Original Paper

MicroRNA-125b Suppresses Ovarian Cancer Progression via Suppression of the Epithelial-Mesenchymal Transition Pathway by Targeting the SET Protein

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Key Words

Epithelial ovarian cancer • MicroRNA-125b • SET protein • Epithelial-mesenchymal transition

Abstract

Background/Aims: MicroRNA-125b (miR-125b) is overexpressed in several types of cancer and contributes to chemotherapy resistance. However, its role in epithelial ovarian carcinoma remains unknown. The goal of this study was to identify the relationship between miR-125b and the epithelial-mesenchymal transition (EMT) in ovarian cancer. **Methods:** In total, 55 patients with epithelial ovarian cancer (EOC) were included in our study. The relative expression of miR-125b was measured using real-time polymerase chain reaction (RT-PCR). The protein expression of SET and EMT-related indicators in cell lines were assessed by Western blot. The regulation of SET by miR-125b was confirmed using luciferase reporter assays. The effect of miR-125b on metastasis was evaluated using an *in vivo* metastasis model. **Results:** miR-125b expression was markedly lower in the EOC specimens. Ectopic expression of miR-125b in EOC cells significantly inhibited tumor invasion. miR-125b expression was negatively associated with both EMT and SET expression, *in vivo* and *in vitro*. Mechanistic studies identified SET as a direct target of miR-125b, and the downregulation of SET, observed during tumor migration, was affected by the overexpression of miR-125b. **Conclusion:** miR-125b suppresses EOC cell migration and invasion by targeting the SET protein, and this study may provide a novel mechanism for understanding the progression of EOC.

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Introduction

Epithelial ovarian cancer (EOC) constitutes the overwhelming majority (90%) of ovarian malignancies and is unique in clinical occurrence and metastasis. Most EOC patients

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are diagnosed with advanced-stage EOC due to a lack of appropriate screening methods to detect early stage EOC [1-4]. Given the difficulty of early stage diagnosis, more sensitive biomarkers should be used for the diagnosis of EOC, especially in the early stages, and the mechanistic relationship between such biomarkers and the development of EOC should be thoroughly explored.

Recently, aside from the traditional transcriptional genes, non-transcriptional RNAs, including lncRNA and miRNA, have gradually been revealed to be of great potential use in the diagnosis of different cancers, including EOC [5, 6].

An increasing number of studies have identified several miRNAs that are remarkably deregulated in EOC, suggesting that miRNAs are involved in the initiation and progression of this disease [7-9]. miR-125b was one such microRNA reported to be seriously associated with tumor biology. The miR-125b family consists of two members: hsa-miR-125b-1 and hsa-miR-125b-2, transcribed from two loci on chromosomes 11q24 and 21q21, respectively [10]. miR-125b is highly conserved in diverse species, from nematodes to humans. It is overexpressed in several types of cancer and has been reported to be associated with tumor resistance to chemotherapy [10, 11]. Veerla et al. reported that the downregulation of miR-125b contributes to aggressive growth behavior in urothelial tumors [12], and Alpini et al. showed that miR-125b may inhibit the invasion, migration and angiogenesis of liver cancer and cutaneous squamous cell carcinoma cells [13, 14]. Moreover, Zhou et al. also demonstrated that the overexpression of miR-125b can inhibit EMT in hepatocellular carcinoma (HCC) [15].

The SET protein was reported to be capable of inhibiting the acetylation of nucleosomes, especially histone H4, via histone acetylases (HAT). This inhibition was most likely accomplished by masking the histone lysines from acetylation, consequently silencing HAT-dependent transcription [16]. The knockdown of SET inhibits cell migration and invasion by increasing the activity and expression of PP2Ac and decreasing the expression of matrix metalloproteinase 9 (MMP-9) [17]; low levels of SET expression are associated with bone metastasis in renal cell carcinoma [18]. Therefore, using a bioinformatics prediction, we found that SET was a potential target of miR-125b; therefore, we sought to assess the expression of miRNA, its effect in human EOC, and its association with SET expression and epithelial-mesenchymal transition (EMT) in human EOC.

Materials and Methods

Tissue samples and cell culture

Human ovarian cancer samples were collected from 55 patients after surgical resection at the Affiliated Nanjing Second Hospital of Nanjing Medical University between 2007 and 2014. No patients had received therapy before resection. Tumor tissues and the corresponding adjacent normal tissues were frozen in liquid nitrogen and stored at -80°C until RNA extraction. Written informed consent was obtained from the patients for the biological studies. This study was approved by the Ethics Committee of Nanjing Medical University. The pathological classification of ovarian cancer was determined according to the International Federation of Gynecology and Obstetrics (FIGO) system. The characteristics of the 55 EOC patients are listed in Table 1.

The EOC cell line SKOV3 was purchased from American Tissue Type Collection (Manassas, VA) and was maintained in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10 % fetal bovine serum (Hyclone) at 37°C and a humidified atmosphere of 5 % CO_2 .

RNA extraction and analysis using quantitative real-time PCR (qRT-PCR)

For miRNA quantification, the total RNA from cell lines and tissues was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The Hairpin-itTM miRNAs qPCR Quantitation Kit (GenePharma, China) was used for miR-125b analysis. This kit contains a highly miRNA-specific RT and PCR primer set with SYBR Green dye included. The RT primer for the stem-loop-like miRNAs and the highly specific primer set for miRNAs ensure that the RT and PCR reaction proceed without interference from the miRNA precursors.

For the detection of SET expression, the following primers were used: SET forward (5'-GUC CCA CUG UCA UGU AAA UTT-3') and reverse (5'-AUU UAC AUG ACA GUG GGA CTT-3'). miRNA and mRNA were normalized to U6 and GAPDH, respectively. All RT-PCR experiments were performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The expression levels relative to U6 and GAPDH were calculated using the $2^{-\Delta\Delta CT}$ formula.

Vector construction and 3'-UTR luciferase reporter assay

Overexpression and inhibition of miR-125b were assessed using miR125b, miR-125b human mimics inhibitor, miR-125b

human inhibitor and the negative control FAM, purchased from GenePharma(China). The 3'-UTR region of SET containing the wild-type or mutant potential target site for miR-125b (SET-wt 3'-UTR or SET-mut 3'-UTR, respectively) was synthesized and inserted into the pEZ-MT01 vector (GeneCopoeia). For the luciferase assay, SKOV3 cells were co-transfected with SET-wt 3'-UTR or SET-mut 3'-UTR and the miR-125b mimics, inhibitor or control (GenePharma, China) using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after transfection for the luciferase assay using a Luc-Pair™ miR Luciferase Assay Kit (GeneCopoeia) according to the manufacturer's protocol.

Western blot analysis

For western blot analysis, cells were lysed in lysis buffer after being washed with PBS containing a protease inhibitor. Proteins were separated by SDS-PAGE on a 10% gel and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After 1h of blocking with 5% non-fat dry milk at room temperature, the blots were incubated with primary antibody overnight at 4°C, followed by four 5 min washes in TBST and incubation with the corresponding secondary antibody for 2 h at room temperature. The proteins were visualized in the membranes using enhanced chemiluminescence solutions (Bio-Rad, Hercules, CA, USA), and Image J software (National Institutes of Health, USA) was used to measure the band intensities. Primary antibodies used and their dilutions are as follows: anti-SET (1:2000), anti-GAPDH (1:5000) (Abcam, Cambridge Science Park, Cambridge, UK), anti-E-cadherin (1:1000), anti-N-cadherin (1:1000), and anti-vimentin (1:1000) (Cell Signaling Technology, Inc., Danvers, MA, USA). The secondary antibody was a goat anti-rabbit antibody (1:2000) (Santa Cruz Biotechnology, Inc.).

Cell invasion and scratch wound assay

In the invasion assay, cell culture inserts (8.0-μm pores, Millipore, USA) were coated with Matrigel diluted 1:6 in serum-free medium. In total, 5000 cells were resuspended in serum-free medium and placed in the upper chamber, and 500 μL medium with 10% FBS was added to the lower chambers. After 24 h, the cells that had not migrated or invaded were removed using a cotton swab; then, the insert was fixed in 4% paraformaldehyde for 30 min at room temperature, stained with 0.1% crystal violet, and counted under an inverted microscope. Cells were seeded in 6-well plates, and the confluent monolayer of cells was scratched using a sterile 100 μL pipette tip. Medium was added, and the culture plates were incubated at 37°C. Images of the scratch wound were taken using a digital camera (Olympus Corporation, Japan) 24 h later. We repeated all experiments three times.

CCK-8 assay for cell proliferation

In each group, cells were seeded at 1×10^4 per well in 96-well plates and were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C and 5% CO₂ for 24 h. CCK-8 reagent (Keygen) (10 μL) was added to the maintenance cell medium at different time points and was incubated with the cells at 37°C for

Table 1. Association of miR125b with the clinicopathological characteristics of epithelial ovarian cancer. (NS; Not statistically significant)

Variables	Cases (n=55)	miR-125b expression		P ^a
		Low/Neg.	High	
Age (years)				NS
≤ 55	18	7	11	
> 55	37	14	23	
FIGO stage				<0.05
Stage I-II	13	10	3	
Stage III-IV	42	11	31	
Histological type				NS
Serous	40	15	25	
Non-serous	15	6	9	
Tumor grade				NS
1-2	20	8	12	
3 or clear cell	35	13	22	

an additional 4 h. Absorbance values were determined by using amicroplate reader (ASYS Hitech GmbH, CliniBio 128C) at 450 nm.

Apoptosis assay

Cell apoptosis was measured using the Annexin V-FITCkit (Miltenyi Biotec). Cells were harvested at 48h post-transfection, washed twice with PBS, resuspended in 100 μ L of 1 $\times$ binding buffer and then stained with 5 μ L of Annexin V-FITC. After 15 min at room temperature in the dark, the cells were resuspended in 500 μ L of the 1 $\times$ binding buffer. A total of 3 μ L of PI solution was added immediately prior to analysis by flow cytometry (FACSCalibur, BD Biosciences, USA).

Mouse model

Nude mice were purchased from Vital River Laboratories (Beijing, China). All animals were used in accordance with the institutional guidelines, and the current experiments were approved by the Use Committee for Animal Care. SKOV3 cells were transfected with the vector control, miR-125b mimic or inhibitor. All cultures used for tail injection were sub-confluent and were fed the day prior to their use. The harvested cell suspension was washed twice by centrifugation in medium containing serum at room temperature and then resuspended in medium without serum at 4°C immediately prior to injection. The cells (1 $\times$ 10⁶ cells) to be injected were suspended in 0.1 ml PBS. The animals were sacrificed 6 weeks after the injections. Pictures were recorded using a Nikon d800 digital camera (Nikon, Tokyo, Japan).

Statistical analyses

All statistical analyses were performed using SPSS for Windows, v.18.0 (SPSS, Chicago, IL). P-values less than 0.05 were considered statistically significant ($P < 0.05$), and graphs were plotted using GraphPad Prism 5.02. Comparisons between groups were performed using the Kruskal-Wallis test for continuous variables and the chi-square (χ^2) test for categorical variables.

Results

miR-125b is downregulated in EOC tissues

The miR-125b expression level was examined using qRT-PCR in ovarian tissues obtained from 55 ovarian cancer patients and was normalized to GAPDH. Our data indicated that miR-125b expression was lower in ovarian cancer tissues (Fig. 1A and B). Furthermore, we analyzed the correlation between the miR-125b expression and the clinicopathological characteristics and found that the expression of miR-125b was significantly higher in tumors at an earlier FIGO stage (Stage I-II). The other clinicopathological characteristics, including age, histological type and tumor grade, did not have a statistically significant effect (Table 1).

To determine the cellular localization of the SET protein and EMT-related indicators in the human ovaries, immunohistochemical analysis was performed, as shown in the typical sample pictures in Fig. 1. Expression of the SET protein in ovarian cancer was clearly higher than that in normal ovaries (Fig. 1C, D). The EOC group was divided into two subgroups according to the expression of miR-125b. The group with high miR-125b expression also had low expression of SET and epithelial cadherin (E-cad), which is a hallmark of the epithelial layer; in contrast, the group with low miR-125b expression had relatively high expression levels of SET and E-cad. Furthermore, a linear correlation test was performed using the expression of SET and E-cad as well as that of SET and miR-125b; the results suggested that SET expression was positively and negatively related to E-cad and miR-125b expression, respectively (Fig. 1C, D).

miR-125b binds directly to the SET 3'-UTR to regulate SET expression

To determine whether miR-125b regulates SET expression, the binding of miR-125b to the 3'-UTR region of SET was first evaluated using online bioinformatics tools (Fig. 2A, B). Then, we co-transfected a miR-125b mimic or inhibitor with a dual-luciferase reporter construct containing either the wild-type or a mutant SET 3'-UTR. The results showed that

Fig. 1. MiR-125b expression is dramatically attenuated in EOC tissues and is negatively correlated with SET and EMT. (A-B) Transcription of miR-125b and SET in EOC and normal ovaries tissues was determined by real-time PCR. The subdivision of EOC patients by miR-125b expression was based on a 95% CI. (C) Expression of SET protein in EOC and normal ovaries by western blot assay. (D) Representative images of IHC staining for SET or EMT markers (E-cad) in the high or low miR-125b subgroups in EOC or normal tissues; linear correlation studies were performed between SET vs. E-cad and miR-125b vs. SET.

the mimic reduced luciferase activity by 54% compared with the inhibitor and also had lower luciferase activity than the empty vector control (Fig. 2C). These data demonstrate that miR-125b can combine with the 3'-UTR region of SET to inhibit gene expression and that the mutated region may eliminate this inhibitory effect.

miR-125b inhibits SKOV3 migration and invasion in vitro

The vast majority of deaths from cancer are due to metastasis, and having ascertained the relationship between miR-125b expression and metastasis, we therefore investigated whether reintroducing miR-125b would decrease the invasive and migration potential of SKOV3 cells. To investigate the effects of miR-125b on EOC invasion, SKOV3 cells were transfected with a miR-125b human mimic, an inhibitor or a negative control (NC). The invasion capacity was measured via transwell. Overexpression of miR-125b can significantly weaken cell invasion capabilities compared with the inhibitor group (Fig. 3A, B). Scratch assays showed that the group transfected with the miR-125b mimic significantly decreased cell invasion, while cell invasion was apparently suppressed by using an miR-125b inhibitor (Fig. 3C, D). Therefore, these data indicate that miR-125b is involved in the regulation of EOC invasion and migration.

Fig. 2. Overexpression of miR-125b inhibits the EMT pathway. Left panel: The sequence alignment of human miR-125b matches the 3'-UTR of SET. Bottom: mutations of the 3'-UTR of SET used to create the mutant luciferase reporter construct. Right panel: Map of the human miR-125b genomic region with the phylogenetically conserved SET-binding site indicated. (B) SKOV3 cells were transfected with miR-125b human mimics, an inhibitor, or their respective negative controls. (D-E) The expression of SET, E-cad, N-cad and Vimentin was analyzed by western blotting. Representative blots and the results of the analyses are shown (* $P < 0.05$).

Fig. 3. Cell invasion and migration are inhibited by miR-125b in SKOV3 cells. (A and C) Matrigel invasion assay and scratch wound healing assay were performed to investigate the changes in cell invasion and migration. (B and D) The bar graph shows quantification of the cell invasion or migration of the miR-125b mimics-transfected and inhibitor-transfected cells. Statistical results of three independent experiments are shown.

miR-125b cannot affect the proliferation and apoptosis of EOC cells

To investigate whether miR-125b affects the proliferation and apoptosis of EOC cells, SKOV3 cells transfected as described above were analyzed by Annexin V-FITC/PI

Fig. 4. MiR-125b has no effect on the proliferation and apoptosis of EOC cells. (A) Apoptosis rate of the cells was detected by flow cytometry. UR: dead cells; LR: apoptotic cells; LL: viable cells. (B) The apoptosis rate was not significantly different among the groups. (C) The fold increase in cell proliferation was tested using CCK-8at 24h intervals. The miR-125b human mimics group did not have a significantly different growth rate compared with the inhibitor or negative control groups in SKOV3 cells. (D) Protein expression of the SKOV3 cells in (A) in each group was detected by western-blot.

staining. The results showed that overexpression or downregulation of miR-125b had no apparent effect on either apoptosis or cell proliferation (Fig. 4A, B). Furthermore, proteins that indicate cell proliferation and apoptosis, such as cyclinD1 and cleaved caspase-3, were detected, revealing that miR-125b has no significant impact on cell proliferation and apoptosis (Fig. 4D). The fold increase in cell proliferation was tested using CCK-8at 24-h intervals. The miR-125b human mimics group did not show a significantly different growth rate compared with the inhibitor or the negative control group in SKOV3 cells (Fig. 4C).

miR-125b inhibits SKOV3 migration and invasion *in vivo*

To investigate the role of miR-125b in inhibiting migration, an *in vivo* assay was performed using tail injections in a nude mouse model; several metastases were found in the WT control group due to the invasive nature of the SKOV3 cells. However, metastasis was almost entirely inhibited when miR-125b was overexpressed in such cells; moreover, significantly increased metastasis was found when the inhibitor of miR-125b was used. In addition, the tumor volume of each group was determined, and no significant difference was found among the three groups (Fig. 5A). The expression of SET- and EMT-related indicators were detected in both the primary tumor and the metastases. Within the primary tumor, as in the *in vitro* assay, SET expression was enhanced in the mimic group and dramatically attenuated in the inhibitor group, and EMT indices, such as the epithelial marker E-cad, were decreased in the mimic group and in the inhibitor group. In contrast, the mesenchymal marker Vim was increased in the mimic group and was lower in the inhibitor group. Within the metastases, the EMT shift was more apparent in the inhibitor group than in the WT control (Fig. 5B).

Fig. 5. MiR-125b inhibited tumor metastasis *in vivo*. (A) Tumorigenesis assay using luciferase-transfected SKOV3 cells via tail vein injection in nude mice. The representative figure is presented in the left panel, and a comparison of metastases in the groups listed is presented in the right panel. (B) Protein expression of the indicated genes in three different subsets was analyzed by western blot in the primary tumor and in the metastases.

Discussion

Ovarian cancer is a highly lethal disease that lacks effective screening tests for early detection [19, 20]. Most ovarian cancers are diagnosed at an advanced stage when the tumor is largely metastatic [1, 21]. Recently, increasing data have indicated that non-coding RNAs, including lncRNA and miRNA, are crucial in the regulation of essential functional genes related to the biological features of invasion, such as EMT [22-24]. This study demonstrates that miR-125b is a tumor suppressor in EOC by inhibiting EMT and directly targeting SET. Moreover, we demonstrated that the downregulation of miR-125b was linked to cell invasion and migration and tumor occurrence, and low expression of miR-125b was associated with advanced FIGO stage (Stage III-IV), which was followed by experimental validation in cell culture studies and clinical data.

MicroRNAs (miRNAs) are endogenous, single-stranded, non-coding, small RNAs that regulate gene expression by preferentially binding to specific sequences in the 3'-untranslated region (3'-UTR) of their target mRNAs [25-27]. In the present study, miR-125b was found to be associated with tumor metastasis by controlling the EMT by regulating the SET gene through 3'-UTR binding. This outcome was similar to the findings of Zhou et al., who indicated that miR-125b may exert inhibitory effects on EMT in liver cancer cells by SMAD2 and that EMT could play an important role in ovarian cancer tumorigenesis [15].

Based on the clinical samples from 55 cases of ovarian cancer, low expression of miR-125b is associated with higher FIGO grades. Interestingly, other researchers have found that miR-125b expression in epithelial ovarian cancer cells and tissues is significantly decreased [23]. In addition, the expression of miR-125b was negatively related to SET expression and the degree of EMT, and the mechanism involved was verified by both *in vivo* and *in vitro* assays showing that miR-125b can downregulate SET transcription and therefore attenuate EMT in ovarian cells. SET overexpression promotes cancer cell invasion and migration and is significantly associated with androgen production by ovarian follicles *in vitro* [28].

SET is widely expressed in many tissues, such as adrenal glands, gonads and the central nervous system [29, 30]. The SET protein belongs to a family of multitasking proteins, also known as TAF-1 β , I2PP2A and INHAT, which are involved in apoptosis, transcription, nucleosome assembly, and histone binding [31, 32]. This nuclear phosphoprotein encoded by the translocation breakpoint has been proven to be associated with acute myelogenous leukemia [16, 33].

Thus, these studies not only substantially broaden our understanding of the complex mechanisms underlying the pathogenesis of EOC but also suggest potential new interventional and therapeutic strategies.

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