Altered expression of miR-152 and miR-148a in ovarian cancer is related to cell proliferation

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Abstract. microRNAs (miRs) are endogenous small non-coding RNAs that are aberrantly expressed in various carcinomas. miR-152 and miR-148a have not been comprehensively investigated in ovarian cancer. Thus, the aim of this study was to identify the role of miR-152 and miR-148a in epithelial ovarian cancer. Total RNA was extracted from tissues of 78 patients with epithelial ovarian cancer, 17 normal ovarian epithelium tissues and two ovarian cancer cell lines. Using quantitative real-time PCR (qRT-PCR) followed by the $2^{-\Delta\Delta CT}$ method for calculating the results, we found that the expression levels of miR-152 were significantly decreased in ovarian cancer tissues compared to normal ovarian epithelium tissues ($p<0.05$). However, although the expression of miR-148a was also decreased in 65% of patients, no statistically significant difference in expression was found. A strong correlation was found between the expression of miR-152 and miR-148a ($p<0.001$, Pearson's correlation). The relationship between miR-152 or miR-148a expression levels in ovarian cancer and clinicopathological features, response to therapy and short-term survival was analyzed and the results showed that no correlation existed. In addition, we found that both miR-152 and miR-148a were down-regulated in ovarian cancer cell lines. After miR-152 or miR-148a mimics were transfected into ovarian cancer cell lines, the MTT cell proliferation assay showed that cell proliferation was significantly inhibited. Taken together, miR-152 and miR-148a may be involved in the carcinogenesis of ovarian cancer through deregulation of cell proliferation. They may be novel biomarkers for early detection or therapeutic targets of ovarian cancer.

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Introduction

Ovarian cancer is one of the most common causes of death from gynecological malignancies. In addition, with an incidence of ~15,000 deaths annually, it is the fifth leading cause of cancer-related deaths among women in the United States (1). More than 90% of ovarian cancers are epithelial ovarian cancers (EOCs), which are derived from the ovarian surface epithelium (2). Due to a lack of effective biomarkers, ineffective tools for ovarian cancer screening, and non-specific symptoms in its early stages, more than two-thirds of patients with ovarian cancer are not diagnosed until the disease is in an advanced stage (3). Despite advances in early detection and the current standard treatment for advanced ovarian cancer, the 5-year survival rate is only 20-25% (4,5), which makes the development of alternative approaches urgent. Understanding the molecular alterations of ovarian cancer will help identify novel diagnostic markers or therapeutic targets, thereby improving the survival rates in this population of cancer patients.

microRNAs (miRNAs or miRs) are a class of highly conserved, non-coding RNAs, approximately 19-25 nucleotides in length. They function as post-transcriptional regulators by binding to the 3'UTR regions of protein-encoding genes, which results in translational repression and gene silencing (6,7). Almost 30% of the human genome is estimated to be regulated by miRNAs (8). These human miRNA genes are frequently located at fragile sites and genomic regions involved in cancer, and 50% of them are known to be frequently amplified or deleted in human cancer cells (9). Specific miRNAs can be classified as oncogenes or tumor suppressors, depending on their expression pattern and function (10).

Several studies have shown that miRNAs are aberrantly expressed in several cancers, indicating that they play an important role in essential processes such as cell growth (11), differentiation, apoptosis (12,13), and carcinogenesis (14,15). In addition, there is evidence specifically implicating miRNAs in ovarian cancer. Iorio *et al*, used miRNA microarray technologies to first describe a complete miRNA expression profile in human EOCs (10). Those study results concluded that miR-141, miR-200a, miR-200b, and miR-200c are significantly up-regulated in ovarian cancer tissue, while miR-125b, miR-140, miR-145, and miR-199a are strongly down-regulated

(10). In addition, Bhattacharya *et al* demonstrated that miR-15a and miR-16 are weakly expressed in ovarian cell lines and in primary ovarian tissues, whereas their expression leads to a significant reduction in ovarian cancer cell proliferation and clonal growth via the down-regulation of Bmi-1 protein levels (16). Recently, Li *et al* used a microarray screen to show that a variety of miRNAs are differentially expressed between paired high-metastatic human serous ovarian cancer cells, SKOV-3ip, and low-metastatic human serous ovarian cells SKOV-3. Particularly, miR-22 was identified as a potential inhibitor of cell migration and invasion (17).

Our previous studies have shown that miR-152 and miR-148a are down-regulated in gastric cancer, when compared to their matched non-tumor adjacent tissues (NATs) (18). In neuroblastoma, miR-152 is involved in cell proliferation, neuroblast differentiation, migration, and apoptosis (19). Here, we present data showing that the expression of miR-152 and miR-148a were down-regulated in ovarian cancer tissues compared to normal ovarian tissues, although the decreased expression of miR-148a was not statistically significant. In addition, our *in vitro* cell experiments showed that miR-152 and miR-148a could inhibit cell proliferation in SKOV3 ovarian cancer cells.

Materials and methods

Patients and tissue samples. Seventy-eight ovarian epithelial carcinoma tissue samples and 17 normal ovarian tissue samples were collected from patients who underwent surgical resection at the First Hospital of China Medical University and the Shengjing Hospital of China Medical University between 2006 and 2011. The samples were diagnosed with ovarian carcinoma based on histopathological evaluation. Fresh samples were snap-frozen in liquid nitrogen and stored at -80˚C after surgery. One section of each sample was stained with hematoxylin-eosin (H&E) and used for subsequent histopathological evaluation. The median age of the patients at surgery was 54 years (range was 31-80 years). The histological classification of tumors was assessed according to the World Health Organization Histological Classification criteria. All cancers were staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Response after chemotherapy was assessed according to RECIST criteria. The study was approved by the Ethics Committee of China Medical University and informed consent was obtained from all the patients.

Cell lines and cell culture. The human ovarian epithelial cancer cell lines SKOV3 and OVCAR3 were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Beijing, China) and were cultured in RPMI-1640 medium (Gibco) and Dulbecco's modified Eagle's medium (DMEM) respectively; they were supplemented with 10% fetal bovine serum (FBS). Cell lines were cultured at 37˚C in a humidified atmosphere of 5% CO_2.

RNA extraction and polyadenylation. Total RNA was isolated from frozen tissues and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Polyadenylation of total RNA was performed with ATP by *Escherichia coli* poly (A) polymerase (E-PAP) at 37˚C for 30 min following the manufacturer's instructions for the poly (A) Tailing kit (Ambion, Austin, TX, USA) (20). RNA was purified by phenol-chloroform extraction and ethanol precipitation and then dissolved in RNase-free water. The concentration of isolated and purified RNA was assessed using a Nano-Photometer UV/Vis spectrophotometer (Implen GmbH, München, Germany).

Reverse transcriptase reaction. Single-stranded cDNA was synthesized from 1 μg RNA in a 20-μl reaction volume using the PrimeScriptR RT reagent kit Perfect Real-Time (Takara Bio, Kyoto, Japan). The 20-μl reverse transcriptase reaction mixture contained 1 μg RNA sample, 1 μl RT primer (Table I), 1 μl RT enzyme, 4 μl 5X RT buffer, and RNase-free water. The reactions were incubated at 42˚C for 15 min, inactivated by incubation at 85˚C for 5 sec, and subsequently held at 4˚C.

Real-time PCR. Real-time PCR was carried out using SYBR Premix Ex Taq™ II kit (Takara Bio) according to the manufacturer's instructions. Amplification and detection of mature miRNAs were performed using a Rotor-gene 6000 system (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. The 25-μl PCR reaction mixture contained 2 μl reverse-transcribed product, 12.5 μl SYBR-Green Supermix, 8.5 μl RNase-free water and 1 μl each forward and reverse primers (Table I). The reaction was incubated in a 36-well optical plate followed by 45 amplification cycles at 94˚C for 5 sec, 58˚C for 20 sec, and 72˚C for 30 sec. The assay was run in triplicate to allow for assessment of technical variability. U6 RNA was used as an endogenous control. Threshold cycle data were determined using default threshold settings. The $2^{-\Delta\Delta CT}$ method (21) was used to calculate the relative abundance of miRNA expression compared to U6 RNA (22) expression, and relative to the normal epithelium tissues. The fold-change of miR-152 and miR-148a was calculated from expression levels between tumor tissues/cells and normal tissues. A relative expression ratio <1.0 was considered low expression in cancer tissues/cells relative to the normal control (23).

Transfection of miR-152 and miR-148a mimics. miR-152 and miR-148a mimics are two separate RNA duplexes, designed as previously described (24). The negative control (NC) RNA duplex consists of non-specific sequences which are non-homologous to any human genome sequences (Table I). All pyrimidine nucleotides in the miR-152 and miR-148a mimics or NC duplex were substituted for 2-O-methyl analogs to improve RNA stability in the MTT assay. All RNA oligoribonucleotides were chemically synthesized by GenePharma (Shanghai, China). Cells were seeded in 6-well plates 24 h prior to transfection in antibiotic-free medium, to reach a confluency of 50-60% by the next day. A final concentration of 50 nM miRNA mimics and NC was transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 6 h post-transfection, the media was replaced with normal media containing 10% fetal bovine serum.

Cell proliferation assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed

Table I. RT-PCR primers for amplification of expression of miR-152 and miR-148a.

Primers	Primer sequences (5'-3')
RT-primer-1	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTA
RT-primer-2	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTG
RT-primer-3	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTTTTTTTTTTTTTTTTTTTTC
miR-152-forward	TCAGTGCATGACAGAACTTGGAA
miR-152-reverse	GCTGTCAACGATACGCTACGT
miR-148a-forward	TCAGTGCACTACAGAACTTTGT
miR-148a-reverse	GCTGTCAACGATACGCTACGT
U6 RNA-forward	CGCTTCGGCAGCACATATAC
U6 RNA-reverse	TTCACGAATTTGCGTGTCAT
miR-152-mimics	UCA GUG CAU GAC AGA ACU UGG AAG UUC UGU CAU GCA CUG AUU
miR-148a-mimics	UCA GUG CAU GAC AGA ACU UGG AAG UUC UGU CAU GCA CUG AUU
Negative control	UUC UCC GAA CGU GUC ACG UTT ACG UGA CAC GUU CGG AGA ATT

in 96-well plates (4,000 cells/well) in SKOV3 cells. At 24 h post-transfection, 20 μl MTT (5 mg/ml) was added to the appropriate wells, followed by a 4-h incubation. The supernatant was then discarded, and 150 μl of DMSO was added to each well to dissolve the precipitate. The resulting colored solution was quantified using the microplate reader SUNRISE RC (Tecan, Switzerland) at a wavelength of 570 nm. Six wells were used for each sample, and three independent experiments were performed.

Transwell cell invasion assay. The invasion assay was performed using 24-well Transwell plates (8.0 μm pore size, Corning-Costar, USA). SKOV3 cells were transfected with miRNA mimics, and then incubated for 24 h; $6x10^4$ cells in 200 μl serum-free media were added to the upper chamber of each Transwell chamber while 550 μl media, supplemented with 10% fetal bovine serum was placed in the lower chamber. Cells were incubated at 37˚C with 5% CO_2 for 24 h and then fixed in 75% ethanol for 30 min. This was followed by cell staining with crystal violet for 5 min for subsequent counting and imaging after cells on top of the membrane were removed with a cotton-tipped swab. Cells in nine random visual fields, as visualized under the microscope at x200 magnification, were counted. Three independent experiments were conducted.

Statistical analysis. Statistical analysis was performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL). The expression levels of miR-152 and miR-148a in epithelial ovarian cancer tissues and cell lines were analyzed using the Student's t-test. Associations between miR-152 or miR-148a expression and clinicopathological features were evaluated using non-parametric tests. The Mann-Whitney U-test was used for comparison between the two groups, and the Kruskal-Wallis test was used to compare three or more groups. Correlation coefficients of miR-152 and miR-148a were evaluated by Pearson's correlation. Short-time survival analysis was performed using the Kaplan-Meyer method. $P<0.05$ was considered statistically significant.

Results

Expression of miR-152 and miR-148a in ovarian cancer tissues and cell lines. The expression of miR-152 and miR-148a was quantitated in 78 epithelial ovarian tumors and 17 normal ovarian epithelium tissues by real-time PCR. We found that miR-152 and miR-148a were weakly expressed in epithelial ovarian cancer tissues compared to normal ovarian tissues. Specifically, among the 78 patients with ovarian cancer, miR-152 was weakly expressed in 52 cases (67%; P=0.008; Fig. 1A and C), and miR-148a was weakly expressed in 49 cases (63%). However, no significant differences in miR-148a expression were found between the ovarian cancer and normal epithelium tissues (P=0.445; Fig. 1B and D). The median fold-change was 0.70 and 0.37, respectively. Moreover, we also found significantly decreased expression of miR-152 and miR-148a in two ovarian cancer cells lines, SKOV3 ($P<0.01$ and $P<0.01$, respectively) and OVCAR3 ($P<0.01$ and P=0.017, respectively), compared to the 17 normal ovarian tissues (Table II). The fold-changes are shown in Fig. 2.

Correlation between miR-152 and miR-148a. In order to understand the correlation between miR-152 and miR-148a, Pearson's correlation analysis was used. As shown in Fig. 3, there was a positive correlation between the expression levels of miR-152 and miR-148a in ovarian cancer tissues ($P<0.001$). The correlation coefficient was 0.770.

Correlation between miR-152 and miR-148a expression and clinicopathological features in patients with epithelial ovarian cancer. A non-parametric test was used to determine if there is a correlation between miR-152 and miR-148a expression levels and clinicopathological features. Unexpectedly, no statisti-

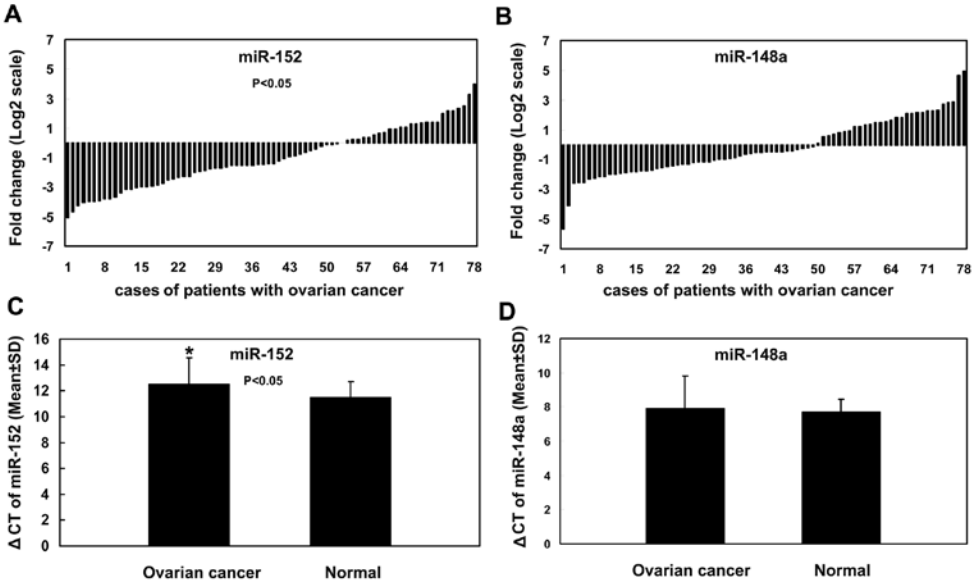

Figure 1. Expression of miR-152 and miR-148a in ovarian cancer patients. (A and B) miR-152 and miR-148a expression levels were quantified using SYBR Green real-time PCR. Data are presented as the log2 fold-change of ovarian cancer relative to normal tissues. Each sample was analyzed in triplicate and experiments were repeated three times. (C and D) miR-152 and miR-148a expression levels were normalized by U6RNA. $\Delta C_T = C_{T\ miR\text{-}148a/152} - C_{T\ U6RNA}$. The ΔC_T of miR-152 was significantly higher in ovarian cancer tissues than normal tissues (P<0.05, independent-sample t-test). There was no significant difference between the expression of miR-148a in ovarian cancer tissues and normal tissues (P=0.445).

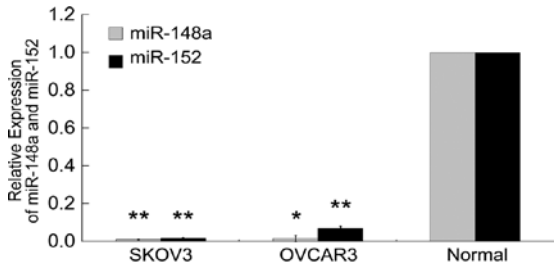

Figure 2. Expression of miR-152 and miR-148a in two ovarian cancer cell lines (SKOV3, OVCAR3). miRNAs were quantitated using SYBR-Green real-time PCR. Data are presented in ovarian cancer cell lines relative to 17 normal ovarian epithelium tissues. For each case, the experiment was analyzed in triplicate, and repeated three times (*P<0.05; **P<0.01).

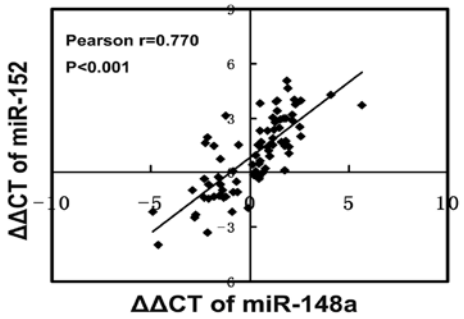

Figure 3. Correlation of miR-152 and miR-148a expression in patients with ovarian cancer. $\Delta\Delta C_{T\ miRNA} = (C_{T\ Tumor\ miRNA} - C_{T\ Tumor\ U6RNA}) - (C_{T\ Non\text{-}tumor\ miRNA} - C_{T\ Non\text{-}tumor\ U6RNA})$. Pearson r=0.770, P<0.001.

Table II. Comparison of ΔC_T of miR-152 and miR-148a in ovarian cancer tissue, cell lines and normal tissue.

	miR-152 (P-value)	miR-148a (P-value)
ΔC_T of cancer tissue (mean ± SD)	12.53±2.03	7.94±1.88
ΔC_T of normal tissue (mean ± SD)	11.51±1.18 (0.008)	7.73±0.70 (0.445)
ΔC_T of SKOV3 cells (mean ± SD)	18.07±1.30 (<0.01[a])	14.63±0.34 (<0.01[a])
ΔC_T of OVCAR3 cells (mean ± SD)	15.79±1.32 (<0.01[a])	14.22±1.59 (0.017[a])

[a]All 17 normal ovarian epithelium tissues were used as control.

cally significant associations were observed between miR-152 and miR-148a expression levels and the clinicopathological factors studied including FIGO stage (stage I, II/III, and IV), histological subtypes and differentiation grade (Table III). We evaluated the relationship between miR-152 and miR-148a expression and response to chemotherapy of EOC patients according to RECIST criteria. Of the 78 patients, 49 patients who were subjected to chemotherapy were followed up. A complete response (CR) to chemotherapy was observed in 31 patients; progressive disease (PD) occurred in 18 patients. The correlation between response and miR-152 and miR-148a expression levels was non-significant (Table III). The

Table III. Correlations between the expression of miR-152 and miR-148a and clinicopathological features in patients with ovarian cancer.

	n	miR-152[a]	miR-148a[a]
Age (years)			
≤54	18	1.07 (0.37-2.23)	1.21 (0.36-3.18)
>54	60	0.34 (0.13-1.19)	0.64 (0.33-2.30)
P-value		0.006	0.213
FIGO stage			
I	16	0.55 (0.12-1.90)	0.82 (0.42-2.19)
II	5	0.36 (0.20-3.31)	3.49 (0.34-5.38)
II	49	0.37 (0.19-1.32)	0.67 (0.32-2.46)
IV	8	0.65 (0.15-2.19)	0.55 (0.27-2.14)
P-value		0.992	0.671
Histological subtypes			
Serous	58	0.43 (0.17-1.59)	0.72 (0.30-2.78)
Mucinous	2	0.28 (0.13-0.43)	0.41 (0.31-0.51)
Endometrioid	3	0.20 (0.20-1.94)	0.54 (0.44-7.46)
Clear cell	7	0.84 (0.37-2.40)	0.74 (0.58-1.87)
Others	8	0.19 (0.13-0.65)	0.58 (0.37-1.32)
P-value		0.585	0.862
Grade			
1	45	0.35 (0.18-1.22)	0.69 (0.36-2.12)
2	28	0.54 (0.12-1.43)	0.73 (0.30-3.64)
3	4	1.62 (0.31-3.94)	1.23 (0.38-2.67)
4	1	5.03	6.56
P-value		0.245	0.465
Response[b]			
CR	31	0.52 (0.2-1.28)	0.73 (0.38-1.52)
PD	18	0.45 (0.17-1.82)	1.52 (0.29-3.00)
P-value		0.959	0.694

[a]Median of relative expression, with 25-75th percentile in parenthesis; [b]49 patients were followed-up. CR, complete response; PD, progressive disease.

Kaplan-Meier estimator was used to determine if there is correlation between miR-152 and miR-148a expression and short-term survival rates, however, the log-rank test P-values obtained were P=0.95 and P=0.392, indicating that there was no correlation between miR-152 and miR-148a expression and short-term survival rates.

Overexpression of miR-152 and miR-148a inhibits proliferation of SKOV3 cells. We detected the expression of miR-152 and miR-148a after transfection of miR-152 and miR-148a mimics and NC, the transfection efficiency was perfect. The proliferation of transfected SKOV3 cells was demonstrated using MTT growth assays. As shown in Fig. 4, overexpression of miR-152 and miR-148a markedly reduced the proliferation of SKOV3 cells compared to the matched NC. This inhibition of cell proliferation was observed 72 and 96 h post-transfection of miR-152, and 48 and 96 h post-transfection of miR-148a.

miR-152 and miR-148a have no effect on invasion of SKOV3 cells. To explore the potential role of miR-152 and miR-148a in ovarian cancer metastasis, Transwell invasion assays were carried out in SKOV3 cells. As shown in Fig. 5, the average numbers of invading cells that were transfected with miR-152 (112±42 cells) or miR-148a (110±43 cells) were not significantly different from the numbers of transfected NC cells (110±44 cells). No significant difference was observed between the control and blank groups (106±45 cells). The data indicate that miR-152 and miR-148a have no effect on the invasion of SKOV3 cells.

Discussion

miRNAs have recently been described as important players in human cancer. Specifically, the aberrant expression of miRNAs in many types of cancers suggests that they function as tumor suppressors or oncogenes (25). In previous studies from our group, we found that miR-152 and miR-148a are down-regulated in gastrointestinal cancer tissues and cancer cell lines compared to non-tumorous controls (18). It has recently been reported that miR-148a expression is down-regulated in several types of cancer, such as pancreatic ductal adenocarcinoma (PDAC) (26), prostate cancer (27), and hepatoblastoma (HB) (28), however, studies on hepatocellular carcinoma (HCC) have shown that miR-148a is up-regulated in this disease (28,29). miR-152, which is one of the miRNA-148/152 family members, was found to be deregulated in many cancers as well. For example, it is down-regulated in endometrial serous adenocarcinoma (30), and HBV-related HCC (31). However, it is overexpressed in neuroblastoma (19). Therefore, conclusions regarding whether miR-152 and miR-148a function as tumor suppressors have been controversial. In this study, we have performed the only large-scale study to date, that assesses the expression levels of both miR-152 and miR-148a in ovarian cancer using real-time PCR. Among all the methods used in miRNAs study, real-time PCR is a more quantitative and more sensitive method, and is thus able to more accurately profile the expression of miR-152 and miR-148a in ovarian cancer and clarify the relationship between miR-152 and miR-148a and clinicopathological characteristics in ovarian cancer.

In our study, we found significantly decreased expression of miR-152 in epithelial ovarian cancer tissues and cell lines. In accordance with our findings, a study also found this trend in 33 epithelial ovarian cancer tissues, using TaqMan qRT-PCR assays (32). We then evaluated whether a correlation existed between miR-152 expression and various clinicopathological features including FIGO stages (stage I, II/III, and IV), as well as different histological subtypes, differentiation grade, response to chemotherapy, and short-time survival. However, no statistically significant associations were observed. These data indicate that reduced miR-152 expression in ovarian cancer may be involved in carcinogenesis, not progression of ovarian cancer and miR-152 may function as a tumor suppressor gene. Deregulation of cell proliferation and invasion is the key mechanism for carcinogenesis and progression. To examine the function of miR-152, we detected the cell proliferation and invasion ability associated with miR-152 expression in an ovarian cancer cell line. Since the down-regulation of miR-152 was

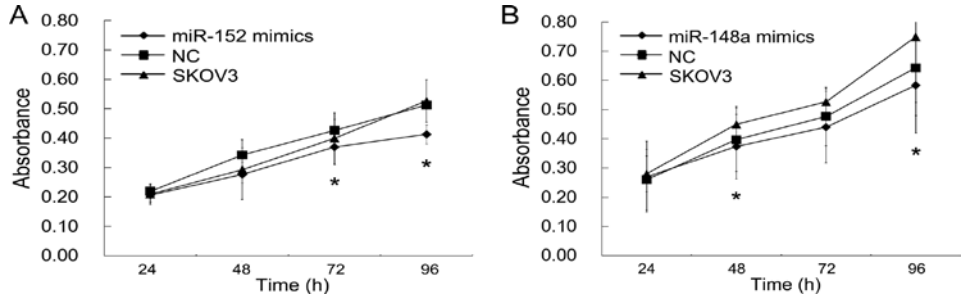

Figure 4. miR-152 and miR-148a inhibit cell proliferation in SKOV3 cells significantly. MTT proliferation assay was used to detect the growth ability of SKOV3 cells transfected with (A) miR-152 or (B) miR-148a mimics. Error bars represent the average and standard deviations of three independent experiments ($^{*}P<0.05$). NC, negative control.

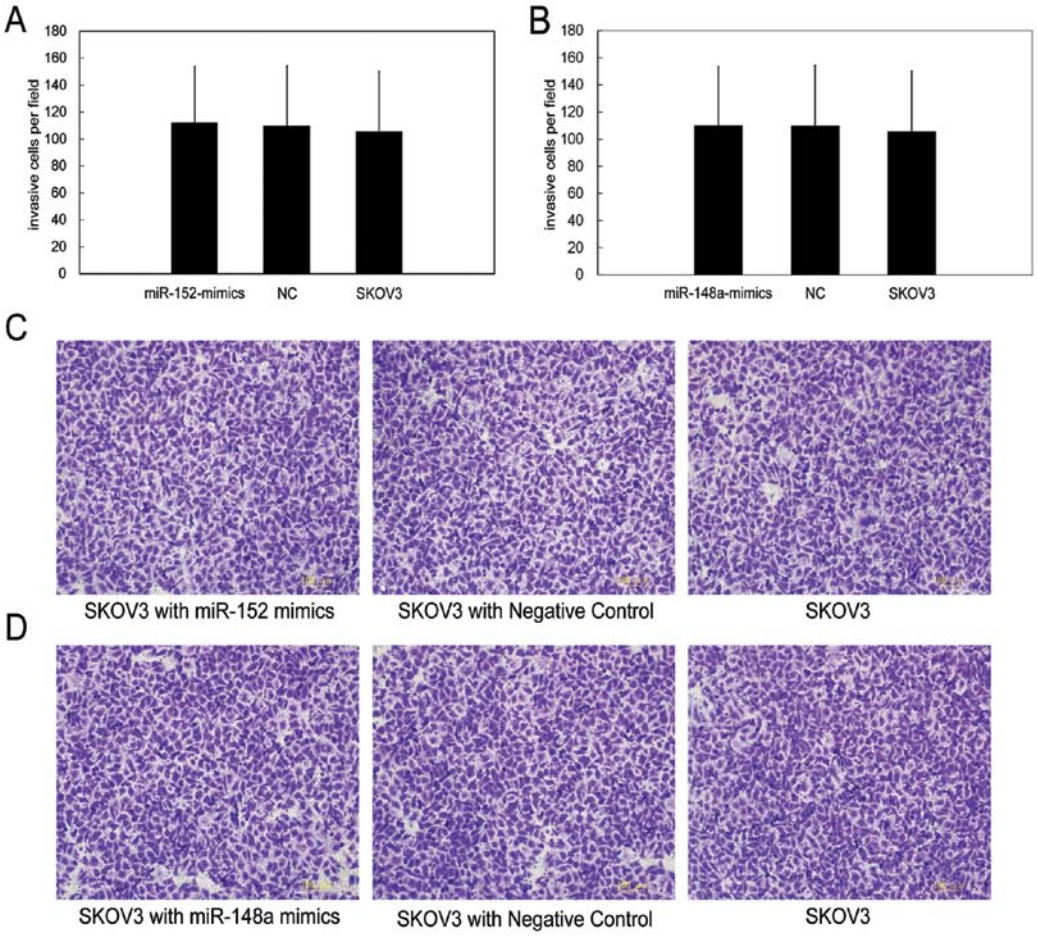

Figure 5. miR-152 and miR-148a do not inhibit cell invasion. The Transwell asssy was used to detect the invasion of SKOV3 cells. There were no significant differences between SKOV3 cells transfected with miR-152 or miR-148a mimics and negative control (NC). (A and B) Cell numbers traversing the membrane were determined by randomly counting in nine random fields under a microscope at a magnification of x200. The bars represent the mean ± SD of three independent experiments. (C and D) Magnification, x100.

more prominent in SKOV3 cells compared to OVCAR3 cells, we chose to use the SKOV3 cell line for subsequent cellular experiments. Our MTT assay indicates that the overexpression of miR-152 is associated with significant growth inhibition in ovarian cancer cells. However, our Transwell assay shows no effect of miR-152 overexpression on cell invasion. Taken together, we speculate that the down-regulation of miR-152 may promote the carcinogenesis through deregulation of cell proliferation in ovarian cancer.

According to our results, although not statistically significant, miR-148a, the other member of the highly conserved miR-148/152 family, also appears to be down-regulated in ovarian cancer cells. Of the 78 patients with ovarian cancer, 49 (63%) cases showed low expression of miR-148a compared to normal ovarian epithelial tissue. We presume that this lack of statistical significance may be due to the low number of tumor specimens evaluated, as well as the relatively small number of patients who were screened. Increases in sample size,

increase the likelihood of observing differences in expression. Recent studies have shown that miR-148a expression levels are significantly associated with differentiation grade (33) lymph node metastasis (34), and drug resistance in several cancers (35,36). Therefore, we next analyzed the relationship between miR-148a and clinicopathological characteristics in ovarian cancer, including chemotherapy prognosis and survival time. Unexpectedly, we did not find any association between miR-148a expression levels and clinicopathological features or disease prognosis. This is likely to be the case for miR-152 as well. Therefore, a microarray-based study of a larger sample size should be carried out in order to fully evaluate the relationship of miR-152 and miR-148a expression and the clinical characteristics of ovarian cancer.

MTT and Transwell assays were then carried out to examine cell proliferation and invasion, respectively. We found that overexpression of miR-148a dramatically decreased the growth of SKOV3 cells, but had no effect on cell invasion. This suggests that there is a proliferation inhibition effect associated with miR-148a expression in ovarian cancer, but no relationship between miR-148a expression and the invasion ability of ovarian cancer cells, which is in agreement with our analysis of clinicopathological features (no relationship with FIGO stages). In light of the results described above, we speculate that miR-152 and miR-148a may play important roles in carcinogenesis of ovarian cancer and may be detected in the early stages of the disease. Thus, our studies indicate that miR-152 and miR-148a may have the potential to be early-detection biomarkers of ovarian cancer, which may thereby lead to detection and diagnosis of this disease in its early stages, and may also open up new venues of treatment.

In this study, we found a close relationship between miR-152 and miR-148a expression, which is consistent with results from our previous studies in gastrointestinal cancer. As shown on the miRBase website, miR-152 and miR-148a have the same seed region, which is 2nt-8nt of miRNA, and is considered the most important recognition site (37). Other studies have shown that miR-148a, miR-148b, and miR-152, all from the same family, have the same seed sequences as well, and also regulate the same target, namely CCKBR, in gastric cancer (38). Stumpel *et al* also demonstrated that miR-148 and miR-152 have the same seed sequence, and target DNMT1 together (39). Therefore, miR-152 and miR-148a may play similar roles in ovarian cancer, although the relationship between them needs further investigation.

Recently, an increasing number of studies have demonstrated that many factors can change the expression of miRNAs, although the precise mechanisms underlying these changes remain largely unknown. First, nuclear Drosha and cytoplasmic Dicer are significant regulatory points in both global and individual miRNA biogenesis (40,41), and thus their altered expression is associated with an altered miRNA profile in many cancers, such as gastric cancer (42) and ovarian cancer (43). Second, the differential expression of miRNA is mostly likely not solely dependent on the differential expression of Dicer and Drosha, but may also be related to inactivation by deletions or mutations. Mutations in precursor proteins can regulate the expression of miR-15a and miR-16-1 in the CLL patients (44). Third, the most important epigenetic mechanism responsible for the aberrant expression of miRNAs is altered DNA methylation. In ovarian cancer, Iorio *et al* showed that DNA hypomethylation resulted in an up-regulation of miR-203 and miR-21 compared to normal ovarian tissue (10). In addition, aberrant methylation of miR-148 and miR-152 has been reported. Promoter CpG island hypermethylation occurs in miR-148 genes, and the transcriptional silencing of miR-148a in metastatic cancer cells is associated with the presence of CpG island hypermethylation in colorectal mucosa, lymphocytes, and skin (45). miR-148 and miR-152 re-express after treatment with the demethylating agent in breast cancer cells (34). We thus speculate that CpG island hypermethylation may be one of the mechanisms of miR-152 and miR-148a down-regulation in ovarian cancer. Further studies will be needed to confirm this hypothesis.

In conclusion, miR-152 and miR-148a may be involved in carcinogenesis of ovarian cancer through deregulation of cell proliferation. They may be novel biomarkers for early-detection or therapeutic targets of ovarian cancer.

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