressing plasmid. Restriction enzyme *Eco*RV was used to insert AB209630. (B) Relative transcript levels of AB209630 in HCCLM3 cells transfected with the AB209630-expressing plasmid. *P<0.05 vs. control. (C) Western blot analysis showed the protein level of the CBP tag, which was connected to the inserted AB209630 transcript. GAPDH served as an internal control.

assays were performed to explore the role of AB209630 in cell migration. As shown in Fig. 4A, the upregulation of AB209630 reduced the migration of HCCLM3 cells through the membrane by >70%. Similarly, ~200 untransfected HCCLM3 cells invaded to the lower surface of the membrane, but only 50 of the AB209630-upregulated cells did so (Fig. 4B).

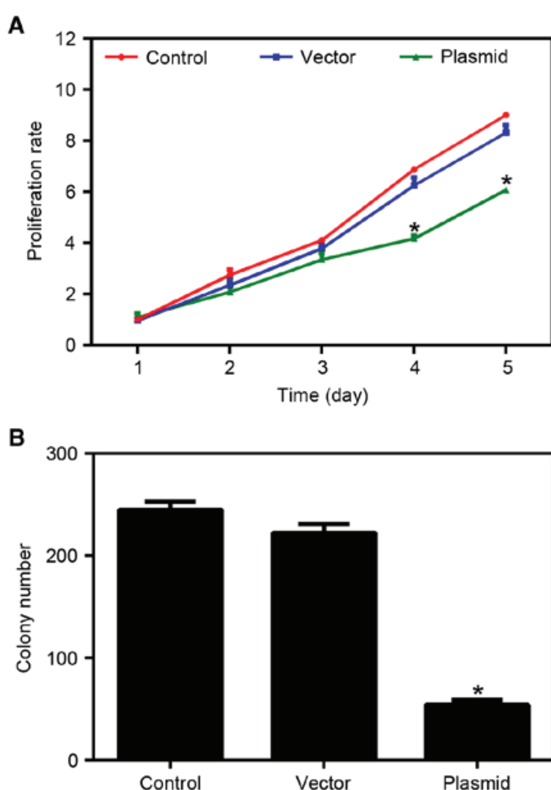

Figure 3. Overexpression of AB209630 inhibited cell proliferation and colony formation in HCC cells. (A) The cell proliferation rate was significantly inhibited upon AB209630 overexpression on days 4 and 5 after transfection. *P<0.05 vs. control. (B) Colony formation assays were performed to assess the effects of AB209630 on HCCLM3 cells. The colonies were notably decreased in number when AB209630 was upregulated. *P<0.05 vs. control. HCC, hepatocellular carcinoma.

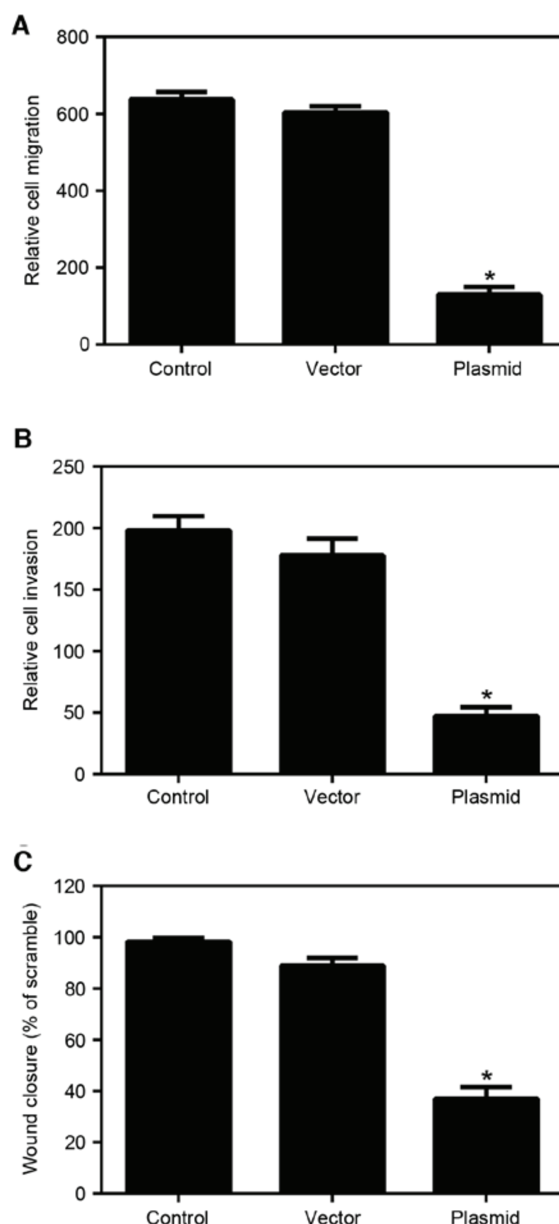

Figure 4. Overexpression of AB209630 suppressed the migration and invasion of HCC cells. (A) AB209630 overexpression inhibited the migration ability of HCCLM3 cells. (B) Cell invasion capacity was suppressed when cells were transfected with AB209630-expressing plasmid, compared with that of untransfected control cells. (C) Wound-healing assays showed cell migration was retarded by >50% when cells were treated with AB209630 plasmid. *P<0.05 vs. control. HCC, hepatocellular carcinoma.

Wound-healing assays demonstrated that cell migration was restrained by 55% in the AB209630-treated cells compared with the control cells (Fig. 4C). These results indicate that overexpression of LncRNA AB209630 suppressed the migration and invasion of HCC cells.

Discussion

Hepatocellular carcinoma (HCC) is the most prevalent malignancy of the liver and the second most common cause of cancer-related mortality, exceeded only by lung cancers (20). A wide variation in liver cancer incidence rates among nations has been observed worldwide, with a considerable geographic disparity in HCC (21). Notably, approximately half of all HCC cases and associated mortalities occur in China; thus, it is a significant health problem for people of different races and ethnicities, but particularly for Chinese individuals (22). The present study aimed to elucidate the role of LncRNA AB209630 in HCC, and to provide new information useful for the clinical diagnosis and treatment of HCC.

Three HCC cell lines, MHCC97-L, MHCC97-H and HCCLM3, were examined in present study. These cell lines are listed in order of increasing metastatic capacity, as established by the Liver Cancer Institute of Fudan University in 2001 (23). MHCC97 L was found to have a relatively low metastatic potential while MHCC97H and HCCLM3 were cell clones derived from MHCC97 cells that had incrementally higher metastatic capacity (23). Since considerable efforts are being made to identify metastasis-related genes, these three cell lines serve as useful materials for researchers. In the present study AB209630 expression was shown to be lower in HCC cells compared with the control liver cells (Fig. 1B). It was also observed that HCCLM3, the HCC cell line with the strongest potential for metastasis, had the lowest expression of AB209630, which further supported the conclusion that AB209630 is decreased in HCC cells and has a negative correlation with cell metastasis.

Another noteworthy feature of this study was the construction of an AB209630-expressing plasmid, which contained a CBP tag in the empty vector pGLUE. Since the transcript level

of LncRNA AB209630 was decreased in HCC patients and cultured HCC cells, an AB209630-expressing plasmid (and not specific shRNA against AB209630), was used to transfect the cells. LncRNAs are non-coding RNAs, and so cannot be detected using western blot analysis. Thus, an empty vector containing a CBP tag was used, which enabled the expression of the CBP tag instead of the LncRNA to be detected. As shown in Fig. 2C, the protein expression of the CBP tag was significantly increased in the AB209630-expressing plasmid group, indicating that the plasmid was successfully transfected into the HCCLM3 cells. This method has also been described in previous literature (17,24-26). With the aid of AB209630-expressing plasmid, it was observed that overexpression of AB209630 significantly inhibited HCCLM3 cell proliferation and colony formation capacity. Likewise, it was further observed that the migration and invasion of HCCLM3 cells were also significantly inhibited by overexpression of AB209630. These functional assays led to the conclusion that AB209630 suppressed cell proliferation and metastasis in human HCC and the suggestion that AB209630 may be a promising tumor suppressor for HCC.

In conclusion, the present study revealed that LncRNA AB209630 was downregulated in HCC tissues and cultured HCC cells, the mechanism of which remains to be discovered. Overexpression of AB209630 inhibited the proliferation, migration and invasion of HCC cells, which is indicative of potential antimetastatic activity. AB209630 is a potential biomarker of HCC for use in clinical diagnosis and treatment, and may provide novel information useful for researchers involved in the study of HCC.

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