

Kindlin-2 suppresses cervical cancer cell migration through AKT/mTOR-mediated autophagy induction

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Abstract. Kindlin-2 plays a carcinogenic or tumor-suppressor role in various tumors. However, its role in cervical cancer remains unclear. In the present study, kindlin-2 expression was first analyzed using public expression data and clinical specimens. It was revealed that kindlin-2 was downregulated in cervical cancer tissues, and low expression of kindlin-2 was associated with poor disease-free survival. In addition, kindlin-2 was overexpressed and knocked down in two cell lines to study its effect in cervical cancer cells. The results revealed that kindlin-2 promoted cell autophagy and inactivated AKT/mTOR signaling. Rescue experiments indicated that the regulation of autophagy by kindlin-2 was dependent on the AKT/mTOR signaling pathway. Furthermore, it was revealed that kindlin-2 inhibited cell migration, and autophagy was required for this process. Collectively, these findings revealed the role and mechanism of kindlin-2 in the autophagy and migration of cervical cancer cells.

Introduction

Cervical cancer is one of the most common gynecologic malignancies and the fourth leading cause of cancer-related deaths in women (1). It is estimated that there were 570,000 cases and 311,000 deaths worldwide in 2018 (1). Early screening, surgery, and radiotherapy have significantly improved the prognosis of patients with cervical cancer; however, tumor metastasis, which is a major cause of death from cervical cancer, can still not be completely prevented (2). Therefore, studying the mechanism of cervical cancer metastasis and understanding the driving factors behind it are crucial for the development of reasonable interventions to improve patient prognosis.

Autophagy is an evolutionarily ancient mechanism which degrades redundant or potentially harmful cytosolic entities to maintain stable cell metabolism (3). While the role of autophagy in tumorigenesis is dual and context-specific (4,5), increasing evidence suggests that autophagy is involved in the metastasis of tumor cells (6-8). The role of autophagy in tumors remains unclear, and further investigation is required.

Kindlin-2 (also known as FERMT2 or MIG-2) is a member of the kindlin family, which consists of three members: Kindlin-1, -2, and -3. Members of this family generally contain F1, F2, and F3 subdomains and characteristically harbor a pleckstrin homeodomain in the F2 subdomain (9). Kindlin-2 has been shown to be involved in the progression of pancreatic cancer, breast cancer, and glioma (10-12). However, to our knowledge, the role of Kindlin-2 in cervical cancer has not been reported. Moreover, the role of Kindlin-2 in autophagy is unknown. Autophagy is a complex process that involves several signaling pathways. AKT/mTOR is a main pathway regulating cell autophagy, and activation of this pathway inhibits autophagy (13,14). However, whether the AKT/mTOR pathway mediates autophagy regulation by kindlin-2 remains to be studied.

In this study, we compared Kindlin-2 expression in cervical cancer tissues and healthy cervical tissues. Further, we found that Kindlin-2 acts as a novel autophagy regulator that regulates the AKT/mTOR pathway, a major autophagy pathway. In addition, we found that Kindlin-2 inhibits the migration of cervical cancer cells by inducing autophagy. Kindlin-2 may be a tumor migration suppressor gene, and has potential as a tumor marker and therapeutic target in cervical cancer.

Materials and methods

Clinical tissue specimens. Normal tissue adjacent to the tumor (NAT), which is usually used as a control in tumor studies, should be collected >2 cm from the tumor margin (15). In the present study, given the small size of the cervix and the differences in gene expression between NAT and normal tissues (15), normal cervical tissue was used as a control. Forty-two cervical cancer tissue samples were obtained from patients (age range, 35-70 years; mean age, 51.8±8.7 years) with cervical cancer diagnosed by histopathology, and 24 normal cervical tissue samples were obtained from patients

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with benign uterine lesions, and all patients underwent hysterectomy at the Guangxi Medical University Cancer Hospital between April 2017 and September 2017. The study was approved by the Ethics Committee of Guangxi Medical University Cancer Hospital, and all patients signed informed consent to participate in the study.

Cell culture and transfection. The SiHa human cervical cancer cell line was purchased from Shanghai GeneChem Co., Ltd., and CaSki and C-33A human cervical cancer cells were purchased from Zhongqiaoxinzhou Biotech. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin solution (Beyotime Institute of Biotechnology) in a humidified 5% CO₂ atmosphere at 37°C. To observe the effect of kindlin-2 expression in cervical cancer cells, SiHa and CaSki cells were transfected with a lentivirus encoding full-length human kindlin-2 cDNA or carrying a short hairpin (sh)RNA targeting kindlin-2, constructed by Shanghai GeneChem Co., Ltd. The siRNA sequence was as follows: 5'-GCGGACAGTTCTTAC AACTTA-3'. Empty lentiviral vector and lentivirus containing nonsense shRNA sequences were used as negative controls.

Reagents and antibodies. Earle's balanced salts solution (EBSS; product no. E2888) was purchased from Sigma-Aldrich (Merck KGaA). 3-Methyladenine (3-MA) and rapamycin were purchased from Shanghai GeneChem Co., Ltd. The AKT inhibitor MK2206 and the AKT activator SC79 were purchased from Beyotime Institute of Biotechnology. Primary antibodies against LC3 A/B (product no. 12741; dilution 1:1,000), SQSTM1/p62 (product no. 88588; dilution 1:1,000), phospho-mTOR (product no. 5536; dilution 1:1,000) and GAPDH (product no. 97166; dilution 1:1,000) were obtained from Cell Signaling Technology, Inc. Antibodies targeting kindlin-2 (cat. no. 11453-1-AP; dilution 1:2,000), mTOR (cat. no. 66888-1-Ig; dilution 1:10,000), phospho-AKT (cat. no. 66444-1-Ig; dilution 1:3,000) were purchased from ProteinTech Group, Inc. and AKT antibody (cat. no. WL0003b; dilution 1:500) was purchased from Wanleibio Co., Ltd.

Transwell migration assay. For the Transwell cell migration assay, 3x10⁴ SiHa cells or 6x10⁴ CaSki cells were seeded in 200 µl of serum-free medium in the upper chamber, whereas 600 µl of medium containing 20% FBS was added to the lower chamber. After incubation for 16 h, the cells on the upper insert surface were removed with a cotton swab. Cells that had successfully migrated to the lower surface of the insert were stained with 0.1% crystal violet at room temperature for 10 min. Cells were counted in five randomly selected high-power fields using a microscope (x20, magnification; IX71; Olympus Corporation).

Wound healing assay. SiHa and CaSki cell migration was evaluated by a wound healing assay. When cells reached 100% confluence, they were treated with mitomycin C (5 µg/ml; cat. no. M4287; Sigma-Aldrich; Merck KGaA) for 1 h. Then, the cell monolayer was scratched using a 200-µl pipette tip, washed with PBS, and supplemented with serum-free medium. The plates were further incubated and images were captured

using a microscope at 0, 24 and 48 h after treatment. Migration ability was assessed by measuring the gap area using the ImageJ software (version 1.52a; NIH).

Western blotting. Cells were lysed using RIPA lysis buffer supplemented with 0.1 mM PMSF. The proteins were quantified using BCA protein assay kit. Next, 30 µg total protein were separated by 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked using 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 h. The membrane was incubated with a primary antibody at 4°C overnight, and then with horseradish peroxidase (HRP)-conjugated anti-rabbit (cat. no. A0208) or anti-mouse (cat. no. A0216) secondary antibodies (dilution 1:1,000; Beyotime Institute of Bioetchnology) at room temperature for 1 h. Specific bands were detected by chemiluminescence using ECL detection reagent (Biokits Tech, Inc.). GAPDH was probed to serve as a control. The protein expression levels were quantified using Image Lab software (version 6.0; Bio-Rad Laboratories, Inc.).

Autophagic flux analysis. To detect autophagic flux, SiHa and CaSki cells were transfected with mRFP-GFP-LC3 lentivirus (Shanghai GeneChem Co., Ltd.). After 72 h, the cells were seeded in confocal dishes, and GFP and RFP puncta were observed using a laser confocal microscope (TCS SP8; Leica Microsystems GmbH). In merged images, yellow spots indicated autophagosomes and red spots indicated autolysosomes. In each group, 30 cells were randomly selected for counting.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were cut into 4-µm sections. Tissue sections were deparaffinized with xylene and rehydrated, subjected to antigen retrieval in EDTA (pH 9.0), and incubated in a 3% hydrogen peroxide solution to block endogenous peroxidase at room temperature for 25 min. The sections were blocked with 3% bovine serum albumin (cat. no. G5001; Servicebio, Wuhan, China) at room temperature for 30 min, incubated with kindlin-2 antibody (cat. no. 11453-1-AP; dilution 1:100; ProteinTech Group, Inc.) at 4°C overnight, washed with PBS, and incubated with HRP-conjugated secondary antibody (cat. no. A0208; dilution 1:50; Beyotime Institute of Biotechnology) at room temperature for 50 min. Then, the sections were immersed in 3,3'-diaminobenzidine for color development. Nuclei were counterstained with hematoxylin at room temperature for 3 min. Finally, the tissue sections were dehydrated and mounted. The images were captured using a light microscope (XSP-C204; Chongqing Optical Instrument Co., Ltd.). Kindlin-2 expression was measured based on the average optical density, using ImageJ version 1.52a.

Bioinformatics and statistical analysis. RNA-seq data and survival data for patients with cervical cancer were downloaded from the Gene Expression Omnibus (GEO) database (GSE44001 and GSE52904) (16,17) and Gene Expression Profiling Interactive Analysis (GEPIA), an online analysis tool based on The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) database (18). R (version 3.5.3) and related packages were used for statistical analysis. Evaluate Cutpoints

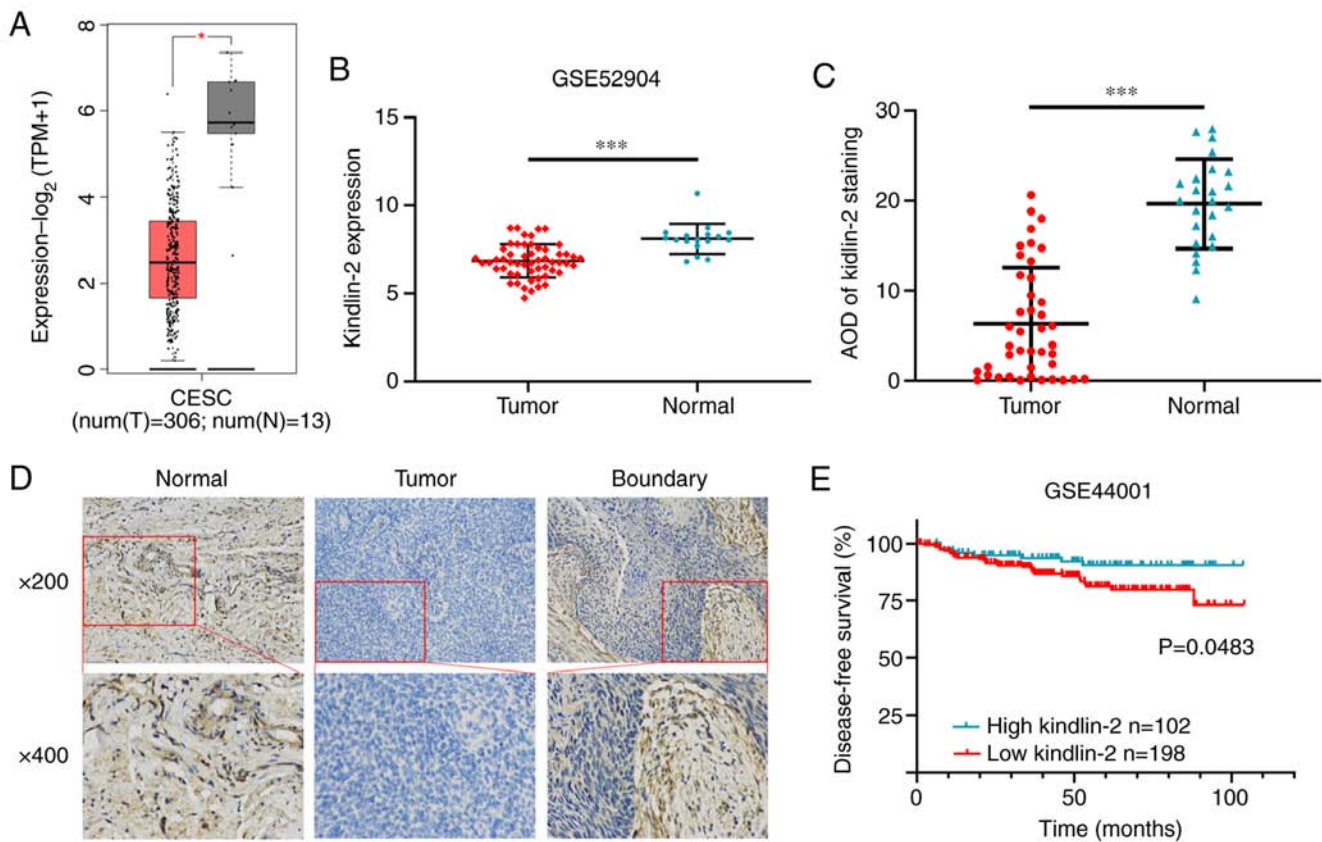


Figure 1. Kindlin-2 expression is reduced in cervical cancer tissues. (A) Kindlin-2 expression in healthy cervical tissues and cervical cancer tissues based on TCGA and GTEx databases. (B) Kindlin-2 expression in healthy cervical tissues and cervical cancer tissues based on the GSE52904 dataset. (C) Quantification of the AOD representing kindlin-2 expression in healthy cervical tissues and cervical cancer tissues. (D) Representative immunostained images (magnification, x200) of kindlin-2 expression in healthy cervical tissues and cervical cancer tissues. (E) Kaplan-Meier analysis of DFS for kindlin-2 expression in cervical cancer from two independent cohorts in the GSE44001 dataset. * $P<0.05$; *** $P<0.001$. TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; AOD, average optical density; DFS, disease-free survival.

(<http://wnbikp.umed.lodz.pl/Evaluate-Cutpoints/>) was used to determine the optimal cut-off for survival analysis (19). All data are reported as the mean \pm SD. Student's *t*-test was used to compare means between two groups. For multiple comparisons, ANOVA and Dunnett's post hoc analysis were used. Disease-free survival time was analyzed using Kaplan-Meier curves, and any differences in survival were evaluated with a log-rank test. Differences were considered statistically significant at $P<0.05$.

Results

Kindlin-2 is downregulated in cervical cancer, and low Kindlin-2 expression is correlated with poor disease-free survival. First, it was determined whether kindlin-2 is differentially expressed between healthy cervical tissues and cervical cancer tissues. To this end, we used Gene Expression Profiling Interactive Analysis (GEPIA) tools to analyze kindlin-2 mRNA expression in the TCGA and GTEx databases, including 306 cervical cancer tissues and 13 healthy cervical tissues. The results revealed that kindlin-2 expression was significantly downregulated in cervical cancer tissues ($P<0.05$, Fig. 1A). In addition, 55 cervical cancer tissues and 17 healthy cervical tissues in the GSE52904 dataset (GPL 6244) were analyzed, and similar results were obtained ($P<0.001$, Fig. 1B). To validate the expression of kindlin-2 in clinical

specimens, the expression of kindlin-2 in 42 cervical cancer tissues and 24 healthy cervical tissues was detected using immunohistochemistry. The results revealed that kindlin-2 expression in cancer tissues was lower than that in healthy tissues ($P<0.001$, Fig. 1C and D). Kaplan-Meier survival analysis was performed using the GSE44001 dataset in the GEO database. The results revealed that disease-free survival was shorter in the low-kindlin-2 expression group than in the high-expression group ($P<0.05$, Fig. 1E).

Kindlin-2 induces autophagy of cervical cancer cells.

To explore the role of kindlin-2 in cervical cancer cells, kindlin-2 expression was assessed in three cervical cancer cell lines. Kindlin-2 expression was the highest in SiHa cells and the lowest in CaSki cells (Fig. 2A). Therefore, kindlin-2 was overexpressed and knocked down in CaSki and SiHa cells, respectively (Fig. 2B). To explore the role of kindlin-2 in autophagy, autophagic protein marker expression was first assessed using immunoblotting. p62/SQSTM1 and LC3 are considered to play important roles in autophagy (20,21). The amount of LC3-II is generally associated with the number of autophagosomes (22). p62 is a selective autophagy substrate and thus, p62 protein levels are generally inversely correlated with the level of autophagy (22). In CaSki cells overexpressing kindlin-2, the p62 and LC3-II levels were decreased and increased, respectively, whereas in kindlin-2-knockdown

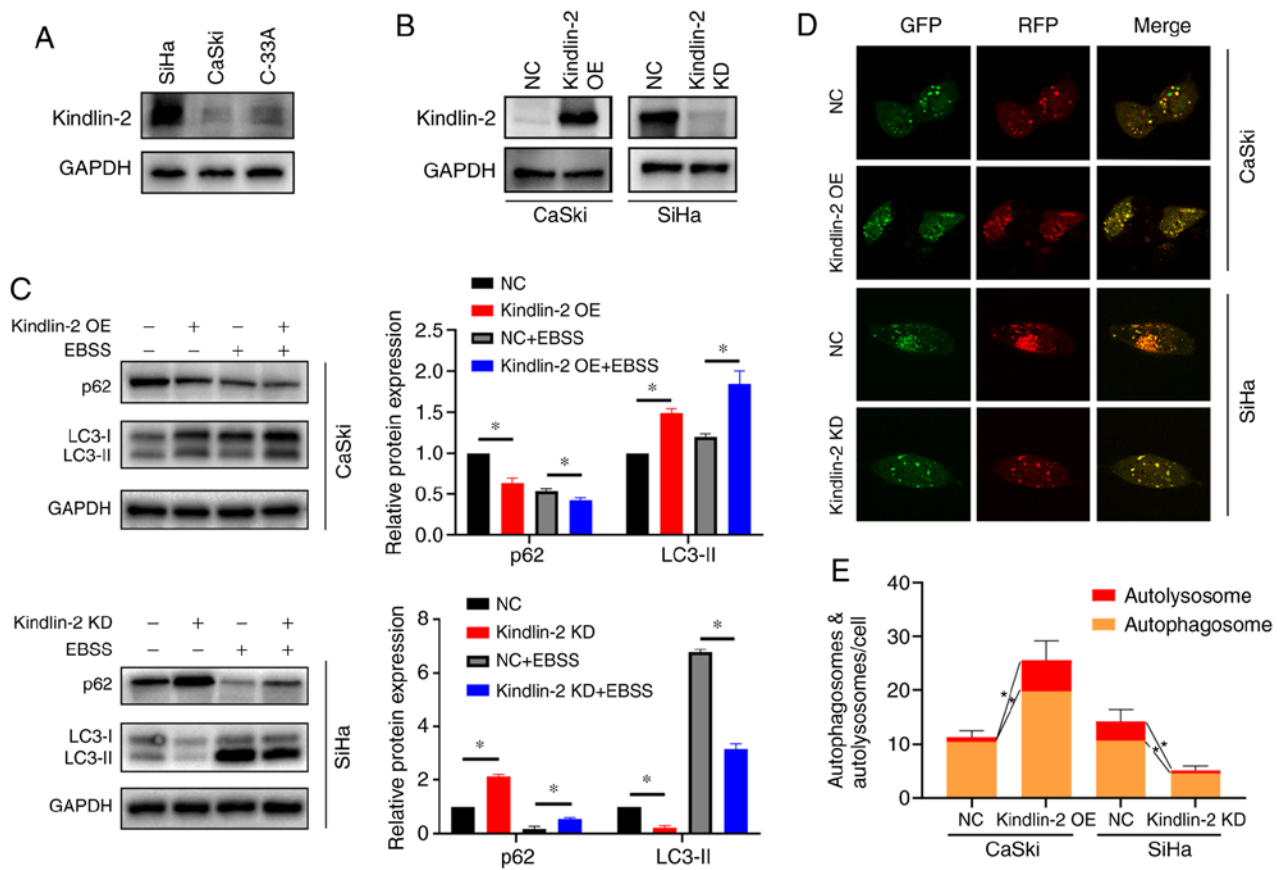


Figure 2. Kindlin-2 induces autophagy in cervical cancer cells. (A) Western blot results of kindlin-2 expression in cervical cancer cell lines. (B) Western blot results of kindlin-2 in the indicated cells. (C) Expression levels of p62, LC3, and GAPDH in the indicated cell lines. Cells were cultured in normal medium or EBSS-containing medium for 6 h. (D) Representative fluorescence images of CaSki and SiHa cells transfected with mRFP-GFP-LC3 lentivirus (magnification, x630). (E) GFP and RFP puncta were counted in 30 cells. * $P < 0.05$. NC, negative control; OE, overexpression; KD, knockdown; GFP, green fluorescent protein; RFP, red fluorescent protein; EBSS, Earle's balanced salts solution.

SiHa cells, p62 and LC3-II levels were increased and decreased, respectively, compared to the NC group. To verify whether kindlin-2 promotes starvation-induced autophagy, cells were cultured with EBSS-containing medium for 6 h to induce autophagy. The results indicated that kindlin-2 enhanced the level of LC3-II and suppressed p62 accumulation under starvation compared to the NC+EBSS group in CaSki cells while the opposite results were obtained in SiHa cells (Fig. 2C).

Autophagy is a dynamic process. Thus, an increase in autophagosomes (as indicated by an increase in the autophagosome marker LC3-II) may indicate an increase in autophagy, blockage of autophagolysosome formation, or both (22). Therefore, to determine the cause of autophagosome accumulation, we transfected cells with an mRFP-GFP-LC3 tandem construct to detect autophagic flux based on the fact that in the acidic environment of autophagolysosomes, GFP fluorescence is quenched while RFP fluorescence remains stable. The results revealed that the numbers of yellow spots (GFP⁺ and RFP⁺, indicating autophagosomes) and red spots (GFP⁻ and RFP⁺, indicating autolysosomes) were increased in kindlin-2-overexpressing CaSki cells and decreased in kindlin-2-knockdown SiHa cells when compared with the respective controls, indicating that kindlin-2 in fact promoted autophagic flux and thus increased the number of autophagosomes ($P < 0.05$, Fig. 2D and E).

Kindlin-2 inactivates AKT/mTOR signaling. The AKT/mTOR pathway is the main pathway associated with autophagy (23,24). Therefore, it was surmised that kindlin-2 may promote autophagy by inactivating the AKT/mTOR signaling pathway. To test this hypothesis, the expression of AKT/mTOR-related proteins was examined by western blotting. Phosphorylated AKT and phosphorylated mTOR were significantly decreased and increased in kindlin-2-overexpressing CaSki cells and kindlin-2-knockdown SiHa cells, respectively, when compared with the respective control cells. Total AKT and total mTOR levels were not affected (Fig. 3A). These findings indicated that kindlin-2 inactivated AKT/mTOR signaling.

Kindlin-2 induces autophagy via the AKT/mTOR pathway. To confirm whether kindlin-2 induces autophagy via the AKT/mTOR pathway, kindlin-2-overexpressing CaSki cells were treated with the AKT activator SC79 (20 μ M) and then autophagy marker expression was assessed. The autophagy of the kindlin-2 OE+SC79 group was significantly attenuated as compared to the kindlin-2 OE group, demonstrating that this activator can attenuate autophagy induction mediated by kindlin-2 overexpression (Fig. 3B). In addition, the AKT inhibitor MK2206 (5 μ M) was added to kindlin-2-knockdown SiHa cells. The autophagy of the kindlin-2 KD+MK2206 group was significantly promoted when compared with kindlin-2 KD group, demonstrating that this inhibitor can reverse the

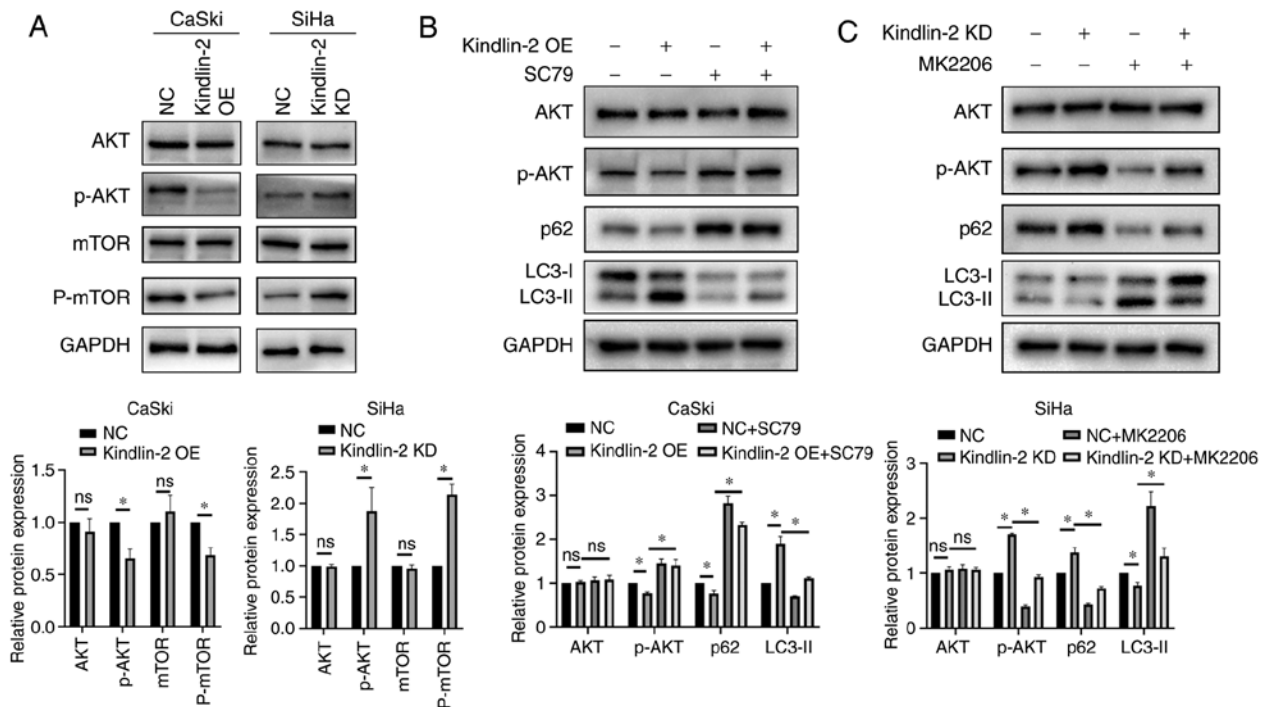


Figure 3. Kindlin-2 induces autophagy by inactivating the AKT/mTOR pathway. (A) Protein levels of AKT, p-AKT, mTOR, p-mTOR, and GAPDH in the indicated CaSki and SiHa cells as detected by western blotting. (B) The indicated CaSki cells were treated with SC79 (20 μ M) for 24 h and the protein levels of AKT, p-AKT, p62, LC3, and GAPDH were determined by western blotting. (C) The indicated SiHa cells were treated with MK2206 (5 μ M) for 24 h and protein levels of AKT, p-AKT, p62, LC3 and GAPDH were detected by western blotting. * P <0.05. NC, negative control; OE, overexpression; KD, knockdown; ns, not significant.

inhibition of autophagy in Kindlin-2-knockdown SiHa cells (Fig. 3C). Collectively, these results indicated that kindlin-2 regulates autophagy via inhibition of AKT/mTOR signaling.

Kindlin-2 inhibits cervical cancer cell migration by promoting autophagy. Wound healing assays revealed that wound closure was slower in kindlin-2-overexpressing CaSki cells than in Lv-NC-infected CaSki cells (Fig. 4A and B), whereas the opposite results were observed in kindlin-2-knockdown SiHa cells (Fig. 4C and D). Transwell assays revealed that kindlin-2 overexpression suppressed migration in CaSki cells, whereas kindlin-2 knockdown enhanced migration in SiHa cells when compared with the respective controls (Fig. 4E-G). To investigate whether autophagy is involved in the regulatory effect of kindlin-2 on cell migration, the autophagy inhibitor 3-MA (5 mM) was added to kindlin-2-overexpressing CaSki cells, which attenuated the inhibitory effect of kindlin-2 on cell migration (Fig. 4A, B, E, and F). Addition of the autophagy activator rapamycin (20 nM) reversed the promoting effect of kindlin-2 knockdown on SiHa cell migration (Fig. 4C-E and G). Collectively, these results indicated that kindlin-2 inhibited cell migration and that this effect at least partly depended on autophagy.

Discussion

In most types of cancer other than cervical cancer, kindlin-2 is expressed at high levels in tumors and acts to promote tumor progression (25-27). In the present study, it was revealed that kindlin-2 plays a different role in cervical cancer. Kindlin-2 was expressed at low levels in cervical cancer, induced

autophagy by inhibiting the AKT/mTOR pathway, and inhibited the migration of cervical cancer cells by promoting autophagy. The present study revealed the role of kindlin-2 in autophagy and migration of cervical cancer cells.

Kindlin-2 exerts multiple and even opposite effects in different tumors, of which tumorigenicity is dominant. For example, kindlin-2 was revealed to interact with epidermal growth factor receptor to promote the migration of breast cancer cells (25) and to promote gastric cancer cell invasion by phosphorylating integrins β 1 and β 3 (26). However, kindlin-2 can also exert a tumor-suppressive effect. For example, it was revealed to inhibit tumor peritoneal dissemination in serous epithelial ovarian cancer (28) and tumor cell invasion in mesenchymal cancer (29). The role of kindlin-2 in cervical cancer remained unclear. In the present study, kindlin-2 overexpression suppressed the migration ability of cervical cancer cells, whereas downregulation of kindlin-2 promoted cell migration, indicating that kindlin-2 inhibits cervical cancer cell migration. These contradictory findings suggest that kindlin-2 acts as either an oncoprotein or a tumor suppressor depending on the cancer type and context.

Autophagy plays an important role in tumor progression (30,31). In cervical cancer cells, hispolon was revealed to inhibit cell migration by activating autophagy (32), and spermidine inhibited cell growth and promoted apoptosis by activating autophagy (33). However, the relationship between kindlin-2 and autophagy remained unclear. In the present study, autophagy-promoting effects were observed after overexpression of kindlin-2 and the opposite effects were observed after knockdown of kindlin-2. This indicated that kindlin-2 can induce autophagy in cervical cancer cells, confirming

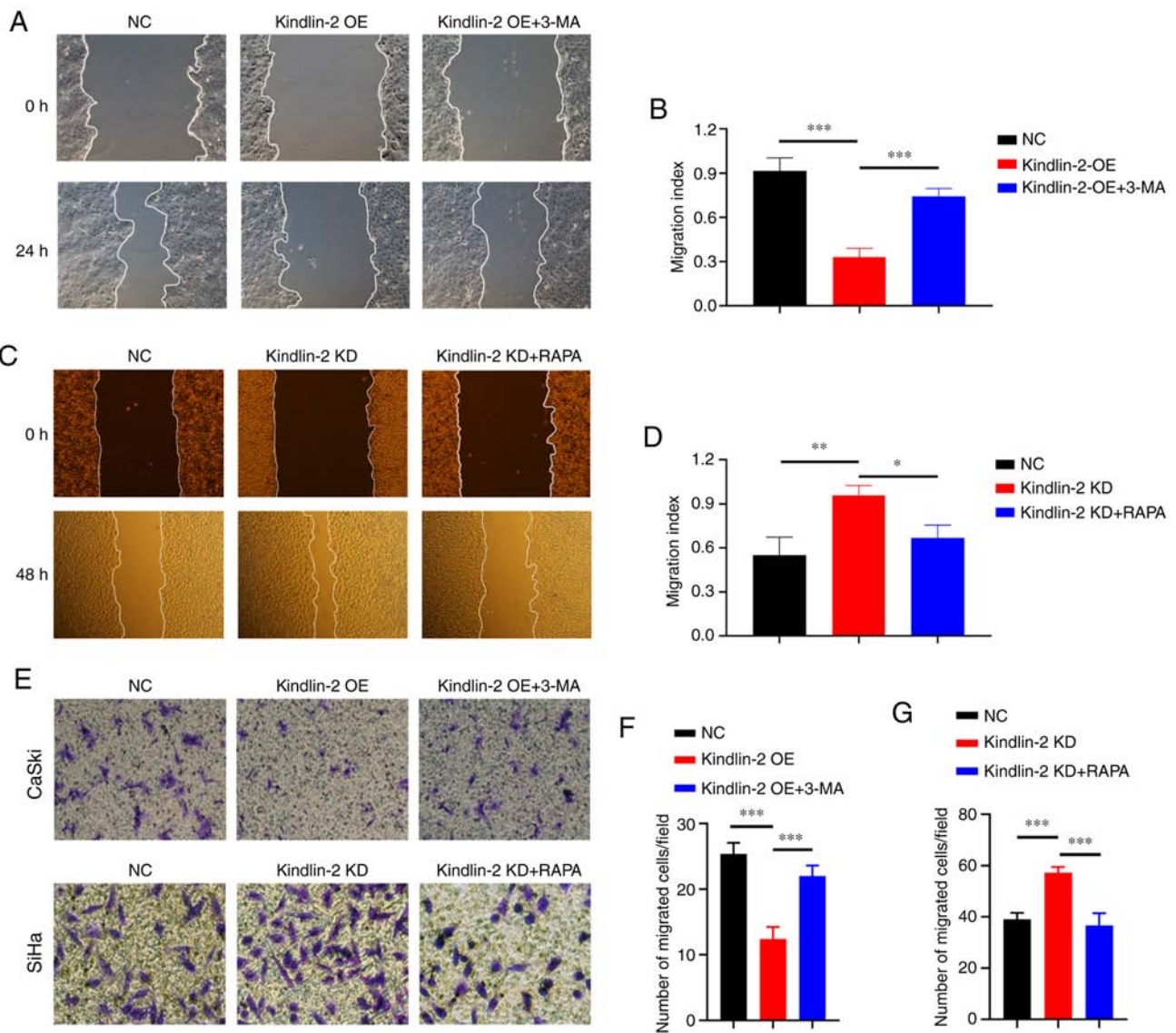


Figure 4. Kindlin-2 impairs the migration of cervical cancer cells by inducing autophagy. CaSki cells were transfected with kindlin-2 lentivirus or NC lentivirus and then treated with 5 mM 3-MA. SiHa cells were transfected with kindlin-2 RNAi or NC lentivirus and then treated with 20 nM RAPA. Wound healing and Transwell migration assays were performed to detect cell migration under different treatments. (A and B) Representative images (magnification, x100) and quantification of CaSki cells subjected to the indicated treatments after scratch wounding. (C and D) Representative images (magnification, x100) and quantification of SiHa cells subjected to the indicated treatments after scratch wounding. The wound area was evaluated using ImageJ software. (E) Representative images (magnification, x200) and quantification of migrated (F) CaSki and (G) SiHa cells. Migrated cells were counted in five random fields. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NC, negative control; OE, overexpression; KD, knockdown; 3-MA, 3-methyladenine; RAPA, rapamycin.

the relationship between kindlin-2 and autophagy in cervical cancer cells.

Autophagy has both metastasis-promoting and -suppressive effects (34,35). It was determined, in the present study, that inhibition of autophagy rescued the decrease in cell migration ability caused by kindlin-2 overexpression, whereas promotion of autophagy attenuated the increase in cell migration ability induced by kindlin-2 knockdown. This indicated that kindlin-2 inhibited cell migration by inducing autophagy. Similar results have been reported in other types of tumors, including hepatocellular carcinoma, renal cell cancer, and colorectal cancer (36-38). The present results also provide new evidence for the role of autophagy in the migration of cervical cancer cells.

The AKT/mTOR pathway negatively regulates autophagy (23,24). Moreover, AKT/mTOR pathway activation has been revealed to promote tumor growth, metastasis, angiogenesis,

and therapeutic resistance (39), and this pathway was revealed to serve as a therapeutic target in various tumors (40-42). In the present study, it was revealed that overexpression of kindlin-2 resulted in inhibition of the AKT/mTOR pathway, whereas downregulation of kindlin-2 had the opposite effect, suggesting that kindlin-2 negatively regulates this pathway. In further experiments, it was revealed that treatment with an AKT activator or inhibitor reversed the effect of overexpression or knockdown of kindlin-2 on autophagy. These results indicated that kindlin-2 regulated autophagy via the AKT/mTOR pathway.

In conclusion, the present study revealed that kindlin-2 impairs the migration of cervical cancer cells by promoting autophagy, which is mediated by the AKT/mTOR pathway. Autophagy plays an important role in the migration of cervical cancer cells. Unfortunately, a mouse model of cervical cancer metastasis has not been established yet, and further studies are

required to confirm the effect of kindlin-2 on tumor metastasis *in vivo*. Another limitation is the small sample size used for the detection of the expression of kindlin-2. A larger sample size is required to confirm the results. Collectively, the present study findings increased our understanding of the role of kindlin-2 in tumors and provides a novel potential target for the treatment of cervical cancer metastasis.

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Availability of data and materials

All data generated or used during this study are included in this published article, still, further details are available from the corresponding author on reasonable request.

Authors' contributions

GW, YaL and DY conceived and designed this study. GW, XX, YF, XY preformed experimentation and data analysis. YiL collected the clinical samples and preformed experimentation. GW wrote the draft manuscript. DY and YaL reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Guangxi Medical University Cancer Hospital, and all patients signed an informed consent to participate in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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