Esophageal cancer (EC) recently has become a common malignancy of digestive system worldwide. RAB11A is a critical member of the small GTPases superfamily and was reported to affect a variety of cellular functions. However, its potential effects on EC progression and the specific regulatory mechanisms are still unclear. In this study, RAB11A was upregulated in human EC tissues and cells and predicted poor diagnosis. RAB11A expression was correlated with clinical-pathological features including pTNM stage (P=0.001*) and recurrence (P=0.000**) in patients with EC. Furthermore, RAB11A knockdown decreased the proliferation, migration, and invasion of EC cells via WNT pathway in vitro. Subsequently, the in vivo experiments confirmed that RAB11A contributed to EC tumor growth via WNT pathway. Therefore, these results provided evidence showing that RAB11A could promote the progression of EC via WNT pathway and might serve as a promising therapeutic target for EC treatment.

Key words: esophageal cancer, RAB11A, proliferation, migration, WNT pathway

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INTRODUCTION

Esophageal cancer (EC) is a common malignancy of digestive system worldwide, ranked seventh in incidence and sixth in mortality (Lutfi et al., 2020). There are over 200,000 new cases of EC in China every year, and the 5-year overall survival rate (OSR) of EC patients is still less than 25% (2002). Esophageal squamous cell carcinoma (ESCC) is the main histological type of EC, accounting for nearly 90% of cases in China (Nam et al., 2020). Although there are many treatment methods for EC, the prognosis of patients is poor due to high metastasis ability and local lymph node metastasis (Su et al., 2020). It is well-accepted that targeted therapy is a promising prospect for EC treatment (Wu et al., 2020). Therefore, to improve the survival rate of patients with EC, novel and effective drugs, and particularly, therapeutic targets are urgently needed.

RAB11A is a critical member of small GTPases superfamily and is involved in a variety of cellular functions, such as the regulation of membrane vesicular sorting and transport, ciliogenesis, and autophagy (Jiang et al., 2017). It was also reported that RAB11A controls intracellular transport of innate immune receptor TLR4 (Husebye et al., 2010). Knockdown of RAB11A can cause serious human diseases, such as the defective formation of basolateral microvilli in neonatal intestinal diseases, infertility, tumors, etc. (Knowles et al., 2015; Feng et al., 2017; Dobiliene et al., 2018). RAB11A is highly expressed in various tumor tissues, such as thyroid cancer, breast cancer, and lung cancer (Calboun et al., 1998; Palmieri et al., 2006; Dong et al., 2017; Jiang et al., 2017).

For decades, the possible regulatory mechanism of RAB11A on tumor progression has been revealed (Palmieri et al., 2006). RAB11A could affect the motility of non-small cell lung cancer cells (NSCLCs) by regulating YAP (Dong et al., 2017). RAB11A was also found to be dysregulated in EC, but its clinical significance remains unclear (Robert et al., 2005; Shrestha et al., 2020). Additionally, the specific regulatory mechanism is also unclear. A previous study showed that RAB11A was regulated by miR-150 and affected the proliferation of thyroid cancer (TC) cells via WNT pathway (Bai et al., 2017). Based on these conclusions, a possibility that RAB11A may contribute to EC progression through WNT pathway was raised.

In this study, we reported the abnormally high RAB11A expression in human EC tissues and cell lines. RAB11A expression was also related to the clinical-pathological features of EC patients. RAB11A knockdown suppressed the proliferation, migration and invasion of EC cells via WNT pathway in vitro, and inhibited EC tumor growth in vivo. Therefore, our findings confirmed the regulatory role of RAB11A in EC progression and revealed the mechanism.

MATERIALS AND METHODS

Antibodies, primers and shRNA plasmids. The following antibodies were used: anti-RAB11A (for IHC assays, 1:100 dilution, for immunoblot assays, 1:1000 dilution, ab128913, Abcam, Cambridge, UK), anti-Ki67 (for IHC assays, 1:500 dilution, for immunoblot assays, 1:2000 dilution, ab16667, Abcam), anti-Cyclin D1 (1:2000 dilution, ab16663, Abcam), anti-MMP2 (1:1000 dilution, ab97779, Abcam), anti-MMP9 (1:2000 dilution, ab16667, Abcam), anti-Cyclin D1 (for IHC assays, 1:1000 dilution, ab97779, Abcam), anti-p-GSK3β (1:500 dilution, ab131097, Abcam), anti-β-catenin (1:1000 dilution, ab16051, Abcam), and anti-β-actin (1:2000 dilution, ab8226, Abcam).

The quantitative real-time PCR assay was performed with RAB11A primers (forward, 5’-TCACAAGGCTGGTGGATAGA-3’ and reverse, 5’-GCACCACCCAACCAGAAG-3’). GAPDH was amplified as an

Abbreviations: EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; OSR, overall survival rate; MTI, 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide NSCLCs, non-small cell lung cancer cells; RIPA, radioimmunoprecipitation; TC, thyroid cancer; PFA, paraformaldehyde; TBST, Tris-buffered saline; WNT, wingless-type integration site; TLR, Toll-like receptor; YAP, Yes-associated protein.

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internal control (forward, 5'-GAGTCAACGGATTG-GTCGT-3' and reverse 5'-TTGATTTTGGAGG-GATCTCG-3'). RAB11A shRNA (pLKO.1-puro-Rab11a) and control plasmids were purchased from Adgene.

**Human samples.** The 50 pairs of human EC tissues and para-carcinoma tissues were acquired from patients diagnosed in The Second Hospital of Dalian Medical University. Patients’ consent was obtained before the experiment. All procedures were performed in accordance with the standards upheld by the Ethics Committee of The Second Hospital of Dalian Medical University (Approval no. 2017014) and the 1964 Helsinki Declaration and its later amendments for ethical research. The clinical-pathological characteristics, such as patient age, gender, pTNM stage, lymph node metastasis, and recurrence were listed in Table 1.

Immunohistochemical (IHC) assays were performed to detect the expression of RAB11A in EC tissues. Briefly, paraffin-embedded tissues were cut into 4 μm sections. Then, the slices were fixed in 4% paraformaldehyde (PFA) for 1 h at room temperature and subsequently immersed in 2% BSA for 30 minutes at room temperature. The slices were incubated with anti-RAB11A antibodies at room temperature for 2 hours. Subsequently, the sections were stained with biotinylated secondary antibody for another 1 hour and visualized with diaminobenzidine. RAB11A was mainly localized in the cytoplasm of EC tissues. The staining scores for RAB11A were evaluated as follows: 0 for negatively stained cells; 1 for 1–20% stained cells; 2 for 21–60% stained cells; 3 for 61–100% stained cells. The intensity of staining was evaluated as 0 (no staining), 1 (weak staining), 2 (medium staining) and 3 (strong staining). The expression levels of RAB11A were scored and calculated using the following formula: score = the score of staining intensity × the score of stained cells percentage. The samples were identified as “high-expression” with score ≥3, while the others were considered “relatively low-expression”.

Cell culture and transfection. The human EC cell lines, TE-10 and Eca109, obtained from ATCC were maintained in Dulbecco’s Modified Essential Medium (DMEM), with 10% fetal bovine serum (FBS, Gibco, CA, USA), in a humidified incubator with 5% CO₂.

For RAB11A knockdown, Lipofectamine 2000 (11668019, Invitrogen, CA, USA) was used to transfect RAB11A shRNA plasmids into TE-10 and Eca109 cells. For the control group, the shRNA targeting sequence was nonsense and could not target any intracellular RNAs. TE-10 cells with shRAB11A stable transfection were screened with puromycin and used for the further in vitro experiments.

Quantitative PCR assays. Extraction of total RNA from tissues or cells was conducted with Trizol (15596026, Invitrogen, CA, USA). Then, RNA was reverse-transcribed using M-MLV reverse transcriptase (M1701, Promega, Wisconsin, USA). SYBR Ex Taq kit (638319, Takara, Japan) was used for quantitative PCR and GAPDH was used as the control.

Immunoblot assays. Proteins from EC cells or tissues were extracted with Radioimmunoprecipitation (RIPA) Buffer (9800, Cell Signaling, Danvers, MA). Then, SDS-PAGE was performed and the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated in 5% milk in Tris-Buff ered Saline with Tween (TBST) buffer and then probed with indicated primary antibodies at room temperature for 2 hours. Then, the PVDF membranes were incubated with HRP-conjugated secondary antibodies for 45 minutes and analyzed using an ECL system.

| Characteristics | Number of patients | RAB11A Low expression (≤ medin) | RAB11A High expression (> medin) | P value |
|-----------------|--------------------|---------------------------------|---------------------------------|---------|
| Ages (years)    |                    |                                 |                                 |         |
| <55             | 26                 | 11                              | 15                              | 0.258   |
| ≥55             | 24                 | 14                              | 10                              |         |
| Gender          |                    |                                 |                                 |         |
| Male            | 18                 | 7                               | 11                              | 0.239   |
| Female          | 32                 | 18                              | 14                              |         |
| T2              | 28                 | 20                              | 8                               |         |
| T3/T4           | 22                 | 5                               | 17                              |         |
| Tumor grade     |                    |                                 |                                 |         |
| Low             | 16                 | 6                               | 10                              |         |
| High            | 34                 | 19                              | 15                              | 0.001   |
| Lymph node metastasis |        |                                 |                                 | 0.390   |
| Yes             | 21                 | 12                              | 9                               |         |
| No              | 29                 | 13                              | 16                              |         |
| Source          |                    |                                 |                                 | 0.000** |
| Yes             | 29                 | 7                               | 22                              |         |
| No              | 11                 | 18                              | 3                               |         |

*P<0.05, **P<0.001
Colony formation assays. EC cells were seeded into 6-well culture plates at a density of 1000/per well. 5 µl transfection reagent, 1 µg plasmid and 250 µL of serum-free medium were mixed for 5 minutes at room temperature. 5 minutes later, the mixed solution was added to the 6-well culture plate and incubated for 6 hours. Then, the cells were cultured for 14 days (medium was replaced every 2 days). The cells were fixed with PFA for 30 minutes at room temperature, rinsed in PBS and stained with 0.2% crystal violet at room temperature for 30 minutes. The colonies were quantified manually.

MTT assays. EC cells were seeded into 96-well plates at a density of approximately 1000 cells each well and incubated for 24 hours. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was added and incubated for 4 hours at room temperature and the culture medium was removed. Subsequently, the cells were washed with PBS and dimethyl sulfoxide (DMSO) was added. The absorbance was analyzed with a microplate reader at 490 nm wavelength.

Wound-healing assays. Cell migration was assessed using wound-healing assays. In brief, cells transfected with the indicated plasmids were scraped with a 20 µL tip to make a wound, followed by rinsing. Subsequently, the cells were maintained in the complete culture medium to stimulate wound healing. Photographs were captured at 0 hours and 24 hours time-point to evaluate the migration of EC cells.

Cell invasion assays. Cell invasion was measured using Transwell chambers (8 µm pore size; Corning) with Matrigel. After transfection, cells in FBS-free DMEM medium were seeded into the upper chamber with 10% Matrigel. The complete medium was added to the bottom chamber. After incubation for 24 hours, the cells in the upper chamber were stimulated to invade into the bottom chamber. Then, the upper chamber medium was removed, and the lower chamber cells were fixed in PFA and visualized with 0.2% crystal violet. The invasive cells were counted under a microscope.

Tumor growth assays. The animal experiments were approved by the Laboratory Animal Ethics Committee of Dalian Medical University. 6 female Balb/C nude mice aged 6 weeks were used in each group, and all mice were given adequate food and water and did not die normally. The mice were sacrificed by cervical dislocation before the tumor tissue was removed. Adequate humanitarian measures were taken. For RAB11A depletion, TE-10 cells were stably infected with control or RAB11A shRNA lentivirus. Subsequently, approximately 5×10⁵ TE-10 cells infected with the indicated lentivirus were implanted into athymic nude mice subcutaneously. Tumor volume was measured every week. After 6 weeks, the tumors were extracted and photographed, and the tumor growth curves were calculated.

Statistics. Statistical analysis was performed with GraphPad 6.0 software. All results were shown as mean ± S.D. The correlation between clinical features of EC patients and RAB11A expression levels was analyzed using the χ² test. Student’s t-test was used for statistical comparisons. *indicated P<0.05, **indicated P<0.01, and ***indicated P<0.001, which was also considered as a statistically significant difference.

RESULTS

RAB11A expression was up-regulated in human esophageal cancer (EC) tissues and predicted poor prognosis

The RAB11A expression was assessed in EC and para-carcinoma tissues from 50 patients. IHC staining results showed that the expression of RAB11A in para-carcinoma tissues (Fig. 1A, left) was lower than that in EC tissues (Fig. 1A, right). In addition, quantitative PCR assays were performed to detect the mRNA levels of RAB11A in EC tissues and para-carcinoma tissues and showed the higher mRNA levels of RAB11A in EC tissues than...
para-carcinoma tissues (Fig. 1B). Based on the RAB11A mRNA levels, 50 patients were divided into 2 groups, low RAB11A group (≤median) and high RAB11A (>median) group (n=25 in each group). Figure 1C shows that the number of patients with high RAB11A expression was larger than that with low RAB11A expression. The K-M survival analysis showed that the expression of RAB11A closely correlated with the survival rate of patients with EC and that high RAB11A expression predicted poor survival of the patients (Fig. 1D).

The expression of RAB11A was significantly related to the clinical-pathological features of EC patients

The correlation between RAB11A expression and clinical-pathological features including age, gender, pTNM stage, tumor grade, lymph node metastasis, and recurrence, was analyzed. There was no significant correlation between RAB11A expression and age (P=0.258), gender (P=0.239), tumor grade (P=0.225), nor lymph node metastasis (P=0.390). However, RAB11A expression was related to pTNM stage (P=0.001*) and recurrence (P=0.000*) of EC patients (Table 1). To further confirm the correlation between RAB11A expression and clinical features, we performed multivariate Cox regression analysis. We also noticed that RAB11A expression correlated with the pTNM stage (P=0.043*) and recurrence (P=0.048*) of patients with EC, consistent with the previous data (Table 2). Therefore, RAB11A expression was related to the clinical features of EC patients.

RAB11A depletion decreased the proliferation of EC cells in vitro

To further clarify the involvement of RAB11A in EC progression, shRNA plasmid targeting RAB11A was used to lower its expression in human EC cell lines, TE-10 and Eca109. The transfection efficiency was confirmed by quantitative PCR assays (Fig. 2A) and western blotting (Fig. 2B).

Subsequently, MTT and colony formation assays were performed to explore the effects of RAB11A on the proliferation of TE-10 and Eca109 cells in vitro. MTT assays showed that the OD value at 490 nm was obviously decreased in TE-10 and Eca109 cells after RAB11A was knocked down compared to the shControl group (Fig. 2C). Similarly, RAB11A knockdown led to the reduction in colony number of TE-10 and Eca109 cells (Fig. 2D). The expression levels of Cyclin D1 and Ki67, two cell cycle- and proliferation-related markers, were detected in TE-10 and Eca109 cells after transfection with RAB11A shRNA or control plasmids. RAB11A depletion decreased the expression of Cyclin D1 and Ki67 in TE-10 and Eca109 cells as compared to the shControl group (Fig. 2E).

RAB11A depletion decreased the migration and invasion of EC cells

Subsequently, the involvement of RAB11A in the migration and invasion of EC cells was determined. RAB11A depletion resulted in the decreased migratory...
RAB11A mediates esophageal cancer progression

RAB11A promoted EC tumor growth via WNT pathway in mice

DISCUSSION

EC has become a common malignancy of the digestive tract that affects about 300,000 people worldwide.
each year (Triantafyllou et al., 2020). Though surgical treatment is the most common treatment for early EC (Yamamoto et al., 2020), in patients with advanced EC high heterogeneity and metastasis are observed, targeted therapies have thus emerged as a new therapeutic direction (Xu et al., 2020). In order to improve the survival rate of patients with EC, it is still urgent to develop new EC therapeutic targets. Importantly, in the present study, RAB11A was upregulated in human EC tissues and predicted poor prognosis. Moreover, the expression of RAB11A was associated with the clinical features, including pTNM stage and recurrence of patients with EC, suggesting that RAB11A was closely related to EC progression. Taken this data together, RAB11A might serve as a promising therapeutic target for EC treatment.

Figure 4. RAB11A depletion suppressed WNT pathway in EC cells. The protein levels of GSK3β, phosphorylated GSK3β (pGSK3β), and β-catenin in TE-10 or Eca109 cells were detected using immunoblot assays. All experiments were repeated three times, and the results were presented as mean ± S.D., **indicates P<0.01.

Figure 5. Knockdown of RAB11A suppressed EC tumor growth via WNT pathway in vivo. (A) TE-10 cells infected with shRAB11A or shControl (n=6 in each group) were implanted into nude mice. Tumor volume was monitored every week. After 6 weeks, tumors were isolated. The silencing efficiency of RAB11A shRNA was shown in panel (A), representative images of tumors were shown in panel (B). Mouse weight (C) and tumor growth curves (D) were calculated. (E) Immunohistochemical assays revealed the expression levels of RAB11A, Ki67 and β-catenin in tumor tissues of mice in shRAB11A or shControl group. Results were presented as mean ± S.D., **indicates P<0.01, ***indicates P<0.001.
Using MTT, colony formation, wound closure and Transwell assays, RAB11A was proved to promote the proliferation, colony formation, migration and invasion of EC cells. These in vitro experiments provided evidence showing that RAB11A contributed to the development of EC in a variety of regulatory pathways, which was also confirmed in the mice model of EC. Similarly, numerous studies indicated that RAB11A could act as an oncogene in multiple types of cancers, such as breast cancer, lung cancer, and pancreatic cancer (Palmieri et al., 2006; Khandelwal et al., 2013; Bai et al., 2017; Dong et al., 2017). A microRNA, miR-320a, suppressed the proliferation and migration of breast cancer cells via targeting RAB11A (Wang et al., 2015). Additionally, RAB11A was regulated by Inc-SNHG1 in invasive pituitary tumors (Wang et al., 2018). RAB11A also contributed to the proliferation and motility of NSCLC via regulating YAP (Dong et al., 2017). These investigations, together with our findings, indicated that RAB11A might act as a promising EC therapeutic target.

RAB11A is known as a member of Ras superfamily of small G proteins which could mediate vesicle transport in cells (Kazemi-Noureini et al., 2004; Baetz & Goldenring 2013). RAB11A promotes the recycling of a variety of receptors to the cell surface and thus affects multiple cellular processes, which suggests its regulatory effects on cancer progression (Kakar-Bhanot et al., 2019). RAB11A could also regulate E-cadherin transport and lumen formation, which also could potentially affect tumorigenesis (Khandelwal et al., 2008; Elias et al., 2015). Further analysis should clarify whether RAB11A promoted EC progression via similar mechanisms. It is widely known that WNT pathway is important for the development, invasion, and metastasis of multiple types of tumors, such as lung cancer, gastric cancer, and breast cancer (Yu et al., 2016). Notably, RAB11A promoted thyroid cancer growth via WNT/β-Catenin pathway (Yu et al., 2016). Another study indicated that RAB11A sustained WNT pathway to promote the progression of pancreatic cancer. We, therefore, speculated that the involvement of RAB11A in EC progression was via WNT pathway (Bai et al., 2017; Wang et al., 2018). Importantly, multiple proteins affected the progression of EC via this pathway, suggesting that WNT pathway could serve as a promising therapeutic target for EC (Yu et al., 2016; Bai et al., 2017; Wang et al., 2018). Here, immunoblot assays showed that RAB11A regulated the WNT pathway in EC, which was also confirmed in mice model. This study thus revealed the regulatory mechanism underlying the role of RAB11A in EC progression.

In conclusion, this study showed the upregulation of RAB11A in human EC tissues and cells. The expression of RAB11A was correlated with poor prognosis and clinical characteristics of patients with EC. Furthermore, RAB11A promoted the proliferation, migration and invasion of EC cells via WNT pathway, which was also confirmed in vivo. Therefore, our findings indicated RAB11A could serve as a promising therapeutic target for EC.

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Not applicable.

Conflict of interest
The authors declare that no conflict of interest is associated with this work.

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Not applicable.

Ethics approval and consent to participate
Patients’ consent was obtained before clinical material collection. All procedures performed in this study involving human participants were in accordance with the standards upheld by the Ethics Committee of The Second Hospital of Dalian Medical University and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects.

Animal assays performed in this study were approved by the Laboratory Animal Ethics Committee of Dalian Medical University.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
BW conceived and designed the experiments, DYZ analyzed and interpreted the results of the experiments, HWC performed the experiments.

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