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Overexpression of Aristaless-Like Homeobox-4 Inhibits Proliferation, Invasion, and EMT in Hepatocellular Carcinoma Cells

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Aristaless-like homeobox-4 (ALX4), a member of the Aristaless-like homeobox family, has been found to be involved in tumor cell proliferation, migration, and invasion. However, the role of ALX4 in hepatocellular carcinoma (HCC) remains largely unclear. Therefore, in this study we investigated the effects of ALX4 on HCC. The study results indicated that the expression of ALX4 was downregulated in HCC tissues and cell lines. Furthermore, we demonstrated that overexpression of ALX4 inhibited the proliferation, invasion, and epithelial–mesenchymal transition (EMT) in HCC cells. We also found that ALX4 had an inhibitory effect on the sonic hedgehog (Shh) signaling pathway. Taken together, the results suggest that ALX4 may be a promising target for HCC treatment.

Key words: Aristaless-like homeobox-4 (ALX4); Proliferation; Invasion; Epithelial–mesenchymal transition (EMT); Hepatocellular carcinoma (HCC)

INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common cancers, is characterized by high morbidity and mortality (1–3). According to the statistics, more than 600,000 deaths are caused by HCC every year, making HCC the third leading cause of cancer-related deaths around the world (1–4). Currently, the therapies for HCC include surgical resection, partial ablation therapy, and liver transplantation (5–7). All of these therapies are insufficient for complete removal of tumors due to tumor recurrence, thus leading to a poor outcome in HCC patients (8). Therefore, it is of great importance to identify new targets for HCC treatment and explore the underlying molecular mechanisms.

Aristaless-like homeobox-4 (ALX4), located on human chromosome 11p11.2, is a member of the Aristaless-like homeobox family (9,10). By combining with other family members to bind to palindromic DNA sequences, ALX4 is capable of activating transcription through promoters containing the homeodomain-binding site P2 (11). There are researchers who proved the restriction of ALX4 expression to mesenchymal condensations during the development of some organs and tissues, such as limbs, whiskers, bones, teeth, hair, and mammary tissue (12). Some studies have also shown the significant role of ALX4 in epithelial and stromal cells during craniofacial development as well as in hair follicle and skin structure (9,11). In addition, there has been evidence demonstrating that the loss of ALX4 expression would exert a disruptive effect on epidermal and craniofacial development and cause damage to mammary epithelial morphogenesis (13,14). Recently, ALX4 expression was found to be lost in breast tumors and the adjacent stromal cells, indicating the possible involvement of ALX4 in carcinogenesis (15). Despite all these reports on ALX4, the role that ALX4 plays in HCC remains unclear.

In this study, we investigated the effects of ALX4 on HCC. The study results indicated that the expression of ALX4 was downregulated in HCC tissues and cell lines. Furthermore, we demonstrated that overexpression of ALX4 inhibited the proliferation, invasion, and epithelial–mesenchymal transition (EMT) in HCC cells. We also found that ALX4 had an inhibitory effect on the sonic hedgehog (Shh) signaling pathway. All in all, we suggest that ALX4 may be a promising target for HCC treatment.

MATERIALS AND METHODS

Clinical Specimen Collection

Twenty HCC patients from the Department of Surgery, Hong-hui Hospital, Xi'an Jiaotong University College of Medicine (China) participated in the study and provided

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written consent. No patients received radiotherapy or chemotherapy before surgical resection. HCC tissue and corresponding noncancerous liver tissue were surgically resected from all the participants and then frozen in liquid nitrogen for future experiments. The study was performed with the approval of the Research Ethics Committee of Hong-hui Hospital, Xi'an Jiaotong University College of Medicine.

Cell Collection and Culture

Human HCC cell lines (Huh7, HepG2, and HCCLM3) and normal liver cell line (L02) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All the cells were cultured in RPMI-1640 medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Quantitative RT-PCR Analysis

Extraction of total RNA from cells or tissues was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription of RNA into cDNA was conducted with PrimeScript Reverse Transcript (Takara, Tokyo, Japan). The RT-PCRs for cDNA amplification were carried out under the following conditions: 95°C for 10 s, 95°C for 30 s, and 40 cycles at 60°C for 30 s. The primers used for RT-PCR were as follows: ALX4, 5′-CAAAGGCAAGAAGCGGCGGAATC-3′ (forward) and 5′-GGTACATTGAGTTGTGCTGTCC-3′ (reverse); β-actin, 5′-AGAAAATCTGGCACCACACC-3′ (forward) and 5′-TAGCACAGCCTGGATAGCAA-3′ (reverse). The relative expression of genes was determined using the comparative CT method ($2^{-\Delta\Delta Ct}$) (16). The expression of β-actin was analyzed for normalization.

Western Blot Analysis

Cells or tissues were lysed in RIPA lysis buffer (Beyotime, Shanghai, China). Following centrifugation at $12{,}000 \times g$ for 15 min, total proteins were separated using 10% SDS-PAGE (Pierce Chemical Company, Rockford, IL, USA) and transferred to PVDF membranes (Millipore, Boston, MA, USA). The membranes were blocked for 1 h in PBS and 5% nonfat milk, followed by overnight incubation at 4°C with the primary antibodies against ALX4, E-cadherin, fibronectin, N-cadherin, Shh, Ptch1, Gli1, or β-actin. After washing three times with TBST, the membranes were incubated for 1 h at 37°C with secondary HRP-conjugated antibodies. All antibodies used in these experiments were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactive protein bands were visualized with enhanced chemiluminescence (Invitrogen). The relative protein expression levels were analyzed using Glyko BandScan 5.1 software (Life Technologies, Gaithersburg, MD, USA). β-Actin was chosen as an internal control.

Establishment of ALX4 Overexpression Cell Lines

The plasmid vector pCMV harboring ALX4 precursor sequence or scramble sequence was purchased from GeneChem Biotechnology (Shanghai, China). After growing to 80%–90% confluence in six-well plates, Huh7 and HepG2 cells were transfected with pCMV-ALX4 or pCMV-scramble vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's procedure. The transfection rate was determined by Western blot assays.

Cell Proliferation Assay

The cell proliferation ability was detected by MTT assay. After 24 h of transfection with ALX4, Huh7 and HepG2 cells were cultured in 96-well plates at a density of 1×10^5 cells/well for 24, 48, 72, or 96 h. MTT (Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by cell culturing for 4 h in a 5% CO_2 incubator at 37°C. The absorbance was measured at 490-nm wave length by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Migration and Invasion Assays

Transwell chambers of 8-μm pore size were used to detect the invasive and migratory capabilities of HCC cells. For the invasion assay, filters were coated with Matrigel (BD Biosciences, San Jose, CA, USA). Transfected cells were suspended in serum-free RPMI-1640 medium. The upper chamber was filled with 100 μl of the cell suspension (containing 1×10^5 cells) and the lower chamber with 600 μl of complete medium. Twenty-four hours later, the cells on the upper side of the filters were removed with a cotton swab, and the cells on the bottom of the filters were fixed and stained with 0.2% crystal violet. Four random areas were selected to count the number of invaded cells under a microscope (200×). For the migration assay, the procedure was similar to that of the invasion assay, except that filters were not coated with Matrigel.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Student's *t*-tests were applied for statistical analysis. A value of $p < 0.05$ was considered statistically significant. All experiments were carried out independently at least three times.

RESULTS

Expression of ALX4 Is Downregulated in HCC Tissues and Cell Lines

The expression levels of ALX4 in HCC tissues and cell lines were analyzed by qRT-PCR and Western blot assays. As shown in Figure 1A and B, the expression of ALX4 was significantly decreased, compared with the

matched noncancerous liver tissues. We also detected the expression of ALX4 in HCC cell lines with the normal liver cell line L02 as an internal control. As shown in Figure 1C and D, ALX4 was lowly expressed in three HCC cell lines (Huh7, HepG2, and HCCLM3) but highly expressed in the normal liver cell line L02.

Overexpression of ALX4 Inhibits the Proliferation of HCC Cells

To investigate the effect of ALX4 overexpression on HCC proliferation, Huh7 and HepG2 cells were transfected with pCMV-ALX4 or pCMV-scramble vector, respectively. Overexpression of ALX4 in Huh7 (Fig. 2A)

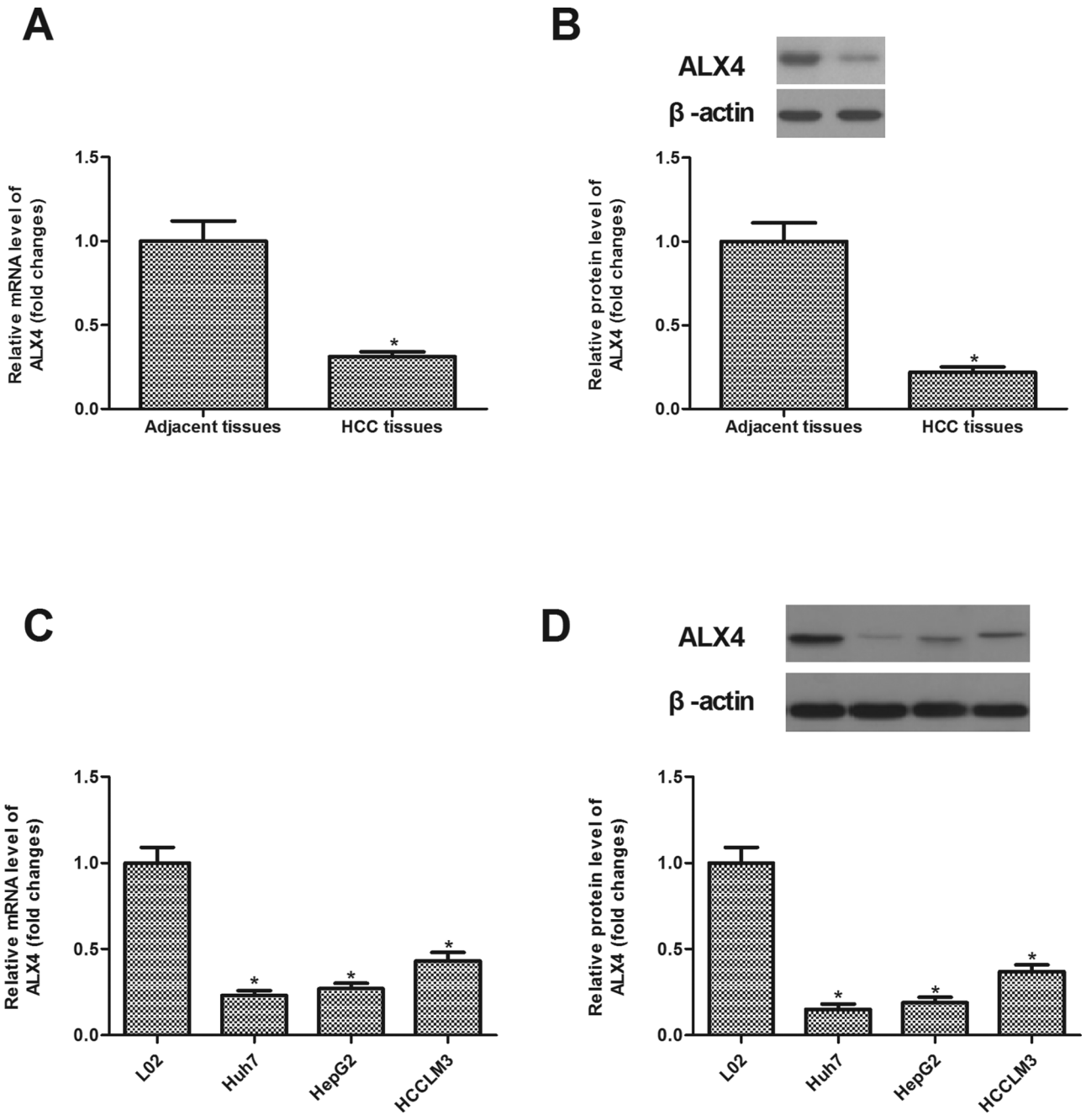

Figure 1. Expression of ALX4 is undetectable in HCC tissues and cell lines. (A, B) qRT-PCR and Western blot assays were conducted, and the results indicated downregulation of ALX4 in HCC tissues in comparison with the matched noncancerous liver tissues. (C, D) Results of the qRT-PCR and Western blot assays showed that ALX4 was lowly expressed in three HCC cell lines (Huh7, HepG2, and HCCLM3) but was highly expressed in the normal liver cell line L02. *$p < 0.05$.

and HepG2 (Fig. 2B) cells was confirmed by Western blot analysis.

We performed MTT assays to detect the effect of ALX4 overexpression on the proliferation of HCC cells. MTT results showed that the proliferation rate of Huh7 (Fig. 2C) cells transfected with pCMV-ALX4 was greatly slowed down in comparison with the control group transfected with pCMV-scramble. Similar results were obtained for HepG2 (Fig. 2D) cells.

Overexpression of ALX4 Inhibits the Migration and Invasion of HCC Cells

To investigate the effect of ALX4 overexpression on the migration and invasion of HCC cells, Transwell assays were performed. As shown in Figure 3A and B, the migratory and invasive capabilities of Huh7 cells were obviously weakened by overexpression of ALX4 in comparison with the control group. In addition, we observed a similar inhibitory effect of ALX4 overexpression on the migration and

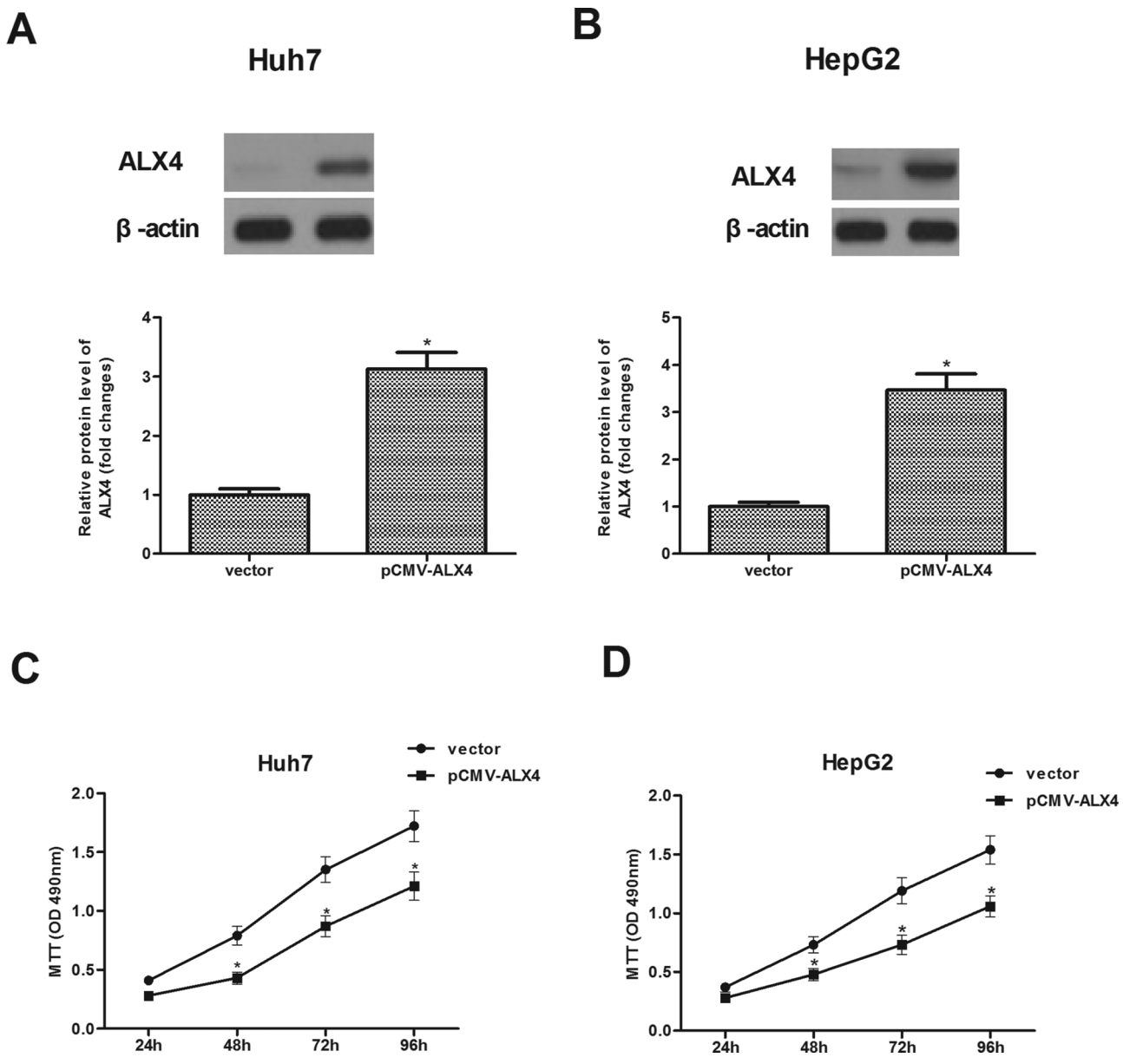

Figure 2. Overexpression of ALX4 inhibits the proliferation of HCC cells. Expression of ALX4 in Huh7 (A) and HepG2 (B) cells after transfection with pCMV-ALX4 was confirmed by Western blot assays. As shown by the MTT assay, overexpression of ALX4 obviously inhibited the proliferation rate of Huh7 (C) and HepG2 (D) cells transfected with pCMV-ALX4 compared to that of the control group transfected with pCMV-scramble. $*p < 0.05$.

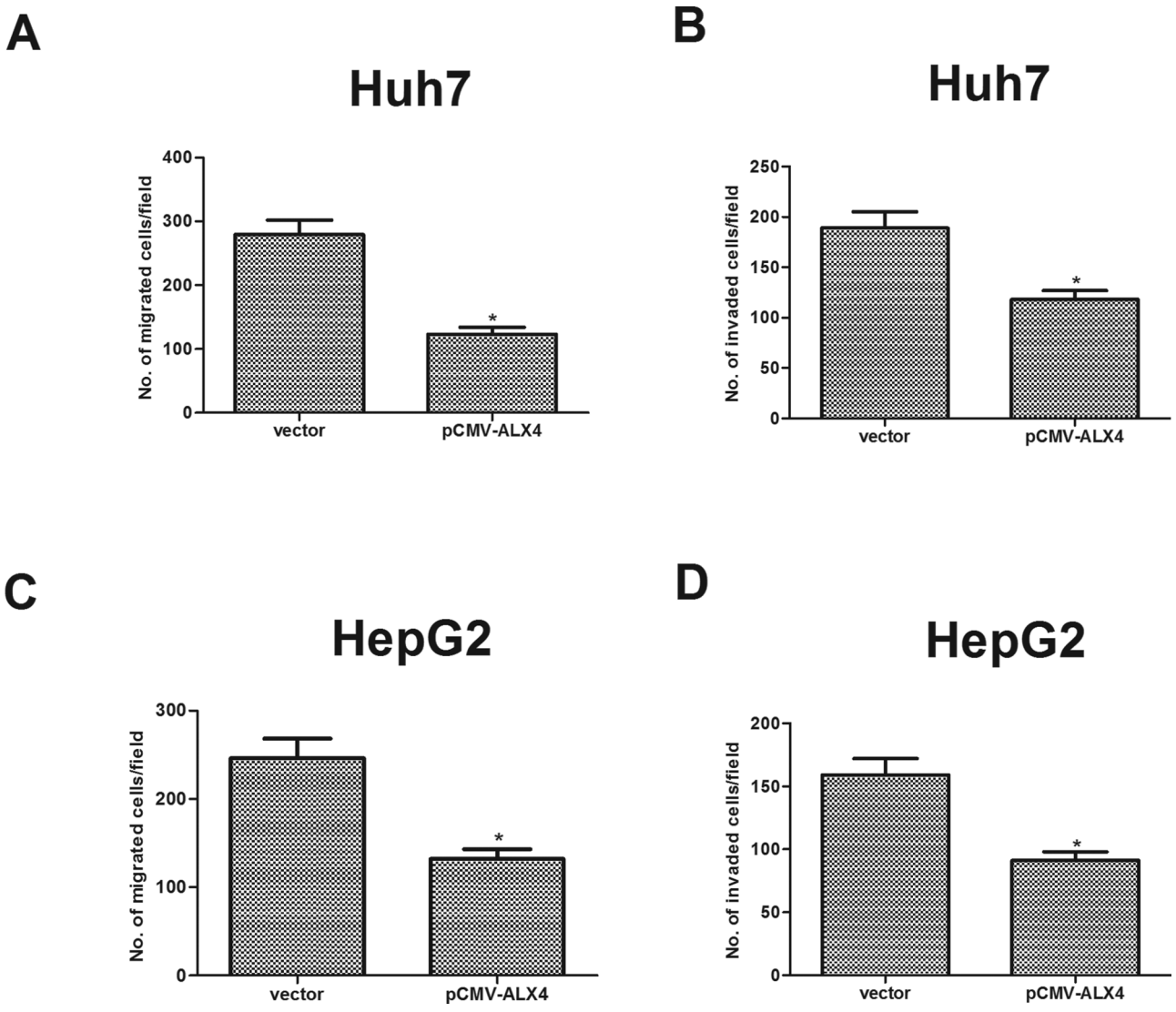

Figure 3. Overexpression of ALX4 inhibits the migration and invasion of HCC cells. Migration and invasion of Huh7 (A, B) and HepG2 (C, D) cells overexpressing ALX4 were measured by Transwell assays. The assay results were quantitated by counting the migrated and invaded cells in four randomly selected areas. *$p < 0.05$.

invasion of HepG2 cells (Fig. 3C and D). These results indicated that ALX4 overexpression played a suppressive role in the migration and invasion of HCC cells.

Overexpression of ALX4 Inhibits the EMT Process in HCC Cells

We conducted Western blot assays to analyze the expression levels of EMT-related markers (E-cadherin, fibronectin, and N-cadherin) in Huh7 cells, aiming to verify whether ALX4 played a functional role in the EMT process. As shown in Figure 4A, overexpression of ALX4 in Huh7 cells led to higher protein expression levels of E-cadherin and lower protein expression levels of fibronectin and N-cadherin. Quantification analysis of E-cadherin, fibronectin, and N-cadherin is shown in Figure 4B.

Overexpression of ALX4 Inhibits the Shh Signaling Pathway

Considering the previous studies on implication of the Shh signaling pathway in HCC (17–19), we determined the effect of ALX4 overexpression on the Shh signaling pathway by examining its significant components Shh, Ptch1, and Gli1 in Huh7 cells overexpressing ALX4 via Western blot analysis. As shown in Figure 5A, the protein expression levels of Shh, Ptch1, and Gli1 were remarkably downregulated in Huh7 cells overexpressing ALX4

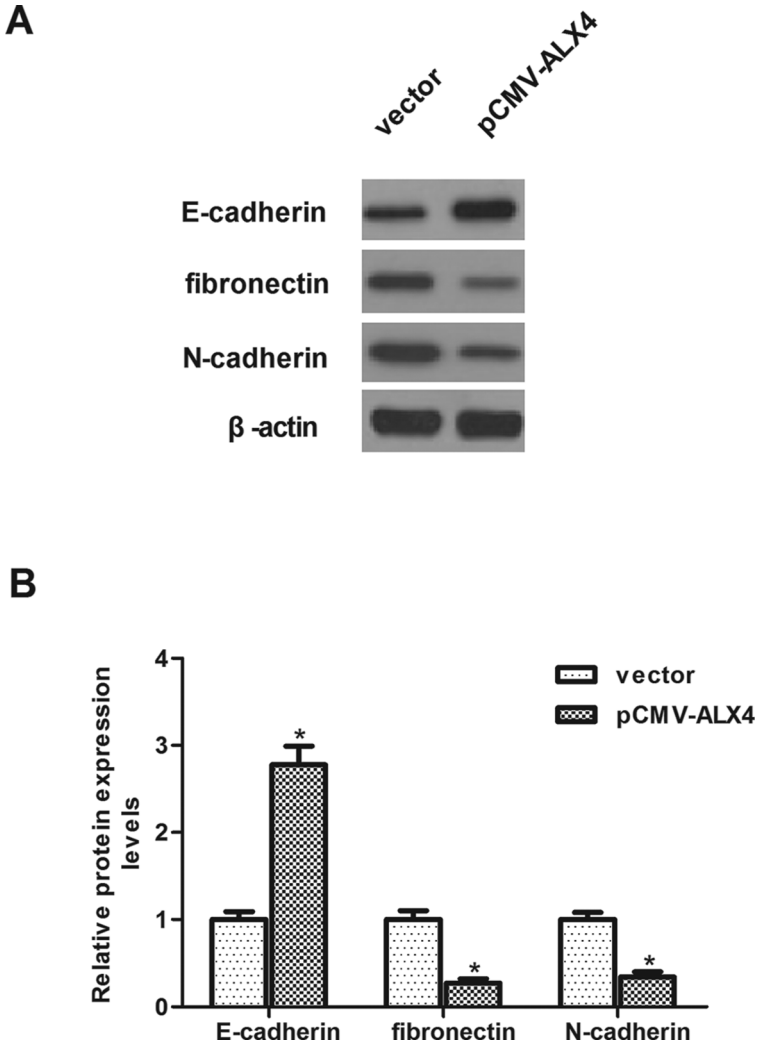

Figure 4. Overexpression of ALX4 inhibits the EMT process in HCC cells. (A) The expression of EMT-related markers E-cadherin, fibronectin, and N-cadherin in Huh7 cells overexpressing ALX4 was detected by Western blot, with β-actin as an internal control. (B) The relative protein expression levels of E-cadherin, fibronectin, and N-cadherin in the different cell groups were analyzed via Glyko BandScan 5.1 software. *$p<0.05$.

in comparison with the control group. Quantification analysis of Shh, Ptch1, and Gli1 is shown in Figure 5B.

DISCUSSION

Characterized by high morbidity and mortality, HCC ranks as the third leading cause of death associated with cancers, thus becoming a worldwide health problem needing to be solved (1–3,20). To improve the poor outcome of HCC patients, new therapeutic approaches are urgently needed. In this study, we investigated the relation between ALX4 and HCC, which may provide a new path to the effective treatment of HCC.

We first examined the expression of ALX4 in HCC tissues and cell lines via qRT-PCR and Western blot assays. The assay results indicated that ALX4 was lowly expressed in HCC tissues. Similarly, we found that ALX4 has little expression in HCC cell lines but a high expression in the normal liver cell line. Consistent with our findings, Liu et al. found that ALX4 expression is downregulated or silenced in lung cancer cell lines (21). Unlike Liu et al., Yuan et al. demonstrated that ALX4 expression is upregulated in ovarian cancer cells (22). Thus, we inferred that the expression of ALX4 may vary between different types of cancers.

We next investigated the effect of ALX4 overexpression on the proliferation, invasion, and EMT of HCC cells. Before the investigation, we established whether HCC cell lines stably overexpressed ALX4 by transfecting pCMV-ALX4 or pCMV-scramble vector. We conducted Western blot assays to confirm that the transfection was

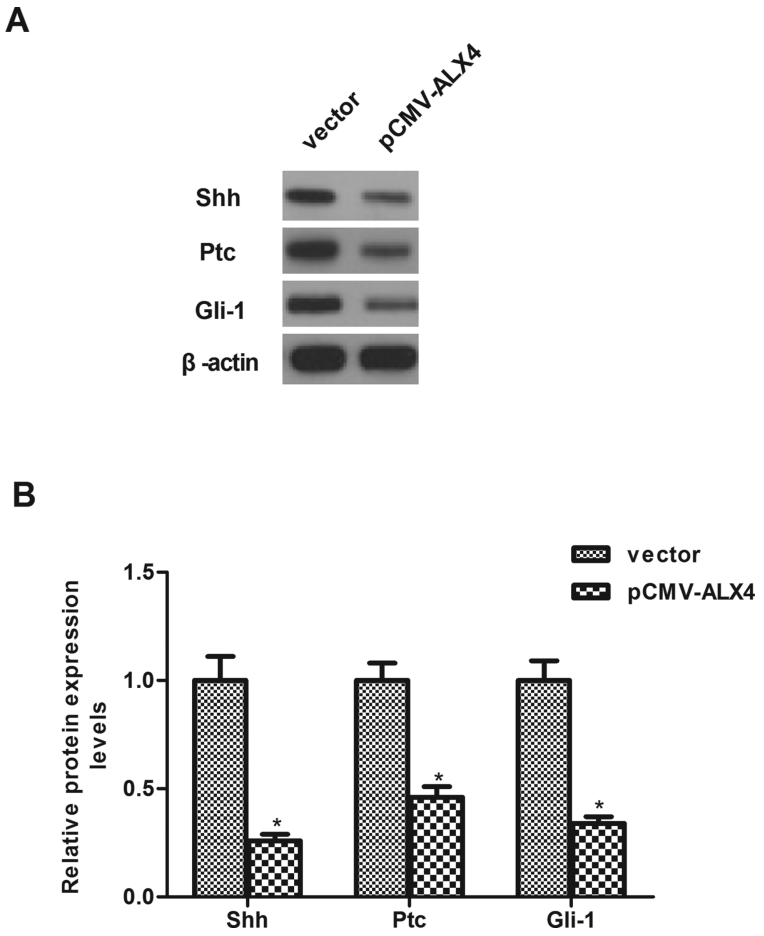

Figure 5. Overexpression of ALX4 inhibits the Shh signaling pathway. (A) As indicated by the Western blot assays, the protein expression levels of Shh, Ptch1, and Gli1 were significantly reduced in Huh7 cells overexpressing ALX4 in comparison with the control group. β-Actin was used as an internal control. (B) The relative protein expression levels of Shh, Ptch1, and Gli1 in the different cell groups were analyzed via Glyko BandScan 5.1 software. $*p<0.05$.

successful. Then we examined the effect of ALX4 overexpression and found the proliferation, invasion, and EMT of HCC cells to be greatly suppressed by overexpression of ALX4. There are researchers who conducted similar experiments on lung cancer cells and demonstrated the inhibitory effect of ALX4 expression on cell growth and migration of lung cancer cells, suggesting the role of ALX4 as a potential tumor-suppressor gene related to lung cancer (21). In contrast, Yuan et al. found that ALX4 could promote cell invasion and EMT in ovarian cancer cells (22). On the basis of all these studies, we believed that ALX4 may function as a tumor suppressor or promoter in different kinds of cancers. In our study, we demonstrated the tumor-suppressive function of ALX4 in HCC and investigated its underlying mechanism.

The Shh signaling pathway, a mammalian counterpart of the Hedgehog signaling pathway, plays a significant role in cell growth and development (23). It has been found that the Shh signaling pathway is implicated in many types of cancers, such as colorectal cancer, prostate cancer, basal cell cancer, ovarian cancer, and pancreatic cancer (24–28). There has also been evidence demonstrating the relation between ALX4 and the Shh signaling pathway (21). Therefore, it is a promising research direction to verify whether ALX4 exerted its inhibitory effect on the development of HCC via the Shh signaling pathway. To test our hypothesis, we conducted Western blot analysis to examine several important components of the Shh signaling pathway, including Shh, Ptch1, and Gli1. As indicated by the assays, the protein expression levels of Shh, Ptch1, and Gli1 were obviously decreased in Huh7 cells overexpressing ALX4, suggesting the suppressive effect of ALX4 overexpression on the Shh signaling pathway.

In conclusion, this study demonstrated that ALX4 was lowly expressed in HCC tissues and cell lines. Furthermore, we put forward that overexpression of ALX4 inhibited the proliferation, invasion, and EMT of HCC cells. We also

found that ALX4 exerted its inhibitory effect on HCC cells via suppressing the Shh signaling pathway. Taken together, our findings could be used in support of the role of ALX4 as a promising target for HCC treatment.

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