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ORIGINAL RESEARCH

The Overexpression of Kinesin Superfamily Protein 2A (KIF2A) was Associated with the Proliferation and Prognosis of Esophageal Squamous Cell Carcinoma

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Aim: Kinesin family member 2A (KIF2A) is a member of the kinesin-13 superfamily protein. KIF2A played a role in the development of many tumors. However, the role of KIF2A in esophageal squamous cell carcinoma (ESCC) remains unclear. In this study, we aimed to investigate the role of KIF2A in ESCC.

Methods: We used bioinformatics analysis to study the expression levels and prognosis of KIF2A in ESCC and normal tissues. We also used our own samples to verify the results by immunohistochemistry. The the biological functions of KIF2A in ESCC was studied by cell experiments and animal experiments.

Results: Both the TCGA database and our samples showed that KIF2A was relatively highly expressed in ESCC tissues and was significantly associated with disease-free survival (P =0.0 in TCGA database. Colony formation assay, CCK8 and Western blotting results showed that knockdown of KIF2A can significantly reduce colony forming ability and proliferation ability. The results of animal experiments showed that knocking down KIF2A can significantly reduce the tumor volume of mice.

Conclusion: KIF2A might be used as a prognostic factor for ESCC, and knockdown of KIF2A could inhibit ESCC proliferation in vitro and in vivo, respectively. KIF2A could serve as a potential prognostic biomarker and therapeutic target for future ESCC.

Keywords: esophageal squamous cell carcinoma, kinesin superfamily protein 2A, ESCC, KIF2A, proliferation

Introduction

Esophageal cancer is very difficult to cure around the world.[1–3] The most representative of these is China, which accounts for about 50% of global cases of esophageal cancer. The main pathological type is esophageal squamous cell carcinoma (ESCC).[4,5] During the last decade, the development of multiple therapeutic strategies for esophageal cancer including radical surgery, chemotherapy, and radiotherapy has improved the prognosis of patients with this malignancy;[6–8] however, the 5-year survival rate of esophageal cancer patients is less than 30%. Thus, there is an urgent need to further understand the molecular mechanisms underlying ESCC tumorigenesis for the treatment of ESCC.

In recent years, studies had successively identified the kinesin 13 family, including the kinesin family member 2A (KIF2A), etc.[9–11] They are related to the spindle

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and are essential for precise chromosome segregation in mitotic and meiotic cell cycles.[10,12] Among them, KIF2A acts as a microtube minus-end depolymerization motor, which is an essential component in assembling ordinary bipolar spindles.[13] In recent years, some evidence suggests that KIF2A may be involved in carcinogenesis.[14–16] For example, aberrant expression of KIF2A is associated with the prognosis of gastric cancer,[17] breast cancer,[15] and glioma.[18] However, there is no clear study on the role of KIF2A in ESCC and its mechanism.

In our study, we analyzed the protein expression level of KIF2A in ESCC patients, revealing that overexpression of KIF2A is associated with poor clinical outcome. We have revealed that knocking down KIF2A could significantly inhibit the proliferation of ESCC in both cell lines and mice. Because KIF2A can promote the proliferation of ESCC tumors and is associated with the prognosis of ESCC.

Methods

Bioinformatics Analysis

The mRNA-seq data for ESCC patients herein is data from the Cancer Genome Atlas (TCGA).[19] Download at: http://firebrowse.org/ (Broad Institute TCGA Genome Da Analysis Center).

Cells Culture

The human ESCC cell lines CaEs-17 and EC-1 used in this study were purchased from Keygen B tech (Nanj China). Among them, CaEs-17 cells w cu red in Du cco's modified Eagle medium (D EM) (Biol cal Industries), while EC-109 cells were ultured in RPMI- 40 medium (Biological Industries). oth me a were then supplemented with 10% fetal bovine se (FBS) (B logical Industries), 100 U/L penici n an 0.1 m mL reptomycin (Biological Industries). e have t eep the ce s in a humidified incubator containing 5% O at 37 ° C for a long time.

Immunohistochemistry (IHC)

The human ESCC organizations used in this article are taken from patients who are self-hospital, and these are surgically cut tissues. The ESCC tissue was first deparaffinized, rehydrated in successively different concentrations of ethanol bath, placed in a pressure cooker and soaked in citrate buffer, and the antigen was recovered after approximately 30 minutes. KIF2A was then immunostained with a polyclonal antibody (ab197988, Abcam plc, Cambridge, UK) at a 1:200 dilution. Enzymatic detection of antibodies. The results were centrally reviewed and scored by two independent pathologists who were blinded. The scores were divided into four grades which are 0, 1, 2, 3 respectively. The use of human samples in this study was approved by the Ethics Committee of Xingtai people's hospital, in accordance with the Declaration of Helsinki. The research involving human participants experiments had been approved by our hospital and our equivalent committee. The participants provided their written informed consents to participate in thi tudy.

Transfection

Commercialized Ready-to- ckage A shRN clones of KIF2A was purchased. e target equenc re as follows: sh1: 5′-GGAATGGCA CTC GAAA 3′ and sh2: 5′--C ACCGGCAAAC GATTG CTGC -3′. RNA iMAX reagent was ed transfect t e ESCC cells. Negative Control group, which ansfected with scrambled sequence. Trans ctions were co cted using lipofectamine 2000 (11 8019, Invitrogen, Carlsbad, CA, USA). Stable knockdow of ESCC c ls was obtained by its shRNA lentivirus infect and se ction was performed by puromycin supplementation and used in animal experiments.

Quantitative Polymerase Chain Reaction (qPCR)

TRIZOL reagent was added to the cultured cells to extract total RNA. The isolated RNA is then reverse transcribed into cDNA. The cDNA was then subjected to qPCR analysis. In these two-steps we used TransScript Green Two-Step qPCR SuperMix (Transgen biotech, beijing, China). Our results are all based on β-actin as an internal reference. The primers were as follows: KIF2A sense 5′- GCC TTTGATGACTCAGCTCC-3′, reverse 5′- TTCCTGA AAAGTCACCACCC-3′; β-actin sense 5′-TGACGTGG ACATCCGCAAAG-3′, reverse 5′-CTGGAAGGTGGAC AGCGAGG-3′. The results were expressed as mean ± standard deviation (SD). The relative quantification of KIF2A expression levels were calculated relative to β-actin using the $2^{-\Delta\Delta CT}$ method.

Western Blotting

We used a protein extraction kit (KeyGEN Biotech, Nanjing, China). The concentration of the protein was measured using a Bio-Rad Protein Assay Kit (KeyGEN Biotech). The protein sample was subjected to

electrophoresis and then electrotransferred to a PVDF membrane (Millipore, USA). After completion, the cells were blocked with 5% skim milk powder in TBST buffer for 1 hour. Then incubate with the corresponding antibody and overnight at 4 °C. The next day, it was incubated with the corresponding secondary antibody for 1 hour at room temperature. Then check the strip condition. This study used β-actin as an internal reference. In addition, the antibody information used herein is as follows: rabbit anti-KIF2A antibody (1:1, 0000 dilution, ab197988, Abcam plc, Cambridge, UK), rabbit anti-β-actin (1:1000 dilution, ab8227, Abcam plc, UK) Cambridge), rabbit anti-Ki67 (1:1000 dilution, ab16667, Abcam plc, Cambridge, UK) and rabbit anti-proliferating cell nuclear antigen (PCNA) (1:500 dilution, ab18197, Abcam plc, Cambridge, UK).

Colony Formation Assay

A total of 500 cells were added to each well and seeded into 6-well plates, followed by incubation for 2 weeks. The resulting colonies were then fixed with methanol and then stained with 0.1% crystal violet to finally obtain the size and number of colonies.

Cell Counting Kit-8 (CCK-8) Assay

We used CCK-8 reagent (Beyotime Technology) to measure cell viability. To each well, 2000 cells were added to the 96-well plate, and finally the survival rate was recorded on the third day.

Experiment of Tumor Growth in Mice

Our institutional animal care committee approved the care and operation of mice. BALB/c nude mice (6–8

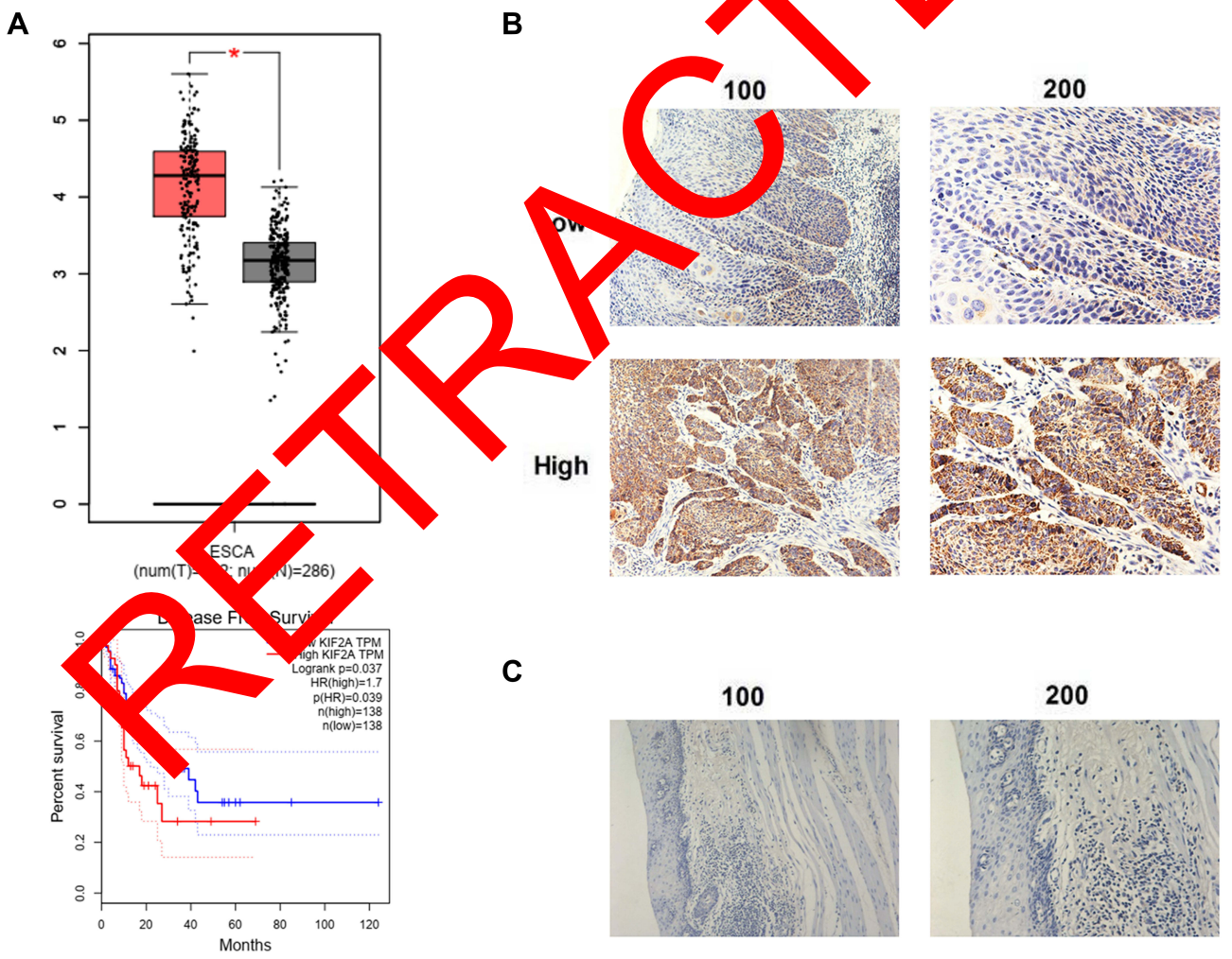

Figure 1 The expression of KIF2A and the disease-free survival in ESCC. (**A**) Upper: the expression of KIF2A mRNA in tumor tissues and normal tissues from TCGA. Below: KIF2A overexpression is associated with poor disease-free survival form TCGA. (**B**) Low and high expressed of KIF2A in tumor tissues. (**C**) The expression of KIF2A in normal tissues. *P<0.05.

weeks, 18–22 g) were purchased from Beijing Life River Experimental Animal Technology Co., Ltd. (Beijing, China). First, 6-week-old BALB/c mice were divided into two groups, and KIF2A shRNA or control cells were injected subcutaneously into mice. The volume of the tumor was measured every 7 days for about 2 weeks after the completion of the injection. The tumor is finally isolated and photographed. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal study was carried out in accordance with the guidelines approved by the Animal Experimentation Ethics Committee of Xingtai people's hospital. The protocol was approved by the Committee of Xingtai people's hospital, all surgery and all efforts were made to minimize suffering.

Statistical Analysis

The statistical analysis results of this paper were completed by SPSS 23.0 software (IBM, Chicago, Illinois, USA). Statistically significant comparisons were made between the two groups using an independent Student's *t*-test. Data are expressed as mean ± SD, $P < 0.05$ is considered statistically significant.

Results

KIF2A was Overexpressed and Associated with Poor Prognosis in ESCC

To investigate the mRNA expression level of KIF2A in ESCC, we obtained ESCC data from the TCGA database for bioinformatics analysis. KIF2A mRNA expression was significantly higher in ESCC specimens (n = 182) compared to normal specimens (n = 286) ($P < 0.001$, Figure 1A). To investigate the relationship between KIF2A expression levels and prognosis in ESCC, we continued to perform bioinformatics analysis of the ESCC dataset in the TCGA database. Kaplan-Meier survival analysis showed that specimens with high KIF2A expression (n = 138) had poor disease-free survival (DFS) compared with specimens with low KIF2A expression (n = 138) (HR = 1.7, P = 0.037, Figure 1A). Then, we performed IHC in our own samples to detect the protein expression level of KIF2A. The results indicate that the KIF2A protein is highly expressed in most ESCC tissues (Figure 1B). We also divided KIF2A in the ESCC organization into two groups according to the level of expression (Figure 1B). In addition, we rarely see high expression of KIF2A in normal tissues (Figure 1C). We analyzed the relationship between the expression of KIF2A and clinicopathological features. The results showed that the expression of KIF2A in ESCC was significantly correlated with tumor size (P=0.003) and T stage (P=0.008) (Table 1). However, there were no significant differences between the high and low KIF2A groups in other aspects of clinicopathological features, such as age, gender, tumor grade, and lymph node metastasis. These results indicate that KIF2A is highly expressed in ESCC and is significantly associated with poor prognosis.

KIF2A Supports ESCC Proliferation in vitro

To investigate the role of KIF2A in ESCC, we used shRNA vectors to establish CaEs-17 and EC-109 cell lines that knockdown KIF2A. The knockdown effect was confirmed by qPCR (Figure 2A) and Western blot (Figure 2B). Among the two short hairpin RNAs we

Table 1 Relationships of KIF2A and Clinicopathological Characteristics in 78 Patients with Esophageal Cancer

Feature	All n=78	KIF2A Expression		χ^2	P
		Low n=28	High n=50		
Age (year)				2.842	0.092
< 60	46	13	33		
≥ 60	32	15	17		
Gender				0.268	0.605
Male	50	19	31		
Female	28	9	19		
Tumor grade				2.457	0.117
Low	48	14	34		
High	30	14	16		
Tumor size				9.018	0.003*
< 3.5 cm	38	20	18		
≥ 3.5 cm	40	8	32		
T stage				6.982	0.008*
T_{1-2}	49	23	26		
T_{3-4}	29	5	24		
Lymph node metastasis				0.997	0.318
Yes	25	7	18		
No	53	21	32		

Note: *P<0.05.
Abbreviations: χ^2, chi-square test; P, P value.

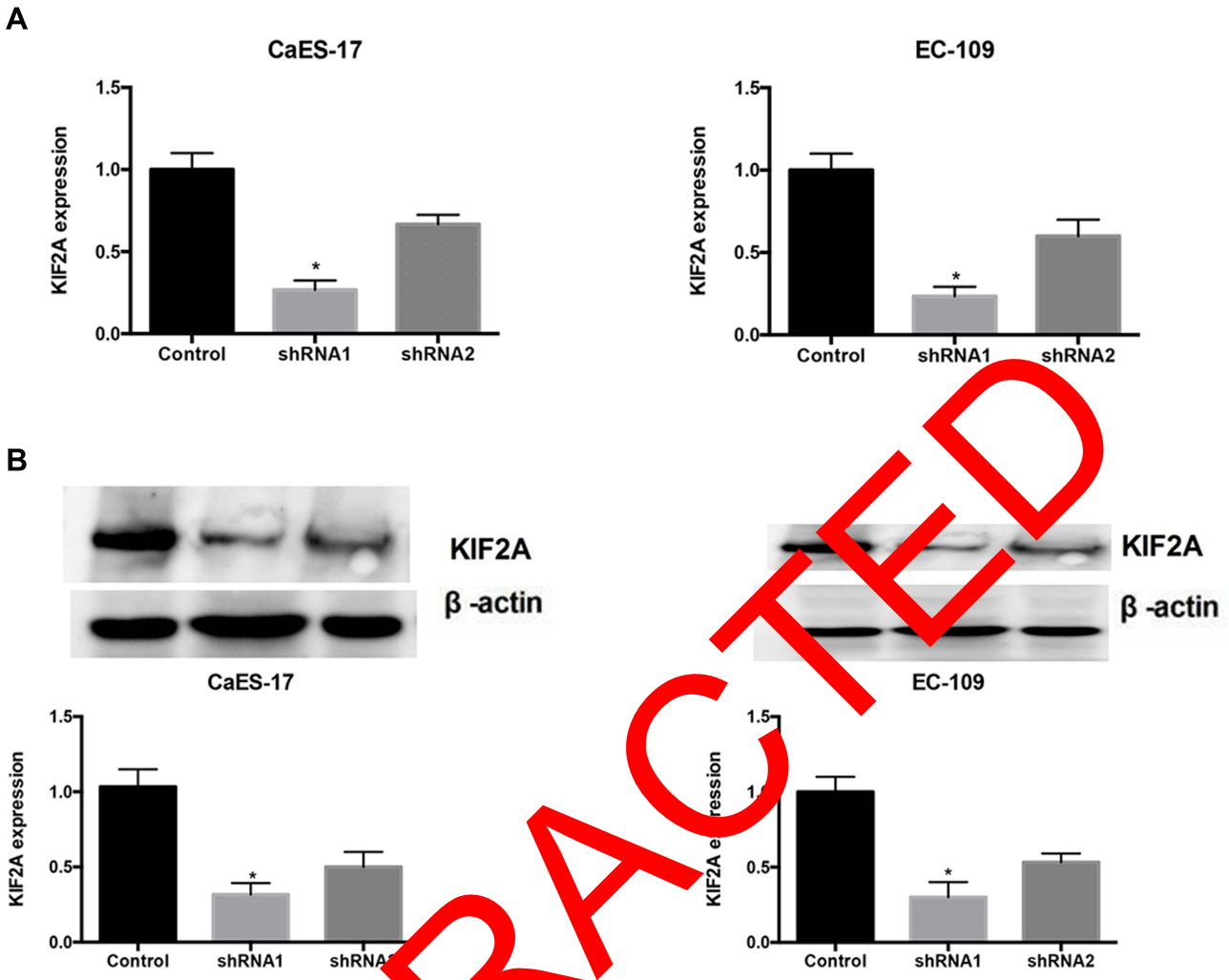

Figure 2 KIF2A knocking down cells for CaEs and EC cell lines. (**A**) RT-qPCR showed shRNA knocked down KIF2A in CaEs-17 and EC-109 cell lines. (**B**) Upper: Western blotting showed shRNA knocked down KIF2A. Below: Histograms with the gray value of the western booting strips. *P<0.05.

selected, shRNA1 (sh showed the most significant knockdown effect, we chose to use sh1 in the next experiments. We first performed a colony formation test. When knocking down KIF2A, we found that its ability to clone formation was significantly reduced (Figure 3A). We then tested CCK8 to assess proliferation and viability. When KIF2A was knocked down, viability and proliferation were significantly reduced (Figure 3B). In addition, we also used Western blotting to detect proliferation and selected two proliferative markers Ki67 (Figure 3C) and PCNA (Figure 3D) in CaEs-17 and EC-109 cells. The results showed that Ki67 and PCNA were significantly decreased in the knockdown group, indicating that proliferation was inhibited after knockdown of KIF2A. These data indicate that KIF2A can significantly promote the proliferation of ESCC cells in vitro.

Downregulation of KIF2A Inhibits Proliferation of ESCC Cells in vivo

To investigate the effect of KIF2A on ESCC proliferation in vivo, we divided 6-week-old BALB/c mice into two groups and subcutaneously injected KIF2A shRNA or shRNA to attenuate lentiviral cells. Measurement of tumor volume was performed every 7 days after about 2 weeks after injection of the cells. The results showed that the tumor volume was significantly smaller than that of the control knockout group (Figure 4A). We then used IHC to detect the expression of KIF2A in mouse tumors. The results showed that KIF2A was significantly lower in the knockdown group than in the control group (Figure 4B). We also used IHC to detect mouse tumor proliferation markers Ki67 and PCNA, and the results showed that both Ki67 and PCNA were significantly reduced in the

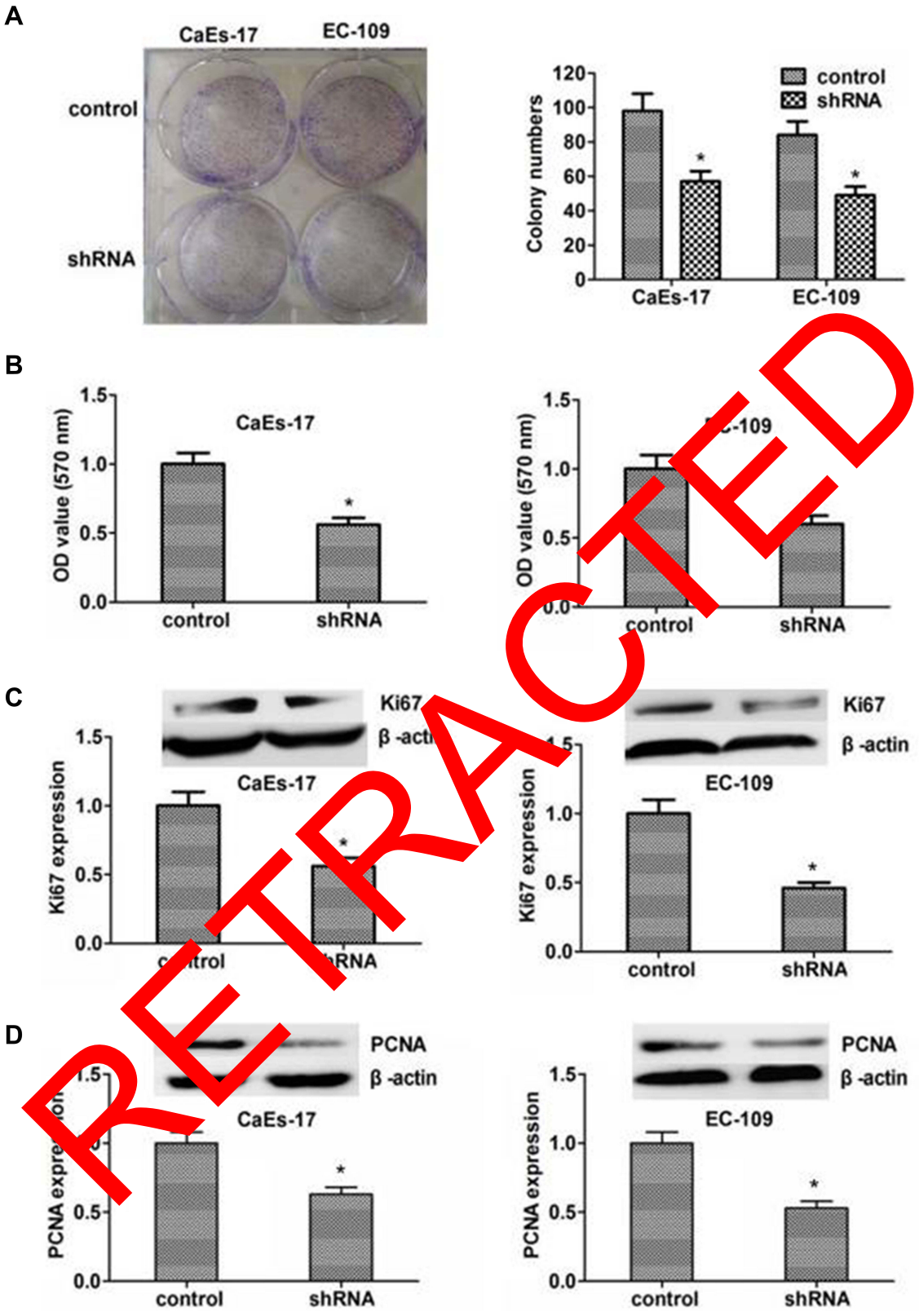

Figure 3 Knocking down of KIF2A can inhibit the proliferation and growth of ESCC cells. (**A**) Left: clone formation assay to assess the role of KIF2A knocking down in ESCC cells. Right: Histograms with the number of colony. (**B**) CCK8 to assess the role of KIF2A knocking down in ESCC cells. (**C**) Upper: Ki67 to assess the role of KIF2A knocking down for proliferation by Western blotting. Below: histograms with the gray value of the western booting strips. (**D**) Upper: PCNA to assess the role of KIFC1 knocking down for proliferation by Western blotting. Below: histograms with the gray value of the western booting strips. *P<0.05.

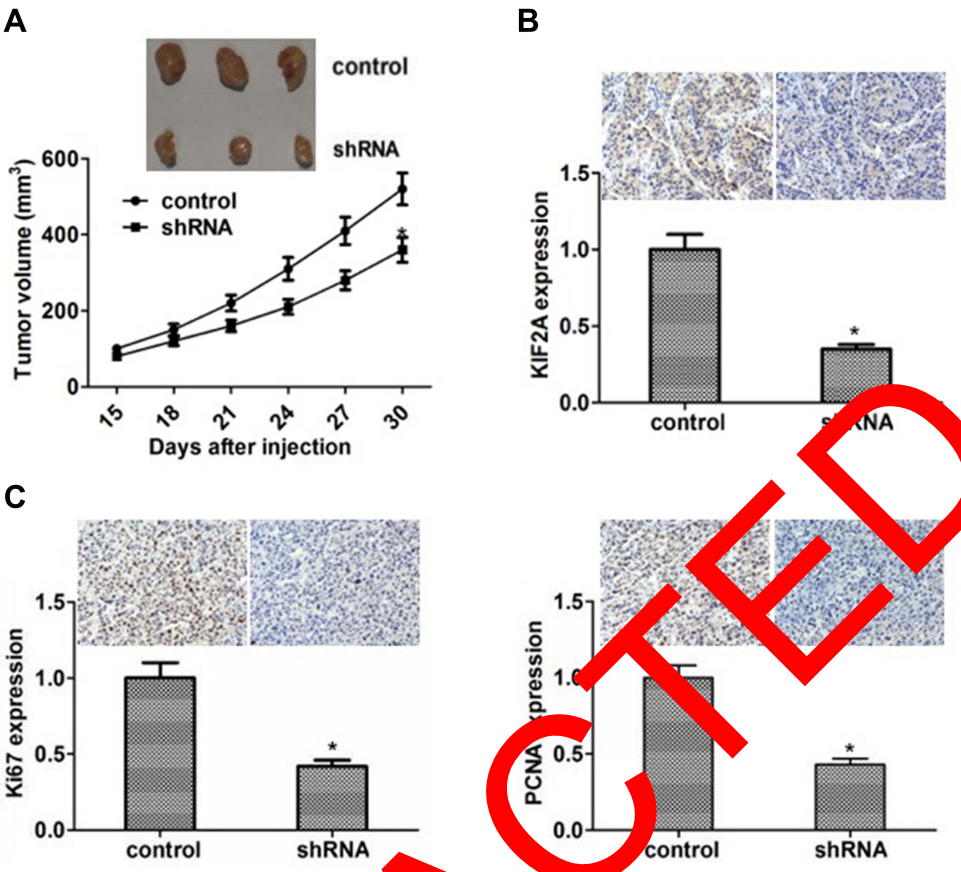

Figure 4 Knocking down of KIF2A can inhibit the proliferation and growth of ESCC cells in vivo. (**A**) Upper: representative images of the tumors in mice. Below: line chart with volume of tumor in mice. (**B**) Upper: detection of KIF2A expression in the tumor of mice by IHC. Below: histograms with the gray value of IHC. (**C**) Upper: representative images of Ki67 and PCNA staining in the tumor. Below: histograms with the gray value of the Ki67 and PCNA staining. *P<0.05.

knockdown group compared to the control group (Figure 4C). These results indicate that KIF2A still promotes the proliferation of ESCC cells in vivo.

Discussion

KIF2A is an important protein for cell mitosis and spindle assembly.[18] It has also been reported to be a carcinogenic protein that is overexpressed in many human malignancies.[10,12–14] The high expression level of KIF2A is associated with poor prognosis of gastric cancer,[17,20] breast cancer[15] and glioma,[18] etc.[21] In our study, KIF2A has been found to be overexpressed in most ESCC patients, and the expression level is correlated with the prognosis of ESCC for DFS. We have reason to believe that KIF2A may have a role in the progression and development of ESCC.

KIF2A is a member of the kinesin-13 family and is a MT depolymerase.[22] MTs are components of the cytoskeleton that play an important role in mitosis and transport.[23] In other cancers, it has been reported that KIF2A can promote proliferation.[24–26] In our study, by establishing two KIF2A knockdown cell line models, the cell proliferation ability of knockout KIF2A was significantly reduced. In in vivo experiments, the results of animal experiments were consistent with cellular experiments. Animal experiments make our conclusions more credible than pure cell experiments.

The study of KIF2A signaling pathways that promote ESCC proliferation is lacking in this paper. Wang et al reported that KIF2A silencing inhibits the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and leads to increased apoptosis.[16] Xie et al showed that the expression levels of phosphorylated AKT and phosphorylated ERK1/2 in the KIF2A knockout group were significantly reduced.[25] In addition, the extracellular signal-regulated kinase (ERK) 1/2 pathway is an effector that controls the expression of these kinesins.[27,28] The Raf/ERK1/2 and PI3K pathways are major factors in Ras transformation and can have a dramatic effect on the cytoskeleton.[29,30] Sheng et al reported that miR-206 inhibited the overexpression of KIF2A[31] and Uchida et al showed that the regulation of KIF2A by anti-tumor miR-451a can inhibit

the aggressiveness of cancer cells.[32] These studies can play a guiding role in our future analysis of mechanisms.

Our research has some limitations that need to be further improved in future research. First, our prognostic results are mainly from public databases, lacking our own follow-up data. Second, the number of tissue specimens is relatively small, requiring multi-center large sample research support. Third, the depth of mechanism research is insufficient, so more and more in-depth mechanisms should be revealed in the future.

Conclusion

In conclusion, the results suggested that KIF2A might be a prognostic factor for ESCC. Moreover, KIF2A could promote ESCC proliferation in vitro and in vivo, respectively. These results provided a theoretical basis for the future use of KIF2A as a potential biomarker and therapeutic target for ESCC.

Disclosure

The authors declare that they have no conflicts of interest regarding this work.

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