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MicroRNA-497 inhibition of ovarian cancer cell migration and invasion through targeting of SMAD specific E3 ubiquitin protein ligase 1

Wei Wang [a], Fang Ren [a], Qinghua Wu [a], Dazhi Jiang [b], Hongjun Li [b,c], Zheng Peng [a], Jinglu Wang [a], Huirong Shi [a,*]

[a] Department of Obstetrics and Gynecology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China
[b] Department of Nuclear Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China
[c] Department of Radiology, Beijing Youan Hospital, Capital Medical University, Beijing 100054, China

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ABSTRACT

Ovarian cancer is the leading cause of death from gynecological malignancies worldwide. Understanding the molecular mechanism underlying ovarian cancer progression facilitates the development of promising strategy for ovarian cancer therapy. Previously, we observed frequent down-regulation of miR-497 expression in ovarian cancer tissues. In this study, we investigated the role of miR-497 in ovarian cancer metastasis. We found that endogenous miR-497 expression was down-regulated in the more aggressive ovarian cancer cell lines compared with the less aggressive cells. Exogenous expression of miR-497 suppressed ovarian cancer cell migration and invasion, whereas reduction of endogenous miR-497 expression induced tumor cell migration and invasion. Mechanistic investigations confirmed pro-metastatic factor SMURF1 as a direct target of miR-497 through which miR-497 ablated tumor cell migration and invasion. Further studies revealed that lower levels of miR-497 expression were associated with shorter overall survival as well as increased SMURF1 expression in ovarian cancer patients. Our results indicate that down-regulation of miR-497 in ovarian cancer may facilitate tumor metastasis. Restoration of miR-497 expression may be a promising strategy for ovarian cancer therapy.

1. Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies [1,2]. Metastasis is responsible for ovarian cancer recurrence and patient death [3]. Therefore, understanding the molecular mechanism underlying metastasis may provide promising strategy for ovarian cancer therapy.

MicroRNAs (miRNAs), a class of small non-coding RNAs, are known to play pivotal roles in cancer development, tumor angiogenesis and metastasis [4,5]. MiR-497, an important member of the micro-15/16/195/424/497 family of miRNAs [6], is aberrantly expressed in many malignant tumors, including ovarian cancer [7,8]. MiR-497 has been shown to inhibit tumor metastasis in colorectal cancer [9] and to affect cell invasion and growth by targeting cyclinE1 in breast cancer [10]. In a study of miRNA profiling, miR-497 was decreased in high-grade astrocytomas compared with normal tissues and serum miR-497 expression was identified as noninvasive biomarker for human malignant astrocytomas [11]. However, its role in ovarian cancer metastasis is unclear.

In this study, we clarified the role of miR-497 in ovarian cancer metastasis using *in vitro* assays and clinical tissues. *In vitro* assays showed that miR-497 was down-regulated in high invasive ovarian cancer cell lines, SKOV-3 and HO-8910PM, compared with the low invasive OVCAR-3 and HO-8910 cells. Exogenous expression of miR-497 in high-invasive ovarian cancer cells suppressed cell migration and invasion, whereas reduction of endogenous miR-497 expression in low invasive ovarian cancer cells induced cell migration and invasion. Ex *in vivo* study showed that down-regulation of miR-497 in human ovarian cancer specimens was associated with lymph node metastasis, advanced clinical stage, and worse overall survival. We further provided evidence that miR-497 exerted its anti-metastatic effect by targeting pro-metastatic factor SMAD specific E3 ubiquitin protein ligase 1(SMURF1). Our results suggested that miR-497 may play an important role in ovarian cancer progression and metastasis.

* Corresponding author. Fax: +86 0371 66862053.
E-mail address: huirongshiww@163.com (H. Shi).

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2. Materials and methods

2.1. Human tissue specimens

Human ovarian cancer tissues were collected from 96 patients who underwent surgery at the department of Obstetrics and Gynecology of the First Affiliated Hospital of Zhengzhou University between 2006 and 2012. Tumor tissues were histologically examined and the tumor histotype was serous cystadenocarcinoma. The tissue collection was used for quantitative real-time PCR analysis. Formalin-fixed, paraffin-embedded tissues, matching the frozen cases, were retrieved for immunohistochemical analysis. The patients had not received any local or systemic anticancer treatments prior to the surgery. The patients were followed-up for a median follow-up time of 60 months 46.9 (rang, 6–60 months). The relevant characteristics of the studied subjects were shown in Table 1. Informed consent was obtained from each patient, and the study was approved by our Institute Research Ethics Committee at the Cancer Center.

2.2. Cell lines and cell culture

The ovarian cancer cell line OVCAR-3, SK-OV-3, HO-8910, HO-8910PM were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cell lines were maintained in RMPI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 units/ml of penicillin, and 100 g/ml of streptomycin at 37 °C, 5% CO_2.

2.3. Lentivirus production and transfection

The human miR-497 precursor sequences were cloned into the lentivirus based expression plasmid plenti-6.3 (Invitrogen, USA). Viral packaging and infection were performed according to standard protocols as recommended by the manufacturer. Cells were infected with 1×10^7 lentivirus transducing units in the presence of 10 μg/ml polybrene (Sigma Aldrich, St. Louis, Missouri, USA). An empty lentiviral vector was used as negative control. The sequence-specific miR-497 inhibitor (anti-miR-497) and its control (anti-NC) were from BioCat GmbH (Heidelberg, Germany).

Table 1
Correlation between miR-497 expression and clinicopathologic features of serous cystadenocarcinoma.

Clinicopathological features		MiR-497 expression		
	Total cases	High	Low	*P* value
Group				
Normal tissue	30	19	11	**0.0047****
Carcinoma tissue	96	38	58	
Age				
⩽60	39	20	19	0.144
>60	57	18	39	
TNM stage				
I-II	31	11	20	**0.030***
III-IV	65	27	38	
Differentiation				
Well	21	9	12	0.130
Moderate	38	17	21	
Poor	37	12	25	
Lymph node metastasis				
Yes	57	17	40	**0.040***
No	39	21	18	
Residual tumor size				
<=1 cm	55	22	33	0.718
>1 cm	41	16	25	

* Significant difference ($p < 0.05$).
** Significant difference ($p < 0.01$).

2.4. 4RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA).To quantitate miR-497 expression, total RNA was polyadenylated and reversely transcribed using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen, USA). To measure the mRNA levels of SMURF1, total RNA was reversely transcribed using PrimeScript RT reagent Kit With gDNA Eraser (TaKaRa, Japan). Quantitative real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Japan) in Agilent Mx3005P. Primers used for miR-497 were 5′-GTG CAGGGTCCGAGGT-3′ (forward) and 5′-TAGCCTGCAGCACACTG TGGT-3′ (reverse). Primers used for SMURF1 were 5′-TGCCTT GTCCAGACACACCTC-3′ (forward) and 5′-CATCATCTCAGTGAAAG CCTCCATA-3′ (reverse). GAPDH was used as an internal control. All samples were normalized to internal controls and fold changes were calculated through relative quantification ($2^{-\triangle\triangle Ct}$).

2.5. Luciferase reporter assay

Double-stranded oligonucleotides corresponding to the wild-type (WT 3′-UTR) or mutant (Mut 3′-UTR) miR-497 binding site in the 3′-UTR of SMURF1 were synthesized and subcloned into the GV272 reporter vector (GV272: SV40-Luciferase-MCS-Poly A was purchased from Shanghai Genechem Co, Ltd.). Cells were transfected with appropriate plasmid and miR-497 duplex. Luciferase assays were done using the luciferase reporter assay system (Promega, USA) 48 h after transfection. Normalized luciferase activity was reported as Luciferase activity/Renilla Luciferase activity.

2.6. Transwell migration and invasion assays

For transwell migration assays, 1×10^5 cells were plated in the top chamber onto the noncoated membrane (24-well insert; pore size, 8 μm; Corning, USA). For invasion assay, 1×10^5 cells were plated in the top chamber onto the Matrigel coated membrane. Each well was coated freshly with Matrigel (60 μg; BD Bioscience, USA) before the invasion assay. The cells were incubated for 24 h (migration assay) or 48 h (invasion assay). Cells on the lower surface of the membrane were fixed with methanol and stained with crystal violet. The number of cells migrating through or invading through the membrane was counted under a light microscope (400×, five random fields per well).

2.7. Western blotting

Proteins were separated on 10% SDS–PAGE gel and then transferred to nitrocellulose membrane (Bio-Rad, USA).After blocked with 5% nonfat milk, the membrane was incubated with SMURF1 antibody (# 2174, 1:1000, Cell Signaling Technology, USA), β-Actin antibody (# 8457, 1:1000, Cell Signaling Technology, USA). The proteins were visualized using ECL reagents (Beyotime, China).

2.8. Immunohistochemistry

SMURF1 expression was examined by immunohistochemistry (IHC) using formalin-fixed, paraffin-embedded human ovarian cancer tissues. Anti-SMURF1 polyclonal antibody (abcam, UK) was used at a dilution of 1/50, and incubated overnight (16 h) at +4 °C, followed by incubation with biotinylated anti-rabbit IgG for 30 min at room temperature (25 °C). Staining was performed in parallel and by using a Vectastain ABC kit (Vector Laboratories).

Then, stained and coded sections were reviewed by pathologists blinded to study groups.

2.9. Statistical analysis

Survival statistical analyses were performed using SPSS, version 13.0 (SPSS, Chicago, IL, USA). Kaplan–Meier curves were constructed to determine patient relapse-free survival (RFS) and overall survival (OS) rates. Patients who were lost to the follow-up or who died from causes unrelated to OC were treated as censored events. The statistical differences in survival among subgroups were compared using the log-rank test.

Data were presented as mean ± standard error of the mean (SEM) of at least 3 independent experiments. The differences between the groups were analyzed by Student's *t* test when two groups were compared or by one-way ANOVA when more than two groups were compared. Analyses were performed with GraphPad Prism, version 5 (GraphPad Software, Inc., San Diego, CA, USA). Correlations between two variables were explored with the Spearman's correlation coefficient. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Expression of miR-497 in ovarian cancer cell lines with different invasion capacity

To investigate the potential role in regulation of ovarian cancer invasion and metastasis, we detected the expression of miR-497 in ovarian cancer cell lines with different invasion capacity [12]. Our data showed that expression of miR-497 was decreased in high-invasive ovarian cancer cell lines SKOV-3 and HO-8910PM compared with low-invasive OVCAR-3 and HO-8910 cells (Fig. 1A).

3.2. Effects of miR-497 on ovarian cancer cell migration and invasion

Next, we explored the potential impact of miR497 in ovarian cancer cell migration and invasion. The cell migration and invasion assay showed that ectopic expression of miR-497 in SKOV-3 cells resulted in decreased cell migration (Fig. 1C) and penetration rate through the matrigel-coated membrane compared with negative control (Fig. 1C). In contrast, depleting endogenous miR-497 in OVCAR-3 cells promoted cell migration and invasion capacity

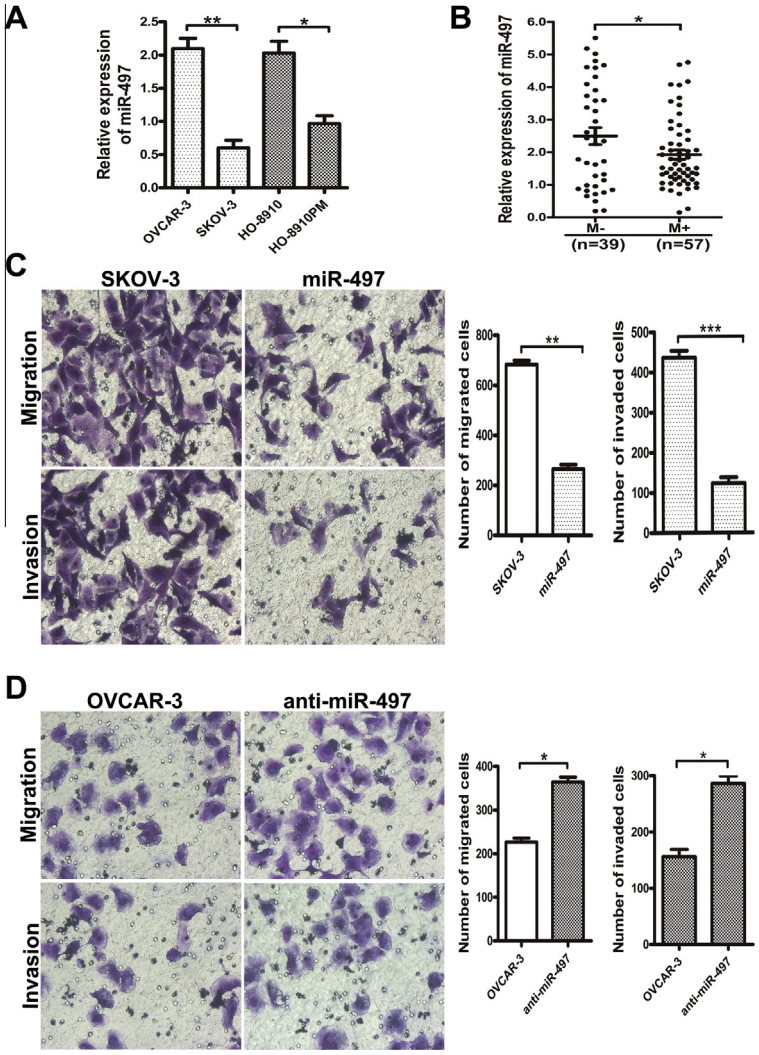

Fig. 1. MiR-497 suppresses ovarian cancer cells migration and invasion. (A) Expression of miR-497 in ovarian cancer cells with different invasion capacity. (B) Real-time PCR analysis of miR-497 expression in patients with or without lymph node metastasis. (C, D) Transwell migration and Matrigel invasion assays are shown. SK-OV-3 (C) and OVCAR-3 (D) cells that were non-transfected (panel 1) or transfected with miR-497 duplex (panel 2) or miR-497 inhibitor (panel 2) were added to transwell chambers without or with Matrigel coatings and incubated for 24 h or 48 h, followed by staining with crystal violet and photographed at 400×. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 1D). These results suggest the suppressive effects of miR-497 on ovarian cancer cell metastasis.

3.3. MiR-497 represses ovarian cancer cell migration and invasion by directly targeting SMURF1

After that, we investigated the targets of miR-497 using the TargetScan database. Among the putative targets of miR-497, we chose SMURF1 for further analysis due to its well-known importance in cell motility and polarity [13,14]. As shown in Fig. 2, both Dual-luciferase reporter analysis (Fig. 2A, 2B) and Western blot assays (Fig. 2C, 2D) confirmed that miR-497 directly suppressed the expression of SMURF1. Moreover, similar to the phenotype induced by miR-497 expression, the silencing of SMURF1 decreased SKOV-3 and OVCAR-3 cell migration and invasion (Fig. 3A, 3B).

3.4. Down-regulation of MiR-497 in ovarian cancer tissues is associated with SMURF1 expression and worse prognosis

Previously, we observed frequent down-regulation of miR-497 in human ovarian cancer tissues. We then associated miR-497 expression with patients' clinicopathologic parameters and found that low miR-497 level was associated with tumor lymph node metastasis ($P = 0.040$, Fig. 1B), advanced clinical stage ($P = 0.030$, Table. 1). To investigate the association between miR-497 and SMURF1 expression in human ovarian cancer tissues, we performed Real-time polymerase chain reaction (PCR) and immunohistochemical analysis of SMURF1 expression on the same panel of ovarian cancer specimens. As shown in Fig. 2, Spearman correlation analysis revealed an inverse correlation between miR-497 and SMURF1 expression ($r = -0.372$, $P = 0.0023$; Fig. 2E and F).

To investigate whether miR-497 expression is associated with 5-year OS and RFS in ovarian cancer patients, we performed Kaplan–Meier survival analyses. Our data revealed that patients with lower miR-497 levels had a mean OS of 45.9 months, whereas patients with higher miR-497 levers had a mean OS of 48.2 months ($P = 0.045$; Fig. 4A). We also found an association of lower miR-497 levels with shorter RFS, although the association was not statically significant (29.2 vs. 36.8, $P = 0.053$; Fig. 4B).

4. Discussion

Metastasis is the major events related to tumor recurrence and poor patient outcome [3]. Mounting evidence suggests that miRNAs may promote or suppress tumor metastasis, thus they provide a new perspective on the tumorigenic process. Herein, we disclosed that miR-497 suppressed ovarian cancer metastasis based on observations from human specimens as well as *in vitro* assays.

Studies from different groups have indicated that miR-497 was down-regulated in ovarian cancer. Flavin et al. characterized the alteration in expression of a select group of microRNAs in primary peritoneal carcinoma relative to matched 34 cases of ovarian serous carcinoma. They found that down-regulation of miR-497 and miR-195 from the microRNA cluster site at chromosome 17p13.1 in primary peritoneal carcinoma relative to ovarian serous carcinoma [7]. Delfino et al. reported that a treatment-dependent association between risk or hazard of ovarian cancer recurrence and miR-497 was identified [15]. The hazard for ovarian cancer recurrence decreased with increasing miR-497 level in patients receiving chemo treatment [15]. Wang et al. and Zhang et al. reported that MiR-497 was down-regulated in ovarian cancer lines versus normal ovarian cell lines [8,16]. However, in these and other reports, miR-497 is not further studies. More extensive

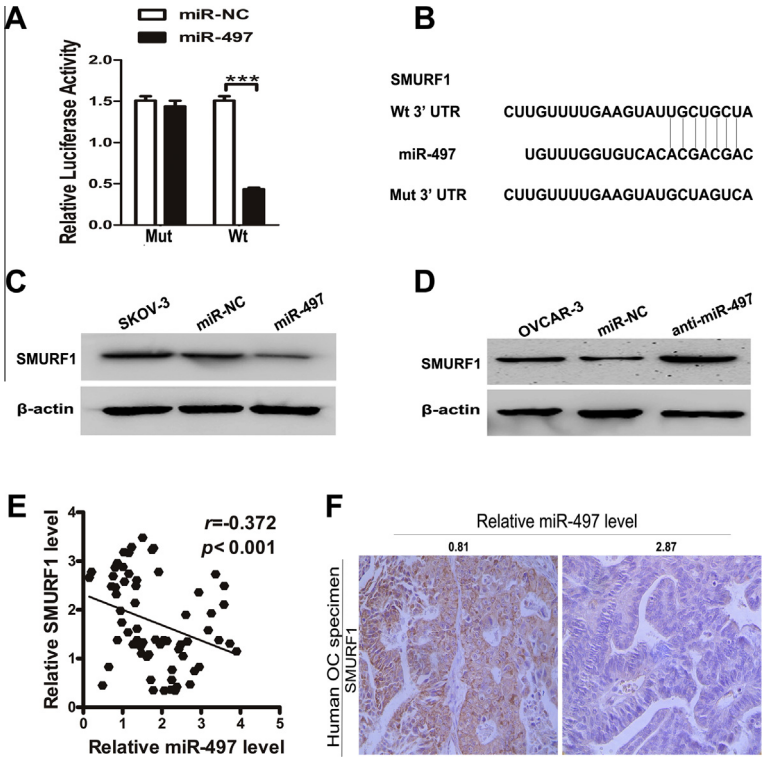

Fig. 2. MiR-497 suppresses SMURF1 expression by targeting its 3'UTR. (A, B) miR-497 inhibited the activity of a luciferase reporter that contained the wild-type 3'UTR of SMURF1. (C, D) Western blot assay show that expression of miR-497 reduced the protein levels of endogenous SMURF1 in SK-OV-3 cells (C) and depletion of endogenous miR-497 enhanced the cellular VEGFA protein level in OVCAR-3 cells (D). MiR-NC, nonspecific control miRNA. (E, F) The levels of miR-497 are inversely correlated with SMURF1 expression in 65 human ovarian cancer tissues. SMURF1 was also detected by immunohistochemical staining. Each data point represents an individual sample, and correlation coefficients (r) indicated. ***, $P < 0.001$.

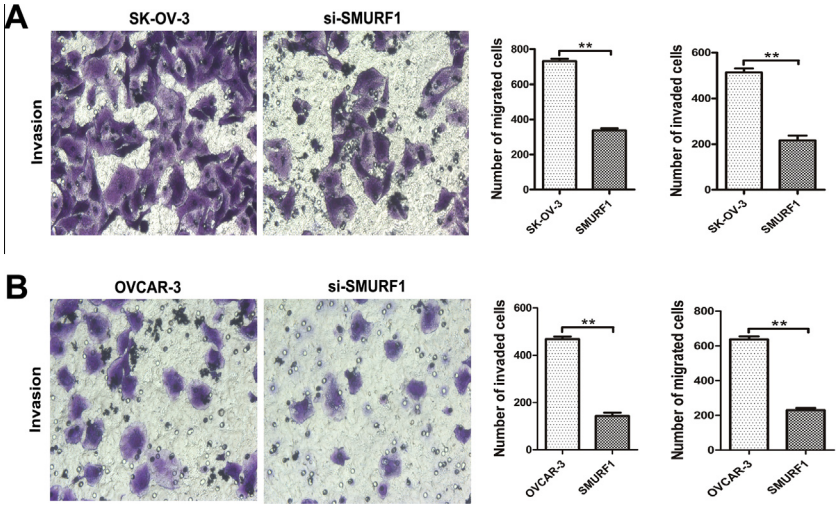

Fig. 3. MiR-497 suppresses SMURF1 expression leading to reduced ovarian cancer metastasis. (A, B)Suppression of SMURF1 by RNAi reduces SK-OV-3(A) and OVCAR-3(B) cells migration and invasion. Photographed at 400×. **, $P < 0.01$.

investigations are required to verify the potential role of miR-497 in ovarian cancer progression. We analyzed miR-497 mRNA expression profiling in ovarian cancer cell lines with different invasion capacity and found that miR-497 was decreased in cells with high invasive potential. Exogenous expression of miR-497 was able to inhibit the capacity of ovarian cancer cells to promote migratory and invasive activities. In human ovarian cancer specimens, down-regulation of miR-497 was associated with ovarian cancer metastasis, advanced clinical stage and short OS of the patients. These results indicate that down-regulation of miR-497 may contribute to ovarian cancer progression by promoting tumor metastasis.

Smurf1, a member of HECT (homologous to E6AP C-terminus) domain E3 ubiquitin ligases, was involved in the regulation of cell adhesion and migration through ubiquitination of Rho guanosine triphosphatases (GTPases) RhoA, talin head domain, and hPEM2 [14,17]. In breast cancer, SMURF1 promotes cell migration and invasion through EGF-mediated ERK1/2 pathways [18]. In human pancreatic cancer, SMURF1 functions as an oncogene to promote cell growth [19]. In colon cancer, cell cycle-regulated SMURF1 promotes cell growth and migration through PI3 K/Akt/mTOR pathways [20]. However, the underlying mechanism of SMURF1 in the regulation of ovarian cancer cell migration is not fully understood. We identified SMURF1 as a novel targets that was at least, partly, responsible for the anti-metastatic function of miR-497. We found that miR-497 down-regulation was correlated with SMURF1 up-regulation in human ovarian cancer cells and ovarian cancer tissues. MiR-497 deficiency in ovarian cancer cells promoted cell migration and invasion via revitalite of SMURF1 activation. However, we detected SMURF1 protein only in two of the ovarian cancer lines, OVCAR-3 and HO-8910PM. We anticipate that future studies will reveal that SMURF1 may play functional roles in ovarian cancer progression.

To date, a few miRNAs have been characterized to possess pro-metastatic or anti-metastatic functions in ovarian cancer.

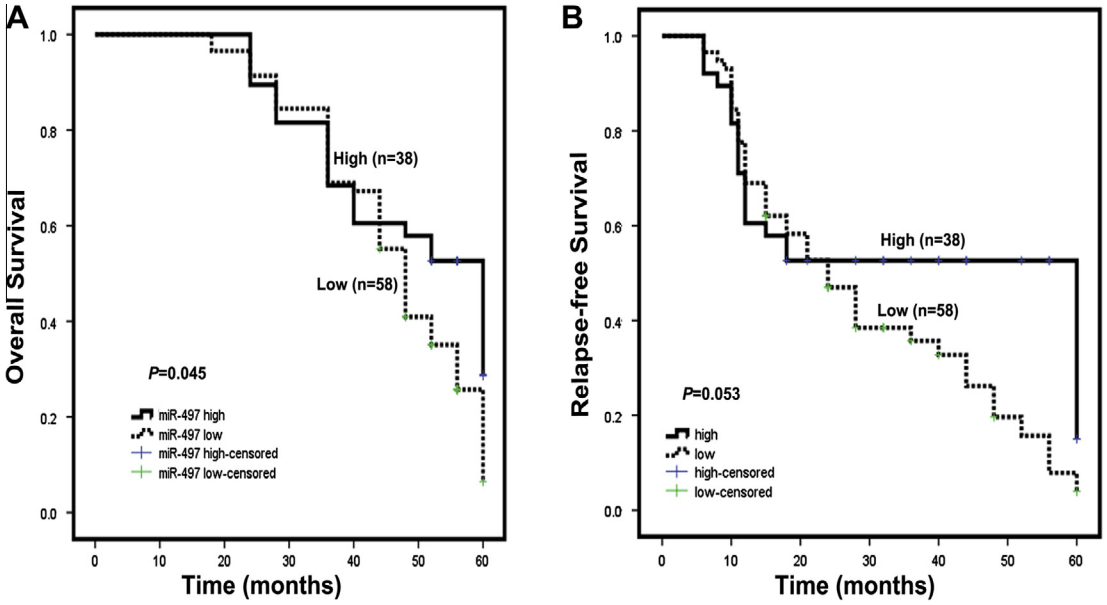

Fig. 4. Down-regulation of miR-497 in ovarian cancer tissues is associated with worse prognosis. (A) Kaplan–Meier survival curves show that median overall survival is higher in miR-497 high group (48.2 vs. 45.9 months, Log Rank = 4.006, $P = 0.045$). (B) Kaplan–Meier survival curves show that miR-497 expression with relapse-free survival (36.8 vs. 29.2 months, Log Rank = 3.742, $P = 0.053$).

MiR-199a suppressed ovarian cancer cell migration and metastasis by targeting HIF-1α and HIF-2α [21]. MiR-181a promoted cellular migration, invasion, and drug resistance by targeting Smad7 in high-grade serous ovarian cancer [22]. MiR-506 augmented E-cadherin expression, inhibited ovarian cancer cell migration and invasion, and prevented epithelial-mesenchymal transition by targeting SNAI2 [23]. MiR-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1α [24]. MiR-182 promotes ovarian cancer cell growth, invasion, and chemo-resistance by targeting PDCD4 [25]. MiR-200a inhibits $CD133/1^{+}$ ovarian cancer stem cells migration and invasion by targeting E-cadherin repressor ZEB2 [26]. Herein, we provide the evidence to show that miR-497 inhibited ovarian cancer cell migration and invasion by targeting pro-metastatic factor SMURF1. Our study, together with those from other groups, suggests a crucial inhibitory function of microRNAs in ovarian cancer metastasis. These results implicate that our findings are clinically relevant. MiR-497 may represent as a promising molecular target for optimizing individual therapy management in ovarian cancer patients.

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