MiR-381 regulates cell motility, growth and colony formation through PIK3CA in endometriosis-associated clear cell and endometrioid ovarian cancer

CHIA-YI HSU[1,2], TSUNG-HUA HSIEH[1], TZE-KIONG ER[3], HUNG-SHENG CHEN[1], CHING-CHOU TSAI[4] and EING-MEI TSAI[1,2]

[1]Department of Obstetrics and Gynecology, Kaohsiung Medical University Hospital; [2]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807; [3]Department of Health and Nutrition Biotechnology, College of Medical and Health Science, Asia University, Taichung 402; [4]Department of Obstetrics and Gynecology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung 833, Taiwan, R.O.C.

Received May 16, 2018; Accepted September 7, 2018

DOI: 10.3892/or.2018.6779

Abstract. Ovarian cancer is the one of the most lethal gynecological cancer types. MicroRNAs (miRs) are noncoding RNAs that modulate the translation of their target mRNAs via binding to a complementary sequence in the target 3' untranslated region, and the dysregulation of certain miRs has been demonstrated to contribute to cancer progression. In this regard, the current study extended our previous work and used next-generation sequencing data to search for upstream regulators of genetic alterations that are common in ovarian cancer, as well as the miRs that are involved in controlling the expression of these regulators. An miR prediction program was used to identify miR-381 as an upstream regulator of phosphatidylinositol 3-kinase catalytic subunit α (PIK3CA) in the context of ovarian cancer. Levels of miR-381 were decreased in clear cell and endometrioid carcinoma ovarian cancer. Experimentally induced upregulation of miR-381 led to a decrease in the level of PIK3CA in ovarian cancer cells. Furthermore, experimentally induced upregulation of miR-381 inhibited the proliferation of ovarian cancer cells *in vitro* and their ability to form colonies and migrate. The observed decrease in miR-381 in ovarian cancer could be reversed upon overexpression of the gene encoding the tumor suppressor homeobox D10. The current results highlight the role of miR-381-mediated regulation of PIK3CA in the development and progression of ovarian cancer and suggest that restoration of miR-381 to normal levels in ovarian cancer cells may constitute a therapeutic strategy for patients.

Correspondence to: Professor Eing-Mei Tsai, Department of Obstetrics and Gynecology, Kaohsiung Medical University Hospital, 100 Zihyou 1st Road, Sanmin, Kaohsiung 807, Taiwan, R.O.C.
E-mail: tsaieing@yahoo.com

Key words: ovarian cancer, microRNA-381, homeobox D10

Introduction

Ovarian cancer is the one of the most lethal gynecological cancer types. Approximately 85-90% of ovarian malignancies manifest as epithelial ovarian cancers (1). Ovarian cancer is a heterogeneous disease, which has been divided into two major subtypes based on histopathological, molecular and genetic criteria (2). Type I tumors comprise low-grade serous, clear cell, endometrioid and mucinous carcinomas, whereas Type II tumors comprise high-grade serous carcinomas, undifferentiated carcinomas and carcinosarcomas. Ovarian cancer remains a highly lethal malignancy, primarily as a result of inability to detect the cancer at an early, and thus more treatable, stage (3). Studies have demonstrated that ovarian cancer development is associated with the accumulation of certain gene mutations, as well as alterations in epigenetic modifications, which are associated with downstream activation of signaling pathways (4,5). Low-grade serous, clear cell, endometrioid and mucinous carcinomas are often associated with mutations in KRAS, BRAF, AT-rich interaction domain 1A (ARID1A), phosphatidylinositol 3-kinase catalytic subunit α (PIK3CA), CTNNB1 and ERBB2 but only very rarely in the tumor-suppressor gene tumor protein 53 (TP53) (6-9). Patients with high-grade serous ovarian cancer present with highly unstable genomes and harbor TP53 mutations in ~95% of cases (10). Although numerous studies have attempted to elucidate the general mechanisms of carcinogenesis in ovarian cancer, the molecular mechanisms, both genetic and epigenetic, that underlie ovarian tumorigenesis are not clearly understood.

MicroRNAs (miRNAs or miRs) are non-coding RNAs, 19-22 nucleotides in length, which are usually involved in the post-transcriptional downregulation of gene expression via base pairing with the 3'-untranslated region (3'-UTR) of target mRNAs. MiRNAs have been demonstrated to regulate both the translatability and turnover of numerous mRNAs involved in various biological processes, and hence miRNAs often serve as oncogenes or tumor suppressors (11). Mounting evidence indicates that miRNAs help define a particular

cancer phenotype via modulating the expression of genes and signaling pathways that are often involved in tumorigenesis (12). The signaling pathways involved in tumorigenesis that are frequently activated in human cancer thus represent attractive targets for therapies based on small molecules. Therefore, defining the networks of genetic and epigenetic interactions that govern tumorigenesis in any particular cancer type could inform molecular strategies for effective treatments across cancer subtypes.

The aim of the current study was to investigate whether epigenetic drivers could cause genetic alterations that typically lead to ovarian cancer. Next-generation sequencing data from our previous study (13) was analyzed to identify potential miRNAs that, upon dysregulation, could significantly impact both growth factor signaling pathways and the rate of genetic mutations that often lead to ovarian cancer.

Materials and methods

Bioinformatics analysis. Target prediction tools TargetScan (version 7.1; http://www.targetscan.org/vert_71/) and miRanda (http://www.mirbase.org/) were used to search potential target miRNAs for PIK3CA, ARID1A, ETS1 and DNA-dependent protein kinase catalytic subunit (PRKDC).

Cell lines and culture. The ovarian cancer cell lines TOV21G and TOV112D were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were grown in a 1:1 mixture of complete Medium 199 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and MCDB 105 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All cells were cultured in a humidified incubator at 37˚C with 5% CO_2. The procedure used to isolate stromal cells from an ectopic endometriotic implant has been described previously (14,15). Ovarian endometrial tissue from 3 patients of reproductive age (aged 21 to 42 years) who underwent laparoscopic surgery was obtained from the Department of Obstetrics and Gynecology, Kaohsiung Medical University Hospital (Kaohsiung, Taiwan). For each case, a final diagnosis of endometriosis was made according to the revised American Society of Reproductive Medicine classification (1997) in female patients that had been surgically resected between June 2014 and April 2016 (16). All samples were histologically confirmed by pathologists. All patients had regular menstrual cycles and had not received any hormone treatment within the 6 months prior to gynecological surgery.

The tissue samples were enzymatically dissociated using collagenase II, then stromal cells were separated from epithelial glands by wire sieves. The procedure used to isolate stromal cells from an ectopic endometriotic implant has been described previously (14,15). Filtered stromal cells were then cultured in Dulbecco's Modified Eagle's Medium Nutrient Mixture F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. All cells were cultured in a humidified incubator at 37˚C with 5% CO_2. The study protocol was approved by the Ethics Committee of the Institutional Review Board of the Kaohsiung Medical University Hospital (approval no. KMUH-IRB-20140031), and informed written consent was obtained from each patient.

Serum collection. Serum samples were obtained from patients who underwent surgery with indications of gynecological disease at the Department of Obstetrics and Gynecology of Kaohsiung Medical University Hospital. A total of 9 subjects aged 21 to 55 years participated in the present study, including 3 healthy controls, 3 patients with endometriosis, and 3 patients with ovarian cancer (1 endometrioid and 2 clear cell carcinoma). In each case of endometriosis-associated ovarian cancer (EAOC), a final diagnosis of endometriosis-associated clear cell cancer, endometrioid cancer or ovarian cancer was made according to FIGO and WHO criteria in patients who were surgically resected between June 2014 and April 2016 (17). Each diagnosis of endometriosis, either by laparoscopy or laparotomy, followed the classification of the revised American Society of Reproductive Medicine, 1997 (16). The patients with endometriosis and EAOC were histologically confirmed by examination of a laparoscopy. All patients had regular menstrual cycles and had not received any hormone treatment in the 6 months prior to gynecological surgery. Healthy controls were diagnosed as having other benign diseases, including urinary incontinence, pelvic organ prolapse or ovarian hemorrhagic cyst. Exclusion criteria included post-menopausal status, endometrial cancer, hyperplasia, endometrial polyps, infectious diseases, adenomyosis, inflammatory diseases, malignancy, autoimmune disease or cardiovascular disease.

The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (approval no. KMUHIRB-G(I)-20150046), and written informed consent was obtained from each participant. Whole blood was drawn in EDTA-coated tubes (BD Biosciences, Franklin Lakes, NJ, USA) and centrifuged at 2,200 x g for 10 min at room temperature. The resultant sera were aliquoted into Eppendorf tubes and stored at -80˚C for use in reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) studies.

RNA extraction, cDNA synthesis and qPCR. RNA was extracted from patient sera using bulk reagents from the MasterPure Complete DNA and RNA Purification kit (Epicentre; Illumina, Inc., San Diego, CA, USA) or purified from cultured cells using TRIzol reagent (Thermo Fisher Scientific, Inc.). RT was performed using the Deoxy+HiSpec RT kit (cat. FYT501-100R; Yeastern Biotech Co., Ltd., Taipei, Taiwan) and the TaqMan MicroRNA Reverse Transcription kit (cat. 4366596; Applied Biosystems; Thermo Fisher Scientific, Inc.). Then, qPCR was performed using the ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). mRNA was amplified using the Eztime Fast Real-Time PCR Premix (2X, SYBR-Green, ROX; Yeastern Biotech Co., Ltd.), and 18S mRNA served as the internal control. The levels of miRNAs were measured using specific primers to miR-381 (000571), miR-203 (000507) and RNU6 (001093; Applied Biosystems; Thermo Fisher Scientific, Inc.). RNU6 served as the internal control. The reaction mixture included 2 μl cDNA, 5 μl TaqMan Universal PCR Master Mix II (2X; cat. no. 4440042; Applied Biosystems; Thermo

Table I. Primer sequences.

Gene name	Sequence
18S	F: 5'-CATGGCCGTTCTTAGTTGGT-3'
	R: 5'-CGCTGAGCCAGTCAGTGTAG-3'
PIK3CA	F: 5'-TTCTTACATTTTCGTAA GTGTTACTCA-3'
	R: 5'-CGAAGGTATTGGTTTAGACAGAAA-3'
PRKDC	F: 5'-TGCTTTTCCATGAGCTATTACA-3'
	R: 5'-TGGCAACTTAACGTGTTTGA-3'
ETS1	F: 5'-GAACGAATTTGGGAACATGC-3'
	R: 5'-CTTCCCTTCATCCACC TCCT-3'
ARID1A	F: 5'-CCAGCAGAACTCTCACGACC-3'
	R: 5'-CTGAGCGA AGGACGAAGACG-3'
RNU6B	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT
miR-381	UAUACAAGGGCAAGCUCUCUGU
miR-203	GUGAAAUGUUUAGGACCACUAG
MiR-381 ChIP (-565 to -574)	F: 5'-GTGTGCTCATCAGCGCTTTTT-3'
	R: 5'-ACAGCTCCCACGGCTCAT-3'
MiR-381 ChIP (-682 to -691)	F: 5'-CGGAGCACTGGCTCTGTCTA-3'
	R: 5'-GGACTGCGAGCTGGATCAT-3'

PIK3CA, phosphatidylinositol 3-kinase catalytic subunit α, PRKDC, DNA-dependent protein kinase catalytic subunit; ARID1A, AT-rich interaction domain 1A; miR, microRNA; ChIP, chromatin immunoprecipitation.

Fisher Scientific, Inc.), 1 μl specific primers and 2.5 μl RNase-free water for a final reaction volume of 10 μl. The thermocycling conditions were initiated by uracil-N glycosylase activation at 50˚C for 2 min and initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, and annealing at 60˚C for 60 sec. Expression levels were normalized to that of an endogenous control. Relative miRNA and mRNA levels were determined using the $2^{-\Delta\Delta Cq}$ method (18). The qPCR primers for HOXD10, 18S, PIK3CA, PRKDC, ETS1 and ARID1A were synthesized by Genemessenger Scientific Co., Ltd. (Kaohsiung, Taiwan), and their sequences are listed in Table I.

Transient transfection of cells. TOV21G and TOV112D cells were transiently transfected with 45 nM of miRNA mimics, inhibitors, and 1 μg of short hairpin RNAs (shRNAs) or other plasmids was performed using Turbofect Transfection Reagent (Thermo Fisher Scientific, Inc.). The miRIDIAN miRNA mimic (miR-381 mimics, cat. no. C-300690-03-0010) and mimic negative control (cat. no. CN-001000-01-05) were purchased from GE Healthcare Dharmacon, Inc. (Lafayette, CO, USA). The mirVana miRNA inhibitor (miR-381 inhibitor, cat. no. MH10242) and inhibitor negative control (cat. no. 4464078) were obtained from Ambion (Thermo Fisher Scientific, Inc.). The shRNAs included shRNA-homeobox D10 (HOXD10)#1 (cat.no.TRCN0000274091),shRNA-HOXD10#2 (cat. no. TRCN0000274093), shRNA-HOXD10#3 (cat. no. TRCN0000274028) and control shRNA (cat. no. TRCN0000274091; National RNAi Core Facility at the Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). pDNA-HOXD10 was purchased from Addgene, Inc. (Cambridge, MA, USA). Following 24 h of transfection, the cells were subjected to assays measuring mRNA and protein expression, cell viability, migration and colony formation.

Cell viability assay. Cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay (Honeywell Fluka; Thermo Fisher Scientific, Inc.). Briefly, ovarian cancer cell lines were seeded into 96-well plates at a density of $5x10^3$ cells/well in a total volume of 100 μl medium with 15% (v/v) FBS, transfected with miRNA mimics, inhibitors or scrambled-sequence controls, and incubated at 37˚C in an atmosphere of 5% CO_2. The following day, 90 μl culture medium and 10 μl CCK-8 reagent were added to each well. Cell viability was assayed after 2 h using an enzyme-linked immunosorbent assay reader (Multiskan EX; Thermo Fisher Scientific, Inc.) at 450 nm (reference, 650 nm). In each case, three independent experiments were performed.

Colony formation assay. Ovarian cancer cells transduced with the miR-381 mimic, miR-381 inhibitor, or scrambled-sequence controls were plated in triplicate into 6-well plates (500 cells/well), cultured in a humidified incubator at 37˚C with 5% CO_2 for 2 weeks, then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet at room temperature for 1 h. Colonies were counted by visual inspection. Digital images of the colonies were obtained using a camera. Colonies were counted using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA). Triplicate wells were measured for each treatment group.

In vitro migration assay. A total of $5x10^4$ cells were seeded into the upper chamber of each Transwell insert (8 μm pore size) in a 24-well plate in serum-free medium. In the lower chambers, medium containing 15% FBS was added as a chemoattractant. Following incubation for 18 h at 37˚C, non-migrated cells in the upper chamber were removed with cotton swabs. The migrated cells on the lower chamber surface were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet at room temperature for 15-20 min. The number of migrated cells was calculated by counting three different fields of view under a phase-contrast microscope. In each case, three independent experiments were performed.

Western blotting. Proteins were extracted using radioimmunoprecipitation lysis buffer (EMD Millipore, Billerica, MA, USA) containing several protease and phosphatase inhibitors (Roche Applied Science, Madison, WI, USA). Protein concentration was determined with the Bio-Rad DC Protein Assay kit from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Cell lysates were analyzed by western blotting. Proteins (30 μg) were resolved on a 10% BIS-TRIS SDS gel) at 100 V and transferred onto nitrocellulose membranes at 90 V for 1.5 h. Non-specific binding was blocked by incubating the membranes in 5% nonfat dry milk in PBS for 1 h. Then, the membranes were incubated with primary antibodies overnight at 4˚C. Primary antibodies were as follows: Mouse monoclonal anti-HOXD10 (cat. no. TA800777; 1:500; OriGene Technologies, Inc., Rockville, MD, USA) or rabbit monoclonal anti-PIK3CA (cat. no. TA302178; 1:1,000; OriGene Technologies, Inc.). Expression of β-actin was used as a loading control and was detected with monoclonal anti-actin (cat. no. A5316; 1:5,000, Sigma-Aldrich; Merck KGaA). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Secondary antibodies were incubated at room temperature for 1 h. Enhanced chemiluminescence reagents (EMD Millipore) were used for immunodetection.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed using the Chromatin Immunoprecipitation Assay kit (Upstate Biotechnology, Inc., Lake Placid, NY, USA). The ALGGEN-PROMO prediction software (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to identify potential transcription factor binding sites within the miR-381 promoter (19). Chromatin was incubated with anti-HOXD10 (cat. no. TA800777; 1:500; OriGene Technologies, Inc.) or goat anti-mouse IgG (control, cat. no. TA130004; 1:500: OriGene Technologies, Inc.). PCR was conducted with primers complementary to the miR-381 promoter region. The primer sequences are presented in Table I. RT semi-quantitative PCR was performed to amplify the transcription factor binding sequence of miR-381. RT-PCR analysis of relative expression was performed with the one-step Emerald Amp GT PCR Master mix (Takara Bio, Inc., Otsu, Japan). Thermocycling was performed under the following conditions: 94˚C for 5 min; 35 cycles of 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec. PCR products were analyzed by electrophoresis with 2% agarose gels and visualized by UV light following staining with EtB'Out' Nucleic Acid Staining Solution (cat. no. FYD007-200P; Yeastern Biotech Co., Ltd).

Statistical analysis. Data are presented as the mean ± standard deviation from at least three independent experiments by GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). For the *in vitro* cell migration assays, groups were compared using the Mann-Whitney U test. For all other results, one-way analysis of variance followed by Tukey's honest significant difference test was used to analyze differences among multiple groups. Student's t-test was used to analyze differences between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of differentially expressed miRNAs and prediction of target genes. In our previous work, the genetic alterations that are commonly observed in endometriosis-associated ovarian cancers were characterized; the genes PIK3CA, ARID1A, ETS1 and PRKDC were identified to be the most frequently mutated genes in patient samples (13). Further examination revealed potential interactions between miRNAs and the mutated mRNAs in ovarian tumors. To characterize the mechanisms by which genetic alterations promote ovarian cancer, TargetScan (version 7.1) and miRanda were used to predict potential upstream regulators of the mutated gene (20). The two most commonly mutated genes encoded mRNAs are regulated by two miRNAs, namely miR-203 and miR-381 (Fig. 1A). Therefore, in the present study, the expression level of miR-203 and miR-381 was determined in tumor samples from patients with ovarian cancer and in ovarian cancer cell lines. The results revealed that miR-381 and miR-203 were expressed at a significantly lower level in the sera of patients with ovarian cancer compared with healthy patients and endometriosis patients, and in ovarian cancer cell lines compared with controls (Fig. 1B and C). Subsequent studies focused on miR-381 because it was the most consistently downregulated miRNA in patient sera (Fig. 1B) and ovarian cancer cell lines (Fig. 1C).

Identification of potential miRNA-mRNA interactions specific to ovarian cancer. To address whether miR-381 affects the cellular levels of mRNAs encoding PIK3CA, ARID1A, EST1 and PRKDC, ovarian cancer cell lines were transfected with an miRNA mimic or miRNA inhibitor specific for these individual mRNAs and then mRNA expression was assessed. The PIK3CA mRNA level was significantly reduced in ovarian cancer cell lines transfected with miR-381 mimics compared with a non-specific control mimic; by contrast, PIK3CA mRNA level was significantly increased following transfection with an miR-381 inhibitor compared with a control non-specific inhibitor (Fig. 2A and B). However, the cellular levels of ARID1A, ETS1 and PRKDC mRNAs did not respond consistently to transfection with various miRNA mimics and inhibitors (Fig. 2A and B). Next, the effect of various concentrations of miR-381 mimics or inhibitors on the cellular level of PIK3CA mRNA was examined in the various transiently transfected ovarian cancer cell lines (Fig. 2C). The results indicated that 45 nM of miR-381 mimic or inhibitor affected the cellular level of PIK3CA mRNA and protein (Fig. 2C and D), suggesting that miR-381 effectively suppresses the cellular level of both PIK3CA mRNA and protein.

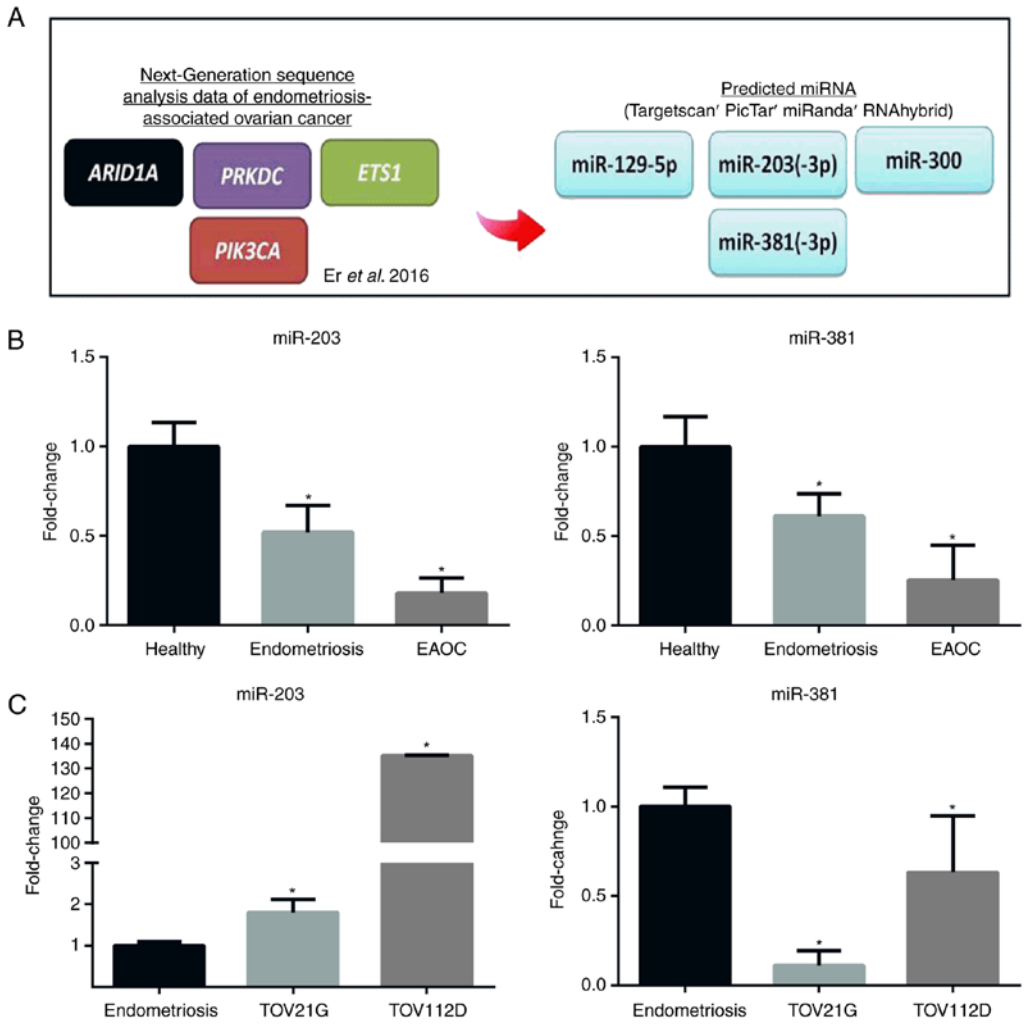

Figure 1. Levels of miR-203 and miR-381 in patient sera and ovarian cancer cell lines. (A) Schematic representation of bioinformatics prediction of candidate miRNAs targeting genes identified by next-generation sequencing as being significantly upregulated or downregulated in patient sera or cell lines. (B) Relative expression of miR-203 and miR-381 in sera from patients with endometriosis or ovarian cancer and in healthy controls, as determined by qPCR. (C) Relative expression of miR-203 and miR-381 in ovarian cancer cell lines (TOV21G and TOV112D) and endometrial stromal cells, as determined by qPCR. *P<0.05 vs. respective control group. EAOC, endometriosis-associated ovarian cancer; miR, microRNA; qPCR, quantitative polymerase chain reaction; PIK3CA, phosphatidylinositol 3-kinase catalytic subunit α, PRKDC, DNA-dependent protein kinase catalytic subunit; ARID1A, AT-rich interaction domain 1A.

Experimental upregulation of miR-381 suppresses cell proliferation, cell migration and colony formation. To investigate how miR-381 suppression promotes ovarian cancer progression, cell proliferation and migration assessed following targeted knockdown or overexpression of miR-381. Cell proliferation was evaluated by CCK-8 and colony formation assays. The downregulation of miR-381 significantly increased cell proliferation, and inhibition of miR-381 activity significantly decreased proliferation (Fig. 3A). Similarly, in a colony formation assay, which is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony (21), clonogenic survival decreased following transfection of cells with miR-381 mimics, whereas survival was enhanced in miR-381 inhibitor-transfected cells (Fig. 3B). Furthermore, cell migration was evaluated by Transwell assays, in which overexpression of miR-381 significantly decreased migration, whereas an miR-381 inhibitor significantly increased migration (Fig. 3C). These results indicated that miR-381 suppresses the growth and migration of ovarian cancer cells.

HOXD10 drives the expression of miR-381. The current results indicated that miR-381 is an upstream regulator of PIK3CA in ovarian cancer cells. Previous studies have identified that dysregulation of the phosphatidylinositol 3-kinase (PI3K) pathway, which is commonly observed in human tumors, drives tumorigenesis; therefore, the PI3K pathway is an appealing therapeutic target (22). Hence, the upstream regulators of miR-381 with respect to the progression of ovarian cancer were investigated. The ALGGEN-PROMO prediction program (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to identify potential transcription factor binding sites within the miR-381 promoter (19,21,23). HOXD10 is a tumor-suppressor gene that suppresses tumor invasion and hence could potentially modulate the cellular level of miR-381 (24). To determine whether miR-381 level is regulated by HOXD10, ovarian cancer cells were transfected with an overexpression plasmid encoding HOXD10. Subsequently, HOXD10 was efficiently overexpressed (Fig. 4A), resulting in a significantly increased cellular level of miR-381 expression (Fig. 4B). Conversely,

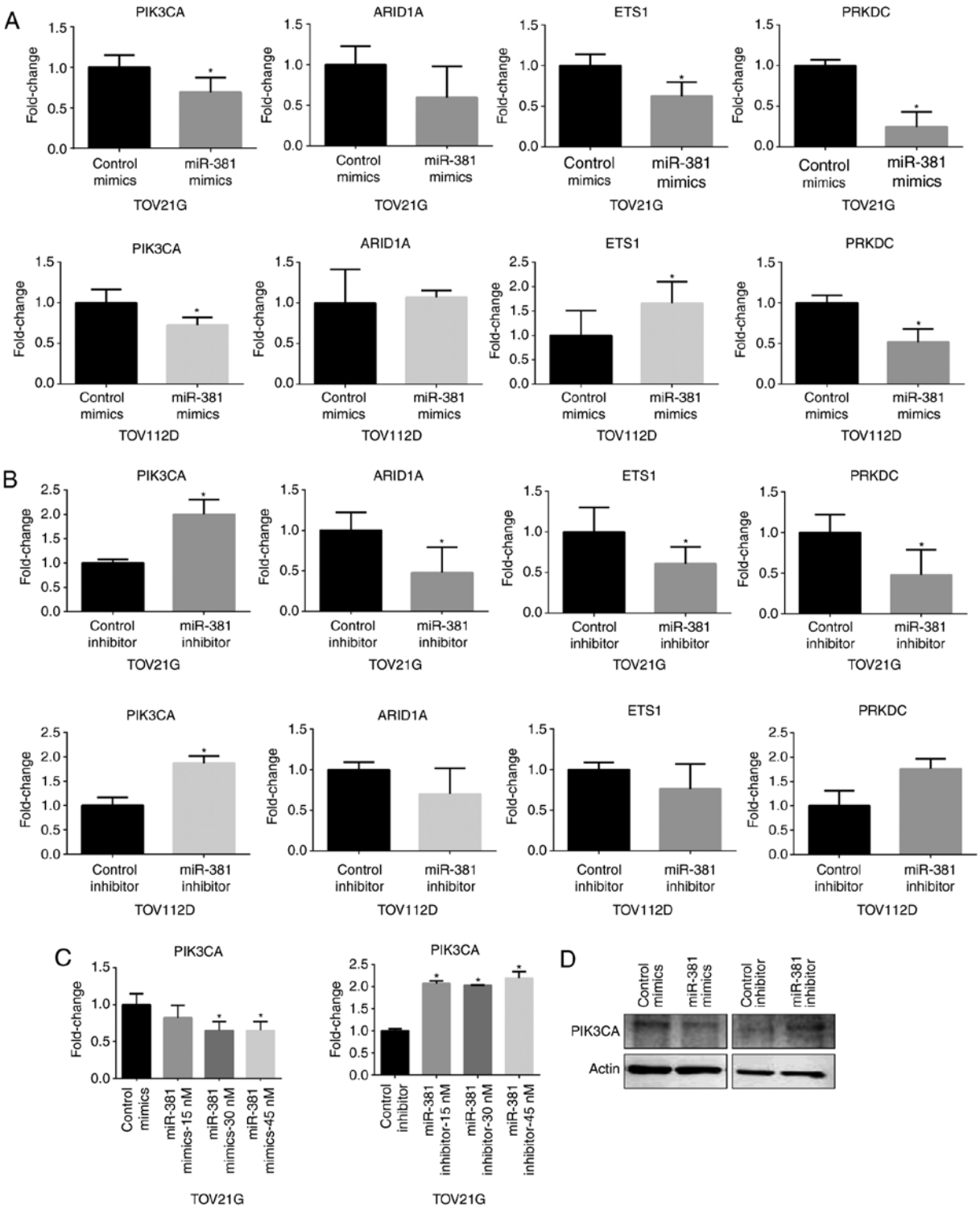

Figure 2. MiR-381 affects the expression of PIK3CA. (A) qPCR analysis of PIK3CA, ARID1A, ETS1 and PRKDC following transfection of cells with miR-381 mimics. (B) qPCR analysis of PIK3CA, ARID1A, ETS1 and PRKDC following transfection of cells with miR-381 inhibitors. (C) qPCR analysis of PIK3CA following transfection of cells with miR-381 mimics or inhibitor at different doses. (D) Western blot analyses of PIK3CA levels following treatment of TOV112D ovarian cancer cells with miR-381 mimics or miR-381 inhibitors. Actin was used a loading control. Data are presented as the mean ± standard deviation from three independent experiments. qPCR, quantitative polymerase chain reaction; miR, microRNA; PIK3CA, phosphatidylinositol 3-kinase catalytic subunit α, PRKDC, DNA-dependent protein kinase catalytic subunit; ARID1A, AT-rich interaction domain 1A. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. control group.

HOXD10 knockdown resulted in a decreased level of miR-381 in cells (Fig. 4B). Furthermore, it was investigated whether HOXD10 modulates the cellular level of miR-381. The ALGGEN-PROMO program was used to predict the promoter region for miR-381. ChIP assays revealed that HOXD10 positively regulated transcription of the miR-381 gene by binding to the region -565 to -574 bp upstream of the miR-381 coding sequence (Fig. 4C).

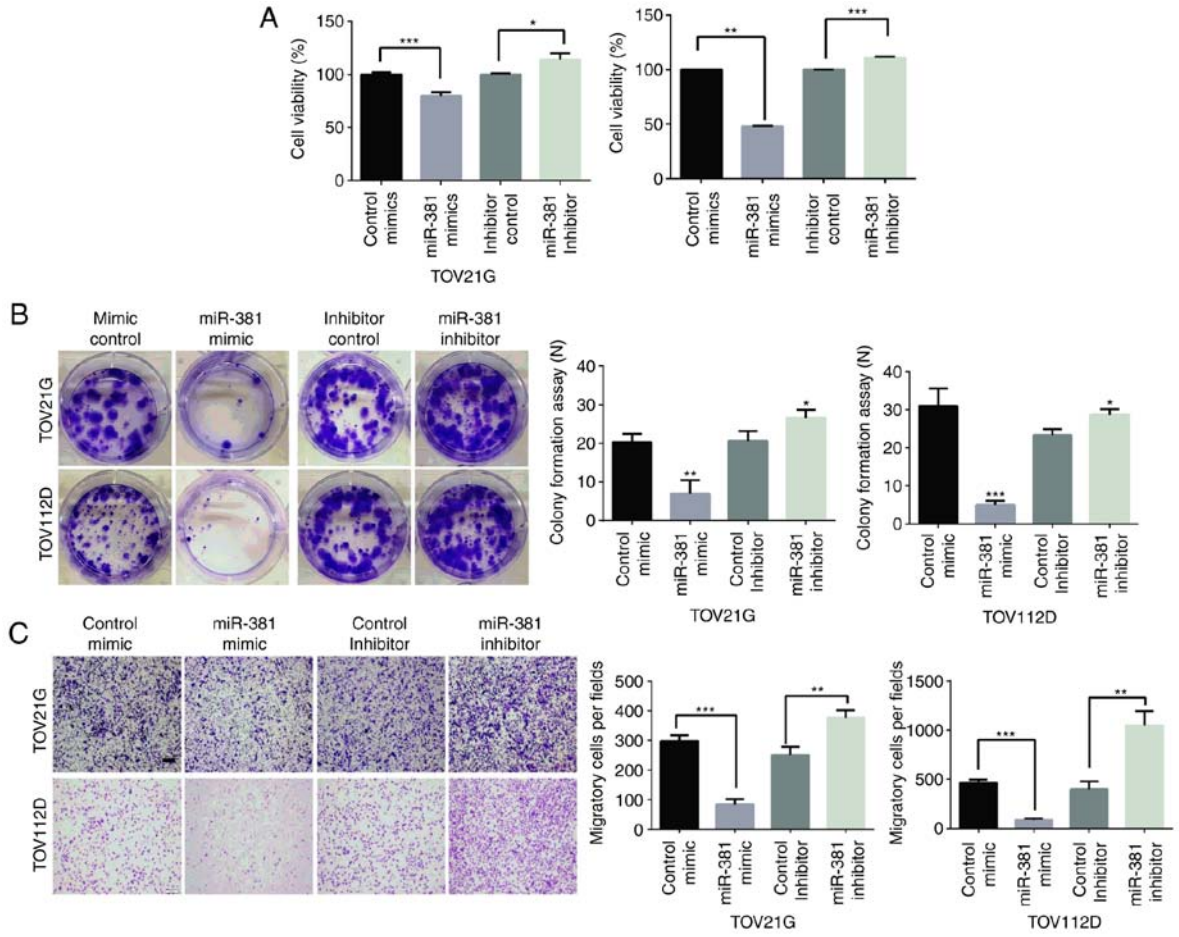

Figure 3. Effect of miR-381 on cell viability and migration *in vitro*. TOV21G and TOV112D cells were transfected with miR-381 mimics or mimic control, or with miR-381 inhibitors or inhibitor control. (A) A Cell Counting Kit-8 assay was performed to determine the viability of ovarian cancer cell lines. (B) Colony formation assays were performed to determine the relative rate of proliferation of ovarian cancer cells. Left panel: Representative images of the colony-forming assay. Right panel: Quantification of the colony formation rate for TOV21G and TOV112D cells. (C) Cell migration was assessed using a Transwell assay, and the cells on the bottom of each Transwell membrane were counted. Magnification, x100. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. respective control group. miR-381, microRNA-381.

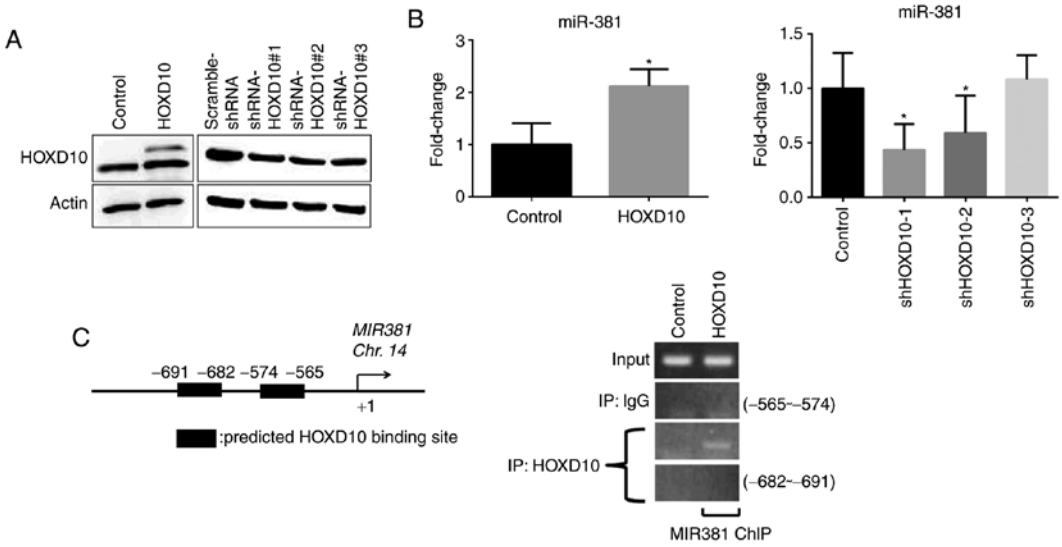

Figure 4. HOXD10 upregulates miR-381 expression. Ovarian cancer cells were transfected with an HOXD10 overexpression plasmid or HOXD10 shRNA. (A) Western blot analysis of HOXD10 in cells following transfection with the HOXD10 plasmid or HOXD10 shRNA. (B) Levels of miR-381 in cells following transfection with the HOXD10 plasmid or HOXD10 shRNA. Expression levels were normalized to that of Run6. (C) Schematic diagram of the HOXD10-binding element identified within the *MIR381* promoter (left panel). ChIP assay of HOXD10 binding to the 5' flanking region (-565 to -574) of the *MIR381* promoter (right panel). IgG was used as the control antibody. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. control group. HOXD10, homeobox D10; ChIP, chromatin immunoprecipitation; miR-381, microRNA-381; shRNA, short hairpin RNA; IP, immunoprecipitating antibody; Input, internal control for the amount of genomic DNA used in the immunoprecipitation.

Discussion

Emerging evidence has demonstrated that several miRNAs are mutated in human cancers, resulting in their dysregulation. These miRNAs may thus act as tumor suppressors or oncogenes. MiRNAs serve as important regulators of gene expression in various human cancer types and hold promise for the development of cancer diagnoses or therapies (25,26). Several studies have investigated the association between miRNAs and the various phenotypes observed in ovarian tumors (27-29). However, the associations between mutations in miRNAs and/or gene-coding regions and cancer risk have not been fully elucidated. The present study was an extension of our previous work, in which next-generation sequencing was used to identify the majority of endometriosis-associated somatic mutations associated with ovarian cancer, including mutations in ARID1A, PIK3CA, ETS1 and PRKDC, among others (13). Based on the results of that previous study, in the present study it was identified that miR-381 is an upstream regulator of PIK3CA using an miR prediction program and the regulation and role of miR-381 in ovarian cancer cells was explored. MiR-381 was observed to be downregulated in ovarian cancer samples and clear cell and endometrioid cancer cells. Exploration of the effect of miR-381 on ovarian cancer cells confirmed that miR-381 inhibits cell migration and proliferation.

MiR-381 has been identified to be involved in a variety of tumorigenic processes, including cell proliferation, apoptosis, migration and invasion, complicating the regulatory network in human cancer. For instance, miR-381 has been identified to be diversely deregulated in several cancer types, including colorectal and breast cancers, and this deregulation could affect epithelial-mesenchymal transition as well as proliferation, invasion, and migration of cancer cells via targeting TWIST and CXCR4 mRNAs (30,31). In addition, Xia *et al* (32) reported the downregulation of miR-381 in ovarian epithelial cancer tissue. The current findings are consistent with these previous studies in that miR-381 was identified to act as a tumor suppressor in various cancer types.

Chemotherapy is the principal treatment for ovarian cancer; however, the acquisition of chemoresistance is a major problem with respect to patient long-term survival. Previous studies have demonstrated that the level of miR-381 correlates with the development of drug resistance following chemotherapy (33,34). Consistent with this, the PI3K-AKT pathway and its components (principally PIK3CA), which target miR-381, are often mutated, and dysregulation of this pathway or its components increases the chemoresistance observed in ovarian cancer (35-37). Therefore, it is proposed that the deregulation of miR-381 may have consequential effects on the PI3K-AKT pathway and thus contribute to chemoresistance. Additionally, it was identified in the current study HOXD10 is a novel transcription factor for miR-381. HOXD10 drives increased transcription of miR-381, perhaps leading to the downregulation of PIK3CA. Previous work has demonstrated that HOXD10 is frequently downregulated in other cancer types, including breast cancer (38), ovarian cancer (24), bladder cancer (39) and cholangiocellular carcinoma (40). Ectopic expression of HOXD10 significantly blocks tumor cell migration and invasiveness, indicating that HOXD10 may serve as a tumor suppressor (38,41). A previous study revealed that HOXD10 is a direct target of miR-10b, which promotes cell invasion through the RhoC/AKT signaling pathway (24).

In addition to the aforementioned results, a role for HOXD10 has been established in angiogenesis and in modulating cell motility in numerous cancer types (24,39,42,43). Similar to the results reported previously, the current results establish a functional connection between HOXD10, miR-381 and PIK3CA.

In summary, the current results demonstrate that miR-381 is significantly downregulated in ovarian cancer cells and that miR-381 overexpression may inhibit the migration and proliferation ability of ovarian cancer cells *in vitro*. Additionally, expression of the miR-381 gene is directly regulated by HOXD10, and HOXD10 may mediate the effect of miR-381 on ovarian cancer cell migration and proliferation. The current results suggest that miR-381 functions as a tumor suppressor through targeting PIK3CA in ovarian cancer.

Acknowledgements

Not applicable.

Funding

This study was supported by the Kaohsiung Medical University Hospital Research Fund (grant no. KMUH106-6R38), Ministry of Science and Technology, Taiwan (grant nos. MOST 105-2314-B-037-052-MY3 and MOST 106-2320-B-037-019) and National Sun Yat-Sen University-KMU Joint Research Project (grant no. NSYSUKMU107-P031).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

CYH and EMT designed the experiments. CYH and EMT conducted the experiments and wrote the manuscript. THH, TKE, CCT and HSC provided the research materials and analyzed the data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research.

Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (approval no. KMUHIRB-G(I)-20150046), and written informed consent was obtained from each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Cho KR and Shih Ie M: Ovarian cancer. Annu Rev Pathol 4: 287-313, 2009.
2. Kurman RJ and Shih Ie M: The origin and pathogenesis of epithelial ovarian cancer: A proposed unifying theory. Am J Surg Pathol 34: 433-443, 2010.
3. Koshiyama M, Matsumura N and Konishi I: Recent concepts of ovarian carcinogenesis: Type I and type II. Biomed Res Int 2014: 934261, 2014.
4. Shen H and Laird PW: Interplay between the cancer genome and epigenome. Cell 153: 38-55, 2013.
5. Berdasco M and Esteller M: Aberrant epigenetic landscape in cancer: How cellular identity goes awry. Dev Cell 19: 698-711, 2010.
6. Shih IeM and Kurman RJ: Ovarian tumorigenesis: A proposed model based on morphological and molecular genetic analysis. Am J Pathol 164: 1511-1518, 2004.
7. Jones S, Wang TL, Shih IeM, Mao TL, Nakayama K, Roden R, Glas R, Slamon D, Diaz LA Jr, Vogelstein B, *et al*: Frequent mutations of chromatin remodeling gene *ARID1A* in ovarian clear cell carcinoma. Science 330: 228-231, 2010.
8. Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, Cristiano BE, Pearson RB and Phillips WA: Mutation of the *PIK3CA* gene in ovarian and breast cancer. Cancer Res 64: 7678-7681, 2004.
9. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConechy MK, Anglesio MS, Kalloger SE, *et al*: *ARID1A* mutations in endometriosis-associated ovarian carcinomas. N Engl J Med 363: 1532-1543, 2010.
10. Ahmed AA, Etemadmoghadam D, Temple J, Lynch AG, Riad M, Sharma R, Stewart C, Fereday S, Caldas C, Defazio A, *et al*: Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. J Pathol 221: 49-56, 2010.
11. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
12. Calin GA and Croce CM: MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857-866, 2006.
13. Er TK, Su YF, Wu CC, Chen CC, Wang J, Hsieh TH, Herreros-Villanueva M, Chen WT, Chen YT, Liu TC, *et al*: Targeted next-generation sequencing for molecular diagnosis of endometriosis-associated ovarian cancer. J Mol Med 94: 835-847, 2016.
14. Kao AP, Wang KH, Chang CC, Lee JN, Long CY, Chen HS, Tsai CF, Hsieh TH and Tsai EM: Comparative study of human eutopic and ectopic endometrial mesenchymal stem cells and the development of an in vivo endometriotic invasion model. Fertil Steril 95: 1308-1315.e1, 2011.
15. Ryan IP, Schriock ED and Taylor RN: Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro. J Clin Endocrinol Metab 78: 642-649, 1994.
16. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril 67: 817-821, 1997.
17. Prat J; FIGO Committee on Gynecologic Oncology: FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: Abridged republication. J Gynecol Oncol 26: 87-89, 2015.
18. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408, 2001.
19. Farre D, Roset R, Huerta M, Adsuara JE, Roselló L, Albà MM and Messeguer X: Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res 31: 3651-3653, 2003.
20. Lewis BP, Burge CB and Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20, 2005.
21. Franken NA, Rodermond HM, Stap J, Haveman J and van Bree C: Clonogenic assay of cells in vitro. Nat Protoc 1: 2315-2319, 2006.
22. Li H, Zeng J and Shen K: PI3K/AKT/mTOR signaling pathway as a therapeutic target for ovarian cancer. Arch Gynecol Obstet 290: 1067-1078, 2014.
23. Ferrara N and Kerbel RS: Angiogenesis as a therapeutic target. Nature 438: 967-974, 2005.
24. Nakayama I, Shibazaki M, Yashima-Abo A, Miura F, Sugiyama T, Masuda T and Maesawa C: Loss of HOXD10 expression induced by upregulation of miR-10b accelerates the migration and invasion activities of ovarian cancer cells. Int J Oncol 43: 63-71, 2013.
25. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, *et al*: Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18: 997-1006, 2008.
26. Ambros V: The functions of animal microRNAs. Nature 431: 350-355, 2004.
27. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, *et al*: MicroRNA signatures in human ovarian cancer. Cancer Res 67: 8699-8707, 2007.
28. Dahiya N and Morin PJ: MicroRNAs in ovarian carcinomas. Endocr Relat Cancer 17: F77-F89, 2010.
29. Mateescu B, Batista L, Cardon M, Gruosso T, de Feraudy Y, Mariani O, Nicolas A, Meyniel JP, Cottu P, Sastre-Garau X and Mechta-Grigoriou F: miR-141 and miR-200a act on ovarian tumorigenesis by controlling oxidative stress response. Nat Med 17: 1627-1635, 2011.
30. He X, Wei Y, Wang Y, Liu L, Wang W and Li N: MiR-381 functions as a tumor suppressor in colorectal cancer by targeting Twist1. Onco Targets Ther 9: 1231-1239, 2016.
31. Xue Y, Xu W, Zhao W, Wang W, Zhang D and Wu P: miR-381 inhibited breast cancer cells proliferation, epithelial-to-mesenchymal transition and metastasis by targeting CXCR4. Biomed Pharmacother 86: 426-433, 2017.
32. Xia B, Li H, Yang S, Liu T and Lou G: MiR-381 inhibits epithelial ovarian cancer malignancy via YY1 suppression. Tumour Biol 37: 9157-9167, 2016.
33. Xu Y, Ohms SJ, Li Z, Wang Q, Gong G, Hu Y, Mao Z, Shannon MF and Fan JY: Changes in the expression of miR-381 and miR-495 are inversely associated with the expression of the MDR1 gene and development of multi-drug resistance. PLoS One 8: e82062, 2013.
34. Boren T, Xiong Y, Hakam A, Wenham R, Apte S, Chan G, Kamath SG, Chen DT, Dressman H and Lancaster JM: MicroRNAs and their target messenger RNAs associated with ovarian cancer response to chemotherapy. Gynecol Oncol 113: 249-255, 2009.
35. Lee S, Choi EJ, Jin C and Kim DH: Activation of PI3K/Akt pathway by PTEN reduction and *PIK3CA* mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. Gynecol Oncol 97: 26-34, 2005.
36. Mitsuuchi Y, Johnson SW, Selvakumaran M, Williams SJ, Hamilton TC and Testa JR: The phosphatidylinositol 3-kinase/AKT signal transduction pathway plays a critical role in the expression of $p21^{WAF1/CIP1/SDI1}$ induced by cisplatin and paclitaxel. Cancer Res 60: 5390-5394, 2000.
37. Astanehe A, Arenillas D, Wasserman WW, Leung PC, Dunn SE, Davies BR, Mills GB and Auersperg N: Mechanisms underlying p53 regulation of PIK3CA transcription in ovarian surface epithelium and in ovarian cancer. J Cell Sci 121: 664-674, 2008.
38. Carrio M, Arderiu G, Myers C and Boudreau NJ: Homeobox D10 induces phenotypic reversion of breast tumor cells in a three-dimensional culture model. Cancer Res 65: 7177-7185, 2005.
39. Xiao H, Li H, Yu G, Xiao W, Hu J, Tang K, Zeng J, He W, Zeng G, Ye Z and Xu H: MicroRNA-10b promotes migration and invasion through KLF4 and HOXD10 in human bladder cancer. Oncol Rep 31: 1832-1838, 2014.
40. Yang H, Zhou J, Mi J, Ma K, Fan Y, Ning J, Wang C, Wei X, Zhao H and Li E: HOXD10 acts as a tumor-suppressive factor via inhibition of the RHOC/AKT/MAPK pathway in human cholangiocellular carcinoma. Oncol Rep 34: 1681-1691, 2015.
41. Reddy SD, Ohshiro K, Rayala SK and Kumar R: MicroRNA-7, a homeobox D10 target, inhibits p21-activated kinase 1 and regulates its functions. Cancer Res 68: 8195-8200, 2008.
42. Myers C, Charboneau A, Cheung I, Hanks D and Boudreau N: Sustained expression of homeobox D10 inhibits angiogenesis. Am J Pathol 161: 2099-2109, 2002.
43. Ma L, Teruya-Feldstein J and Weinberg RA: Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 449: 682-688, 2007.