ORIGINAL RESEARCH

RAB43 Promotes Gastric Cancer Cell Proliferation and Metastasis via Regulating the PI3K/AKT Signaling Pathway

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Background: Ras-related GTP-binding protein 43 (RAB43) plays a key part in the progression of many human cancers. However, the role and functional mechanisms of RAB43 in gastric cancer (GC) remain unknown.

Purpose: To elucidate the function and mechanism of RAB43 in the progression of GC.

Patients and Methods: One hundred patients with histologically confirmed GC were recruited for this study. Tumor samples and GC cell lines were used to detect RAB43 levels. Cell Counting Kit8 (CCK8) and colony formation assays were used to analyze cell proliferation. Cell migration and invasion ability were examined by wound healing and transwell assays. Western blot assays and quantitative real-time PCR (qRT-PCR) were performed to examine related mRNA and protein expression. In vivo experiments were used to examine the effect of RAB43.

Results: Patients with RAB43-positive tumors had worse overall survival than patients with RAB43-negative tumors. Downregulation of RAB43 significantly inhibited cell proliferation and cell metastasis. In contrast, RAB43 overexpression promoted proliferation and metastasis in normal gastric epithelial GES-1 cells. In vivo studies confirmed that RAB43 promoted tumor growth. In addition, the knockdown of RAB43 significantly inhibited cell proliferation and metastasis via phosphatidylinositol-3-kinases/protein-serine-threonine kinase (PI3K/AKT) pathway.

Conclusion: RAB43 promotes GC cells proliferation and migration in vivo and in vitro and probably served as a novel potential therapeutic biomarker for GC.

Keywords: gastric cancer, RAB43, cell proliferation, metastasis, PI3K/AKT pathway

Introduction

As the fourth most common cancer and the second highest cause of cancer-related death worldwide, especially in East Asia, gastric cancer (GC) seriously threatens patients' lives.[1,2] Surgical resection combined with chemotherapy and radiotherapy has greatly decreased GC mortality; however, GC patient outcomes remain poor.[3] Therefore, it is essential to identify effective early markers and explore novel therapeutic and diagnostic method to improve the survival rate of GC patients.

Ras-related GTP-binding protein 43 (RAB43) is a member of the Ras superfamily.[4] Previous investigators showed that RAB43 associates with a variety of compartments within cells, including an early compartment of the Golgi, where it may be involved in regulating the association of pre-Golgi intermediates with microtubules.[5] Recently, researchers have focused on its function in

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cancer; for example, RAB43 participates in the regulation of multiple signal transduction pathways related to cell invasion, cell apoptosis and immune response.[6] Li revealed that high RAB43 expression predicts poor prognosis and is related with epithelial-mesenchymal transition in gliomas.[7] However, the biological function and molecular mechanisms of RAB43 in GC are still explored. In this study, we aimed to elucidate the function and mechanism of RAB43 in GC. We found that RAB43 is upregulated in GC. In addition, the functions of RAB43 in promoting the metastasis and growth of GC, as well as the underlying mechanism related to its biological behavior, were investigated.

Materials and Methods
GC Samples and Cell Lines
This study was approved by the Ethics Committee of Xinhua Hospital (Approval No. XHEC-F-2019-044/ XHEC-D-2019-082), and all patients provided written informed consent, and this was conducted in accordance with the Declaration of Helsinki. The GC tissue samples used were collected between 2011 and 2012 at the Department of General Surgery, Xinhua Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, China. We collected GC samples from 100 patients with radical gastrectomy (without prior radiotherapy or chemotherapy). The paired adjacent nontumor tissues were more than 5 centimeters (cm) away from the tumor edge and were estimated to have no tumor invasion. All diagnoses of GC and lymph node metastasis were confirmed by histopathological examination, and adjacent control samples were confirmed to be free of tumor cells. All tissue samples were flash frozen in liquid nitrogen within 5 min immediately and were stored at $-80\,°C$.

The human GC cell lines HGC27, MGC803, SGC7901, and MGC823 and the normal gastric epithelial cell line GES-1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI 1640 medium supplemented with 10% (v/v) bovine calf serum. All cells were cultivated at 37°C in a humidified incubator with 5% CO2.

Plasmid Transfection
RAB43 and an empty vector (pcDNA 3.1) were purchased from Era Biotech (Shanghai, China). Cells were seeded on 6-well plates and transfected for 48 h using Viafect transfection reagent according to the manufacturer's protocol.

RNA Interference and Transfection
The strand sequence of human small interfering RNA (siRNA) of RAB43 is 5′-CCATTGAGACGTCTGCCAA-3′, and the negative control sequence was 5′-TTCTCCGA ACGTGTCACGT-3′. $5×10^5$ cells/well were seeded into 6-well plates and transfected with the relevant siRNA (50 nmol/well) using Lipofectamine 2000 reagent according to the manufacturer's protocol.

RNA Extraction and qRT-PCR
Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was amplified by qRT-PCR with SYBR Green (TaKaRa, Tokyo, Japan). The expression of target genes was normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The following primers were used to detect the expression of RAB43 and GAPDH:

RAB43: 5′- CTGCTGATCGGGAACAAGTCA −3′
5′- CAATGGCACACAGGATGTCATA −3′
GAPDH: 5′-GCCGCATCTTCTTTTGCGTCGC-3′
5′-TCCCGTTCTCAGCCTTGACGGT-3′

Cell Viability Assay
Cell viability was evaluated using a CCK8 assay following the manufacturer's instructions. Human GC cells were cultured in 96-well plates at a density of $1 × 10^3$/well for different times. Optical density (OD) 450 values were analysed by spectrophotometry (BioTek, USA) 3 h after being incubated with $10\,\mu L$ of CCK8 reagent. All data were determined from three independent experiments.

Colony Formation Assay
A density of 500 cells/well of human GC cells were seeded into 6-well plates for approximately 2 weeks in RPMI-1640 medium. Then, the cells were cleaned and fixed with 10% formalin and stained with a 0.1% crystal violet solution (Sigma-Aldrich, MO, USA). After staining, the plates were dried and observed under a microscope. The clones with more than 50 cells were numbered.

Transwell Assays and Wound Healing Assays
Transwell assays were performed to analyse cell migration and invasion ability. $5×10^4$ GC cell were suspensions in 200ul serum-free medium, and then were seeded onto the

upper chambers. In the lower chambers, we placed 500 μL of medium containing 10% fetal bovine serum (FBS). After 24h, the cells were collected. The filters were fixed with 4% paraformaldehyde for 30 min and stained with a 0.1% crystal violet solution for 15 min at 37°C.

GC cells were cultured in 6-well plates until 90% confluency. Then we inhibit cell division with mitomycin C (10 μg/mL) at 37°C in a 5% (v/v) CO2 incubator for 1 h. Then we used a sterile 200-μL pipet tip perpendicular to divided two wounds every plates. The cells were cleaned with PBS and incubated with 2 mL serum-free media. The cells were photographed at 0 h, 24h and 48 h using a microscope. All the experiments were performed three times.

Western Blot Analysis

Total protein was extracted using RIPA buffer (Beyotime, Shanghai, China) supplemented with Protease Inhibitor Cocktail and PhosSTOP (Beyotime, Shanghai, China). The total cell protein concentrations were measured with bicinchoninic acid assays (Beyotime, Shanghai, China) with bovine serum albumin (BSA) as a standard. The lysates were separated in a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and incubated with antibodies against RAB43, Snail, N-cadherin, E-cadherin, vimentin, GAPDH, Bax, Bcl-2, PI3K, AKT and P-AKT (all from Cell Signaling Technology, MA, USA) diluted at 1:1000.

Subcutaneous Xenograft

All animal treatments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Shanghai Jiaotong University. Male nude mice (aged 4–6 weeks) were purchased from Shanghai SLAC Laboratory Animal Co Ltd. (Shanghai, China). Sh-NC and sh-RAB43 cells were resuspended in phosphate buffer saline (PBS). The mice were injected subcutaneously with 5×10^5 cells in 200 μL PBS into their right flank regions. After about 2 weeks, mice were euthanized. For tissue morphology evaluation, hematoxylin and eosin staining was performed on sections of the embedded samples. Immunohistochemistry (IHC) staining for RAB43 and Ki-67 was performed on sections from the xenograft tumors.

Immunohistochemistry

Immunohistochemical staining was performed using a standard immunoperoxidase staining procedure, and the expression and score of RAB43 in the GC specimens was measured as Li described.[8] The sections were scored according to the extent of immunoreactivity as follows: 0% immunoreactive cells were scored as 0; <5% immunoreactive cells were scored as 1; 5–50% immunoreactive cells were scored as 2; and >50% immunoreactive cells were scored as 3. Additionally, the staining intensity was scored as follows: 0, negative; 1, weak; 2, intermediate; and 3, strong. We defined the final immunoreaction score as the sum of extension and intensity, and the samples were classified as negative (0), weakly stained (1–2), moderately stained (3), and strongly stained (4–6). In the final statistics, we defined moderate and strong final immunoreaction scores as positive; the other final scores were considered negative.

Statistical Analysis

All experiments were performed at least 3 times, and the results are expressed as the mean ± standard deviation unless otherwise stated. Student's t-test was used to compare the differences between the treatment groups and the corresponding control groups. P < 0.05 was considered statistically significant.

Results
RAB43 Is Highly Expressed in GC and Correlates with Poor Prognosis in GC Patients

To examine the potential effect of RAB43 in GC progression, we measured its expression in GC and adjacent control tissues by IHC (Figure 1A). As shown in Figure 1A and B, the GC tissues had relatively higher RAB43 expression than the normal tissues. In tumor samples, approximately 70.0% (70/100) of the GC cases had positive RAB43 staining. In contrast, only 31.0% (31/100) of the cases had positive staining in the adjacent samples. Our results demonstrated that the expression of RAB43 was higher in GC tissues than in the matched adjacent tissues (Figure 1C).

Due to high expression of RAB43 in GC, we hypothesized that high levels of RAB43 may predict poor survival in GC patients. Thus, we examined the correlation between RAB43 expression levels and histopathological parameters in GC patients. As we can see in Table 1, RAB43 overexpression was significantly correlated with TNM stage (P<0.001), while RAB43 overexpression was not corrected
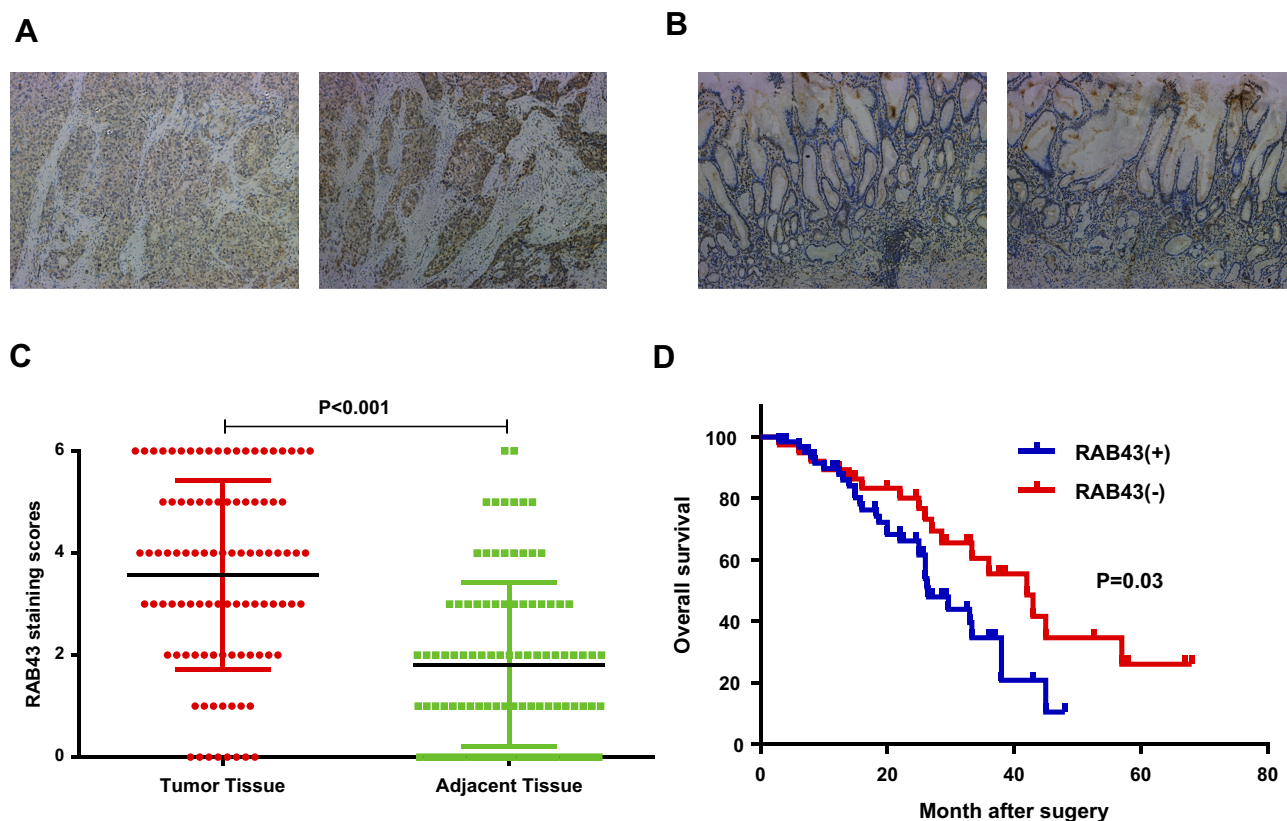
Figure I RAB43 is highly expressed in GC and correlates with poor prognosis in GC patients. (**A, B**) Immunohistochemistry for RAB43 in tumor and adjacent tissues from GC patients. (**C**) Kaplan–Meier plots of the overall survival of GC patients based on RAB43 expression. (**D**) Multivariate Cox analysis for RAB43 in GC.

with sex, age, histopathological subtype and tumor location. A subsequent multivariate Cox analysis (Table 2 and Figure 1D) showed that high RAB43 expression was an independent prognostic factor for poor survival in GC patients. The results confirmed that RAB43 expression predicted a postoperative survival, suggesting that high RAB43 expression is a independent risk factor of GC.

RAB43 Downregulation Attenuates GC Cell Proliferation and Metastasis in vivo and in vitro

To verify the oncogenic activity of RAB43 in GC, we first examined its expression in GC cells. As Figure 2A and B shows, RAB43 expression is higher in GC cells than in normal epithelial GES-1 cells at both the mRNA and protein levels. Next, we knocked down RAB43 expression in MGC803 and HGC27 cell lines using retroviral transduction and then assessed cell proliferation (Figure 2C). As shown in Figure 2D, knockdown of RAB43 significantly reduced the number of colonies by colony formation assay. Next, we explored the role of

RAB43 on GC cell proliferation in vivo. As the results shown in Figure 2E, the tumor volume and weight of RAB43-depleted mice were obviously inhibited compared with the control group. Moreover, IHC analysis revealed that RAB43 and Ki67 levels were substantially decreased in the shRAB43 group (Figure 2F). The above results indicated that RAB43 promotes cell growth in vitro and in vivo.

Furthermore, we investigated the invasion and migration ability of GC cells to understand the molecular basis of RAB43-regulated cancer metastasis. As shown in Figure 3A and B, the invasion and migration ability of shRAB43 cells were significantly reduced according to transwell experiments. Consistent with the above results, wound healing assays revealed that RAB43 knockdown in GC cells moderately decreased the migration rate (Figure 3C). Thus, we believe that RAB43 regulates GC cell proliferation and metastasis in vitro and in vivo.

RAB43 Overexpression Promotes Proliferation and Metastasis in GES-1 Cells

As normal gastric mucosal epithelial GES-1 cells have weaker expression of RAB43, we overexpressed RAB43

Table 1 Association Between RAB43 Expression with the Clinicopathological Parameters of GC

Parameter	Category	No. of Cases	RAB43 Expression		
			No. of Positive Cases (%)	χ^2	P value
Age	<60 ≥60	45 55	32(71.1) 41(74.5)	0.148	0.822
Sex	Male Female	52 48	36(69.2) 37(77.1)	0.781	0.499
Histopathological Subtypes	High Middle Low	22 43 35	12(54.5) 33 (76.7) 28(80.0)	4.977	0.083
TNM Stage	I II III IV	6 15 65 14	2(33.3) 7(46.7) 51(78.5) 13(92.9)	13.852	0.003*
Tumor Location	Antrum Cardia + body	58 42	38 (65.5) 35(83.3)	3.928	0.067
Lymph Node Metastasis	Positive Negative	66 34	49(74.2) 24(70.6)	0.152	0.813

Note: *$P<0.05$.

Table 2 Multivariate Analysis of Overall Survival (OS)

Parameter	Category	HR	95% CI	P value
Age	<60 ≥60	0.550	1.357 (0.833–2.211)	0.211
Sex	Male Female	0.480	0.910 (0.557–1.481)	0.705
Histopathological subtypes	High Middle Low	1.870	0.824 (0.584–1.167)	0.276
TNM stage	I–II III–IV	1.790	2.081 (1.073–4.035)	0.030*
Tumor Location	Head Body/tail	0.420	0.735 (0.443–1.222)	0.236
Lymph node Metastasis	Negative Positive	0.340	0.744 (0.445–1.244)	0.26
RAB43 expression	Negative Positive	0.710	0.530 (0.306–0.917	0.023*

Note: *$P<0.05$.
Abbreviation: HR, hazard ratio.

in GES-1 cells to further confirm the role of RAB43 on GC progression. As shown in Figure 4A, the proliferation of RAB43-overexpression group was obviously increased than the control group. Moreover, RAB43 overexpression increased colony formation than control group

in GES-1 cells (Figure 4B). Additionally, the RAB43-overexpression cells showed stronger invasive and migratory capacities than the control cells (Figure 4C and D). Above all, we concluded that high expression of RAB43 could induce increased proliferation, migration and invasion ability of normal gastric mucosal epithelial GES-1 cells.

RAB43 Was Involved in PI3K/AKT Signaling Pathway in GC Cells

We measured the regulatory pathways related to tumor metastasis and growth to illuminate the molecular mechanisms of RAB43 regulating GC cell metastasis and growth. As shown in Figure 5A, RAB43 knockdown dramatically increased the expression of E-cadherin and decreased the expression of N-cadherin and vimentin, which play important roles in cell metastasis.

In previous studies, researchers reported that PI3K/AKT pathway was a major signaling pathway associated with cancer progression and invasion.[9,10] Thus, we next examined the expression of PI3K/AKT signaling related molecules. As shown in Figure 5B, the expression of AKT, p-AKT, and PI3K in GC cells was lower in the shRAB43 group than in the control group (Figure 5B). All these results demonstrated that RAB43 was involved in PI3K/AKT pathway in GC cells.
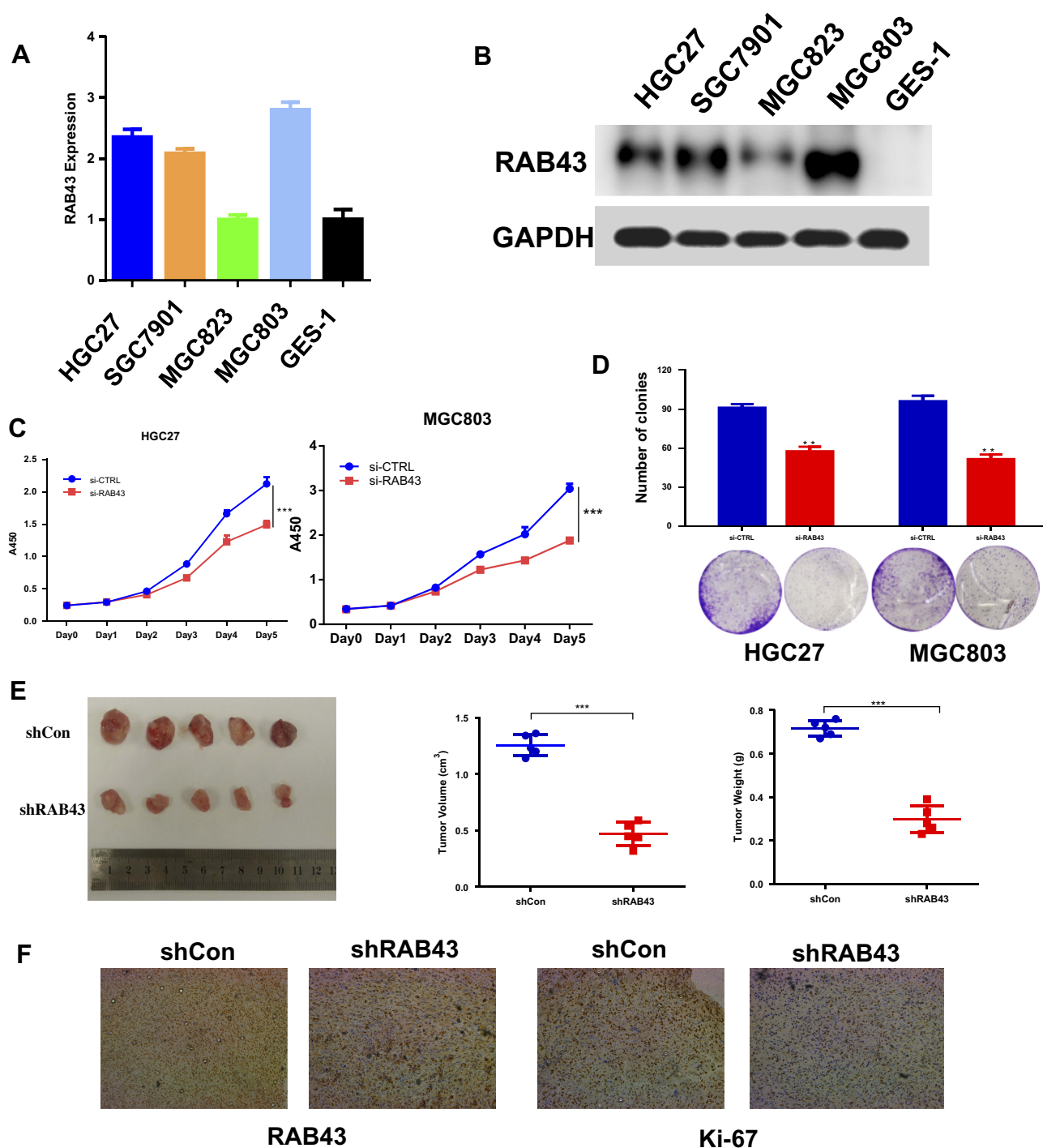
Figure 2 RAB43 downregulation attenuates GC cell proliferation and metastasis in vivo and in vitro. (**A, B**) Protein and mRNA expression of RAB43 in GC and normal gastric epithelial cells according to Western blot analysis and RT-PCR. (**C**) Cellular proliferation of untransfected and transfected GC cells was measured daily for 5 days using a CCK8 assay. ***<0.001. (**D**) Colony formation assay of untransfected and transfected GC cells. Colony numbers were counted and recorded. **<0.01. (**E**) Mice were treated with Lv-shCon and Lv-shRAB43 GC cells for 4 weeks. Tumor volumes and weights were measured. ***<0.001. (**F**) Immunohistochemical analysis showed a decrease in Ki67 and RAB43 expression.

Discussion

The function of RAB43, a member of the Ras-related small GTPase superfamily, is poorly characterized compared with that of many other secretory RAB GTPases.[4] RAB43 localizes at the Golgi and is important for maintaining Golgi structure and function and transporting Shiga toxin from the cell surface to the trans-Golgi network.[11–13] Moreover, RAB43 interacts directly with G Protein-Coupled Receptors (GPCRs) in an
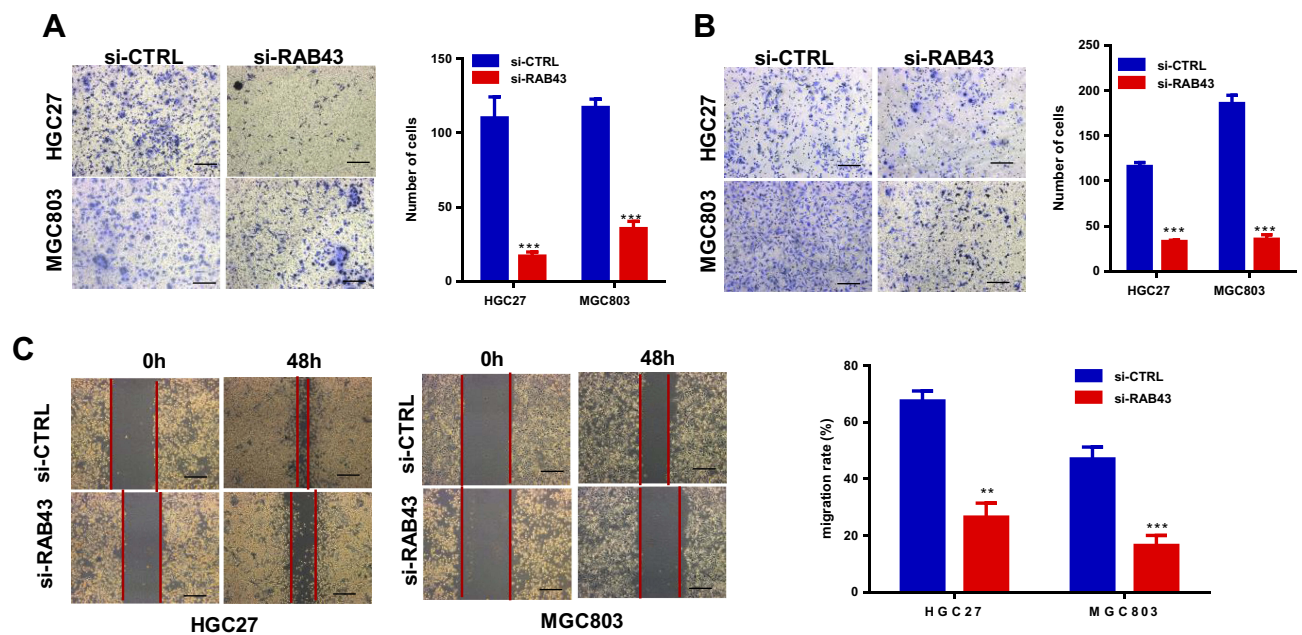
Figure 3 RAB43 downregulation attenuates GC cell proliferation and metastasis in vivo and in vitro. (**A, B**) Migration and invasion in GC cell lines measured by transwell assays decreased after transfection. The number of migrated cells was calculated and is depicted in the bar chart. ***<0.001. (**C**) Wound closure was delayed in shRAB43 cells compared to shCon cells after 48 h. **<0.01, ***<0.001.

activation-dependent pattern. The RAB43-binding domain identified in the receptors effectively converts nonGPCR membrane protein transport into a RAB43-dependent pathway.[14] Recently, researchers focused on the RAB43's anti-cancer effects. High expression of RAB43 predicts poor prognosis and is associated with epithelial-mesenchymal transition in gliomas.[7] However, the effect and mechanism of RAB43 on gastric cancer is still unrevealed.

In our study, we found that the expression of RAB43 was significantly upregulated in GC tissues compared with adjacent control samples. We also found that high expression of RAB43 predicted poor prognosis of GC patients. Increased RAB43 protein expression correlated with poor patient survival, suggesting that RAB43 is a prospective biomarker for GC diagnosis and therapy, although more work still needed further verification.

Additionally, the effect of RAB43 on the biological behavior of GC cells was explored. Decreased migration, invasion, and proliferation were observed in RAB43 knockdown cells both in vitro and in vivo. The proliferation and viability of shRAB4 group was measured by CCK8 and in GC cells. The results demonstrated that knockdown of RAB43 significantly reduced GC cell proliferation. In vivo, compared with those of tumors from the control group, the volumes and weights of tumors from lv-shRAB43 group were significantly decreased. We further studied the effects of RAB43 on GC cell

metastasis. Moreover, the invasion and migration ability of the shRAB43 group was sharply reduced than in control group according to transwell study. In contrast, RAB43 overexpression promoted proliferation and metastasis in normal gastric epithelial GES-1 cells. These results were further confirmed by enhanced expression of E-cadherin and reduced expression of vimentin and N-cadherin, key metastasis-related factors. All these results might explain the RAB43-associated aggressive biological behaviors of GC.

Recently, more and more studies have shown that various signaling pathways are involved in the progression of GC.[15] PI3K/AKT is a common signaling pathway downregulated in human cancers.[16] PI3K/AKT has extremely important biological functions in cell growth, proliferation, apoptosis, angiogenesis, autophagy, and other processes in GC.[17] For example, Linc00152 promotes GC growth through activation of the epidermal growth factor receptor (EGFR)-dependent PI3K/AKT pathway;[18] microRNA-28 promotes cell proliferation and invasion in GC via the gene of phosphate and tension homology deleted on chromosome 10 (PTEN)/PI3K/AKT signaling pathway;[19] and the CXCL10/CXCR3 axis promotes GC invasion via PI3K/AKT pathway-dependent MMP production.[20] In present study, the expression of AKT, p-AKT, and PI3K in shRAB43 cells were reduced compared with the control group. These results suggested that
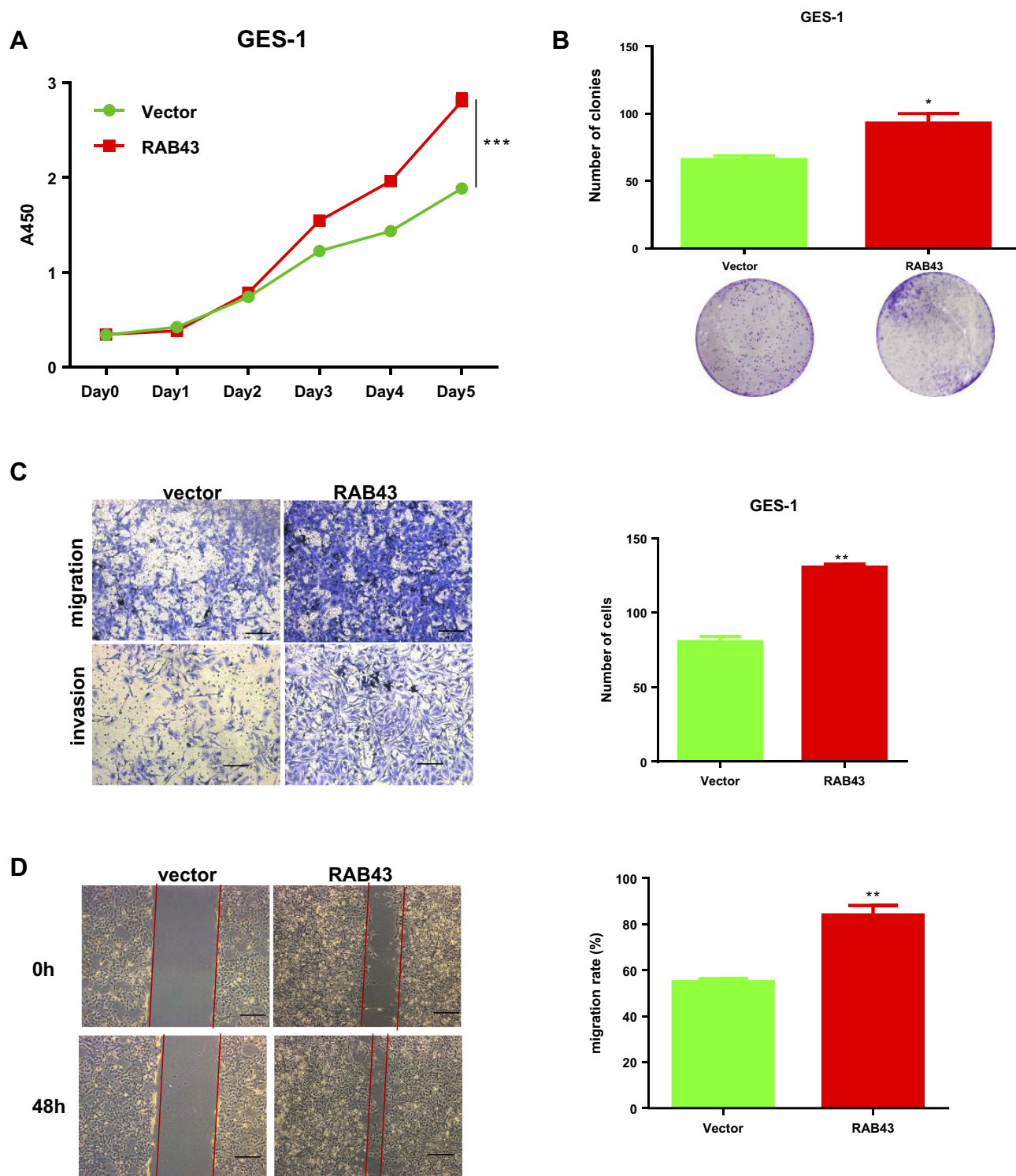
Figure 4 RAB43 overexpression promotes proliferation and metastasis in GES-1 cells. (**A**) Cellular proliferation of GES-1 control and overexpression cells was measured daily for 5 days using a CCK8 assay. ***<0.001. (**B**) Colony formation assay of GES-1 control and overexpression cells. Colony numbers were counted and recorded. *<0.05. (**C, D**) Migration and invasion in GES-1 cell lines measured by transwell assays decreased after transfection. The number of migrated cells was calculated and is depicted in the bar chart. **<0.01.

RAB43 regulated PI3K/AKT signaling in GC cells. Unfortunately, the direct link between RAB43 and this potential downstream pathway remains elusive.

In conclusion, our present study demonstrated that RAB43 is overexpressed in GC tissues and that high expression of RAB43 is associated with poor prognostic
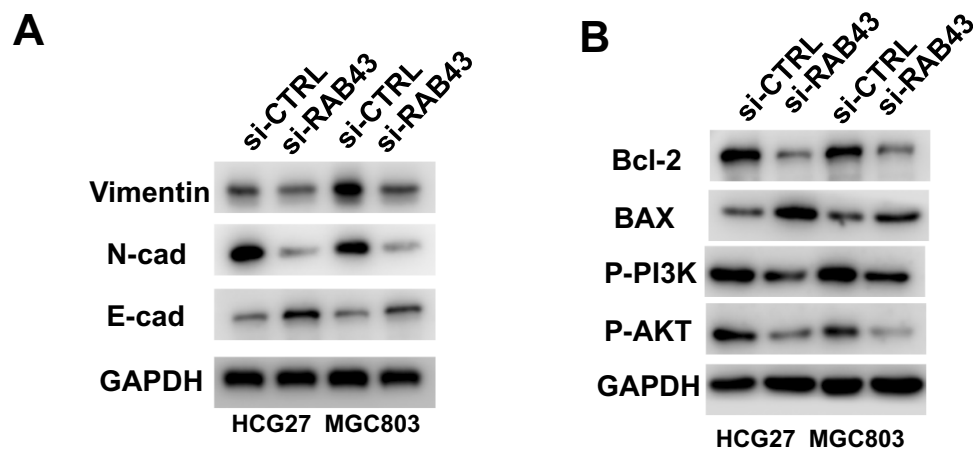
A

Vimentin
N-cad
E-cad
GAPDH

si-CTRL si-RAB43 si-CTRL si-RAB43

HCG27 MGC803

B

Bcl-2
BAX
P-PI3K
P-AKT
GAPDH

si-CTRL si-RAB43 si-CTRL si-RAB43

HCG27 MGC803

Figure 5 RAB43 regulates PI3K/AKT signaling in GC. (**A**) Protein expression of metastasis-related molecules according to Western blot analysis. (**B**) Western blotting analysis of PI3K/AKT signaling-related proteins in GC cell lines. GAPDH was used as a loading control.

signature. Moreover, RAB43 regulates GC cell proliferation and metastasis in vivo and in vitro. In addition, we revealed that knockdown of RAB43 inhibited GC cell proliferation and migration via the PI3K/AKT signaling pathway. Thus, RAB43 may served as a novel potential therapeutic biomarker for GC.

Conclusion
RAB43 promotes GC cells proliferation and migration in vivo and in vitro and probably served as a novel potential therapeutic biomarker for GC.

Abbreviations
GC, gastric cancer; RAB43, ras-related GTP-binding protein 43; CCK8, cell counting kit 8; qRT-PCR, quantitative real-time PCR; PI3K/AKT, phosphatidylinositol-3-kinases/protein-serine-threonine kinase; min, minute; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OD, optical density; FBS, fetal bovine serum; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; PVDF, polyvinylidene difluoride; PBS, phosphate buffer saline; IHC, immunohistochemistry; SD, standard deviation; GPCRs, G Protein-Coupled Receptors; EGFR, epidermal growth factor receptor; PTEN, gene of phosphate and tension homology deleted on chromosome ten; GBC, gallbladder cancer; OS, overall survival.

Data Sharing Statement
The data are included within the manuscript.

Ethics and Consent Statement
All operations as described above were approved by the Institutional Animal Care and Use Committee of Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University. All animal treatments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for Publication
All the authors agree to the publication clause.

Author Contributions
Lei Chen and Haibin Liang designed the study; Haibin Liang and Zhiye Huang conducted the experiments; Haibin Liang and Zhiye Huang collected tissue samples; Lei Chen and Haibin Liang purchased reagents; Zhiye Huang collected and arranged the data. Zhiye Huang wrote the main manuscript text. All authors read and approved the final manuscript. All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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