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Original Article

SEC24A stimulates oncogenicity of human gastric cancer cells

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Abstract: Gastric cancer (GC) is a worldwide health concern and is the second common malignancy. Despite rapid progression in diagnostic and therapeutic approaches over the past decades, the molecular mechanisms underlying the development and progression of GC remain unclear. SEC24 homolog A, COPII coat complex component (SEC24A) belongs to a protein family that are homologous to yeast SEC24, and is critical for neural tube closure. Thus, we focus on the relation of SEC24A and human GC cells. In our study, we found that SEC24A was highly expressed in tumor tissue compared to non-tumor tissue. Furthermore, less aggressive behavior was observed in the si-SEC24A transfected human GC cells (SGC-7901 and BGC-823). On the other hand, we have also found that overexpression of miR-101-3p down-regulated the expression of SEC24A. SEC24A played a role in promoting invasion and metastasis of human GC cells.

Keywords: SEC24A, gastric cancer, oncogene, miR-1

Introduction

Emerging evidence has suggested that gastric cancer (GC) is the second most common cancer and the third leading cause of cancer deaths worldwide [1-3]. Although improved screening techniques now help detect GC and advanced diagnostic and therapeutic approaches significantly improves patients survival, GC is the second most common cancer in men and third most common in women in china [4]. GC, like other cancers, occurs through multi-step genetic alterations, including activation of oncogenes or inactivation of tumor-suppressor genes. Better understanding of the molecular mechanisms and gene alterations could reveal GC initiation and progression and lead to more effective control of GC clinically.

Coat protomer complex II (COPII) is a coatomer, generates intermediate carriers for protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus [5, 6]. SEC24 homolog A, COPII coat complex component (SEC24A) is a cytosolic protein, belonging to the COPII secretory machinery, which assembles in a processive and stepwise fashion [7, 8]. Until now, research has been focused on correlation of a receptor-mediated lipoprotein clearance mechanism with SEC24A [9]. Unfortunately, it is hard to find data on the literature thar clearly demonstrate the relationship between SEC24A and malignant behavior in GC. Hence, more studies are needed to elucidate the biological functions of SEC24A in carcinogenesis, and provide novel perspectives into the development and progression of human GC.

The aim of this study was to determine the role of SEC24A in GC, and its downstream mechanisms. We report herein that SEC24A stimulates oncogenicity and metastasis of human GC cells and the fragments of SEC24A 3'UTR harboring the binding sites for miR-101-3p. However, the role of SEC24A in the multi-step process of human GC by miR-101-3p remains to be further studied.

Materials and methods

Patients and specimens

There are two cohorts of clinical samples included in current study. The study was consti-

tuted by 100 paraffin-embedded surgical tissue specimens obtained from GC cases, and 100 paraffin-embedded surgical control samples collected from a morphologically estimated normal gastric tissue. Tissue specimens were collected from patients attending the First Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China) presenting between 2009 and 2015. All patients who had undergone chemotherapy or radiation therapy before surgery were excluded from this study, and no evidence of rheumatic disease, acute infection, human immunodeficiency virus (HIV) or other types of cancer. The pathological tumor stage of the specimens was consistent with the sixth edition of the tumor-node-metastasis (TNM) classification in accordance with the 2003 World Health Organization (WHO) classification of tumors.

The protocol in this study for the use of tissue samples from patients were approved by our Institutional Review Boards of the First Affiliated Hospital of Anhui Medical University, and the informed consent form was signed by each patient or their guardians.

Immunohistochemistry (IHC) analyses

For immunohistochemical analysis of SEC24A expression in tissue samples, a rabbit polyclonal antibody against SEC24A was obtained from Proteintech Group (Proteintech Group, Chicago, USA) and used at a dilution of 1:50 according to manufacturer's protocol. A Two-Step Histostaining kit (Maixin Biotech Co., Ltd., Fuzhou, China) was performed in our study. IHC analysis of paraffin-embedded specimens was performed as described previously [10]. In brief, the sections were deparaffinized in xylene and rehydrated in graded series of ethanol solutions. Slides were then heated in a microwave oven in 0.01 M sodium citrate buffer (PH 6.0) for 20 min for antigen retrieval. Expression of SEC24A protein in GC or normal tissues were reviewed and photographed by a light microscope (Olympus).

Cell lines and culture

Four human GC cell lines (BGC-823, AGS, MGC-803 and SGC-7901) and one human gastric epithelial cell (GES-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were cultured in their optimal growth medium, recommended by the supplier, that is, GES-1, BGC-823 and SGC-7901 cells were cultured in serum- or antibiotic (ampicillin and streptomycin) -supplement Roswell Park Memorial Institute (RPMI) 1640, AGS was grown in serum- or antibiotic -supplement Dulbecco's modified Eagle's medium (DMEM)/F-12, and MGC-803 was grown in serum- or antibiotic-supplement Dulbecco's modified Eagle's medium (DMEM). All cell culture medium contain 10% fetal bovine serum, and all cell lines were maintained in a humidified incubator under 5% CO_2 at 37°C.

Western blot analysis

Cultured cells were lysed by RIPA lysis buffer (Beyotime, China) for 15 min at 4°C and total protein was extracted according to the manufacturer's instructions. Sample containing 60 ug of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then and transferred to NC or PVDF membranes [11-13]. After that, the membranes were washed with secondary water and blocked with 5% defatted milk at 37°C for 1 hour. After being washed three times with Tris-buffered saline with Tween-20 (TBST), specific primary antibodies were applied to incubate the membranes overnight at 4°C to detect corresponding proteins. After washing, secondary antibodies (goat anti-rabbit or anti-mouse antibodies) were used for the membranes for 1 hour. The membranes were again washed three times with TBST and protein bands were identified using an ECL system (Millipore, Bedford, MA, USA).

The antibodies used were as follows: a rabbit anti-SEC24A polyclonal antibody (Proteintech Group, Chicago, USA) and a mouse anti-β-actin monoclonal antibody (Proteintech Group, Chicago, USA).

RNA oligonucleotides and transfection

MicroRNA (miRNA, miR-101-3p) mimic, small interfering RNAs (SEC24A) and negative control RNA were purchased from GenePharma (Shanghai, China). BGC-823 and SGC-7901 were seeded into 6-well plates and selected for miR-101-3p mimic, si-SEC24A or negative control transfection. Briefly, transfection was performed in the final concentration of 50 nM by LipofectamineTM 2000 Transfection Reagent (Invitrogen, USA) according to the manufacturer's protocol. The efficiency of RNA trans-

Table 1. Expression of SEC24A in gastric cancer and normal tissues

Group	*n*	SEC24A expression	
		Negative, *n* (%)	Positive, *n* (%)
Cancer	100	32 (32.0)	68 (68.0)*
Normal	100	56 (56.0)	44 (44.0)

Note: *$P = 0.001$.

fection was confirmed by Western blot analysis.

Cell proliferation assay

GC cells (BGC-823 and SGC-7901) were harvested after transient transfection with Anti-SEC24A#1 siRNA, Anti-SEC24A#2 siRNA or control siRNA (GenePharma, Shanghai, China) for up to 48 hours. Then cells were seeded in 60-mm dishes at a density of 1×10^5 cells/plate. The cell numbers were counted using a cell counting chamber at a different time. Cell counting assay was carried out at day 2, 3, 4, 5. All experiments were performed in triplicate and repeated once. The result are plotted as mean ± standard deviation.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay

For cell viability, a total of 1×10^3 siSEC24A or siNC (GenePharma, Shanghai, China) transfected GC cells were seeded in 96-well plates in RPMI-1640 medium (100 ul) containing 10% fetal bovine serum and cultured desired period for exposure. Then 10 ul yellow MTT reagent was added in the cells per well. The cultures were incubated for 4 hours at 37°C until purple precipitate was visible. After 100 ul Dimethyl Sulphoxide (DMSO) was added into each well to dissolve formazan crystals, and mixed it to ensure complete solubilization at percussion table for 15 minutes. Absorbance at 570 nm was detected using an automatic microplate reader (Thermo, USA).

Cell migration and invasion assay

A transwell insert (8 um pore size, Corning Costar, USA) was used to determine the effect of siSEC24A (GenePharma, Shanghai, China) on GC cells (BGC-823 and SGC-7901) migration and invasion *in vitro*. Briefly, GC cells were first transfected with siSEC24A or control siRNA (GenePharma, Shanghai, China). 48 hours later, the cells were starved in a medium without fetal bovine serum overnight. Transfected cells (1×10^5) were resuspended in the upper chamber. Upper chamber of each well was filled with 300 ul of culture medium. Nevertheless, RPMI-1640 containing 10% fetal bovine serum was provided to the lower chamber as a chemoattractant and incubated for 24 hours [13, 14]. For the invasion assay, the inserts were coated with 100 ul extracellular matrix gel previously. After visual fields of each insert were fixed and stained with 0.1% crystal violet for 30 minutes, the cells per plate were randomly chosen and photographed by a light microscope.

Luciferase reporter gene assay

The 3'-untranslated region (3'UTR) of human SEC24A (2817bp, Genebank access number: NM_001252231.1) was amplified from human genomic DNA and inserted into psiCHECK2 vector (Promega). For luciferase assay, BGC-823 (1×10^6) were seeded in 6-well plates, cultured overnight, and then co-transfected with 0.2 ug of either SEC24A 3'UTR-WT or SEC24A 3'UTR-MUT and 20 pmol miR-101-3p or its negative control (GenePharma, Shanghai, China) by using Lipofectamine 2000. The cells broke open and luciferase activity was measured by Dual Luciferase Assay (Promega) after 48 hours. Firefly luciferase activity of this mixture was then quantified using a Dual-Luciferase Reporter Assay System (Promega).

Bioinformatic and statistical analyses

Bioinformatic analysis was performed using the miRNA database Targetscan [15] (Release 7.1, http://www.targetscan.org). To assess the differences between experimental groups, statistical analyses were carried out using Microsoft Excel software and GraphPad Prism. Chi-squared (X^2) test was used to analyze the difference in the expression levels among different samples. Statistical significance was assesses by Student's t-test ($P < 0.05$ was considered as statistically significant). All numerical data are expressed as mean ± standard error of the mean from a representative experiment performed in triplicate.

Result

Expression of SEC24A in gastric tissue specimens and gastric cell lines

To explore the effect of SEC24A on development and progression of GC, we first performed

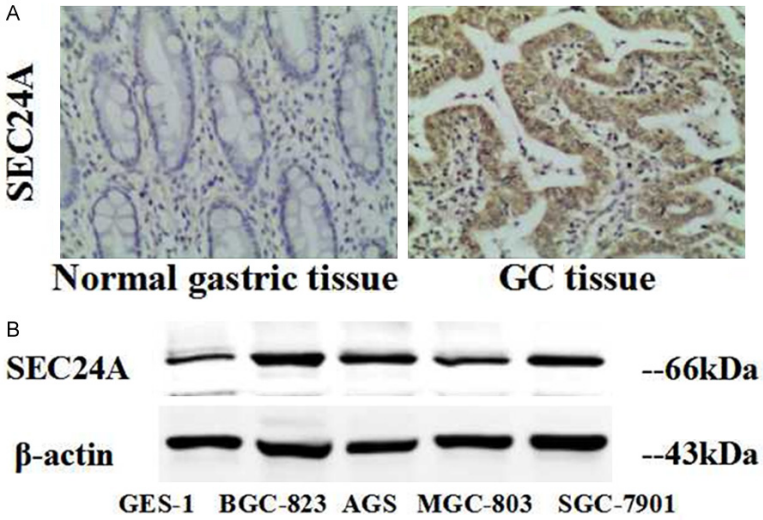

Figure 1. Detection of SEC24A protein in gastric tissue specimens and cell lines. A. Immunohistochemical staining of SEC24A in gastric tissue samples. Left panels, low expression of SEC24A protein in normal tissue. Right panels, high expression of SEC24A protein in GC. All photos at ×200 original magnification. B. GES1, BGC-823, AGS, MGC-803 and SGC-7901 cells lysates were subjected to Western blot analysis with the indicated antibodies. β-actin was used as loading control for cell lysates. The sizes of detected protein bands in kiloDalton (kDa) are shown on the right.

Table 2. Correlation of SEC24A expression with clinicopathological parameters from gastric cancer patients

Parameter	*n*	SEC24A expression	
		Positive, *n* (%)	*P* value
Age (years)			
≤ 60	53	34 (64.2)	0.381
> 60	47	34 (72.3)	
Gender			
Male	54	34 (63.0)	0.242
Female	46	34 (73.9)	
Tumor size (cm)			
≤ 5	67	41 (61.2)	0.038
> 5	33	27 (81.8)	
Lymph node metastasis			
No	37	29 (51.4)	0.006
Yes	63	49 (77.8)	
Grade			
I	7	3 (42.9)	0.097
II	62	40 (64.5)	
III	31	25 (80.6)	
Stage			
I-II	54	32 (59.3)	0.042
III-IV	46	36 (78.3)	

utilized IHC analyses in gastric tissue from benign gastric disease and GC. SEC24A protein expression was observed in 68 (68%) of the 100 GC tissue samples. In contrast to GC, 44 (44%) benign gastric disease specimens were positive for SEC24A, which was a significantly lower than that observed in GC tissues (P = 0.001, **Table 1**). As shown for us, in the case of GC tissue, significantly higher levels of SEC24A protein was demonstrated and localized in the cytoplasm of the epithelium than in normal gastric tissue (**Figure 1A**).

Furthermore, we utilized Western blot assay to determine the protein level of SEC24A in different gastric cell lines. We chose one normal human gastric epithelial cell GES-1 and four GC cell lines (BGC-823, AGS, MGC-803 and SGC-7901). We found that SEC24A expression was significantly higher in GC cell lines than that of the normal GES-1 cells (**Figure 1B**). In addition, BGC-823 and SGC-7901 cells were selected as a couple of model cells for further study.

Correlation between expression of SEC24A and clinicpathological features of GC

We then observed potential association of tumor expression of mRNA or protein for SEC24A with the clinicopathologic features of GC. We found that patients whose tumors expressed SEC24A protein exhibited a positive association with tumor size (P = 0.038), lymph node metastasis (P = 0.006) and GC stage (P = 0.042). No significant association was observed between tumor expression and patient age, gender or GC grade (all P > 0.05) (**Table 2**).

Depletion of endogenous SEC24A significantly down-regulated proliferation, viability, migration, and invasion in BGC-823 and SGC-7901 cells

To assess the potential impact of SEC24A on cell biological behaviors, cell function assays were performed in BGC-823 and SGC-7901 cell lines. First, we depleted BGC-823 and SGC-7901 cells of endogenous SEC24A with siRNA. The efficacy of SEC24A knockdown was verifi-

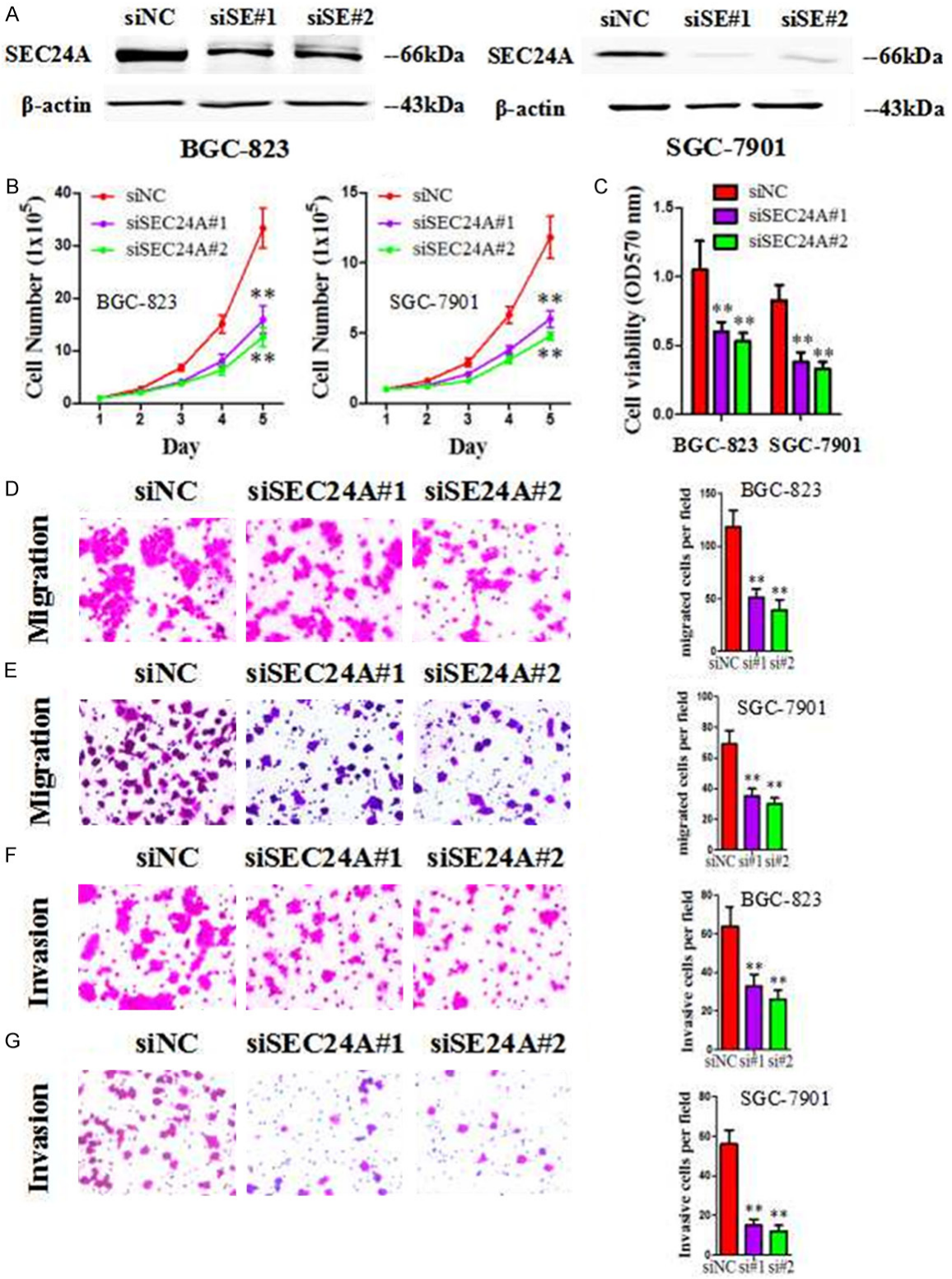

Figure 2. Repression of SEC24A inhibits malignant biological properties of GC. A. The efficiency of SEC24A silencing was checked by Western-blotting in BGC-823 and SGC-7901 cells. B. SEC24A siRNA inhibits cell proliferation. The graphics show the cell proliferation rate at different time points. C. GC cells were plated in 96 well plates. Cell viability was examined by microplate reader after transiently transfected with SEC24A siRNA. D-G. Tumor cell migration and invasion assay. BGC-823 and SGC-7901 cells were grown and transiently with SEC24A siRNA or NC for 48 hours. Then cells were counted and placed in the chambers. Cell invasion was examined using Matrigel-coated transwell chambers. Cells in the upper chamber were removed and those cells that migrated to the lower layer of the inner chamber were stained by crystal violet and counted. **, $P < 0.01$.

Table 3. The sequence of the oligonucleotide primers used for transfection

Gene	Sense Strand (5'-3')	Antisense Strand (5'-3')
SEC24A siRNA 1	GCAUGUGCAAGAACUGUAAGG	UUACAGUUCUUGCACAUGCAG
SEC24A siRNA 2	GGAAGUGUAACUUAUGUUAUC	UAACAUAAGUUACACUUCCAU
NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
miR-101-3p mimics	UACAGUACUGUGAUAACUGAA	

ed by Western blot assays (**Figure 2A**). Next, effects of SEC24A knockdown on BGC-823 and SGC-7901 cellular proliferation were assessed using cell counting. In cell proliferation assay, we observed that depletion of SEC24A decreased cell proliferation in a time-dependent manner compared with the control (**Figure 2B**). After that, we indicated that viable cell number of the cells was significantly decreased with transfection of SEC24A siRNA (the sequences were shown in **Table 3**) compared to the controls (**Figure 2C**). Finally, the transwell assay data showed that GC cells with SEC24A knockdown resulted in decrease of migratory and invasive rate of GC cells (**Figure 2D-G**).

miR-101-3p is a direct target binding site of SEC24A

To examine whether SEC24A exerted their biological functions by modulating miRNAs, we analyzed the potential targets of these miRNAs. Bioinformatic analysis utilized the algorithm of Targetscan showed that SEC24A mRNA contains a 3'UTR element complementary to the miR-101-3p binding site. Each luciferase construct was paired with the corresponding mutant. We generated two luciferase constructs, one of them contained fragments of SEC24A 3'UTR harboring binding sites for miR-101-3p. On the other hand, site-directed mutagenesis was performed to generate SEC24A 3'UTR mutant containing mutations in the conserved miR-101-3p binding site (**Figure 3A**). We purchased miR-101-3p mimics and negative control (the sequences were shown in **Table 3**), We observed that miR-101-3p reduced the activity of firefly luciferase in the 3'UTR luciferase assay, while miR-101-3p could no longer affect the expression of firefly luciferase if miR-101-3p seed sequence were mutated in the SEC24A 3'UTR mutant luciferase assay, suggesting that miR-101-3p would be relevant to SEC24A 3'UTR (**Figure 3B**). We next treated BGC-823 and SGC-7901 cells as model cell lines for Western blot analysis to verify the correlation of SEC24A and miR-101-3p. We found that there was a significant repression of SEC24A protein level by miR-101-3p mimics, compared with negative control (**Figure 3C**).

Discussion

In the last decade, a growing body of evidence indicates that gastric cancer is associated with high morbidity and mortality worldwide [16]. The outcome of gastric cancer may be improved by advanced medical technology. However, gastric cancer remains the second most common cause of cancer-related deaths [17]. GC is a multistep and multifactorial process [18]. Wang W reported that Trefoil Factor 1 (TFF1, also named pS2), which serves as the gastrointestinal mucosal protector, suppress the progression of GC [19]. Nectin-4 had a promoter effect on human GC cell growth and motility by Zhang Y et al. [20]. Although discovery of biomarkers to monitor GC invasion and metastasis could to a large extent help clinicians to be effectively applied in therapy, our understanding of its molecular and cellular mechanisms about SEC24A is still limited.

SEC24 is a component of coat protein II (COPII)-coated vesicles that mediate protein transport from the endoplasmic reticulum. SEC24A/B and SEC24C/D, sharing 60% sequence identity within but only 25% identity across subfamilies constitute mammalian SEC24 paralogs [21, 22]. The secretory pathway of eukaryotic cells packages cargo proteins into COPII-coated vesicles for transport from the endoplasmic reticulum (ER) to the Golgi [6]. More research studies were done in mouse and plant models, but not in human cells [9, 23]. Based on the above findings, we wanted to examine whether SEC24A functions as a promoter or inhibitor in development and progression of GC cell lines.

Herein we documented a significant association of SEC24A expression with clinicopathological parameters (tumor size, lymph node

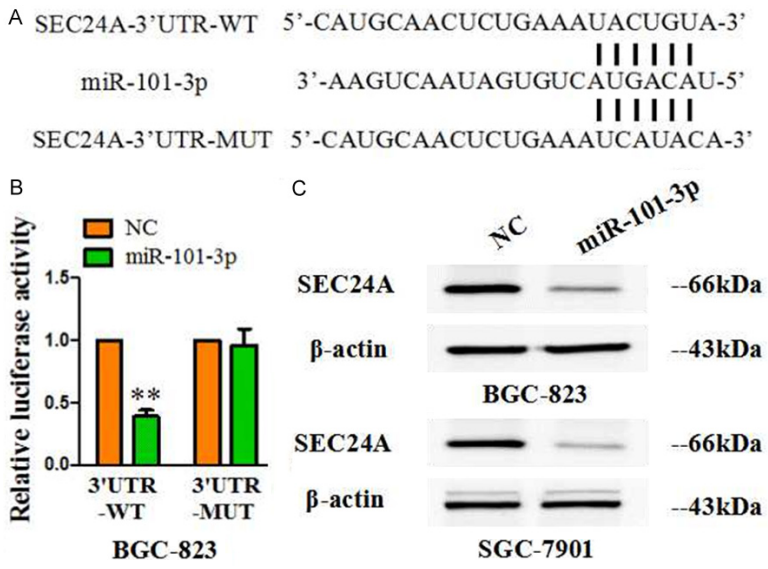

Figure 3. Targets of miR-101-3p interacting with SEC24A. A. Predicted binding site between miR-101-3p and the 3'-UTR of SEC24A or the mutant 3'-UTR of SEC24A used in our study was presented. B. Cells were collected and luciferase activity was measured by a Dual-Luciferase Reporter Assay System. C. Lysates prepared from miR-101-3p treated BGC-823 and SGC-7901 cells were subjected to Western blot analysis. Level of SEC24A decreased as a result of miR-101-3p targeting SEC24A.

metastasis and the status of clinical stage) from gastric cancer patients, but no age, gender, and tumor grade. We have also observed that expression of SEC24A in GC or normal gastric tissue predicted a large and significant difference. In the 200 samples of human gastric tissues (containing 100 cancers and 100 normal tissues), this study supported that SEC24A was upregulated in GC clinical samples compared to normal tissues (68% vs. 44%, P = 0.001). To further corroborate these results, endogenous expression of SEC24A was analyzed in a number of non-cancer and cancer cell lines. As expected, we detected elevated expression of SEC24A in GC cell lines, as compared with the control non-cancer cell. Meanwhile, BGC-823 and SGC-7901 cell lines were chosen for further cell function assays. After that, we have systematically examined the oncogenic role of SEC24A in human BGC-823 and SGC-7901 cell lines. In our study, knockdown of SEC24A inhibited GC cells malignant biological behavior, including the capacity of cell proliferation, viability, migration and invasion according to cell function assays *in vitro*.

miRNAs are a class of endogenous 19- to 25-nucleotide non-coding RNAs, that contribute to regulate gene expression by directly base-pairing to the 3'UTR of mRNAs, and thus modulate mRNA degradation or inhibit translation [15, 24, 25]. Recent studies have shown that down-regulation of miR-101-3p promoted proliferation and migration of hepatocellular carcinoma (HCC) cells [26] 1,25D3 suppresses bladder cancer cell migration and invasion through the induction of miR-101-3p [27]. However, future studies in GC cell lines would be needed to assess this issue about the role of miR-101-3p. Based on the results presented earlier, miR-101-3p is a target binding site of SEC24A 3'UTR by luciferase reporter assay and western blot assay. Notably, this signaling pathway contributed to the promoting role of SEC24A in GC.

In summary, here we have outlined a novel gene-expression pathway in which a protein-coding gene, SEC24A, stimulates GC oncogenicity as a promoter, and the novel perspectives which we proved potentially can be significantly applied to gene therapy.

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Disclosure of conflict of interest

None.

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