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Original Article

ARHGAP9 siRNA inhibits gastric cancer cell proliferation and EMT via inactivating Akt, p38 signaling and inhibiting MMP2 and MMP9

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Abstract: Objective: ARHGAP9 is a RhoGTPase activating protein. This study aimed to investigate the effect of ARHGAP9 on cell proliferation of gastric cancer cell. Methods: Human gastric cancer cell line SGC7901 were transfected with ARHGAP9 siRNA and the expression of epithelial to mesenchymal transition (EMT) factors, MMPs, and activated status of Akt and p38 signaling were detected. Moreover, the migration, invasion, and viability of SGC7901 cells were determined. Results: ARHGAP9 siRNA successfully inhibited cell viability, migration and invasion. The expression of E-cadherin was significantly upregulated, and expression of Snail, Vimentin, as well as MMP2 and MMP9 were obviously downregulated. Moreover, ARHGAP9 siRNA promoted inactivation of Akt and p38 signaling by inhibiting expression of p-Akt and p-p38. Conclusion: This study showed that ARHGAP9 contributed to the viability, migration, invasion as well as EMT in gastric cancer cell line SGC7901. ARHGAP9 might be used as a therapeutic target for gastric cancer.

Keywords: ARHGAP9, epithelial to mesenchymal transition, E-cadherin, SGC7901

Introduction

ARHGAP9, Rho GTPase-activating protein 9, is a member of Rho family of small guanosine triphosphatases (RhoGTPases) activating protein [1]. RhoGTPases take critical roles in the cell epithelial to mesenchymal transition (EMT), migration, and invasion [2, 3]. All these cell functions contributed to cancer cell metastasis and overall survival of cancer patients [4, 5].

Moreover, ARHGAP9 has been identified as a novel mitogen-activated protein (MAP) kinase docking protein [6]. ARHGAP9 interacts with and inhibits activation of Erk2 and p38α. Erk2 is a MAP kinase, and the activation of Erk2 as well as MAPK/ERK2 phosphorylation are essential for cancer cell proliferation and cancer development [7-9]. For example, activated ERK2 pathway induces cell proliferation of various human cancers, including gastric cancer [10], breast cancer [11], and so on.

Gastric cancer lists in the top tumors with high incidence on the world [12, 13]. Evidence had showed that gastric cancer, as other cancers, associates with numerous abnormalities of genetic factors, including A hypoxia-inducible factor-1 [14], MAPK/ERK signaling [10], p38 signaling pathways [15], HOXD10 and Akt pathway [16]. However, there was no study focusing on the relationship of ARHGAP9 with gastric cancer, as well as the association of ARHGAP9 with signaling pathway.

To investigate the association of ARHGAP9 with gastric cancer and signaling pathways such as p38, Akt, and EMT, we suppressed the expression of ARHGAP9 in human gastric cancer cell line SGC7901. The expression of MMP2 and MMP9, and corresponding actors in EMT, p38, and Akt signaling pathways in transfected cells would be detected. This study would provide us with direct information focusing on the association of ARHGAP9 with proliferation of gastric cancer cells and the mechanism involving in it.

Materials and methods

Cell line and culture conditions

Human gastric cancer cell line SGC7901 was purchased (American Type Culture Collection,

Manassas, VA, USA) and Cells were cultured in complete DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc. Waltham, MA, USA) at 37°C in a humidified incubator with 5% CO_2. Cells were prepared for the transfection, extraction of RNA for PCR, and separation of protein for Western blotting analysis.

ARHGAP9 siRNA

SiRNA against ARHGAP9 were chemically synthesized (GeneChem, Shanghai, China), and ARHGAP9 siRNA or control were transiently transfected into SGC7901 cells, seeded into 24-well plates, using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). Cells transfected with ARHGAP9 siRNA or control were incubated at 37°C for 48 h. Next, cells were harvested for the following experiments.

Cell viability assay

Cell viability of transfected SGC7901 cells was assessed using Cell Counting Kit 8 (CCK8, Beyotime, Shanghai, China) assay. In brief, after transfected with ARHGAP9 siRNA or control for 0, 12, 24, and 48 h, cells were trypsinized. Cell suspension were transferred to 96-well plates and allowed to make cells attached to the plate wall. Then, cells were additionally incubated in CCK8 solution for 2 h. The optical density of cells was determined at 450 nm absorbance (A450) using a microplate spectrophotometer (Bio-Rad Labs, Sunnyvale, CA). Each experiment was performed in triplicates.

Migration and invasion assay

For the detection of cell migration and invasion abilities, the Transwell assay were used (Coring, NY, USA). To analyze the cell migration and invasion ability, the upper surface of filter membrane of the Transwell chambers, respectively, were coated with BD Matrigel (BD Biosciences, San Jose, CA, USA) or not [17]. Cells of 1×10^5 were seeded into the serum-free medium in upper compartments and incubated for 24 h. Next, non-migrated cells on the upper surface of the filters were removed and migrated cells on the undersurface of the filter membranes were left. Migrated cells on the fixed with ethanol, stained with Crystal violet for 30 min at 37°C, and counted under a microscope. Cell numbers in five random fields were averaged. All assays were repeated for 3 times.

Western blot analysis

Cultured SGC7901 cells were harvested, and lysed, and quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Cell lysates at equal amount of 20 g protein were separated by 10% SDS-PAGE gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen Corp., Carlsbad, CA, USA), which were then blocked with 5% skimmed milk. Next, blocked membranes were incubated with the primary antibodies against ARHGAP9 (dilution, 1:1000; Abcam Inc., Cambridge, MA, USA), MMP2 (dilution, 1:1000; Abcam), MMP9 (dilution, 1:500; Abcam), Sail (dilution, 1:1000; Cell Signaling Technology, CST, Danvers, MA, USA), E-cadherin (dilution, 1:1000; CST), Vimentin (dilution, 1:1000; CST), p-Akt (dilution, 1:1000; CST), Akt (dilution, 1:1000; CST), p-P38 (dilution, 1:1000; CST), P38 (dilution, 1:1000; CST), and GAPDH, (1:2000 in dilution; CST) at 4°C overnight. Next, membranes were incubated with secondary antibodies (Biyuntian Biotech Co., Ltd., Shanghai, China) at room temperature for 1 h. The polypeptide bands were detected using an enhanced chemiluminescence (Pierce, Rockford, IL, USA) and quantified with AlphaEase software (Alpha, U.S.A.).

Statistical analysis

SPSS version 17.0 (Chicago, IL, USA) was used for all the statistical analysis. All quantitative data are presented as mean ± standard deviations (SD). Comparison among groups was performed by t test and ANOVA. $P < 0.05$ was regarded as significant.

Results

ARHGAP9 siRNA successfully reduced expression of ARHGAP9

Using the siRNA technology, we successfully reduced the expression of ARHGAP9 in the human gastric cancer cell line SGC7901. The SGC7901 cells were transfected with ARHGAP9 siRNA and expression of ARHGAP9 protein were significantly reduced compared to the control and Mock transfection (**Figure 1**, $P < 0.01$).

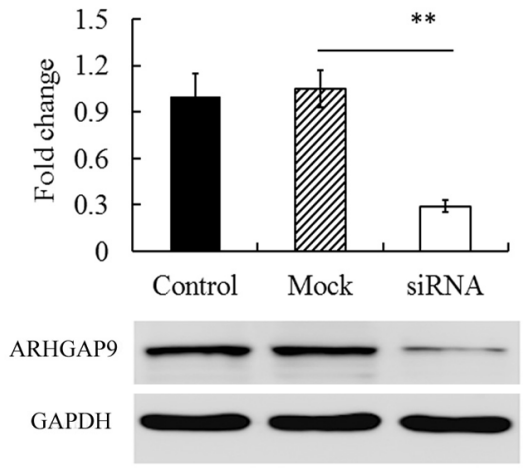

Figure 1. Expression of ARHGAP9 in transfected cells. SiRNA successfully reduced the expression of ARHGAP9 protein the human gastric cancer cell line SGC7901. Expression was detected using Western blotting analysis. **indicates significant level at P < 0.01 vs. Control and Mock.

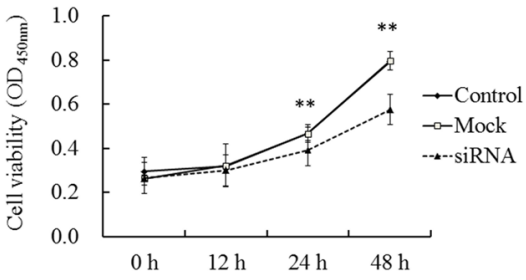

Figure 2. *ARHGAP9* benefits to cell viability. The cell viability was tested using MTT assay. ARHGAP9 siRNA transfected cells showed significant reduction on cell viability, comparing with control. **indicates significant level at P < 0.01 vs. Control and Mock.

ARHGAP9 siRNA inhibits cell viability

In order to investigate the effect of *ARHGAP9* on cell viability, the viability of ARHGAP9 siRNA transfected were detected using MTT assay. The results showed that siRNA obviously reduced the viability of transfected cells at 24 h and 48 h post transfection (**Figure 2**, P < 0.01). However, there were no differences in cell viability between Control and Mock groups during the 48 h post transfection (P > 0.05). This showed ARHGAP9 had influence on cell viability.

ARHGAP9 siRNA inhibits cell migration and invasion

To test the effect of *ARHGAP9* on cell migration and invasion, Transwell assay was employed. The results showed that *ARHGAP9* siRNA obviously reduced the percentage of migrated and invasive cells comparing with control. As **Figure 3** shown, the percentages of migrated and invasive cells of *ARHGAP9* siRNA transfected SGC7901 cells were successfully lower than those of Control or cells transected with Mock vector (**Figure 3A** and **3B**, P < 0.01). There were no differences in the percentages of migrated and invasive cells of *ARHGAP9* siRNA transfected SGC7901 cells between Control and Mock groups (**Figure 3A** and **3B**, P > 0.05). These data showed *ARHGAP9* contributed to cell migration and invasion.

ARHGAP9 changes expression of EMT, Akt and p38

The effects of *ARHGAP9* on epithelial to mesenchymal transition (EMT), Akt and p38 expression were tested using Western blotting analysis. The fold change of MMP2, MMP9, Snail, and Vimentin proteins' expression in transfected cells were significantly inhibited by *ARHGAP9* siRNA transfection in comparison to those in control cells (**Figure 4A**, P < 0.01). On the contrary, the expression of E-cadherin was obviously upregulated by *ARHGAP9* siRNA transfection. The expression E-cadherin in transfected cells was over three fold than those in Control or Mock transfected cells (**Figure 4A**, P < 0.01). Moreover, the expression of activated status Akt and p38 that are p-Akt and p-p38, in siRNA group were significantly reduced by *ARHGAP9* siRNA transfection in comparison with Control and Mock cells (**Figure 4B**, P < 0.01). There were no difference in the expression of total Akt and p38 protein among the three groups (**Figure 4B**, P > 0.05).

Discussion

Gastric cancer is one of the most common cancers on the world, with higher incidence [12, 13]. The pathogenesis of gastric cancer associated with numerous abnormalities of genetic factors [18]. This study aimed to investigate the effect of ARHGAP9 on gastric cancer cells. Just as expected, ARHGAP9 showed great importance on the viability, migration, invasion, as well as expression of EMTs, Akt, p38 signaling pathways.

ARHGAP9 is a novel MAP Kinase docking protein [6], and a RhoGTPases activating protein [1]. RhoGTPase members play great roles in the cell EMT, migration, invasion and adhesion by

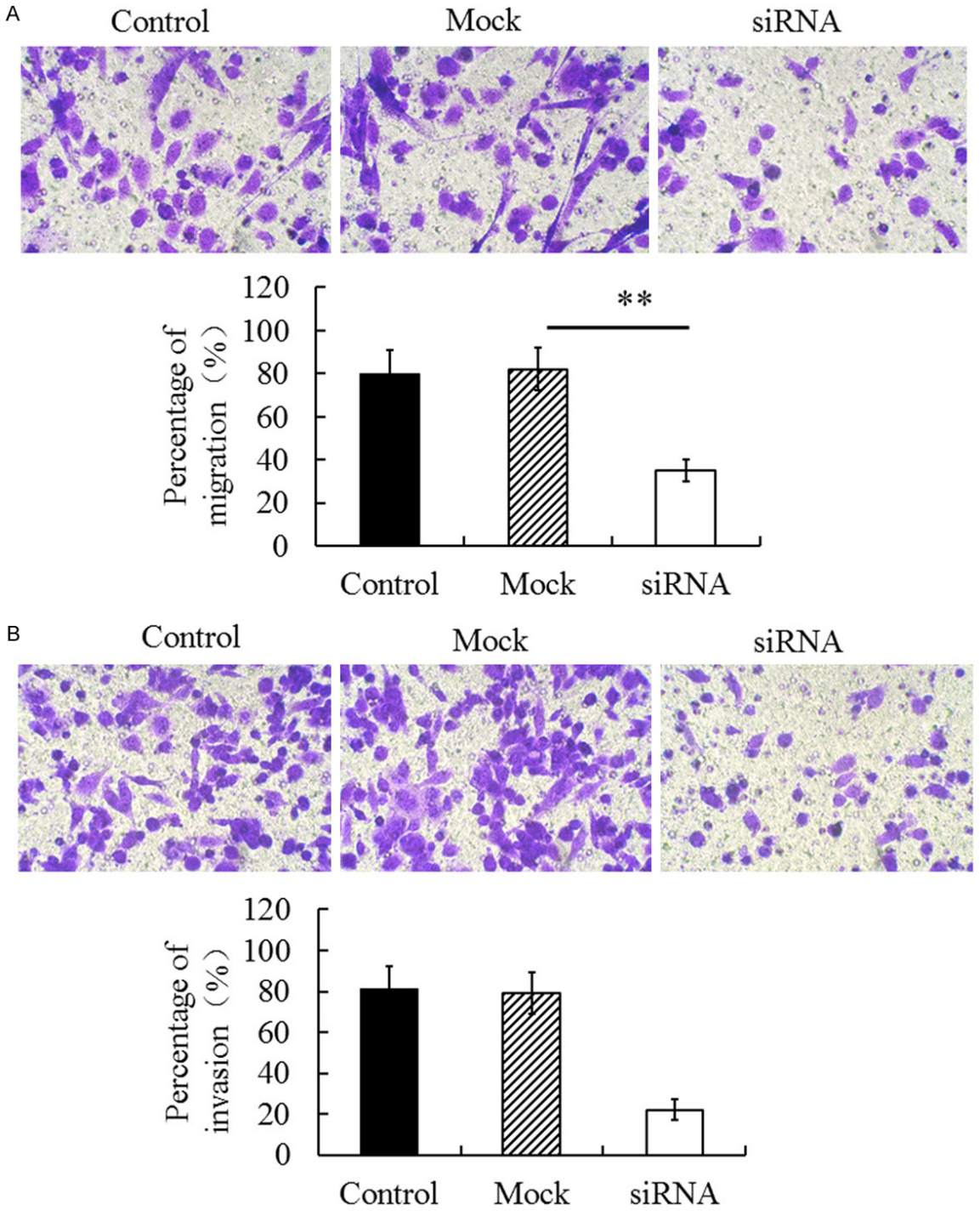

Figure 3. *ARHGAP9* contributes to cell migration and invasion. Cell invasion and migration were tested using Transwell assay. Cells on the undersurface of the filter membranes stained with Crystal violet and cell number in five random fields were averaged. **indicates significant level at $P < 0.01$ vs. Control and Mock.

remodeling the actin cytoskeleton, which tightly related to cell events [2, 3], via acting as coordinators of signaling pathways [1]. Furukawa *et al* firstly demonstrated ARHGAP9 encoded a RhoGTPase activating protein [1]. Bond *et al* had verified that RhoGTPase inhibition with Clostridium difficile Toxin B inhibited the proliferation of vascular smooth muscle cell [19]. In this study, we revealed that the inhibition of ARHGAP9 by siRNA suppressed cell viability,

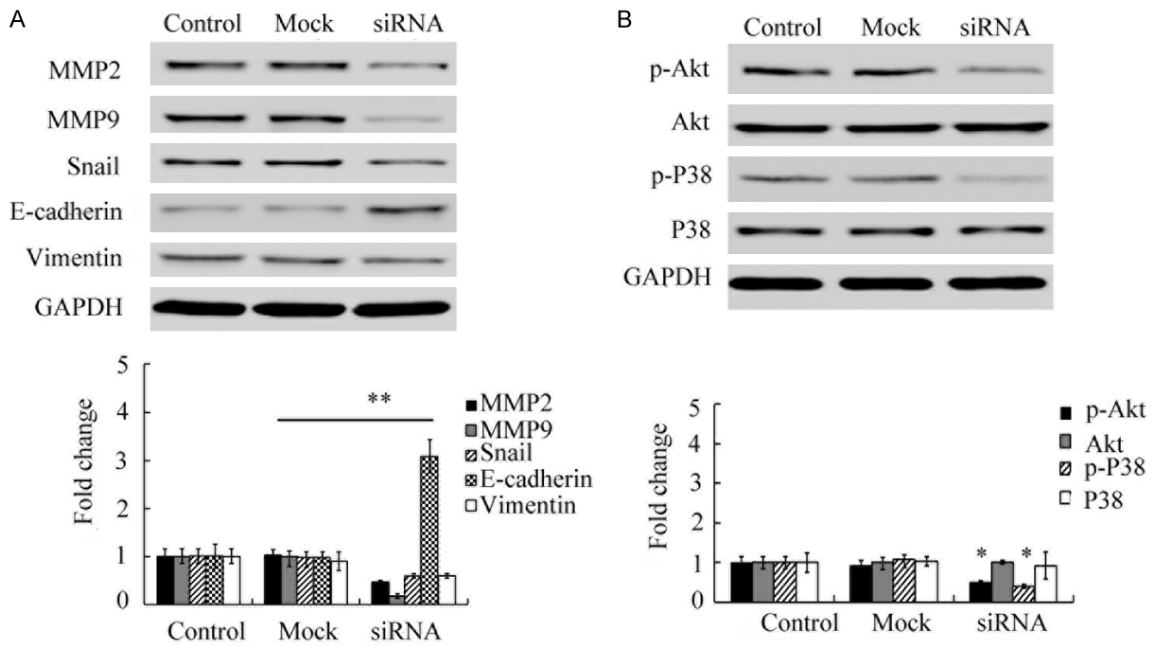

Figure 4. *ARHGAP9* changes expression of EMT, Akt and p38. Protein expressions were detected using Western blotting analysis. * and ** indicates significant level at $P < 0.05$ and $P < 0.01$, respectively, vs. Control and Mock.

migration, and invasion of SGC7901 cells, showing the critical roles of ARHGAP9 in modulating cell proliferation.

Both RhoA and Cdc42 are small RhoGTPases [20]. RhoA pathway had been proven to negatively induce expression of MMP2 and MMP9, as well as MMP2- and MMP9-depended cell invasive behavior [21-23]. The expression of MMP2 and MMP9 always accompany with the induction of EMT [24]. RhoGTPase activation is proven to be a key step and contribute renal epithelial mesenchymal transdifferentiation [25]. Patel *et al* showed that RhoA and Cdc42 expression vector infection significantly downregulated E-cadherin [25], a EMT inhibitor [26, 27]. In this study, we verified that the expression of MMP2 and MMP9, as well as Snail and Vimentin, were significantly reduced by ARHGAP9 siRNA, while the expression of E-cadherin was upregulated by ARHGAP9 siRNA. These showed the inhibition of ARHGAP9 downregulated EMT as well as MMP2 and MMP9.

As reported, EMT is activated by the transcription factor Snail via repressing E-cadherin expression [27], which might result in promotion of cell metastasis [28]. Moreover, inhibition of activated status Akt, p-Akt, inhibited EMT with restoration of E-cadherin expression [29]. Additionally, interaction of Snail and p38 MAP kinase was revealed to result in shorter overall survival of cancer patients [30]. These showed the fact that the expression of p-Akt, p-p38, Snail, and EMT were inhibited by ARHGAP9 siRNA contributed the reduction of cell invasion, migration, and viability of gastric cancer cell line SGC7901.

Conclusion

This study showed that ARHGAP9 contributed to the viability, migration, invasion as well as EMT in gastric cancer cell line SGC7901. The inhibition of ARHGAP9 expression suppressed cell viability, migration and invasion, as well as inhibition of EMT, inactivation of Akt and p38 signaling. All these results demonstrated that ARHGAP9 might be used as a therapeutic target for gastric cancer. However, this assumption and the mechanism of ARHGAP9 modulating cell proliferation, as well as the interaction between proteins should be investigated by more than one experiment.

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Disclosure of conflict of interest

None.

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