

# MiR-381 inhibits epithelial ovarian cancer malignancy via YY1 suppression

Bairong Xia<sup>1</sup> · Huiyan Li<sup>2</sup> · Shanshan Yang<sup>1</sup> · Tianbo Liu<sup>1</sup> · Ge Lou<sup>1</sup>

Received: 24 November 2015 / Accepted: 6 January 2016 / Published online: 14 January 2016  
© International Society of Oncology and BioMarkers (ISOBM) 2016

**Abstract** Epithelial ovarian cancer (EOC) is a common type of gynecologic cancer, which accounts for the majority of deaths among all gynecologic malignant tumors in developed countries. A series of recent studies suggested that miR-381 might play important roles in the development of various cancer types. However, the biological function of miR-381 in EOC remains to be investigated. We examined the levels of miR-381 expression in EOC tissues and cell lines. We identified miR-381 target genes by bioinformatic prediction. We also characterized the phenotype regarding cell proliferation, cell migration, and cell invasion in EOC cells lines with altered expression levels of both miR-381 and its target gene, YY1. The expression levels of miR-381 were downregulated in EOC tissues and cell lines. Overexpression of miR-381 significantly inhibited EOC cell proliferation, migration, and invasion. Restoration of YY1 expression partially reversed the phenotype induced by miR-381 overexpression. Knockdown of miR-381 target gene, YY1, mimicked the phenotype induced by miR-381 overexpression. MiR-381 regulated EOC

cell through miR-381/YY1/p53 and miR-381/YY1/Wnt signaling axis. We concluded that miR-381 inhibited EOC cell proliferation, migration, and invasion, at least in part, via suppressing YY1 expression.

**Keywords** miR-381 · YY1 · Epithelial ovarian cancer · Cell proliferation · Cell migration · Cell invasion

## Introduction

Epithelial ovarian cancer (EOC) is a common type of gynecologic cancer, which accounts for the majority of deaths among all gynecologic malignant tumors in developed countries [1]. The diagnosis and treatment of EOC are faced with a series of challenges, including the incomplete understanding of its pathology, the lack of reliable diagnostic biomarkers, and the resistance to chemotherapy drugs [2–4]. Because of these reasons, most of the patients with advanced EOC suffer from relapse and ultimately develop chemotherapy resistance and die from the disease [5, 6]. This situation prompts the urgent need for the development of novel therapeutic approaches in EOC to achieve better treatment outcome.

MicroRNAs (miRNAs), a class of 19–22 nucleotide RNAs, have recently emerged as novel regulators of cellular activities. They regulate gene expression in a post-transcriptional manner via binding to the 3'-UTR of target miR-381 expression (miRNA), resulting in mRNA degradation or translational inhibition [7, 8]. Accumulating evidence has implicated miRNAs in the development of many cancer types, as either oncogenes or tumor suppressors [9–11]. Some of the fundamental biological processes that are essential for cancer progression, including cell differentiation [12], proliferation [13], invasion [14], and tumor cell apoptosis [15], have been associated with the function of miRNAs.

Bairong Xia and Huiyan Li contributed equally to this work.

**Electronic supplementary material** The online version of this article (doi:10.1007/s13277-016-4805-8) contains supplementary material, which is available to authorized users.

✉ Ge Lou  
lougehyd@163.com

<sup>1</sup> Department of Gynecology, the Affiliated Tumor Hospital, Harbin Medical University, 150 Haping Rd, Nangang, Harbin, Heilongjiang 150020, China

<sup>2</sup> Department of Nursing, the Affiliated Tumor Hospital, Harbin Medical University, Harbin 150020, China

The role of miR-381 in cancer has been characterized by a series of recent studies. In glioma cells, miR-381 was shown to stimulate cell proliferation both in vitro and in vivo, and this function was associated with the regulation of the MEK/ERK and Akt signaling pathways [16]. Paradoxically, downregulation of miR-381 was shown to promote cell proliferation and invasion in colon cancer through upregulation of LRH-1 [17]. In addition, miR-381 was found to be significantly downregulated in lung cancer, and low levels of miR-381 expression were correlated with poor prognosis [18]. miR-381 was found to inhibit invasion of renal carcinoma by targeting Wnt signaling pathway components [19]. Moreover, miR-381 sensitized kidney cancer cells to chemotherapy drugs by upregulating Cdc2 activities [20]. Taken together, these studies suggested that miR-381 might play important roles in the development of various cancer types. However, the biological function of miR-381 in EOC remains to be investigated.

Yin Yang 1 (YY1) is a ubiquitously expressed transcription factor that plays essential roles in embryogenesis [21, 22]. The binding of YY1 to DNA is mediated through the recognition of a specific consensus sequence, which leads to the activation or repression of a wide variety of genes including c-Myc, c-Fos,  $\beta$ -actin, IFN- $\gamma$ , CREB, and Sp1 [23, 24]. Importantly, YY1 has been shown to interact with a number of oncogenes and tumor suppressors that are essential for the regulation of cell growth, apoptosis, and cancer progression, including Rb, Mdm2, p53, and Ezh2 [23, 25]. YY1 was reported to determine cell fate by repressing p53 signaling [25, 26]. In addition, several investigators have found that YY1 promotes Wnt signaling pathway in colorectal cancer development [27, 28]. Knockdown of YY1 inhibited gastric cancer cell proliferation by inhibiting Wnt signaling pathway [29]. Meanwhile, miR-381 was found to inhibit brain tumor by regulating p53 signaling pathway and reduced renal carcinoma invasion by targeting components of Wnt signaling pathway [19, 30, 31]. These information motivated us to study the miR-381/YY1/p53 and miR-381/YY1/Wnt axis in EOC tumorigenesis.

In this study, we demonstrated that the expression levels of miR-381 were downregulated in EOC tissues and cell lines. Overexpression of miR-381 significantly inhibited EOC cell proliferation, migration, and invasion. We also identified transcription factor YY1 as a target of miR-381. Importantly, as knockdown of YY1 mimicked the phenotype of miR-381 overexpression in the regulation of EOC cell proliferation, migration, and invasion, and miR-381 regulated EOC cell through miR-381/YY1/p53 and miR-381/YY1/Wnt signaling axis. Restoration of YY1 expression reversed the phenotype induced by miR-381, we concluded that miR-381 inhibited EOC cell proliferation, migration, and invasion, at least in part, via suppressing YY1.

## Materials and methods

### Human samples

Tissues were collected from patients who underwent surgery at the Department of Obstetrics and Gynecology of Harbin Medical University Cancer Hospital between 2012 and 2013, including 40 epithelial EOC tissues and 20 normal epithelial ovarian tissue sections. None of the patients were treated with chemotherapy or radiotherapy before they were subjected to operation. The histopathological diagnoses were performed according to the World Health Organization criteria. The percentage of tumor cells in all primary EOC samples are more than 50%. All fresh specimens were stored at  $-80^{\circ}\text{C}$  for further use. This study was approved by the Medical Ethics Committee of Harbin Medical University Cancer Hospital and all patients were provided informed consent. The clinical characteristics of all of EOC patients were recorded in Table 1.

### GSE and DLANA tool analysis

The method of GSE analysis was discussed in details in the website (<http://www.ncbi.nlm.nih.gov/geo/info/geo2r.html>).

**Table 1** Association of miR-381 and clinical feature of 40 EOC patients

Characteristics	Total	miR-381 expression		<i>p</i> value
		Low	High	
Age (years)				0.204
>55	22	9	13	
$\leq 55$	18	11	7	
Stage				0.022
I+II	15	4	11	
III+IV	25	16	9	
Grade				0.008
1	9	1	8	
2+3	31	19	12	
Tumor size				0.337
>1	23	10	13	
$\leq 1$	17	10	7	
Histology				1.000
Serous	20	13	7	
Non-serous	20	7	13	
Lymph node				0.077
Negative	29	12	17	
Positive	11	8	3	
YY1 expression				0.011
Low	20	6	14	
High	20	14	6	

We analyzed miR-381 expression in GSE47841 microarray, which included data from nine clear cell ovarian carcinomas (CCOC), 12 high-grade serous ovarian carcinomas (HGSC), and nine normal ovarian surface epithelial cells (OSES) [32]. Potential miR-381 targets were predicted in the website ([http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT\\_CDS/index](http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index)).

### Cancer cell lines and primary normal epithelial cells

The human EOC cell lines (OVCAR3, Caov3, OVCA429, SKOV3, A2780, and COV644) and normal human ovarian surface epithelial (HOSE) cells were supplied by China Center for Type Culture Collection (CCTCC). EOC cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10 % fetal bovine serum and antibiotics (Gibco). HOSE cells were cultured in medium containing 1:1 mixture of MCDB 105 and M199 medium (Sigma, St. Louis, MO). All cells were incubated at 37 °C under a humidified atmosphere containing 5 % CO<sub>2</sub>.

### Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). To quantitate miR-381 expression, total RNA was polyadenylated and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit and TaqMan miRNA assay (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. U6 small nuclear RNA was used as the internal control. Quantitative real-time PCR (qRT-PCR) analyses for mRNA of Yin Yang1 (YY1) were performed by using QIAGEN OneStep RT-PCR kits (Qiagen, Valencia, CA) and SYBR Green real-time PCR. The mRNA level of  $\beta$ -actin was measured as an internal control. RT-PCR was performed in triplicates. Relative expression of the tested genes was calculated and normalized using the  $2^{-\Delta\Delta C_t}$  method. Primers were as follows: YY1 forward, 5' GCGGAGCCCTCAGCCATGGCCTCG 3', and reverse, 5' CAGCGGCTGCAGAGCGATCATGG 3'; cyclin D1 forward, 5' AGCTCCTGTGCTGCGAAGTGGAAC 3', and reverse 5' AGTGTTCAATGAAATCGTGCGGGGT 3'; LEF-1 forward, 5' CGACGCCAAAGGAACACTGACATC 3', and reverse, 5' GCACGCAGATATGGGGGGAGAAA 3'; Axin2 forward 5' ACAACAGCATTGTCTCCAAGCAGC 3', and reverse 5' GCGCCTGGTCAAAACA TGATGGAAT 3'; and  $\beta$ -actin forward, 5' TGACGGGGTCACCCACACTGTGCCCATCTA3', and reverse, 5' CTAGAAGCATTTGCGGTGGACGATGGAGGG 3'.

### Transfection and luciferase assay

Oligonucleotides including miR-381 mimics and non-specific miRNA negative control (miR-Ctrl) were synthesized and

purified by GenePharma (Shanghai). All oligonucleotides were transfected into EOC cells at a final concentration of 50 nM using HiPerFect transfection reagent according to the product manual (Qiagen). The full-length 3'-UTR of YY1 gene containing the putative miR-381 binding sites was amplified by PCR and was inserted into the pGL3 control vector subcloned Xba I with CMV promoter (Promega, Madison, WI). The primer sequence is as follows: forward primer, 5'-A G T T C T A G A A A G A A G A G A G A G A A G A C C C T -3', and reverse primer, 5'-A G T T C T A G A A A G A A G A A T T A A G C T -3'. The coding sequences of YY1 were generated by PCR and cloned into pCDNA3.1(+) vector (Invitrogen) to generate pCDNA3.1-YY1. The luciferase reporter vector (YY1 3'-UTR, p53, and TOPflash), pCDNA3.1-YY1 and Renilla plasmid were all transfected using Lipofectamine LTX according to the manufacturer's instructions.

Cells were seeded in triplicate in 24-well plates 1 day before transfection for the luciferase assay. Forty-eight hours after transfection, the cells were harvested and lysed, and the luciferase activity assayed using the dual-luciferase assay kit (Promega). Normalized luciferase activity was reported as luciferase activity/Renilla luciferase activity. Three independent experiments were performed.

### Cell counting and MTT assay

The cell viability and proliferation were determined by cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Promega). For cell counting, at 48 h after transfection,  $0.25 \times 10^4$  cells were seeded into 24-well plates. Then, cell numbers were counted at 0, 1, 2, 3, and 4 days. For MTT assay, at 48 h after transfection, the cells were plated in 96-well plates at 2000 per well in a final volume of 100  $\mu$ l. Then at 0, 1, 2, 3, and 4 days, 25  $\mu$ l of MTT stock solution was added to each well and incubated for 4 h. The absorbance was measured at 570 nm. The assays were performed in triplicates.

### Colony formation assay

The transfected EOC cells were seeded in 6-well plates (300 cells per well) and incubated for 10 days for the colony formation assay. The cells were then washed with PBS, fixed with 10 % formalin, and stained with 0.5 % crystal violet (Sigma). The assay was repeated in five replicates.

### Transwell migration and invasion assays

In vitro cell migration and invasion assays were performed using 24-well transwell chambers (8- $\mu$ m pores, BD Biosciences, San Jose, CA). The transfected EOC cells ( $5 \times 10^4$  cells per well) were cultured in the top chamber with 100  $\mu$ l 1 %

FBS medium. Five hundred microliters complete media with 10 % FBS was added into the lower chamber. After 24 h of cultivation, the medium from the chamber and the transwell were removed, and the chamber was gently wiped with a cotton swab. The migrated cells were fixed in 4 % paraformaldehyde, stained with crystal violet solution, and counted under a microscope in six fields. The procedure for the cell invasion assay was similar to the cell migration assay, except that the transwell membranes were precoated with Matrigel (BD Biosciences). The assays were performed in triplicates.

### Western blot

Western blot was performed as described previously [33]. Briefly, total protein was extracted by RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholic acid, 0.1 % SDS, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail; Santa Cruz Biotechnology). The total extracts were separated using 10 % SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad, Hercules, CA). The membranes were probed with a primary antibody against human YY1 (sc-281), p53 (sc-126),  $\beta$ -catenin (sc-1496), or  $\beta$ -actin (sc-47778) from Santa Cruz, followed by (HRP)-conjugated secondary antibody (Santa Cruz). Bound antibody was detected using the Supersignal West Pico ECL chemiluminescence kit (Thermo scientific, Rockford, IL).

### Statistical analysis

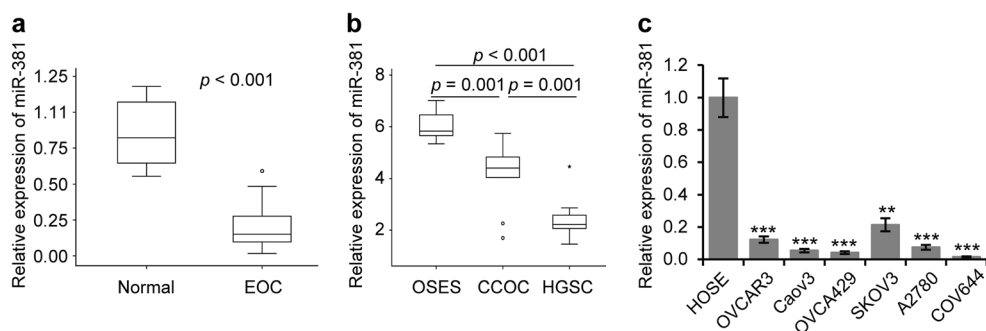
Statistical analyses were performed by SPSS Windows version 19. Data was expressed as mean  $\pm$  SEM of the experiments performed in triplicate. One-way ANOVA followed by Tukey's post hoc was performed to determine the

significance of difference among groups. Differences were considered significant at \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

## Results

### MiR-381 is downregulated in EOC tissues and cell lines

In order to determine the clinical relevance of miR-381 in human EOC, we first examined the expression levels of miR-381 in samples from primary EOC tumor tissues ( $n = 40$ ) and normal ovarian epithelial tissues ( $n = 20$ ). We found that compared to those in normal ovarian epithelial tissues, the levels of miR-381 expression were significantly lower in EOC tissues (Fig. 1a,  $p < 0.001$ ). We also analyzed miR-381 expression in GSE47841 microarray, which included data from nine clear cell ovarian carcinomas (CCOC), 12 high-grade serous ovarian carcinomas (HGSC), and nine normal ovarian surface epithelial cells (OSES) [32]. Consistently, we found that the expression of miR-381 was significantly downregulated in CCOC and HGSC tissues (Fig. 1b). In addition, we determined the levels of miR-381 in six EOC cell lines (OVCAR3, Caov3, OVCA429, SKOV3, A2780, and COV644). The expression of miR-381 was significantly lower in all six cell lines, compared to those in human ovarian surface epithelial (HOSE) cells (Fig. 1c). Among the six cell lines, SKOV3 had the highest and COV644 has the lowest miR-381 expression. The relationship between miR-381 and clinical feature of EOC patients was summarized in Table 1. Reduced expression of miR-381 was significantly associated with tumor stage ( $p = 0.022$ ), grade ( $p = 0.008$ ), and lymph node metastasis ( $p = 0.077$ ). However, there was no significant association between miR-381 expression and the other clinical features, such as patient's age, tumor size, and histology.



**Fig. 1** MiR-381 is downregulated in EOC tissues and cell lines. **a** Relative miRNA levels of miR-381 was determined by qRT-PCR in 40 EOC tissues and in 20 normal epithelial tissues samples.  $p < 0.001$ . **b** Relative expression levels of miR-381 in clear cell ovarian carcinomas (CCOC,  $n = 9$ ), high-grade serous ovarian carcinomas (HGSC,  $n = 12$ ), and ovarian surface epithelial cells (OSEC,  $n = 9$ ) (GSE47841,  $p = 0.001$

for OSEC and HGSC,  $p < 0.001$  for OSEC and CCOC, and  $p = 0.001$  for CCOC and HGSC). **c** Relative miRNA levels of miR-381 in 6 EOC cell lines (OVCAR3, Caov3, OVCA429, SKOV3, A2780, and COV644), as compared to those in human ovarian surface epithelial (HOSE cells). U6 snRNA was used as the internal control. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared to normal tissue samples or cell lines



Taken together, these data indicated that miR-381 was down-regulated in EOC tissues and cell lines.

### MiR-381 inhibits EOC cell proliferation, migration, and invasion

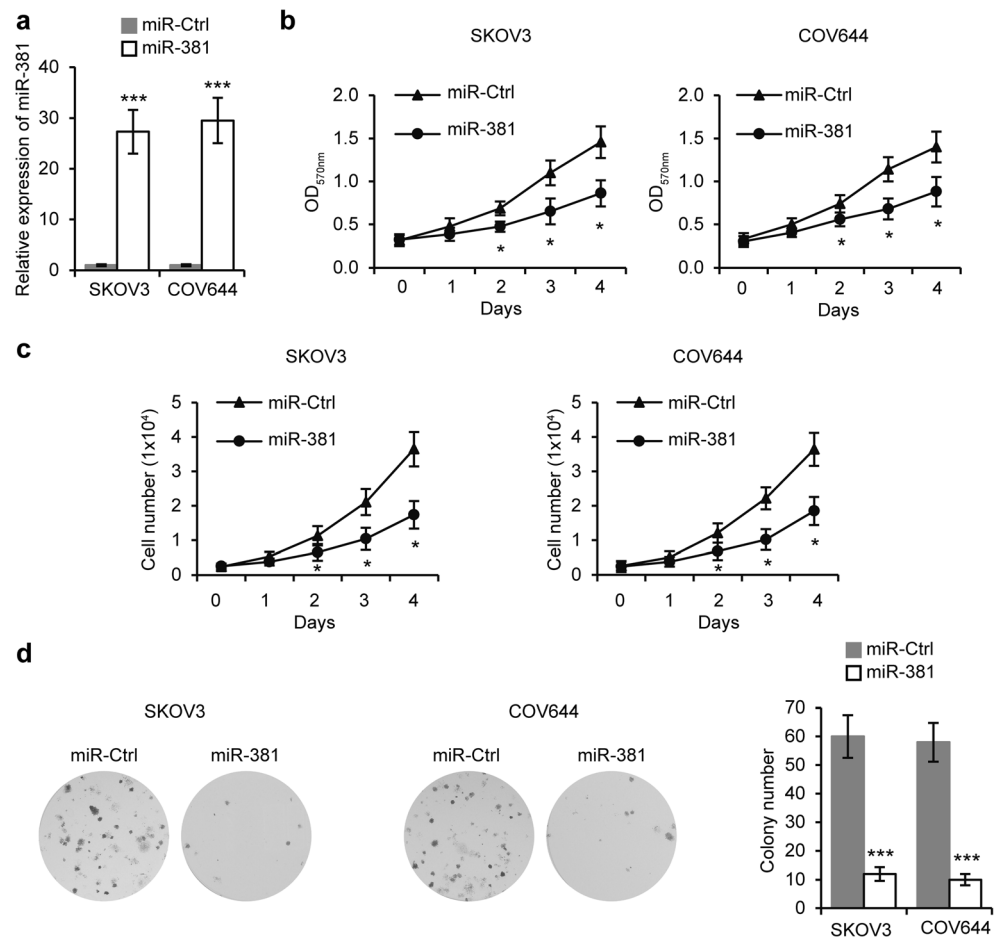
Next, we sought to determine whether the upregulation of miR-381 expression could affect the proliferation, migration, and invasion of EOC cells. We transfected miR-381 or control miRNA into SKOV3 and COV644 cells, and confirmed the upregulated expression of miR-381 to more than 25 folds after 48 h (Fig. 2a). We discovered that in both cell lines, the cells transfected with miR-381 showed significantly reduced growth rate compared to those transfected with control miRNA, as determined by MTT cell proliferation assay (Fig. 2b) and cell counting assay (Fig. 2c). Additionally, colony formation assays demonstrated that miR-381 significantly inhibited EOC cell proliferation (Fig. 2d). Furthermore, we examined the migration and invasion ability of SKOV3 and COV644 cells with upregulated miR-381 expression using both transwell migration assay and invasion assay. In both cell

lines, we found that overexpression of miR-381 led to significantly inhibited cell migration (Fig. 3a) and cell invasion (Fig. 3b). Collectively, these results indicated that miR-381 suppressed EOC cell proliferation, migration, and invasion.

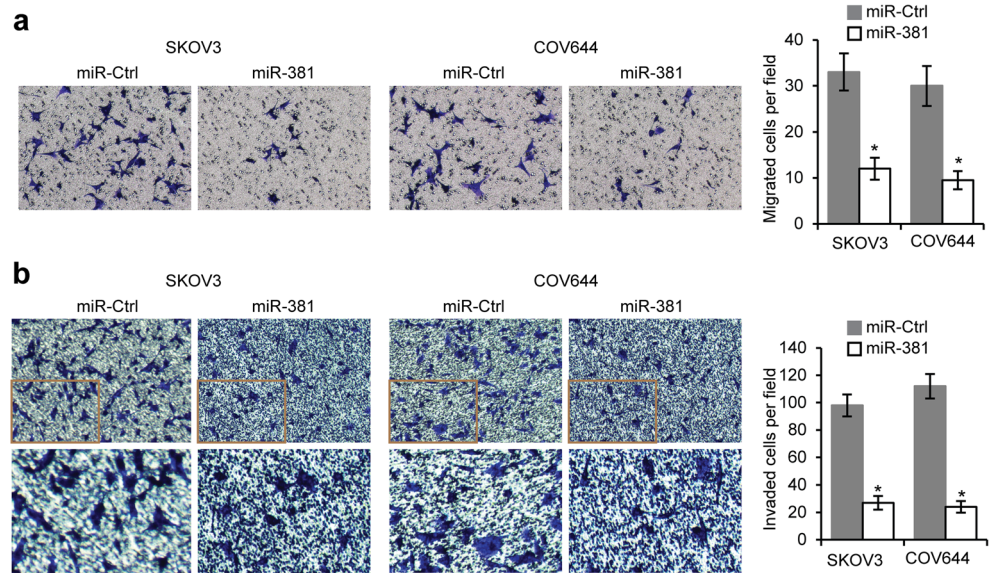
### YY1 is a direct target of miR-381

We employed the bioinformatic DIANA tools to predict the potential target genes of miR-381, and found that in the context of cancer development [34], YY1 was one of the functionally relevant target genes. Besides DLANA tool, we used other bioinformatic tools including targets can and microRNA.org to confirm that YY1 was predicted as a potential target of miR-381. There were three miR-381 binding sites in YY1 3'-UTR (Fig. 4a). In order to validate that YY1 was a direct target gene of miR-381, we constructed luciferase reporter plasmid with the YY1 3'-UTR region. We then co-transfected these plasmids into SKOV3 and COV644 cells with either miR-381 or control miRNA. Results showed the transfection of miR-381 significantly reduced the luciferase activity in both EOC cell lines to less than 30 % (Fig. 4b).

**Fig. 2** MiR-381 inhibits EOC cell proliferation. **a** Relative expression levels of miR-381 was determined by qRT-PCR in SKOV3 and COV644 cells transfected with miR-381 or control miRNA (miR-Ctrl). **b** MTT assay of SKOV3 and COV644 cells transfected with miR-381 or miR-Ctrl. **c** SKOV3 and COV644 cells were transfected with miR-381 or miR-Ctrl for 48 h, then seeded on 24 well plates (2500 cells/well). Numbers of alive cells were counted at the indicated time points. **d** Colony formation assay of SKOV3 and COV644 cells transfected with miR-381 or miR-Ctrl. \* $p < 0.05$ , \*\*\* $p < 0.001$  compared with miR-Ctrl transfected cells

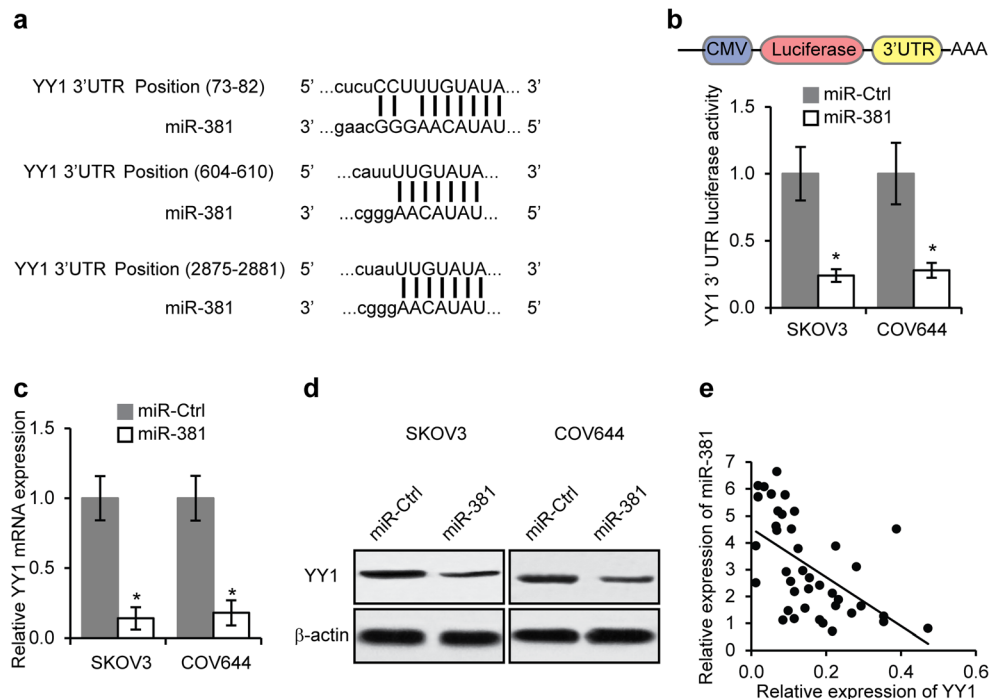


**Fig. 3** MiR-381 inhibits EOC cell migration and invasion. **a** Transwell migration assay and **b** invasion assay in SKOV3 and COV644 cells transfected with miR-381 or miR-Ctrl. \* $p < 0.05$  compared with miR-Ctrl transfected cells. Orange boxes indicate area magnified in top row of (b)



In addition, we examined the YY1 levels in SKOV3 and COV644 cells transfected with miR-381. Consistently, miR-381 remarkably reduced both the mRNA levels (Fig. 4c) and the protein levels (Fig. 4d) of YY1 in both EOC cell lines.

Furthermore, we examined the correlation between the expression levels of YY1 and miR-381 in 40 EOC tissues using qRT-PCR. With the Spearman correlation analysis, we found that there was a strong inverse correlation between the



**Fig. 4** YY1 is a target gene of miR-381. **a** The putative binding sites of miR-381 on the YY1 3'-UTR region (positions 73–82, 604–610, and 2875–2881). **b** Luciferase activities in SKOV3 and COV644 cells 48 h after co-transfected with YY1 3'-UTR luciferase plasmid and miR-381 or miR-Ctrl. \* $p < 0.05$  compared to miR-Ctrl transfected cells. **c** Levels of YY1 mRNA was determined by qRT-PCR in SKOV3 and COV644 cells

transfected with miR-381 or miR-Ctrl. \* $p < 0.05$  compared to miR-Ctrl transfected cells. **d** Levels of YY1 protein was determined by Western blot in SKOV3 and COV644 cells transfected with miR-381 or miR-Ctrl. **e** The correlation of the expression levels of YY1 and miR-381 in 40 EOC tissue samples

expression levels of miR-381 and YY1 (Fig. 4e,  $r=-0.558$ ). This negative correlation was also confirmed by immunohistochemistry (Table 1). Collectively, these results indicated that miR-381 inhibited the expression of YY1 by targeting its 3'-UTR region.

### YY1 deletion inhibits EOC cell proliferation, migration, and invasion

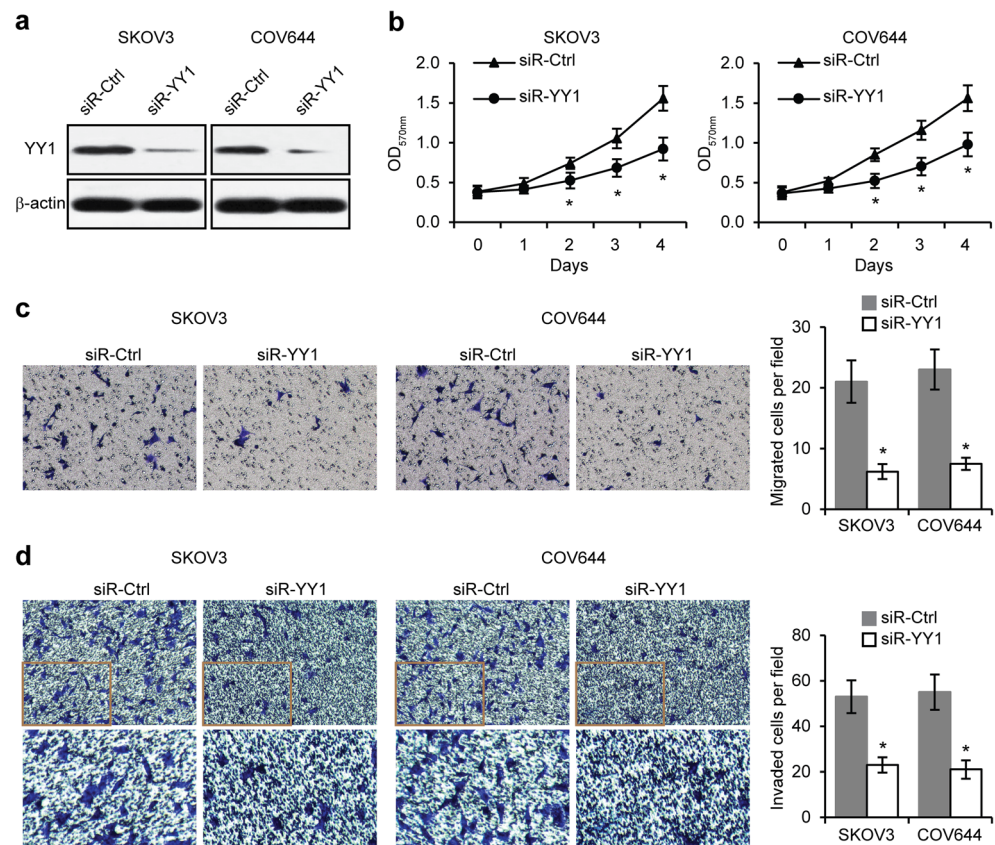
Since YY1 was a target gene for miR-381, we next investigated whether downregulation of YY1 would mimic the phenotypes of miR-381 overexpression in regulating EOC cell proliferation, migration, and invasion. We first knocked down YY1 expression in both SKOV3 and COV644 cells using small interfering RNA (siRNA) targeting YY1, and confirmed the efficiency of knockdown by Western blot analysis (Fig. 5a). Significantly, we found that in both SKOV3 and COV644 cell lines, the rate of cell growth was significantly lower in cells transfected with siRNA targeting YY1, compared to those transfected with control siRNA, as determined by MTT cell proliferation assay (Fig. 5b). Furthermore, we also performed transwell migration assay and invasion assay on SKOV3 and COV644 cells with YY1 knockdown to examine the migration and invasion activities of these cells. In

both cell lines, we found that knockdown of YY1 led to significantly reduced activities of both cell migration (Fig. 5c) and cell invasion (Fig. 5d). These results indicated that knockdown of YY1 inhibited EOC cell proliferation, migration, and invasion.

### MiR-381 inhibits EOC cell proliferation, migration, and invasion via suppressing YY1

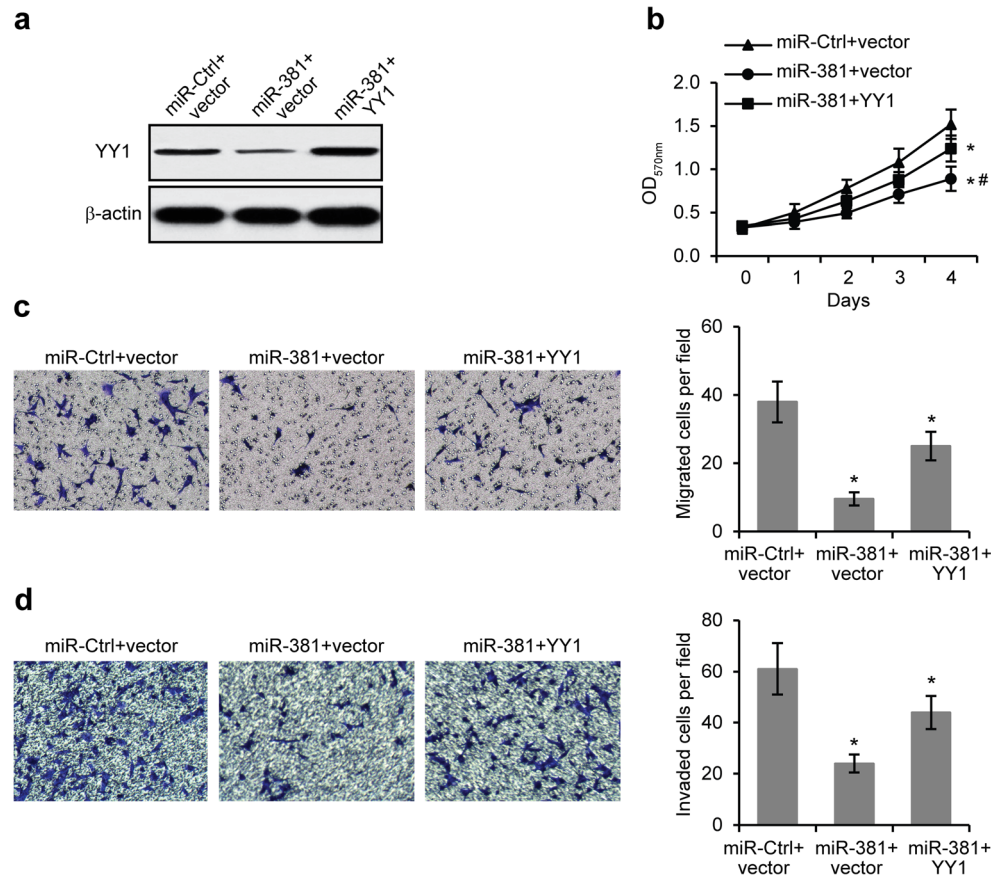
Our previous data showed that miR-381 inhibited EOC cell proliferation, migration, and invasion and YY1 was a target gene of miR-381. It is possible that miR-381 exerted its functions on EOC cells via suppressing the expression of YY1. If this is the case, then the relief of YY1 suppression would reverse the regulation of miR-381 on EOC cells. To test this hypothesis, we simultaneously co-transfected plasmids encoding YY1 into miR-381 overexpressing SKOV3 cells. These plasmids did not contain the 3'-UTR region of YY1 and were therefore resistant to miR-381 regulation. We found that while overexpression of miR-381 reduced the expression of YY1, co-transfection of YY1-overexpressing plasmids completely restored the levels of YY1 protein, as determined by Western blot analysis (Fig. 6a). In these cells, we performed MTT assay to assess cell proliferation and transwell

**Fig. 5** Knockdown of YY1 inhibits EOC cell proliferation, migration and invasion. **a** Levels of YY1 protein was determined by Western blot in SKOV3 and COV644 cells transfected with siRNA targeting YY1 (siR-YY1) or control siRNA (siR-Ctrl). **b** MTT assay, **c** transwell migration assay, and **d** invasion assay in SKOV3 and COV644 cells transfected with siRNA targeting YY1 (siR-YY1) or siR-Ctrl. Orange boxes indicate area magnified in top row of (d). \* $p < 0.05$  compared with siR-Ctrl transfected cells





**Fig. 6** MiR-381 inhibits EOC cell proliferation, migration, and invasion via suppressing YY1 expression. **a** Levels of YY1 protein was determined by Western blot in SKOV3 cells transfected with miR-381 or miR-Ctrl, together with either vector control or plasmids encoding YY1. **b** MTT assay, **c** transwell migration assay, and **d** transwell invasion assay in SKOV3 cells transfected with miR-381 or miR-Ctrl, together with either vector control or plasmids encoding YY1. \* $p < 0.05$  compared with cells transfected with control miRNA (miR-Ctrl) together with vector control; # $p < 0.05$  compared with cells transfected with miR-381 together with vector control



assays to assess cell migration and invasion. We found that transfection of YY1-expressing plasmid significantly reversed the inhibition of EOC cell proliferation (Fig. 6b), cell migration (Fig. 6c), and cell invasion (Fig. 6d) induced by the miR-381. Taken together, these data suggested that miR-381 inhibited EOC cell proliferation, migration, and invasion, at least in part, via suppressing the expression of YY1.

### MiR-381 and YY1 regulates p53 and Wnt signaling in EOC cells

YY1 was reported to regulate p53 and Wnt signaling to determine cell fate [23, 27]. In order to elucidate the molecular mechanisms of YY1's function in EOC, we investigated p53 expression in YY1 silencing SKOV3 and COV644 cells by Western blot. The result revealed that inhibition of YY1 up-regulated p53 expression (Fig. 7a). A wild-type p53 reporter luciferase assay revealed the p53 transcriptional activity was stimulated by YY1 inhibition (Fig. 7b). Next, we examined  $\beta$ -catenin levels in YY1 knockdown EOC cells and observed decreased expression of  $\beta$ -catenin (Fig. 7c). Consistently, TOPflash activity was suppressed after YY1 was knocked down (Fig. 7d). Furthermore, we examined whether miR-

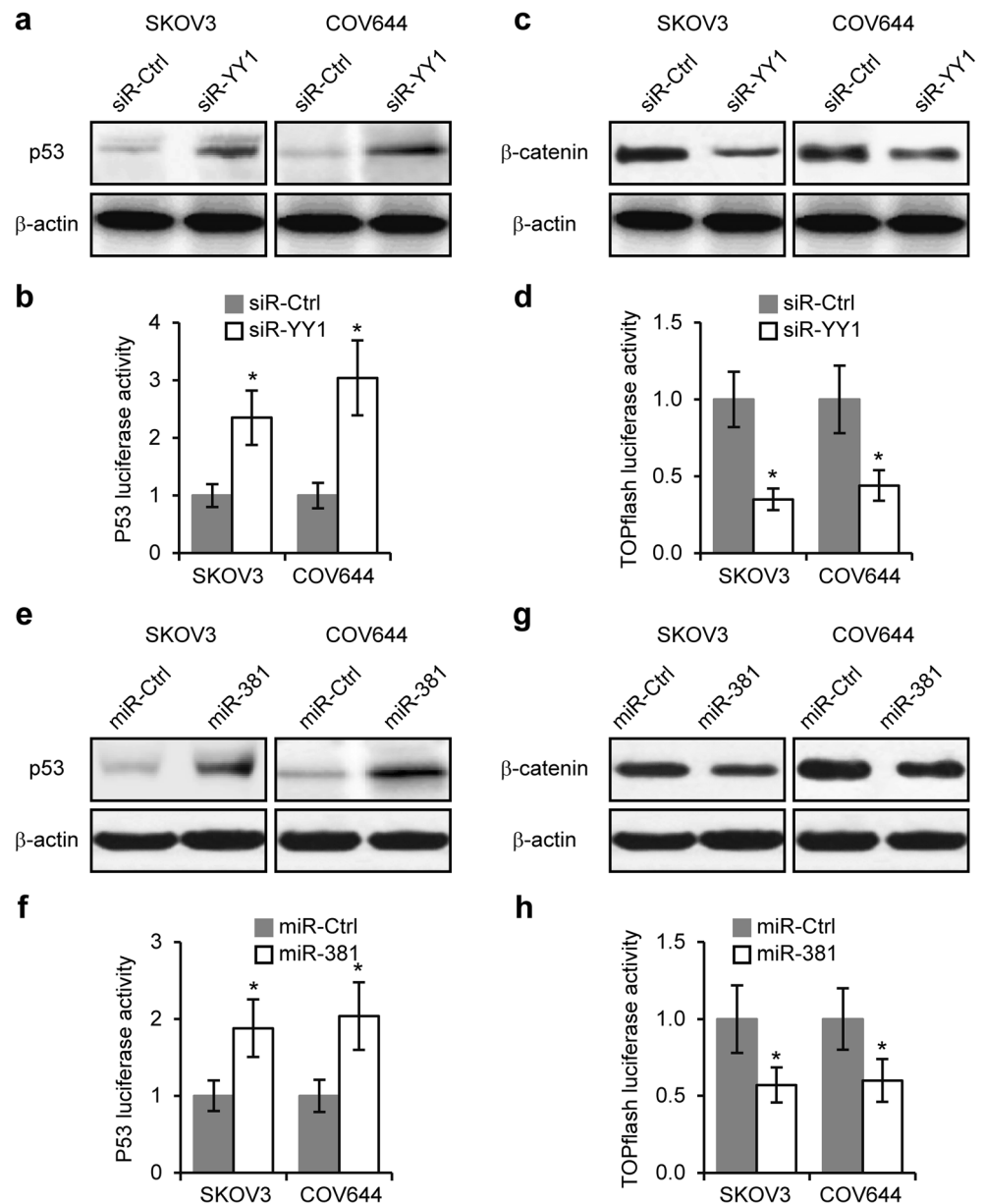
381 also affects p53 and Wnt signaling. We performed expression assays and reporter assays in SKOV3 and COV644 cells overexpressed miR-381. We noticed that miR-381 increased p53 and inhibited  $\beta$ -catenin expression (Fig. 7e–h). miR-381 was also decreased downstream genes of Wnt signaling including cyclin D1, Axin2, and LEF-1 (Supplemental figure 1). These finding further provided the evidences that miR-381 regulates EOC cell function via targeting YY1 and indicated p53 and Wnt are two potential downstream signaling pathways.

### Discussion

In this study, we investigated the function of miR-381 in the regulation of EOC cell proliferation, migration, and invasion. The physiological relevance of miR-381 with EOC was first established by the discovery that the expression levels of miR-381 were significantly reduced in EOC tissues. This finding was supported by data both from patient tissue samples obtained by our group and from publicly available microarray data. The fact that miR-381 was downregulated in cancerous tissue suggested that miR-381 may play a role as a tumor



**Fig. 7** YY1 and miR-381 regulate p53 and  $\beta$ -catenin. **a** Levels of p53 protein was determined by Western blot in SKOV3 and COV644 cells transfected with siR-YY1 or siR-Ctrl. **b** Luciferase activities in SKOV3 and COV644 cells transfected with wild-type p53 luciferase plasmid and siR-YY1 or siR-Ctrl. **c** Levels of  $\beta$ -catenin protein was determined by Western blot in SKOV3 and COV644 cells transfected with siR-YY1 or siR-Ctrl. **d** Luciferase activities in SKOV3 and COV644 cells 48 h after co-transfected with TOPflash luciferase plasmid and siR-YY1 or siR-Ctrl. **e** Levels of p53 protein was determined by Western blot in SKOV3 and COV644 cells transfected with miR-Ctrl or miR-381. **f** Luciferase activities in SKOV3 and COV644 cells 48 h after co-transfected with wild type p53 luciferase plasmid and miR-Ctrl or miR-381. **g** Levels of  $\beta$ -catenin was determined by Western blot in SKOV3 and COV644 cells transfected with miR-Ctrl or miR-381. **h** Luciferase activities in SKOV3 and COV644 cells 48 h after co-transfected with TOPflash luciferase plasmid and miR-Ctrl or miR-381. \* $p < 0.05$  compared with control cells



suppressor, which further suggested that its inhibitory targets were likely to be genes stimulating tumor malignancy. Indeed, this idea was actually supported by a series of recent studies concerning cancers in other tissues. For example, loss of miR-381 was shown to promote cell proliferation and invasion in colon cancer through upregulation of LRH-1 [17]. MiR-381 was also found to be downregulated in lung cancer, and low levels of miR-381 expression were correlated with poor prognosis, partially due to de-repression of ID1 [18]. In addition, miR-381 could sensitize kidney cancer cells to chemotherapy drugs by upregulating Cdc2 activities [20]. Moreover, the expression levels of miR-381 were also inversely correlated with the expression of the MDR1 gene and the development of drug resistance in chemotherapy [35].

In our study, we assessed the function of miR-381 on cell proliferation, cell migration, and cell invasion in in vitro cultured EOC cells. We chose two independent cell lines for these assays, so that we could confirm that the effects of miR-381 were not restricted to just a certain cell line. We found that overexpression of miR-381 in EOC cells significantly inhibited the proliferation, migration, and invasion of these cells. These aspects of cell activities represented the key steps in cancer malignancy, and these results therefore were consistent with the findings that the expression levels of miR-381 were down-regulated in EOC tissues in vivo.

YY1 was identified as a target of miR-381 by bioinformatic prediction. The prediction was confirmed by

multiple lines of evidence. First, miR-381 was able to inhibit the 3'-UTR activity of YY1 gene in a luciferase assay. Second, miR-381 overexpression reduced the expression of endogenous YY1, but the transfected YY1 lacking the 3'-UTR region appeared to be resistant to miR-381 overexpression. Our functional study on YY1 further supported its role as a target for miR-381, as knockdown of YY1 almost perfectly mimicked the phenotype on cell proliferation, cell migration, and cell invasion induced by the overexpression of miR-381. Furthermore, downregulation of YY1 or ectopic miR-381 stimulated p53 and suppressed Wnt signaling. Collectively, the lines of evidence consistently supported that YY1 was a target for miR-381.

An important question we were trying to address in our study was whether or not miR-381 exerted its function on EOC cells via the suppression of YY1 expression. To address this question, we reasoned that when we restore the levels of YY1 in miR-381 overexpressing cells, if the phenotype induced by miR-381 was reversed, then it was a strong indication that miR-381 exerted its function on EOC cells via the suppression of YY1 expression. In our experiments, we were able to completely restore the protein levels of YY1 in miR-381 overexpressing cells by co-transfection of plasmids encoding YY1. In these cells, however, although we were able to partially reverse the inhibition of cell proliferation and migration induced by overexpression of miR-381, the reversal was not complete. These results suggested that there were other factors regulated by miR-381 that also contributed to the inhibition of cell proliferation, migration, and invasion. In this study, SKOV3 had the highest and COV644 has the lowest miR-381 expression. We chose these two representative cell lines to perform functional experiments. Loss p53 was reported in both SKOV3 and COV644 cells [36, 37]. Deletion of YY1 was noted to promote p53 expression, which indicated negative regulation of p53 by YY1 in EOC. INK4a, cyclin D1/CDK4, and RB were important regulators for SKOV3 and COV644 cell progress [33, 38–40]. Majority of ovarian cancer cells showed telomerase activity [41]. However, telomerase activity is not related to p53 gene status, and intensity of telomerase activity is not an independent factor for ovarian cancer patients' prognosis [42]. Wnt signaling pathway plays a pivotal role in ovarian cancer tumorigenesis [43]. A given miRNA may have hundreds of various mRNA targets, and a given target might be regulated by multiple miRNAs [44, 45]. From published results and our data, it seems that miR-381 is related to Wnt signaling pathway.

Actually, this finding was not surprising, considering miR-381 regulated the expression of many other proteins in different types of cancers as mentioned earlier in the discussion. More investigation needs to be carried out in the future to identify other targets of miR-381 in EOC cells that regulate the process of malignancy.

**Acknowledgments** This study was supported by grants from National Natural Science Foundation of China (no. 81472028).

**Compliance with ethical standards** This study was approved by the Medical Ethics Committee of the Affiliated Tumor Hospital of Harbin Medical University and all patients were provided informed consent.

**Conflicts of interest** None

## References

1. Granato T, Midulla C, Longo F, Colaprisca B, Frati L, Anastasi E. Role of he4, ca72.4, and ca125 in monitoring ovarian cancer. *Tumour Biol J Int Soc Oncodevelopmental Biol Med.* 2012;33:1335–9.
2. Bristow RE. Surgical standards in the management of ovarian cancer. *Curr Opin Oncol.* 2000;12:474–80.
3. Chang SJ, Bristow RE. Evolution of surgical treatment paradigms for advanced-stage ovarian cancer: redefining 'optimal' residual disease. *Gynecol Oncol.* 2012;125:483–92.
4. Harter P, Muallem ZM, Buhmann C, Lorenz D, Kaub C, Hils R, et al. Impact of a structured quality management program on surgical outcome in primary advanced ovarian cancer. *Gynecol Oncol.* 2011;121:615–9.
5. Harries M, Gore M. Part i: chemotherapy for epithelial ovarian cancer-treatment at first diagnosis. *Lancet Oncol.* 2002;3:529–36.
6. Matei DE, Nephew KP. Epigenetic therapies for chemoresensitization of epithelial ovarian cancer. *Gynecol Oncol.* 2010;116:195–201.
7. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microma biogenesis pathways and their regulation. *Nat Cell Biol.* 2009;11:228–34.
8. Osada H, Takahashi T. Micromas in biological processes and carcinogenesis. *Carcinogenesis.* 2007;28:2–12.
9. Zhang Q, Tang Q, Qin D, Yu L, Huang R, Lv G, et al. Role of microma 30a targeting insulin receptor substrate 2 in colorectal tumorigenesis. *Mol Cell Biol.* 2015;35:988–1000.
10. Sui X, Wang X, Han W, Li D, Xu Y, Lou F, et al. Micromas-mediated cell fate in triple negative breast cancers. *Cancer Lett.* 2015;361:8–12.
11. Iorio MV, Croce CM. Microma involvement in human cancer. *Carcinogenesis.* 2012;33:1126–33.
12. Lee SH, Jung YD, Choi YS, Lee YM. Targeting of runx3 by mir-130a and mir-495 cooperatively increases cell proliferation and tumor angiogenesis in gastric cancer cells. *Oncotarget.* 2015.
13. Guo M, Zhang X, Wang G, Sun J, Jiang Z, Khadarian K, et al. Mir-603 promotes glioma cell growth via wnt/beta-catenin pathway by inhibiting wif1 and ctnnbip1. *Cancer Lett.* 2015;360:76–86.
14. Liu YN, Yin J, Barrett B, Sheppard-Tillman H, Li D, Casey OM, et al. Loss of androgen-regulated microma 1 activates src and promotes prostate cancer bone metastasis. *Mol Cell Biol.* 2015;35:1940–51.
15. Li X, Li H, Zhang R, Liu J. Microma-449a inhibits proliferation and induces apoptosis by directly repressing e2f3 in gastric cancer. *Cell Physiol Biochem Int J Exp Cell Physiol, Biochem Pharmacol.* 2015;35:2033–42.
16. Tang H, Liu X, Wang Z, She X, Zeng X, Deng M, et al. Interaction of hsa-mir-381 and glioma suppressor lrre4 is involved in glioma growth. *Brain Res.* 2011;1390:21–32.
17. Liang Y, Zhao Q, Fan L, Zhang Z, Tan B, Liu Y, et al. Down-regulation of microma-381 promotes cell proliferation and invasion

- in colon cancer through up-regulation of lrh-1. *Biomed Pharmacother Biomed Pharmacotherapie*. 2015;75:137–41.
18. Rothschild SI, Tschan MP, Jaggi R, Fey MF, Gugger M, Gautschi O. MicroRNA-381 represses *id1* and is deregulated in lung adenocarcinoma. *J Thorac Oncol : Off Publ Int Assoc Study Lung Cancer*. 2012;7:1069–77.
  19. Chen B, Liu B. [mirna-381 inhibits the invasion of renal carcinoma and the underlying mechanisms]. *Zhong nan da xue xue bao Yi xue ban J Cent South Univ Med Sci*. 2015;40:1053–9.
  20. Chen B, Duan L, Yin G, Tan J, Jiang X. Simultaneously expressed mir-424 and mir-381 synergistically suppress the proliferation and survival of renal cancer cells—cdc2 activity is up-regulated by targeting *wee1*. *Clinics*. 2013;68:825–33.
  21. Donohoe ME, Zhang X, McGinnis L, Biggers J, Li E, Shi Y. Targeted disruption of mouse *yn yang 1* transcription factor results in peri-implantation lethality. *Mol Cell Biol*. 1999;19:7237–44.
  22. Shi Y, Seto E, Chang LS, Shenk T. Transcriptional repression by *yy1*, a human gli-kruppel-related protein, and relief of repression by adenovirus *e1a* protein. *Cell*. 1991;67:377–88.
  23. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor *yy1*: structure, function, and therapeutic implications in cancer biology. *Oncogene*. 2006;25:1125–42.
  24. Zhang Q, Stovall DB, Inoue K, Sui G. The oncogenic role of *yn yang 1*. *Crit Rev Oncog*. 2011;16:163–97.
  25. Sui G, el Affar B, Shi Y, Brignone C, Wall NR, Yin P, et al. *Yn yang 1* is a negative regulator of p53. *Cell*. 2004;117:859–72.
  26. Yakovleva T, Kolesnikova L, Vukojevic V, Gileva I, Tan-No K, Austen M, et al. *Yy1* binding to a subset of p53 DNA-target sites regulates p53-dependent transcription. *Biochem Biophys Res Commun*. 2004;318:615–24.
  27. Yokoyama NN, Pate KT, Sprowl S, Waterman ML. A role for *yy1* in repression of dominant negative *lef-1* expression in colon cancer. *Nucleic Acids Res*. 2010;38:6375–88.
  28. Zhang N, Li X, Wu CW, Dong Y, Cai M, Mok MT, et al. MicroRNA-7 is a novel inhibitor of *yy1* contributing to colorectal tumorigenesis. *Oncogene*. 2013;32:5078–88.
  29. Kang W, Tong JH, Chan AW, Zhao J, Dong Y, Wang S, et al. *Yn yang 1* contributes to gastric carcinogenesis and its nuclear expression correlates with shorter survival in patients with early stage gastric adenocarcinoma. *J Transl Med*. 2014;12:80.
  30. Tang H, Wang Z, Liu Q, Liu X, Wu M, Li G. Disturbing mir-182 and -381 inhibits *brd7* transcription and glioma growth by directly targeting *lrrc4*. *PLoS One*. 2014;9, e84146.
  31. Liang HQ, Wang RJ, Diao CF, Li JW, Su JL, Zhang S. The pttg1-targeting mirnas mir-329, mir-300, mir-381, and mir-655 inhibit pituitary tumor cell tumorigenesis and are involved in a p53/pttg1 regulation feedback loop. *Oncotarget*. 2015;6:29413–27.
  32. Vilming Elgaaen B, Olstad OK, Haug KB, Brusletto B, Sandvik L, Staff AC, et al. Global mirna expression analysis of serous and clear cell ovarian carcinomas identifies differentially expressed mirnas including mir-200c-3p as a prognostic marker. *BMC Cancer*. 2014;14:80.
  33. Xia B, Yang S, Liu T, Lou G. Mir-211 suppresses epithelial ovarian cancer proliferation and cell-cycle progression by targeting cyclin d1 and *cdk6*. *Mol Cancer*. 2015;14:57.
  34. Wang CC, Chen JJ, Yang PC. Multifunctional transcription factor *yy1*: a therapeutic target in human cancer? *Expert Opin Ther Targets*. 2006;10:253–66.
  35. Xu Y, Ohms SJ, Li Z, Wang Q, Gong G, Hu Y, et al. 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*. 2013;8, e82062.
  36. Ramos YF, Stad R, Attema J, Peltenburg LT, van der Eb AJ, Jochemsen AG. Aberrant expression of hdmx proteins in tumor cells correlates with wild-type p53. *Cancer Res*. 2001;61:1839–42.
  37. Ryu SY, Kim K, Lee WS, Kwon HC, Lee KH, Kim CM, et al. Synergistic growth inhibition by combination of adenovirus mediated p53 transfer and cisplatin in ovarian cancer cell lines. *J Gynecol Oncol*. 2009;20:48–54.
  38. Liu Z, Gersbach E, Zhang X, Xu X, Dong R, Lee P, et al. Mir-106a represses the *rb* tumor suppressor p130 to regulate cellular proliferation and differentiation in high-grade serous ovarian carcinoma. *Mol Cancer Res : MCR*. 2013;11:1314–25.
  39. Lee MH, Choi BY, Cho YY, Lee SY, Huang Z, Kundu JK, et al. Tumor suppressor p16(*ink4a*) inhibits cancer cell growth by down-regulating *eef1a2* through a direct interaction. *J Cell Sci*. 2013;126:1744–52.
  40. Huang L, Wang HY, Li JD, Wang JH, Zhou Y, Luo RZ, et al. *Kpna2* promotes cell proliferation and tumorigenicity in epithelial ovarian carcinoma through upregulation of *c-myc* and downregulation of *foxo3a*. *Cell Death Dis*. 2013;4, e745.
  41. Gan Y, Mo Y, Johnston J, Lu J, Wientjes MG, Au JL. Telomere maintenance in telomerase-positive human skov-3 cells cannot be retarded by complete inhibition of telomerase. *FEBS Lett*. 2002;527:10–4.
  42. Oishi T, Kigawa J, Minagawa Y, Shimada M, Takahashi M, Terakawa N. Alteration of telomerase activity associated with development and extension of epithelial ovarian cancer. *Obstet Gynecol*. 1998;91:568–71.
  43. Gatliffe TA, Monk BJ, Planutis K, Holcombe RF. Wnt signaling in ovarian tumorigenesis. *Int J Gynecol Cancer : Off J Int Gynecol Cancer Soc*. 2008;18:954–62.
  44. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009;19:92–105.
  45. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005;37:495–500.