Kinesin Family Member C1 (KIFC1) Accelerates Proliferation and Invasion of Endometrial Cancer Cells Through Modulating the PI3K/AKT Signaling Pathway

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Abstract

Endometrial cancer (EC) is one of the most common cancers among women worldwide. Kinesin family member C1 (KIFC1) has been demonstrated to play crucial roles in various tumors. However, the function of KIFC1 in EC remains to be revealed. In this study, upregulation of KIFC1 expression in human EC tissues was found from analysis on data from The Cancer Genome Atlas (TCGA), and positively correlated with short survival outcome of EC patients. In addition, the mRNA and protein levels of KIFC1 were confirmed to be up-regulated in EC cells (Ishikawa, HEC-1B, HEC-1A and KLE) compared to human normal endometrial stromal cells (hESCs) by quantitative real time PCR and western blot. *In vitro* functional experiments showed that overexpression of KIFC1 promoted proliferation, migration and invasion of EC cells, while KIFC1 depletion showed the opposite results. Moreover, KIFC1 knockdown suppressed tumor growth in mice. Further mechanism analysis showed that KIFC1 participated in the regulation of EC progression through regulating the PI3K/AKT signaling pathway. Collectively, KIFC1 promoted proliferation and invasion through modulating PI3K/AKT signaling pathway in EC, implying that KIFC1 might provide a promising therapeutic target for the therapy of EC.

Keywords

endometrial cancer (EC), kinesin family member C1 (KIFC1), proliferation, invasion, PI3K/AKT

Abbreviation

EC, Endometrial cancer; hESCs, human normal endometrial stromal cells; HSET, humans, namely HsKIFC1; KIFC1, Kinesin family member C1; TCGA, The Cancer Genome Atlas

Received: April 14, 2019; Revised: July 16, 2020; Accepted: September 15, 2020.

Introduction

Endometrial carcinoma (EC), is a group of epithelial malignant tumors that occur in the endometrium, accounting for 20% to 30% of the tumors in the female reproductive system.[1] It is one of the major causes of morbidity and mortality in women.[2] EC often occurs in postmenopausal women, with an average age of onset of 60 years.[3,4] The 5-year survival rate of patients with early EC and distant metastases is more than 90% and less than 20%, respectively, and particularly, the prognosis of advanced, poorly differentiated or special types of EC is extremely poor.[5,6] Therefore, exploring the pathogenesis and effective treatment targets of EC is of great significance to improve the prognosis of EC.

KIFC1, also known as HSET, is a type C terminal kinesin belonging to the Kinesin-14 family.[7-9] To date, only 3 members

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of the Kinesin-14 family have been found in humans, namely HsKIFC1 (HSET), HsKIFC2 and HsKFC3.[7,10,11] Among them, KIFC1 plays an extremely important role in spindle mitosis,[7,12] which has attracted much attention. It has been discovered that KIFC1 plays a role in vesicle and organelle transport,[13] oocyte development,[14] spermatogenesis,[9,15] and double-stranded DNA transport.[16] It has been recently considered that KIFC1 is redundant in normal somatic cells and essential in cancer cells, and KIFC1 has the role of gathering additional centrosomes to avoid multipolar cell division and maintain cancer cell survival.[17-19] These investigations indicate that anti-KIFC1 therapy may have significant research prospects in highly selective antitumor methods. It has been reported that KIFC1 is widely expressed in breast cancer,[20,21] ovarian cancer,[22] lung cancer,[23] stomach cancer,[24] prostate cancer[25] and other cancers.[25-27] Thus, KIFC1 is a reasonable target for selective tumor treatment and deserves further discussion. However, few studies focused on the functional role of KIFC1 in the pathogenesis of EC and the specific regulatory mechanism remains unclear.

Therefore, the aim of the current study was to elucidate the role of KIFC1 in the modulation of EC and reveal the underlying molecular mechanisms both *in vivo* and *in vitro*. Our findings suggested that KIFC1 promoted EC cell proliferation and invasion through regulating PI3K/AKT signaling pathway.

Materials and Methods

Cell Culture

Human EC cell lines (Ishikawa, HEC-1B, HEC-1A and KLE) and human normal endometrial stromal cells (hESCs) were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C with 5% CO_2. Cells were cultured in flasks or dishes containing cell medium supplemented with 10% FBS (Thermo Fisher Scientific) in a humidified atmosphere with 5% CO_2 at 37°C.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cells via TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reversed transcribed into cDNA (Roche, Germany). Quantitative PCR was performed on the ABI 7500 real-time PCR system (Applied Biosystems, USA). β-actin was selected as internal controls for standardization. The expression level was calculated by using relative quantitative method ($2^{-\Delta\Delta Ct}$). All the primers used were as follows:

KIFC1, forward, 5′-GACGCCCTGCTTCATCTG-3′, reverse, 5′-CCAGGTCCACAAGACTGAGG-3′; β-actin, forward, 5′-TTCCGAATGCTGACGTTGC-3′, reverse, 5′-CCA ACTGCCAAACGTGCTG-3′.

Western Blot

Total protein was extracted from EC cells using RIPA buffer (Beyotime Biotechnology, Shanghai, China) and quantified by a BCA protein assay kit (Beyotime). Protein lysates were separated on a sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and transferred onto PVDF membrane. After blocked with skim milk (5%), membranes were incubated with the primary antibodies overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated secondary antibodies were subsequently incubated for 1 h. The signal blots were visualized by an ECL kit (Millipore, Billerica, MA, USA). GAPDH was used as a loading control. The primary antibodies were as follows: KIFC1 (ab172620, Abcam, Cambridge, MA, USA), E-cadherin (ab1416, Abcam), N-cadherin (ab98952, Abcam), Vimentin (ab7752; Abcam), t-PI3K (ab40755; Abcam), p-PI3K (ab182651; Abcam), t-AKT (ab18785; Abcam), p-AKT (ab38449; Abcam) and GAPDH (ab8245, Abcam).

Cell Transfection and Treatment

The KIFC1 cDNA sequence was cloned into pcDNA3.1 vector to up-regulate its expression and then transfected into HEC-1A cells. PcDNA3.1 vector (Vector) or pcDNA3.1-KIFC1 (OE-KIFC1) were purchased from Gene Pharma (Shanghai, China). The shRNA sequences targeting KIFC1 (sh-KIFC1#1, sh-KIFC1#2) were synthesized from Gene Pharma (Shanghai, China) and transfected into Ishikawa cells to knock down its expression. Nonsense shRNA was used as a negative control (sh-NC). Cells were cultured to 80% confluence in 6-well plates and then were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 48 h, the transfection efficiency was determined by RT-qPCR. After that, cells were treated by AKT inhibitor LY-294002 (10 μM, Sigma Aldrich, St. Louis, MO, USA) for 24 h. Cell infection were performed using the lentivirus in Ishikawa cells. Stable cells were then sorted out by puromycin and used in animal study.

Cell Counting Kit (CCK)-8 Assay

The cells were plated into 96-well plates at a density of 1×10^4 cells/well and cultured in DMEM with 10% FBS for indicated time points (24 h, 48 h, 72 h, 96 h), followed by adding CCK-8 solution (10 μl). Next, the incubation was continued at 37°C for another 4 h. Optical density (OD) was determined using a microplate reader at 450 nm.

Colony Formation Assay

1×10^3 cells were seeded into a 6-well culture plate and incubated to form colony for 2 weeks. For visualization of colonies, cells were fixed with methanol for 10 min, and then stained with 0.1% crystal violet for 1 h. The numbers of cell clones were counted and photographed manually.

Transswell Assay

For the migration assay, cells suspended in serum-free DMEM medium were added to the upper chamber of Transwell chamber (8 μm pore size; Corning, New York, USA). The DMEM medium with 10% FBS was added to the bottom chamber. Cells were incubated at 37°C for 24 h and then the migrated cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature. After washing with PBS, the samples were photographed under a light microscope (Olympus BX51, Tokyo, Japan). For the invasion assay, the upper chamber of Transwell chamber was pre-coated with Matrigel (Bection Dickinson, California, USA).

In vivo *Xenograft Mouse Model*

The animal study was approved by the Ethics Committee of People's Hospital of Quzhou City (approval no. ZQA2018044). Experiments were operated according to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health.[28] Female BALB/c nude mice (7-week-old, ~20 g). 1×10^6 Ishikawa cells that stably express sh-KIFC1 or sh-NC were inoculated subcutaneously into the mice (6 mice/group). Tumor sizes were determined every week, each tumor's volume was respectively calculated using the formula of $V = 1/2 \times Length \times Width^2$. At 35 days after inoculation, mice were euthanized and tumors were photographed and weighted, then collected for the subsequent analysis.

Immunohistochemistry (IHC)

Paraffin-embedded tissues were sectioned at 4μm thick. After treated with 3% hydrogen peroxide to quench peroxidase activity, the sections were incubated with anti-KIFC1 antibody (ab172620, Abcam), anti-ki-67 antibody (ab15580, Abcam). Thereafter, the sections were rinsed with phosphate buffered saline (PBS), incubated with a secondary antibody for 1 h and stained with 3, 3′-diaminobenzidine substrate kit (DAB, Thermo Scientific Pierce). After the completion of hematoxylin redyeing, section was dehydrated and sealed.

Statistical Analysis

The statistical data were from 3 independent experiments and presented as mean $\pm$ standard deviation (SD). Statistical differences were analyzed using Student's-t test for 2 groups and by ANOVA for multiple groups by GraphPad Prism 7 software. The difference was regarded as statistically significant when p value < 0.05.

Results

KIFC1 Is Up-Regulated in EC Tissues and Cells

MERAV database (http://merav.wi.mit.edu/SearchByGenes. html)[29] was used to summarize the expression of KIFC1 in primary tumor tissues, tumor cells, and normal tissues from the female reproductive system. Results showed that the expression level of KIFC1 in the primary tumor tissues was significantly higher than that in the normal tissues (Figure 1A). The consistent results were found through analyzing data from UALCAN database (http://ualcan.path.uab.edu/analysis.html), as displayed in Figure 1B-E. In detailed, KIFC1 expression level in EC tissues was significantly up-regulated (Figure 1B), which was related to clinical stage Figure 1C), menopause status (Figure 1D) and histological subtypes (Figure 1E, all *P*<0.001). Interestingly, through the analysis of TCGA data at Oncolnc dataset (http://www.oncolnc.org/), it was found that patients with high KIFC1 expression predicted poor prognosis (Figure 1F, $P = 0.0202$). Moreover, the mRNA and protein levels of KIFC1 were confirmed to be up-regulated in EC cell lines (Ishikawa, HEC-1B, HEC-1A and KLE) as compared to human normal endometrial stromal cells line hESCs by quantitative real time PCR and western blot (Figure 1G-H). Compared to other EC cell lines, Ishikawa cells with highest expression of KIFC1 were used for loss-of-function experiments and SK-HEP-1 cells that expressed lowest KIFC1 level were used for Gain-of function experiments. Taken together, these findings provided the potential role of KLF1 in EC progression.

KIFC1 Promotes the Proliferation and Colony Formation of EC Cells *in vitro*

Next, we explored the biological function of KIFC1 in EC cells. The transfection efficiencies were confirmed by qRT-PCR and western blot, respectively (Figure 2A-B). Since sh-KIFC#1 showed lower expression of KIFC than sh-KIFC#2, which was chosen for subsequent experiments. CCK-8 and colony formation assays (Figure 2C-D) revealed that KIFC1 depletion prohibited viability and led to the reduction of colony numbers of Ishikawa cells, whereas overexpression of KIFC1 increased the viability and colony formation of HEC-1A cells, suggesting KIFC1 could act as a promoting factor in EC cell growth *in vitro*.

KIFC1 Promotes the Migration and Invasion of EC Cells *in vitro*

Next, we explored the effect of KIFC1 on cell migration and invasion using transwell assay *in vitro*. As shown in Figure 3A and B, KIFC1 knockdown inhibited the migration and invasion of Ishikawa cells, while overexpression of KIFC1 increased the migration and invasion of HEC-1A cells. Accumulating evidences have demonstrated epithelial-mesenchymal transition (EMT) is closely associated to cancer cell growth and exerts a critical role in the progression of EC.[30,31] Consistent with the effect of KIFC1 on cell migration and invasion, the expression of the epithelial marker (E-cadherin) was significantly increased, while the expression levels of mesenchymal makers (N-cadherin and Vimentin) were decreased in Ishikawa cells that transfected with KIFC1-knockdown (Figure 3C). On the
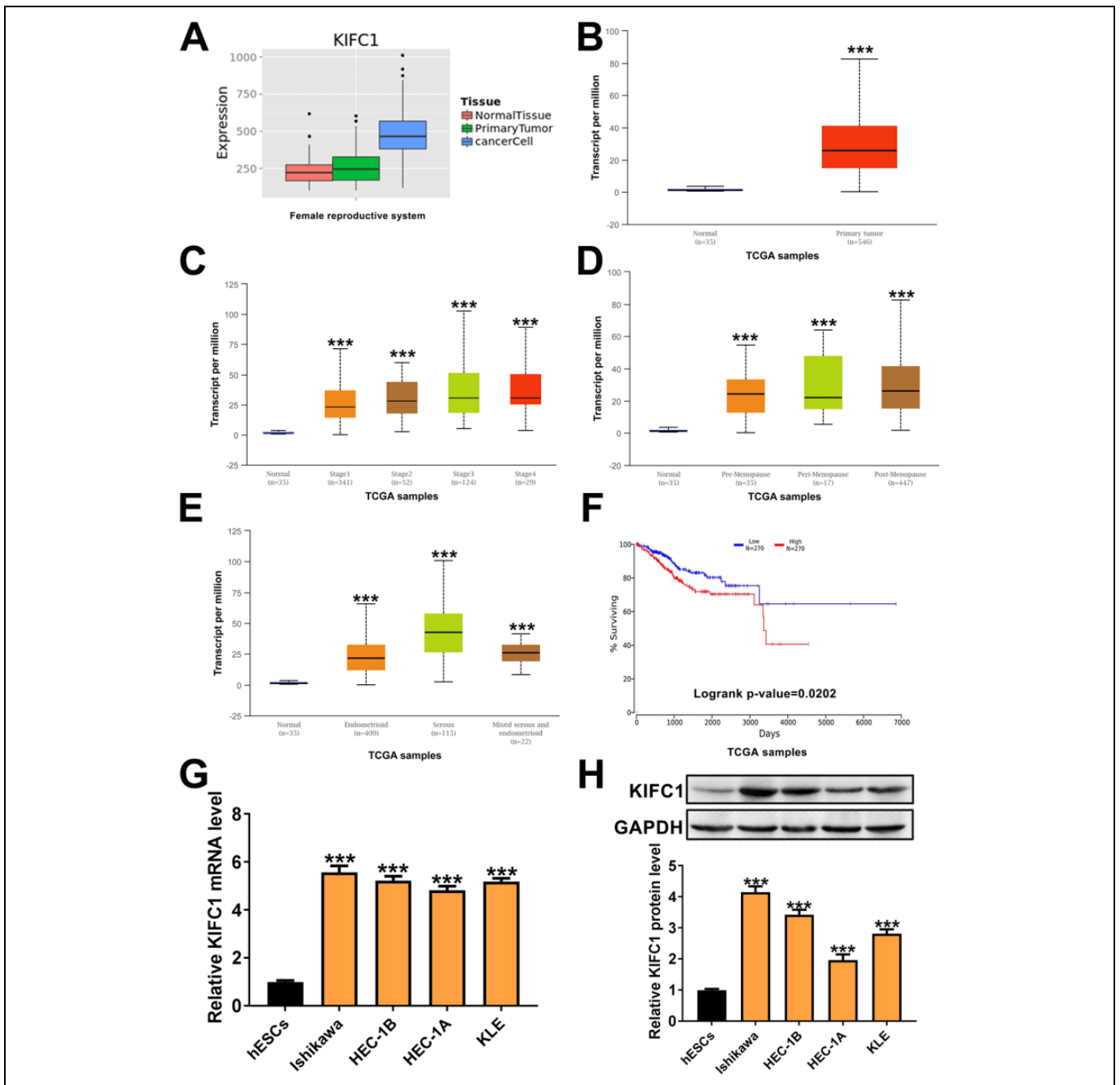
Figure 1. KIFC1 is up-regulated in EC tissues and cells. (A) KIFC1 expression in primary tumor tissues, tumor cells, and normal tissues from the female reproductive system using the MERAV web server (http://merav.wi.mit.edu/). (B) KIFC1 expression in546 EC and 35 controls samples from the TCGA dataset from UALCAN: http://ualcan.path.uab.edu/index.html. (C-E) KIFC1 expression in EC samples for each stage (C), menopause status (D) and histological subtypes (E) were assessed in TCGA samples from UALCAN. (F) KIFC1 expression was related to the overall survival of EC patients. Cox regression analysis was performed by the OncoLnc tool to obtain the survival of the patients (http://www. oncolnc.org). Red line: high expression, blue line: low expression. The mRNA (G) and protein (H) levels of KIFC1 were measured by qRT-PCR and western blot in EC cells (Ishikawa, HEC-1B, HEC-1A, and KLE) and human normal endometrial stromal cells (hESCs). Data were shown as means $\pm$ SD. ***$P < 0.001$.

contrary, the E-cadherin was significantly decreased, whereas N-cadherin and Vimentin expression were increased in KIFC1-overexpressed HEC-1A cells (Figure 3C). Taken together, these findings indicated that KIFC1 promoted the migration, invasion and EMT of EC cells.

Knockdown of KIFC1 Represses Tumor Formation in vivo

To further verify whether KIFC1 influences tumorigenesis *in vivo*, Ishikawa cells stably down-expressing KIFC1 were
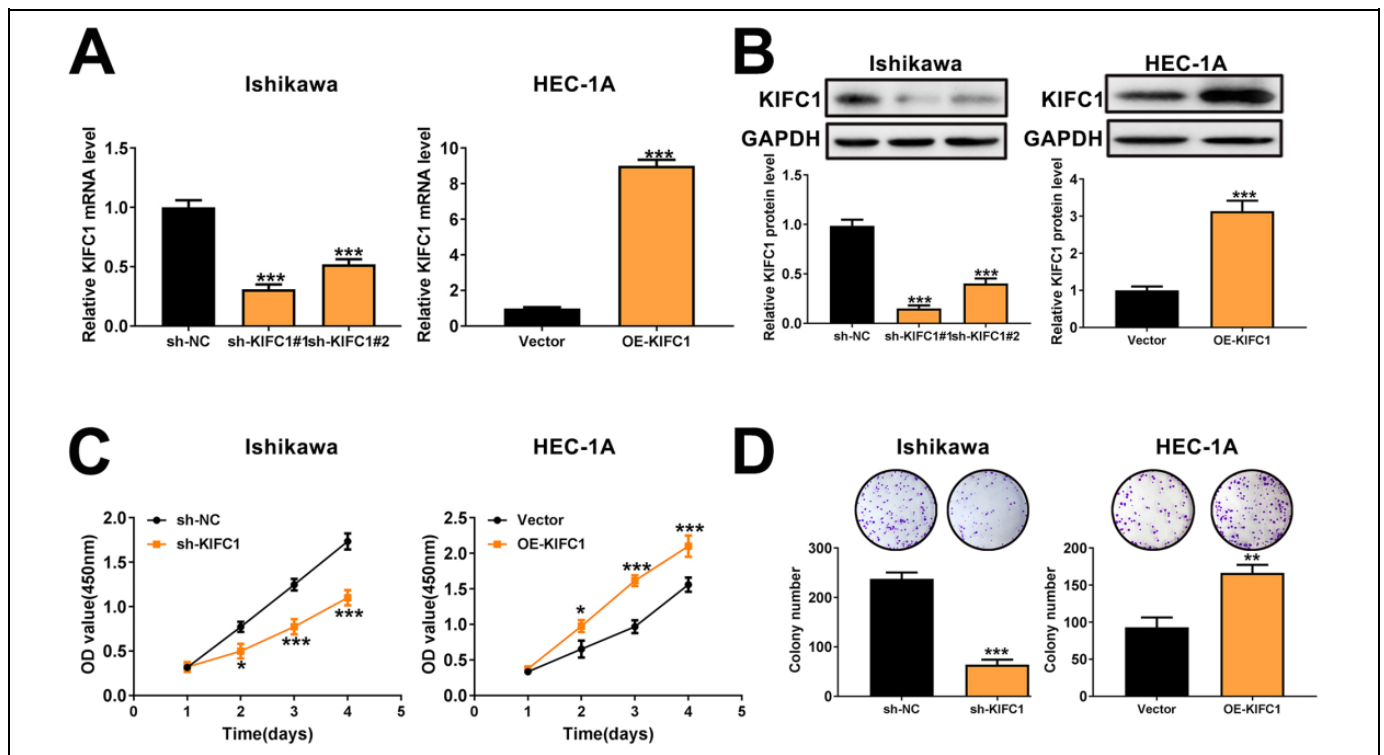
Figure 2. KIFC1 promotes cell proliferation of EC *in vitro*. (A-B) Transfection efficiency of KIFC1 was evaluated by qRT-PCR (A) and western blot (B). (C) The proliferation of NSCLC cells was assessed by CCK-8 assay. (D) Colony formation assay was performed to evaluate cell proliferation. Data were shown as means $\pm$ SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
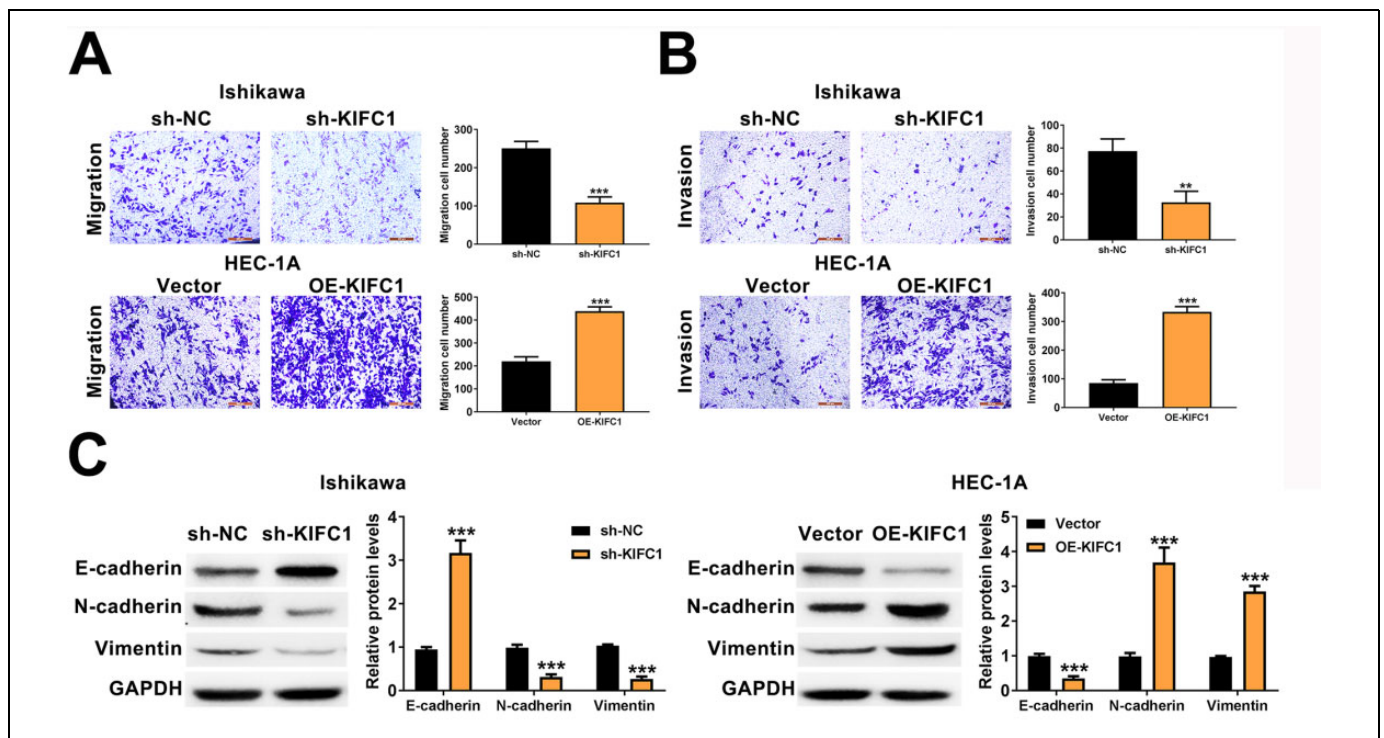

Figure 3. KIFC1 promotes cell migration and invasion of EC *in vitro*. The migration (A) and invasion (B) of EC cells were measured by transwell assay (Scale bar: 200 μm). (C) The protein levels of E-cadherin, N-cadherin and Vimentin were measured by western blot. Data were shown as means $\pm$ SD. **$P < 0.01$, ***$P < 0.001$.
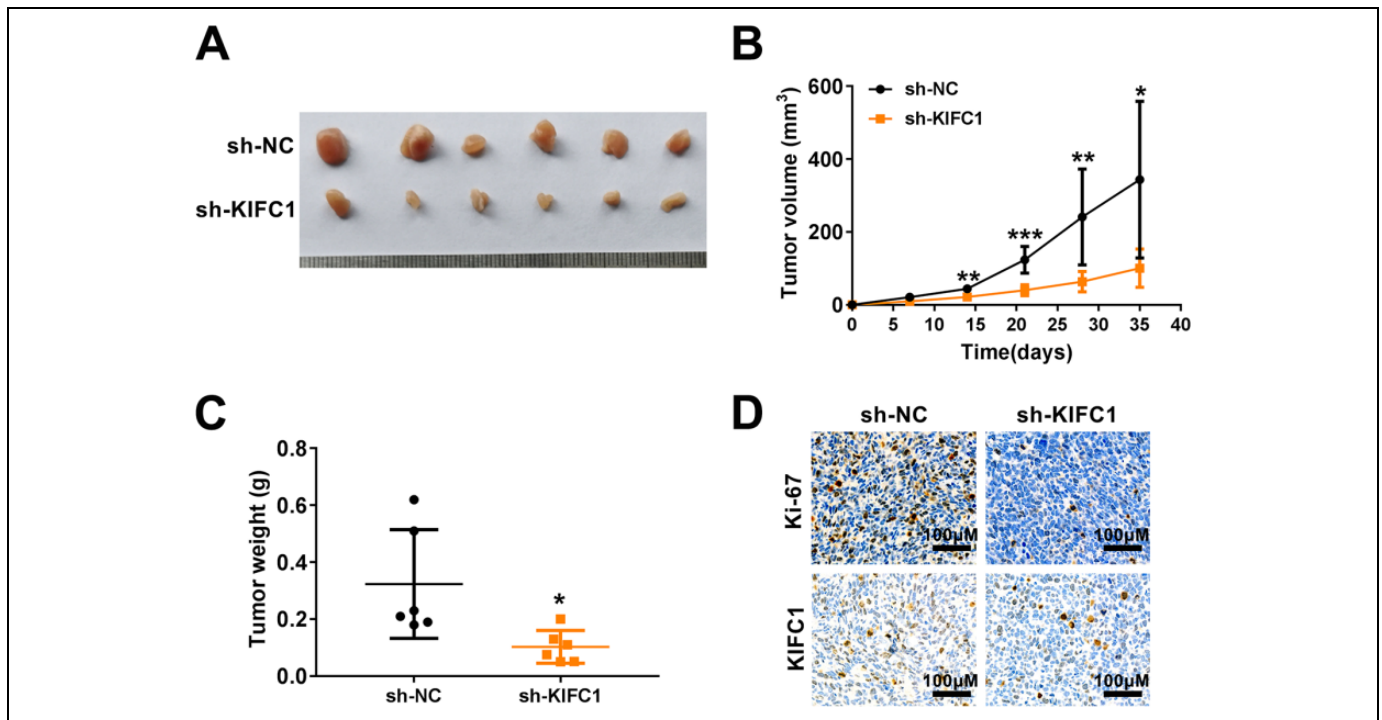
Figure 4. Knockdown of KIFC1 represses tumor formation *in vivo.* (A) After 35 days, the tumor nodules were removed and photographed. Tumor volume (B) and tumor weight (C) were measured after KIFC1 was knocked down. (D) The expression of KIFC1 and Ki-67 in tumors was determined by IHC (Scale bar: 100 μm). Data were shown as means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

subcutaneously implanted into the flank of nude mice. At 35days after implantation, the tumors were excised and the representative image was presented in Figure 4A. Tumor volume was monitored, as shown in Figure 4B-C, the tumors in the sh-KIFC1 group exhibited smaller sizes and lighter weight as compared to that in the sh-NC group. Additionally, KIFC1 inhibition led to decreased Ki67 and KIFC1 positive cell numbers in xenograft tumors as compared to the sh-KIFC1 group (Figure 4D). These results indicated that knockdown of KIFC1 suppressed EC tumor growth o *in vivo.*

KIFC1 Plays a Carcinogenic Role in EC Cells Through the PI3K/AKT Pathway

The PI3K/AKT signaling plays a pivotal role in the development of EC by regulating cell proliferation, invasion and EMT.[32-34] Hence, we further explored the role of KIFC1 in the regulation of PI3K/AKT signaling cascade. As displayed in Figure 5A, knockdown of KIFC1 reduced the protein expression of p-AKT and p-PI3 K, but had no significant effects on the protein expression of AKT and PI3K in Ishikawa cells. Inversely, overexpression of KIFC1 activated PI3K/AKT pathway by increasing the phosphorylation of AKT and PI3K in HEC-1A cells (Figure 5B). Moreover, after treatment with LY294002, a specific antagonist of PI3K/AKT pathway, the expression levels of p-AKT and p-PI3K were decreased in HEC-1A cells, which abrogated the promotive effects of KIFC1 overexpression on PI3K/AKT pathway (Figure 5B).

Therefore, these findings demonstrated that KIFC1 knockdown blocked PI3K/AKT signaling pathway while KIFC1 overexpression activated this pathway. Collectively, these results suggested that PI3K/AKT pathway might participate in KIFC1-mediated EC cell proliferation, invasion and EMT.

Discussion

EC is an epithelial tumor in the endometrium, which develops insidiously and tends to invasion and metastasis.[35] The treatment of EC has been improved due to the progression in surgical resection and postoperative chemoradiotherapy, and the overall endometrial carcinoma has a favorable prognosis. However, the therapeutic options are limited for patients with advanced metastatic or recurrent EC, which have poor survival outcomes.[36] Therefore, better understanding on the molecular mechanism underlying EC progression is necessary. In this study, we found that KIFC1 expression was upregulated in EC tissues and cells, which was closely related to poor prognosis of EC patients. This is the first evidence identifing that upregulated KIFC1 could promote cell proliferation, migration, invasion and EMT in EC. In addition, KIFC1 might regulate EC progression by activating PI3K/AKT pathway.

KIFC1 expression has also been shown to predict poor prognosis and overall survival of ovarian cancer and serve as a potential marker for predicting metastasis.[37] In NSCLC, KIFC1 expression can also serve as a potential marker for brain metastasis.[38] KIFC1 is upregulated in gastric cancer and
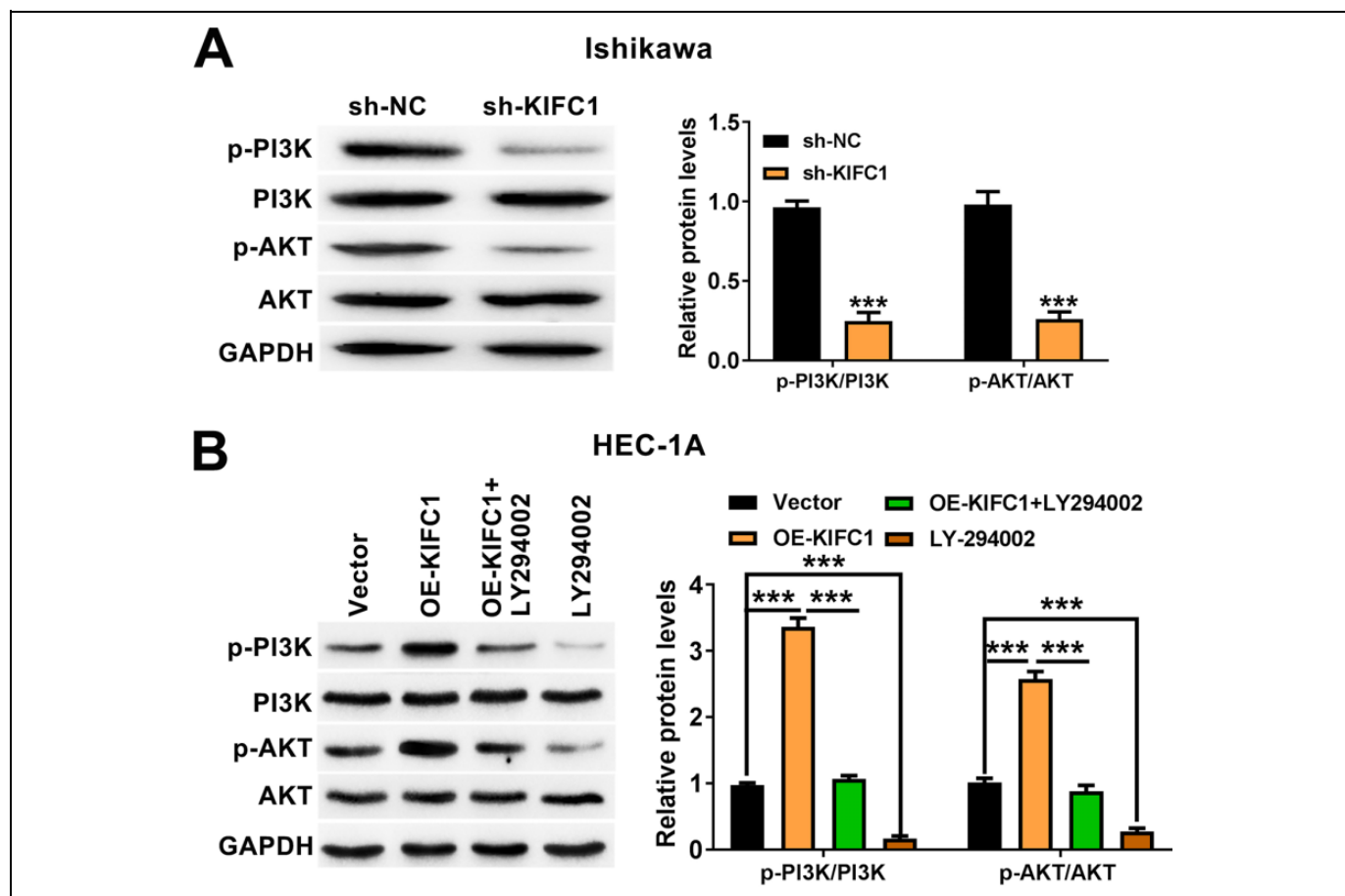
Figure 5. KIFC1 plays a carcinogenic role in EC cells through the PI3K/AKT pathway. (A-B) The protein levels of PI3K, p-PI3K, AKT and p-AKT in Ishikawa (A) and HEC-1A cells (B) were measured by western blot. Data were shown as means $\pm$ SD. ***$P < 0.001$.

knockdown of KIFC1 inhibits sphere formation.[24] A recent study demonstrated that KIFC1 was expressed at high levels in hepatocellular carcinoma, and was significantly associated with tumor emboli, metastasis, recurrence, time of recurrence and tumor-free survival rates.[39] Therefore, it is suggested that KIFC1 may have potential as a tumor diagnostic marker. Through the analysis on the data from MERAV and UALCAN, we found that KIFC1 expression levels in tumor tissues of the female reproductive system and in EC tissues were both significantly up-regulated, which was related to clinical stage, suspension status and pathological type. Further analysis on TCGA dataset demonstrated that EC patients with high KIFC1 expression had short survival outcome. Consistently, the expression of KIFC1 in EC cells was also upregulated. The results suggested that KIFC1 may be used as a biomarker for clinical diagnosis of EC.

A previous study has reported that interfering with the expression of KIFC1 in breast cancer cells could significantly inhibit the cell growth activity.[20] KIFC1 knockdown was demonstrated to decrease cell viability, migration and invasion and induce apoptosis in hepatocellular carcinoma.[39] Consistently, our data demonstrated that KIFC1 could promote cell proliferation, migration and invasion in EC cells, indicating

that KIFC1 could affect the progression of EC. Notably, a novel study proved that KIFC1 induced EMT and metastasis both *in vitro* and *in vivo* in hepatocellular carcinoma via activation of the gankyrin/AKT/TWIST1 signaling pathway.[26] As expected, our mechanism analysis also showed that KIFC1 could activate the PI3K/AKT signaling pathway to promote the progression of EC.

In conclusion, we found the upregulated KIFC1 expression in human EC tissues and cells. KIFC1 expression was obviously correlated with clinical stage, suspension status, pathological type, and prognosis. KIFC1 was involved in the regulation of the proliferation, migration, and invasion of EC cells, and the underlying mechanism was, at least partially, through mediating PI3K/AKT axis. Therefore, our study highlights KIFC1 as a novel and promising therapeutic target for the treatment of EC.

Authors' Note

MMD conceived and designed the experiments, KNZ and JZ analyzed and interpreted the results of the experiments, LFQ, YYH, and JGX performed the experiments. All data generated or analyzed during this study are included in this published article. The animal study was approved by the Ethics Committee of People's Hospital of Quzhou

City (approval no. ZQA2018044). Experiments were operated according to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Declaration of Conflicting Interests

Funding

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