Pathology - Research and Practice 215 (2019) 152452

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

DAAM1-mediated migration and invasion of ovarian cancer cells are suppressed by miR-208a-5p

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ARTICLE INFO

Keywords:
DAAM1
miR-208-5p
Ovarian cancer
Metastasis

ABSTRACT

Ovarian cancer (OvCa) has the highest morbidity among all gynecologic cancers worldwide, and its distant metastasis is one of main causes for the poor prognosis of OvCa patients. Our previous studies have reported that DAAM1-involved signaling pathways play vital roles in metastasis of breast cancer. However, whether DAAM1 participates in OvCa migration and/or invasion is still unknown. The impact of DAAM1 on cell migration and invasion in OvCa was evaluated by wound healing assay and Boyden chamber assay. The specific miRNA targeting *DAAM1* was predicted by bioinformatics methods and verified by dual-luciferase activity assay. The miR-208a-5p expression levels in OvCa tissues and the impacts of miR-208a-5p on cell migration and invasion were also assessed, respectively. High expression of DAAM1 was associated with distant metastasis in OvCa. Silence of DAAM1 by siRNA blocked the migration and invasion of OVCAR-3 cells. MiR-208a-5p directly targeted *DAAM1* and was shown a decreased expression in metastatic OvCa tissues. Elevated expression of miR-208a-5p inhibited the migration and invasion of OVCAR-3 cell which can be rescued by DAAM1 overexpression. Our data suggest that miR-208-5p/DAAM1 axis participates in OvCa migration and invasion and may be a novel clinical target to limit OvCa metastasis.

1. Introduction

Ovarian cancer (OvCa) is a common cancer which has the highest morbidity among females' malignancies and causes 14,070 cancer-related deaths in USA in 2018. OvCa accounts for 2.5% of all gynecologic cancers, but 5% of cancer-related deaths due to its poor prognosis [1,2]. Although the continuous improvement of surgery and comprehensive therapy for primary lesions of OvCa has been significantly improved in recent years, there is no effective method to limit the metastasis of OvCa. Thus, it is still significant to uncover the specific mechanism of invasion and metastasis of OvCa.

DAAM1 is an actin-associated regulatory factor and participates in regulating cell motility *via* mediating noncanonical Wnt signaling [3–5]. DAAM1 is essential for various actin-dependent cellular events and has been identified to function as a critical regulator in vertebrate gastrulation, filopodia formation, centrosome reorientation, *etc* [3,6,7]. DAAM1 is a midstream signal transducers of Wnt5a and type IV collagen to activate small G protein RhoA, thus resulting in the elevated cell migration and haptotaxis in breast cancer [5,8]. Nevertheless, the function of DAAM1 on OvCa metastasis is still largely unknown.

MicroRNAs (miRNAs) are endogenous and non-coding small RNAs with 21–23 nucleotides in length, binding to the 3′ non-coding region (3′-UTR) of the target gene, leading to the degradation of mRNA and/or repression of protein translation [9]. MiRNAs is essential for numerous biological behaviors of cancer cells *via* targeting cancer-related genes expression on post-transcriptional level [10–12]. Nonetheless, the potential mechanisms of miRNAs in regulating DAAM1 expression in OvCa has not been explored up to data.

Here, we demonstrate that DAAM1 is essential for cell migration and invasion in OvCa through activating RhoA to regulate the

Abbreviations: DAAM1, dishevelled-associated activator of morphogenesis 1; OvCa, ovarian cancer; SiRNA, small interfering RNA; UTR, untranslated region; MiRNA, microRNA; QRT-PCR, quantitative real-time PCR

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https://doi.org/10.1016/j.prp.2019.152452
Received 28 February 2019; Received in revised form 30 April 2019; Accepted 12 May 2019

microfilament formation. On the other hand, miR-208a-5p restrains the OvCa cells migration and invasion *via* directly targeting DAAM1.Taken together, these findings definite the significance of miR-208a-5p/DAAM1 axis in cell migration and invasion in OvCa and suggest this signaling as a novel clinical target in controlling OvCa metastasis and improving the prognosis of this disease.

2. Materials and methods

2.1. Clinical specimens

We total collected 61 OvCa tissues in this research and all the patients were recruited by the First Affiliated Hospital with Nanjing Medical University and the Affiliated Jiangyin Hospital of Southeast University Medical College from 2016 to 2019. OvCa tissues were histopathologically confirmed by a pathologist *via* hematoxylin-eosin (HE) staining for selected areas of higher tumor cell density. The clinicopathological characteristics information of each patient is obtained by consulting his medical documents. Ethical approval for the study was granted by the Clinical Research Ethics Committee, Affiliated Jiangyin Hospital of Southeast University Medical College, and our research was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant.

2.2. Cell culture and transfection

HEK-293 T and HeLa cell lines were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). OVCAR-3 cells were gifted from Dr. Jinhui Liu (Nanjing Medical University). OVCAR-3 cells were grown in RPMI-1640 medium (Hyclone) supplemented with 20% (v/v) FBS and 10 μg/mL bovine insulin. HEK-293 T and HeLa cells were grown in DMEM medium (Hyclone) supplemented with 10% (v/v) FBS. All cultured cells were verified to be mycoplasma negative monthly. The use of the gifted OVCAR-3 cell line in our study was approved by the Clinical Research Ethics Committee, Affiliated Jiangyin Hospital of Southeast University Medical College.

Special siRNA targeting *DAAM1* was applied to silence DAAM1 [5]. The overexpression of DAAM1 in DAAM1-knockdown OVCAR-3 cells was performed by transfection of siRNA targeting *DAAM1* and exogenous siRNA-silent DAAM1 construct at the same time. MiR-208-5p mimic and mimic NC (miR-Ctrl) were purchase from RiboBio (Guangzhou, China). Wildtype or mutant fragments of Daam1 (DAAM1-3′-UTR-WT and DAAM1-3′-UTR-mutant) were sub-cloned into pCS2 vectors. All cells were transfected by Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA).

2.3. Western blotting

OVCAR-3 cells were placed into 35-mm dishes (6×10^5 cells/dish) for extracting total proteins. Seventy-two hours later, OVCAR-3 cells were harvested and homogenized with lysis buffer. Cellular lysates were separated by denatured 10% SDS-PAGE. DAAM1 and β-actin primary antibodies (Proteintech, Wuhan, China) were used. β-actin was used to normalize relevant protein levels.

2.4. Dual-luciferase activity assay

The 3′-UTR of *DAAM1* which contained the binding sequence for the miR-208a-5p was synthesized and placed at the *Xba*I site in the pGL3-control vector (Promega, Madison, WI) by Integrated Biotech Solutions Co., Ltd (Shanghai, China). OVCAR-3 cells were seeded in 24-well plates (1.5×10^5 cells/well). Two hundred ng of pGL3-DAAM1-3′-UTR-WT and 100 ng pRL-TK (Promega) were transfected with 50 pmol miR-208a-5p or control by Lipofectamine 3000. Dual-Luciferase Reporter Assay System (Promega) was performed to measure luciferase activity 24 h after transfection. The *photinus pyralis* luciferase activity of individual sample was normalized to *Renilla reniformis* luciferase activity.

2.5. Wound healing assay

For wound healing analysis, OVCAR-3 cells were seeded in 96-well plates (Costar, Corning, NY) and cultured to confluence. The monolayers of cell were wounded by removing the culture-insert and rinsed with PBS to remove cell debris. After the 6 h migration, cells were stained with 0.2% (v/v) crystal violet for 20 min in room temperature. The images were acquired at time 0 h and 6 h after migration using a Nikon optics microscope conjugated with PowerShot G10 camera (Canon, Tokyo, Japan). The migratory area was calculated by the minus of the edge of the wound closure between 0 h and 6 h.

2.6. Boyden chamber assay

For cell invasion assays, 5×10^4 cells in serum-free medium supplemented with 5 mg/mL BSA were inoculated to the upper sides of modified Boyden chamber (8.0-μm, Costar, Corning, NY). The polycarbonate membranes of Boyden chambers were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ). After 6 h, the invasive cells on the lower sides of Boyden chambers were fixed and stained with 0.2% crystal violet. The stained cells were photographed and five microscopic fields per sample were randomly selected for quantification.

2.7. Quantitative real-time PCR

Total RNA and miRNA of cells and/or tissues were extracted using Trizol reagent and mirVana™ miRNA isolation kit (Ambion, Austin, TX), respectively. Then, we performed qRT-PCR to measure miR-208a-5p and DAAM1 mRNA expression in OvCa tissues and cells. The detail protocol was described as previously [13].

2.8. Immunofluorescence and actin cytoskeleton staining

For immunofluorescent analysis of OVCAR-3 cells, cells were seeded on glass coverslides. After the fixation, OVCAR-3 cells were stained with phalloidine and 4′,6-diamidino-2-phenylindole (DAPI) [9]. The fluorescent pictures were taken by Zeiss LSM710 confocal microscope (Oberkochen, Germany).

2.9. RhoA activation assay

RhoA activation assay was performed to examine the activation level of RhoA in OvCa cells. In RhoA activation assays (Cytoskeleton Inc., Denver, CO, USA), cells were seeded into 35-mm dishes and then were subjected to RhoA activation assays. The protocol and modified methods were described previously [14].

2.10. Statistical analysis

Student's *t*-test or one-way ANOVA followed by Dunnett's multiple posthoc tests were performed to statistical analyses in SPSS 25.0 software (Chicago, IL). The correlations of the clinicopathological characteristics, *DAAM1* expression, and miR-208a-5p expression were analyzed by $\chi 2$ test. Bar charts show means ± SDs of five independent experiments if not noted. For all analyses, differences were defined significant if P value < 0.05.

3. Results

3.1. DAAM1 mediates the migration and invasion of OvCa cells

Several studies revealed that DAAM1 mediates the reorganization of microfilaments and leads to enhanced migration capacity of breast

Table 1
Association between *DAAM1* mRNA expression and patients' characteristics in OvCa.

Characteristics	n	*DAAM1* mRNA[a]		χ^2	*P* value[b]
		low	high		
Tumor size					
≤2cm	21	10	11	0.131	0.717
> 2cm	40	21	19		
Distant metastasis					
M0	29	19	10	4.778	0.029
M1	32	12	20		
ER status					
negative	19	9	10	0.132	0.717
positive	42	22	20		
PR status					
negative	32	15	17	0.419	0.517
positive	29	16	13		
P53 status					
negative	35	18	17	0.012	0.912
positive	26	13	13		
Ki67 status					
negative	30	15	15	0.016	0.900
positive	31	16	15		

[a] The *DAAM1* mRNA expression levels were divided at a cutoff point of 50%.
[b] *P* value for χ^2 test.

cancer cells [5,8]. However, whether DAAM1 participates in the regulation of microfilaments formation and migration of OvCa cells has not been reported. To investigate whether DAAM1 was associated with OvCa cells migration and invasion, we examined *DAAM1* mRNA expression in 61 OvCa tissues using quantitative PCR. High expression of DAAM1 was significantly associated with distant metastasis of OvCa (Table 1). We further examined whether DAAM1 mediated the migration and invasion of OvCa cells. To knockdown DAAM1 expression in OVCAR-3 cells were transfected with a special siRNA (DAAM1-siRNA). Quantitative PCR and Western blotting were conducted to examine the knockdown efficiency (Fig. 1A and B). The migration and invasion capacity of OVCAR-3 cells were evaluated based on wound healing assays and Boyden chamber assays, respectively (Fig. 1C and D). We found that OVCAR-3 cells exhibited repressed migration and invasion capacity after transfection of DAAM1-siRNA. When DAAM1-knockdown OVCAR-3 cells were transfected with exogenous siRNA-silent DAAM1 constructs, the migration and invasion capacity were significantly enhanced (Fig. 1C and D). Overall, these results reveal that DAAM1 plays a significant role in cell migration and invasion in OvCa.

3.2. MiR-208a-5p directly targets DAAM1

Considering the specific miRNAs regulating DAAM1 expression in OvCa is still less clear, we predicted potential specific miRNA targeting DAAM1 on MiRBD (http://mirdb.org/) [13,14]. Considering both conservation and innovation, we finally chose miR-208a-5p to further research. We measured the expression level of miR-208a-5p in clinical

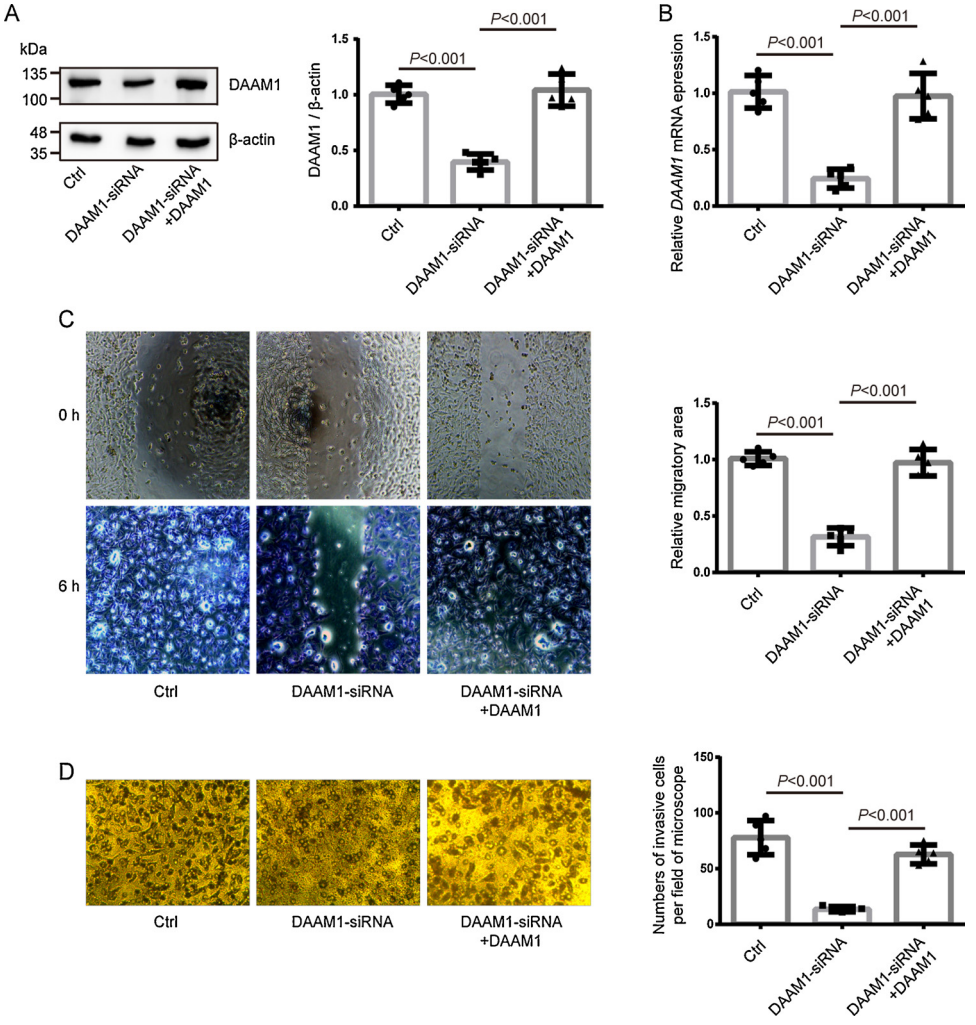

Fig. 1. DAAM1 mediates OvCa cell migration and invasion.
(A, B) Relative mRNA and protein expression level of DAAM1 in OVCAR-3 cells after transfection with Mock (Ctrl), DAAM1-siRNA or DAAM1-siRNA plus siRNA-silent DAAM1 were determined by qRT-PCR and Western blotting, respectively. (C) The migration capacity of OCVAR-3 cells after transfection with Mock (Ctrl), DAAM1-siRNA or DAAM1-siRNA plus siRNA-silent DAAM1 was analyzed by wound healing assay. DAAM1-siRNA suppressed the migration of OVCAR-3 cells, which could be rescued by DAAM1 overexpression. Magnification, ×20. (D) The invasion capacity of OCVAR-3 cells transfected with Mock (Ctrl), DAAM1-siRNA or DAAM1-siRNA plus siRNA-silent DAAM1 was evaluated by Boyden chamber assays. DAAM1-siRNA inhibited the invasion of OVCAR-3 cells, which could be rescued by DAAM1 overexpression. Magnification, ×20.

Table 2
Association between miR-208a-5p expression and patients' characteristics in OvCa.

Characteristics	n	miR-208a-5p[a]		χ^2	*P* value[b]
		low	high		
Tumor size					
≤2cm	21	10	11	0.131	0.717
> 2cm	40	21	19		
Distant metastasis					
M0	29	9	20	8.658	0.003
M1	32	22	10		
ER status					
negative	19	9	10	0.132	0.717
positive	42	22	20		
PR status					
negative	32	17	15	0.143	0.705
positive	29	14	15		
P53 status					
negative	35	20	15	1.314	0.252
positive	26	11	15		
Ki67 status					
negative	30	11	19	4.731	0.030
positive	31	20	11		

[a] The miR-208a-5p expression levels were divided at a cutoff point of 50%.
[b] *P* value for χ^2 test.

samples. As shown in Table 2, miR-208a-5p expression was negatively correlated with distant metastasis and Ki67 status, indicating the anti-tumor effect of miR-208a-5p on OvCa. Subsequently, we constructed the luciferase reporter vectors at the putative *DAAM1* 3′-UTR target site for the miR-208a-5p downstream of the luciferase gene (Fig. 2A). We next measured miR-208a-5p level in various cell lines and the results showed that OVCAR-3 cells expressed low miR-208a-5p than HEK-293 T and HeLa cells (Fig. 2B). Dual-luciferase activity assay showed an obvious decrease of luciferase activity when miR-208a-5p mimic was transfected into OVCAR-3 cells (Fig. 2C). Furthermore, a notable decrease expression of DAAM1 was exhibited in miR-208a-5p-overexpressed OVCAR-3 cells (Fig. 2D and E). We furtherly examined whether miR-208a-5p controlled the expression of DAAM1 in different cell lines. The results showed that miR-208a-5p negatively regulated DAAM1 expression in HEK-293 T cells, but not in HeLa cells (Additional file Fig. S1). Besides, miR-208a-5p expression in metastatic OvCa tissues was less than that in non-metastatic OvCa tissues (Fig. 2F). MiR-208a-5p was negatively associated with DAAM1 mRNA expression in 61 OvCa tissues (Fig. 2G). These results suggest that miR-208a-5p targets DAAM1 in OvCa.

3.3. MiR-208a-5p restrains the migration and invasion of OvCa cells

Then, the effect of miR-208a-5p on cellular events of OvCa cells was evaluated. At first, we measured the DAAM1 level in OvCa cells transfected with miR-208a-5p and miR-208a-5p/DAAM1 to guarantee the transfection efficiency (Additional file Fig. S2). Then, wound healing assays and Boyden chamber assays were conducted to evaluate the migration and invasion capacity of OVCAR-3 cells, respectively. We found that the overexpression of miR-208a-5p largely blocked the migration and invasion of OVCAR-3 cells, while the overexpression of DAAM1 could rescue miR-208a-5p-mediated declining capacity of migration and invasion (Fig. 3A and B). Taken together, both findings uncover that miR-208a-5p suppresses cell migration and invasion *via* downregulating DAAM1 in OvCa.

3.4. DAAM1 activates RhoA to mediate microfilaments' formation in OvCa cells

RhoA had been identified as a downstream factor interacting with DAAM1 and inducing the reorganization of microfilaments in both breast cancer and glioblastoma cells in our previous research [5,14]. However, the function of DAAM1/RhoA axis has not been assessed in OvCa. We measured RhoA activity in DAAM1-downregulated OVCAR-3 cells and found an obvious decrease activity of RhoA, and this decrease could be rescued by exogenous DAAM1 (Fig. 4A). Furthermore, fluorescent phalloidin was used to stain filamentous actin (F-actin) and display their distribution in OVCAR-3 cells. DAAM1-siRNA or CCG-

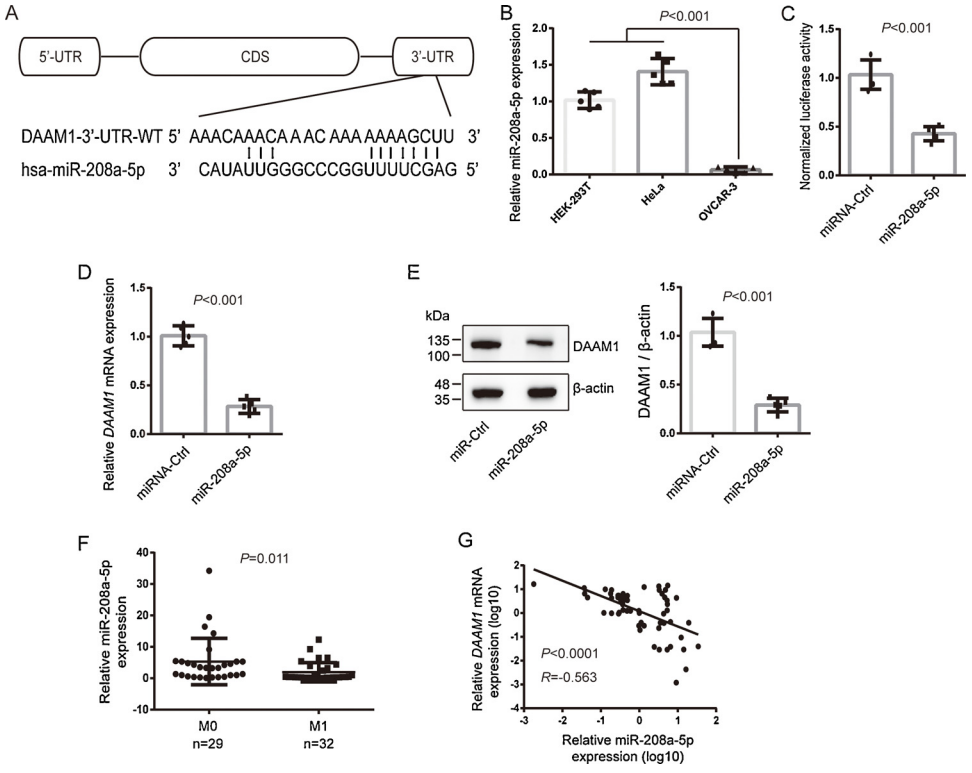

Fig. 2. MiR-208a-5p directly targets *DAAM1*.
(A) The predicted miR-208a-5p binding sites in wild type *DAAM1* 3′-UTR (*DAAM1* 3′-UTR-WT). (B) Relative miR-208a-5p expression was determined in HEK-293 T, HeLa, and OVCAR-3 cell lines by qRT-PCR. (C) The luciferase activity in OCVAR-3 cells transfected with miRNA-Ctrl or miR-208a-5p. (D, E) QRT-PCR and Western blotting showed downregulated DAAM1 expression both in mRNA and protein levels after miR-208a-5p overexpression. (F) Different expression of miR-208a-5p in metastatic OvCa samples and non-metastatic OvCa samples was detected by qRT-PCR. M0, without distant metastasis. M1, with distant metastasis. (G) Correlation between the expression levels of miR-208a-5p and *DAAM1* mRNA in OvCa tissues.

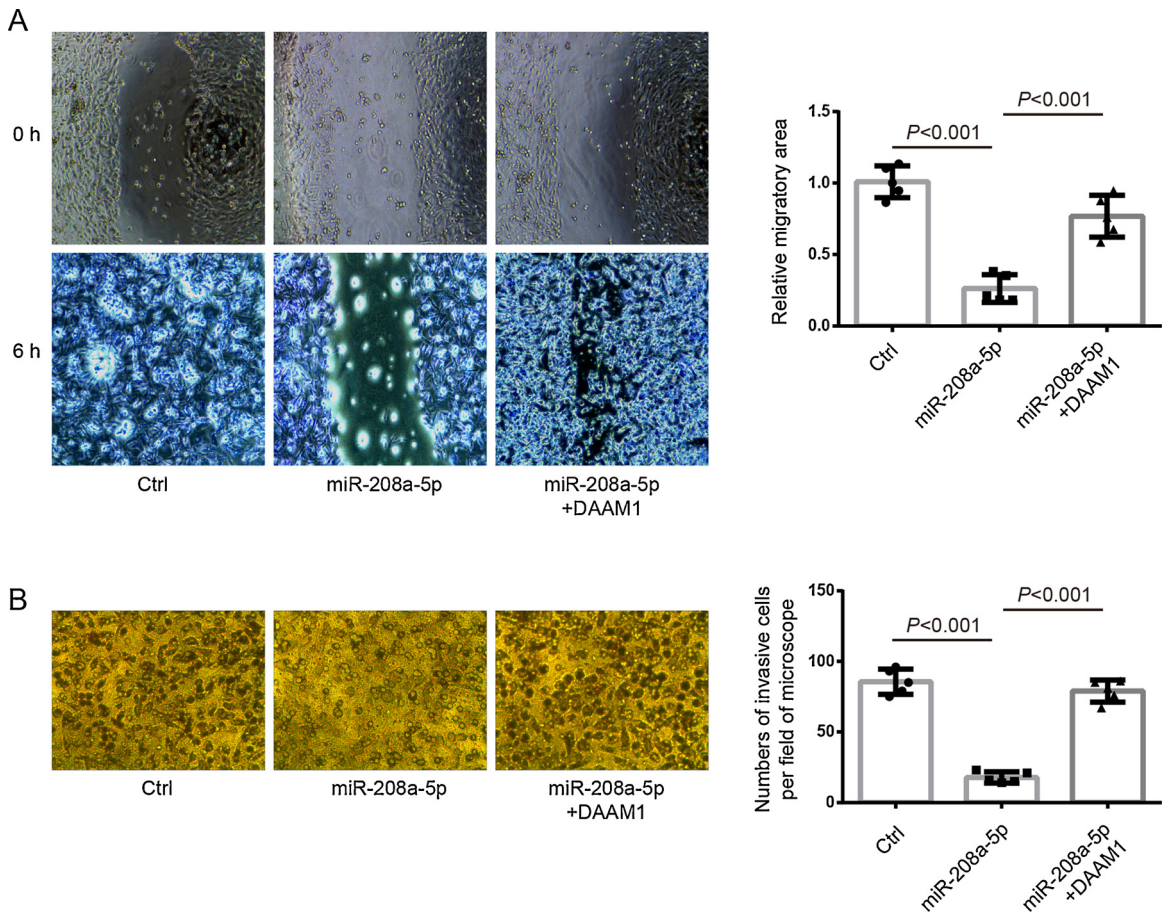

Fig. 3. MiR-208a-5p suppresses the migration and invasion of OvCa cells.
(A) The migration capacity of OCVAR-3 cells after transfection with Mock (Ctrl), miR-208a-5p or miR-208a-5p plus DAAM1 was analyzed by Wound healing assay. MiR-208a-5p suppressed the migration of OVCAR-3 cells, which could be rescued by DAAM1 overexpression. Magnification, ×20. (B) The invasion capacity of OCVAR-3 cells after transfection with Mock (Ctrl), miR-208a-5p or miR-208a-5p plus DAAM1 was evaluated by Boyden chamber assays. MiR-208a-5p inhibited the invasion of OVCAR-3 cells, which could be rescued by DAAM1 overexpression. Magnification, ×20.

1423 (a specific inhibitor for RhoA) treatment disrupted the formation of microfilaments (Fig. 4B, 4C, 4D), while the overexpression of DAAM1 sustained the formation of microfilaments in DAAM1-knockdown OVCAR-3 cells (Fig. 4B–D). Thus, the findings of cellular biological assays indicate that DAAM1 is essential for activation of RhoA and microfilaments' formation in OvCa cells.

3.5. MiR-208a-5p disrupts microfilaments' formation in OvCa cells via inhibiting RhoA activity

We further investigated whether miR-208a-5p decreased the RhoA activation in OvCa cells. A largely decreasing activity of RhoA was shown in OVCAR-3 cells after the overexpression of miR-208a-5p which can be rescued by the overexpression of DAAM1 (Fig. 4A). The overexpression of miR-208a-5p blocked the formation of microfilaments in OVCAR-3 cells, however, the overexpression of DAAM1 could partly reverse miR-208a-5p-induced depolymerization of microfilaments (Fig. 4B, 4C, 4D). Thus, these findings suggest that the microfilament formation can be interdicted by miR-208a-5p *via* blocking DAAM1 and RhoA signaling in OvCa cells.

4. Discussion

In our previous study, DAAM1 participates in regulating breast cancer cells motility through activating RhoA and enhancing the reorganization of microfilaments in response to Wnt5a and collagen [5,8]. Here, we found that high expression of DAAM1 is significantly associated with distant metastasis of OvCa and the silence of DAAM1 significantly blocks the migration and invasion of OvCa cells. Besides, DAAM1/RhoA signaling participates in the formation of microfilament, which is similar to our previous findings in breast cancer and glioblastoma [5,14]. Both results revealed that DAAM1 plays a significant role in migration and invasion of OvCa cells.

Proteins translated from its corresponding mRNA are the basic units that perform biological functions and the post-transcriptional regulation of mRNA is widespread in eukaryotic cells, thus leading to various protein expression. MiRNAs, which participates in downregulating the expression level of the target gene *via* the degradation of mRNA and/or inhibiting the translation of the protein from the mRNA, are the most common post-transcriptional regulators. In this research, we found miR-208a-5p binds to *DAAM1* 3′-UTR and downregulates DAAM1 expression on both mRNA and protein level.

MiR-208a, thought of as a biomarker for myocardial infarction previously, has been rarely researched in progression of cancers [15,16]. Yin et al. revealed that miR-208a-3p suppresses cell apoptosis through decreasing PDCD4 expression in gastric cancer [17]. Tang et al. reported that miR-208a promotes the proliferation and radio-resistance *via* directly downregulating p21 in human lung cancer [18]. However, the function of miR-208a-5p has never been studied in OvCa. Here, we report that miR-208a-5p is notably downregulated in distant metastatic OvCa tissues than that in non-distant metastatic OvCa tissues. The overexpression of miR-208a-5p significantly suppresses the migration and invasion of OVCAR-3 cells. The results indicate the promising antitumor role of miR-208a-5p in controlling OvCa metastasis *via* blocking

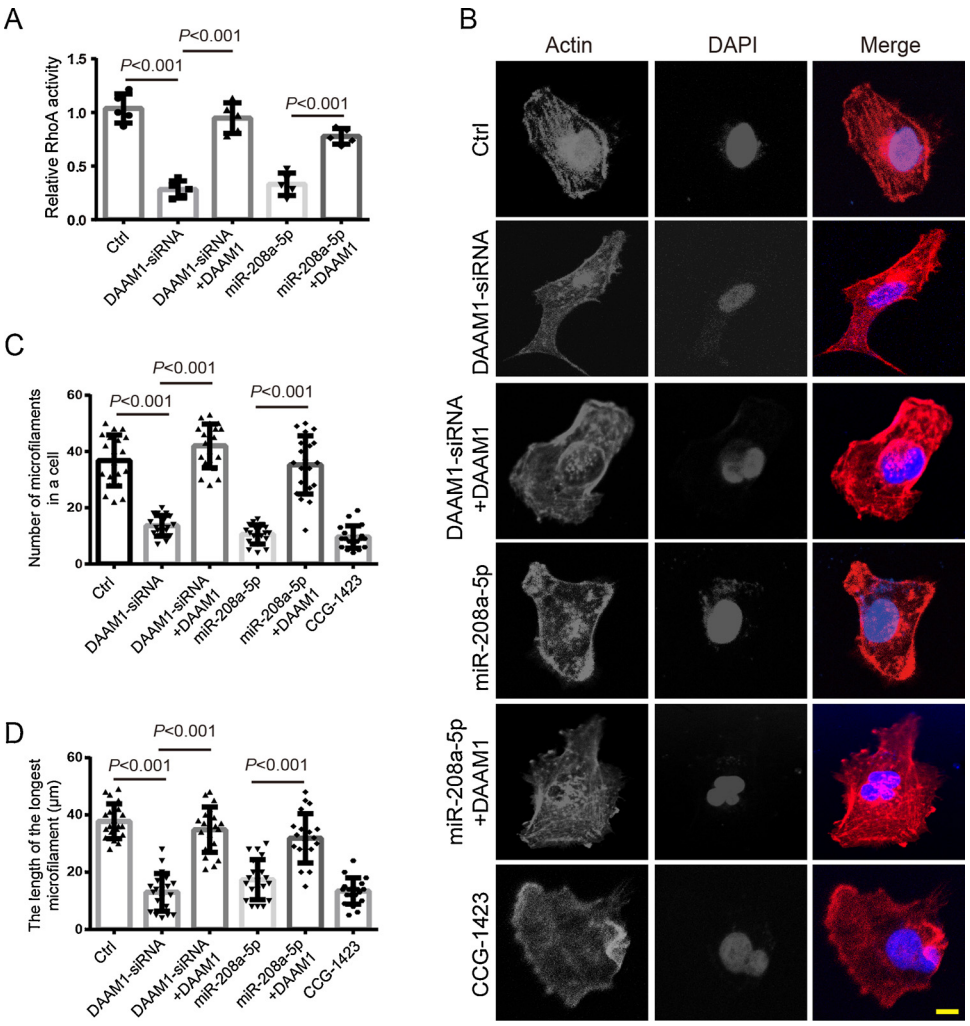

Fig. 4. MiR-208a-5p inhibits DAAM1-mediated RhoA activation and the formation of microfilament in OvCa cells.
(A) RhoA activity of OCVAR-3 cells in various treatment groups was analyzed by RhoA GTPase activation assays. DAAM1-siRNA and miR-208a-5p inhibited RhoA activation, while the overexpression of DAAM1 rescued the suppressed activation of RhoA in OCVAR-3 cells. (B) OCVAR-3 cells were transfected with DAAM1-siRNA, DAAM1, or treated with 1 μmol/L CCG-1423 for 1 h. OCVAR-3 cells in various treatment groups were subjected to actin cytoskeleton staining for immunofluorescent analysis. DAAM1-siRNA and miR-208a-5p disrupted the formation of microfilament, while the overexpression of DAAM1 sustained the formation of microfilament in DAAM1-downregulated OCVAR-3 cells. Bar = 10 μm. Magnification, ×40. (C and D) The number of microfilaments in each OCVAR-3 cell and the length of the longest microfilament were measured (n = 20).

DAAM1 signaling.

To conclude, our study demonstrates that DAAM1 has significantly impact on OvCa migration and invasion. MiR-208a-5p, which directly targets DAAM1, functions as a metastatic suppressor in OvCa *via* blocking DAAM1/RhoA signaling. Overall, we summarize miR-208a-5p and DAAM1 axis as a novel clinical target in controlling the metastasis of OvCa.

Authors' contributions

YZ and JM conceived the study and participated in the study design, performance, coordination and manuscript writing. JM, YH, LH, YL, TY, TQ, RX, BX, ZX, XJ, and KH carried out the assays and analysis. YZ, YH, JM and LH revised the manuscript. All authors reviewed and approved the final manuscript.

Disclosure

The authors declare that they have no competing interests.

Acknowledgments

We thank Dr. Jinhui Liu (Nanjing Medical University) and the Laboratory Center for Basic Medical Sciences (Nanjing Medical University) for providing OVCAR-3 cell line and technical support, respectively.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152452.

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