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Expression and prognostic significance of microRNA-451 in human epithelial ovarian cancer

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Summary

Objective: MicroRNA-451 has been proved to be downregulated in many human malignancies and correlated with tumor progression. However, its expression and clinical significance in epithelial ovarian cancer (EOC) is still unclear. The aim of this study was to explore the effects of miR-451 in EOC tumorigenesis and development. *Materials and Methods:* The expression levels of miR-451 were quantified by qRT-PCR in 115 EOC and 34 normal ovarian tissues, and correlated with clinicopathological factors and prognosis. MTT, flow cytometric assay, and transwell invasion assay were used to test the proliferation, apoptosis, and invasion of SKOV-3 EOC cells transfected with miR-451 mimics or negative control (NC) RNA-oligonucleotides. *Results:* MiR-451 expression was significantly downregulated in EOC compared with normal ovarian tissues. Low level of miR-451 was associated with advanced FIGO stage (p = 0.005), higher serum CA125 expression level (p = 0.005), and lymph node metastasis (p = 0.002). Multivariate Cox regression analysis identified decreased miR-451 expression as an independent factor predicting poor prognosis for EOC patients. In addition, transfection of miR-451 mimics in SKOV-3 was able to reduce cell proliferation, promote cell apoptosis, and inhibit cell invasion. *Conclusions:* miR-451 may act not only as a novel diagnostic and prognostic marker, but also as a potential target for molecular therapy of EOC.

Key words: MicroRNA-451; Epithelial ovarian cancer; Prognosis; Proliferation; Apoptosis; Invasion.

Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women and the most lethal gynecologic malignancy in the world [1]. Early symptoms of EOC patients are generally minor and easily overlooked. At initial diagnosis, most of the women suffer from advanced stage disease. Although improvement of the quality of cytoreductive surgery as well as the development of novel drugs and new chemotherapy regimens for EOC have been made, the prognosis of patients with advanced stage EOC remains poor. The five-year overall survival (OS) is approximately 20% for Stage III disease and 5% for Stage IV disease [2]. Previous studies have demonstrated diverse genetic alterations in EOC [3-7], but the highly complex molecular mechanisms underlying EOC carcinogenesis and progression remain obscure. Therefore, it is necessary to search novel markers for EOC, which can accurately identify biological characteristics of tumors, improve therapeutic strategies, and predict clinical outcome.

MicroRNAs (miRNAs) are single-stranded, small non-coding RNAs with 18–25 nucleotides in length [8]. They can negatively regulate gene expression through base-pairing to the 3' untranslational region (3'UTR) of target messenger RNA (mRNA), resulting in translation inhibition or mRNA degradation [9, 10]. It is now clear that miRNAs play key roles in almost all biological processes, including differentiation, development, gene regulation, cell proliferation and apoptosis, and the development of various diseases, such as cancer. They function as tumor suppressors or oncogenes according to the roles of their target genes. Gene expression profiling studies have revealed that miRNA expression may be an excellent biomarker for cancer diagnosis and prognosis estimation [11, 12]. In terms of EOC, abnormal expression of several miRNAs such as miR-21 [13], miR-125b [13], miR-34a [14], and miR-15 [15] have been reported. Peng *et al.* showed decreased miR-100 expression in EOC and its correlation with advanced clinical stage, higher serum CA125 level, lymph node involvement, and shorter OS [16]. Additionally, miR-100 could affect the growth of EOC cells by post-transcriptionally regulating polo-like kinase 1 (PLK1) expression. By performing miRNA expression profiling analysis, Hu *et al.* found that three miR-200 miRNAs (miR-200a, miR-200b and miR-429) in the miR-200b–429 cluster were significantly associated with cancer recurrence and OS [17]. Furthermore, overexpression of this miR-200 cluster could inhibit ovarian cancer cell migration. These findings suggest that miRNAs act not only as diagnostic and prognostic markers, but also as potential therapeutic targets of EOC.

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One of the tumor suppressive miRNAs is miR-451. miR-451 expression is decreased in gastric cancer [18], breast cancer [19], lung cancer [20], glioma [21], and head and neck squamous cell carcinoma [22]. miR-451 has been reported to be correlated with chemosensitivity of MCF-7 breast cancer cells to doxorubicin (DOX) and A549 non-small cell lung cancer (NSCLC) cells to cisplatin [19, 23]. Recent studies have demonstrated that miR-451 may modulate the process of tumorigenesis and the behavior of cancer cells by suppressing a series of oncogenes. However, expression of miR-451 in EOC and a link between miR-451 and clinicopathological tumor features have not been clearly understood.

In this study, the authors examined the expression of miR-451 in EOC and normal ovarian tissues. Then, the clinicopathological and prognostic significance of miR-451 expression in human EOC were statistically analyzed. Furthermore, the effects of miR-451 on EOC cell line SKOV-3 proliferation, apoptosis, and invasion were investigated.

Materials and Methods

Patients and tissue samples

A total of 149 fresh surgical tissue samples (115 EOC and 34 normal ovarian tissues) were collected at the Department of Obstetrics and Gynecology, The First Affiliated Hospital of Shantou University Medical College between 2005 and 2007. Normal ovarian tissues were obtained from tumor-free participants during surgery for other gynecological diseases. For example, they underwent surgery for a total hysterectomy, bilateral salpingo-oophorectomy, and pelvic and para-aortic lymphadenectomies. The ethical committees of the present hospital approved this study, and informed consent was obtained from all patients. No patient had undergone chemotherapy or radiotherapy before surgery. All tissue samples were flash frozen and stored at -80°C in liquid nitrogen until processed. Clinical tumor stage was classified according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. All EOC patients had been followed up from the time of surgical intervention to 2012. OS was measured up to the date of death due to any cause or, for living patients, the date of last follow-up.

Cell culture

Human ovarian cancer cell line SKOV-3 was obtained from the Beijing Institute for Cancer Research (Beijing, China). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified air at 37˚C with 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was isolated using a reagent according to the manufacturer's instructions. RNA quality and quantity were assessed using a bioanalyzer system. Reverse transcription reaction was carried out starting from 10 ng of total RNA using the looped primers. Real-time PCR was performed using the standard Taqman MicroRNA assays protocol on ABI7500 real-time PCR detection system. U6 small nuclear RNA was used as an internal control. The RT primers were 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAACTCAG-3' for miR-451 and 5'-TGGTGTCGTGGAGTCG -3' for U6. The PCR primers for mature miR-451 or U6 were designed as follows: miR-451 sense, 5'-ACACTCCAGCTGGGAAACCGTTACCATTACT-3' and reverse, 5'-CTGGTGTCGTGGAGTCGGCAA-3'. U6 sense, 5'- CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Each sample was measured in triplicate, and the relative amount of miR-451 to U6 was calculated using the equation 2^{-DCt}, where DCT = ($CT^{miR-451}$ - CT^{U6}).

miR-451 transfection

MiR-451 mimics and negative control (NC) RNA-oligonucleotides were utilized. The day before transfection, SKOV-3 cells were seeded in antibiotic free medium. Transfection was carried out using lipofectamine in accordance with the manufacturer's procedure. The level of miR-451 mimics expression in SKOV-3 cells was assayed by real-time PCR.

Cell proliferation assay

The proliferation capacity of SKOV-3 cells transfected with miR-451 mimics or NC was evaluated with an MTT assay, which was performed following standard procedure in 96-well plates. In brief, cells were seeded at a density of 2,000 cells per well containing 100 ul of culture medium and cultured overnight. Twenty ul of five mg/ml MTT (dimethyl thiazolyl diphenyl tetrazolium) reagent was added to each well and cells were further incubated for four hours at 37˚C. Then the medium was removed, and 100 ul of DMSO (dimethyl sulfoxide) was added to each well to dissolve the formazan. Cell proliferation was assessed daily for fourconsecutive days. The absorbance of the samples was measured with a spectrophotometer reader at 490 nm.

Detection of apoptosis by flow cytometry

Apoptosis was detected by flow cytometric analysis as previously reported [24]. Briefly, the cells were washed and resuspended at a concentration of 1×10^6 cells/ml. Then, the cells were stained with Annexin V and propidium iodide (PI), using the Annexin V apoptosis detection kit. After incubation at room temperature in the dark for 15 minutes, the cells were immediately analyzed with a flowcytometer.

Cell invasion assay

Cell invasion assay was performed using 24-well transwell chambers (eight μm) and the upper chambers were first covered with one mg/ml Matrigel. After transfection, 2×10^5 SKOV-3 cells resuspended in 100 *u*l serum-free medium were seeded into the upper chambers. 0.5 ml of 10 % FBS-RMPI-1640 was added to the lower chambers. Following a 24-hour incubation, non-invaded cells on the top of the membrane were removed with a cotton swab, and the invaded cells were fixed with 95 % ethanol and stained with 0.1% crystal violet. The number of invaded cells was determined by counting five random fields on each membrane.

Statistics

Statistical analyses were performed with SPSS software (version 16.0). Data were expressed as mean ± standard deviation (SD). The differences between groups were analyzed using the Student's t-test or chi-square test. Survival probabilities were described by Kaplan–Meier curves and compared using the log-rank test. Cox regression (Proportional hazard model) was adopted to assess the independence of different prognostic factors. All tests were two-tailed, and the significance level was set at $p < 0.05$.

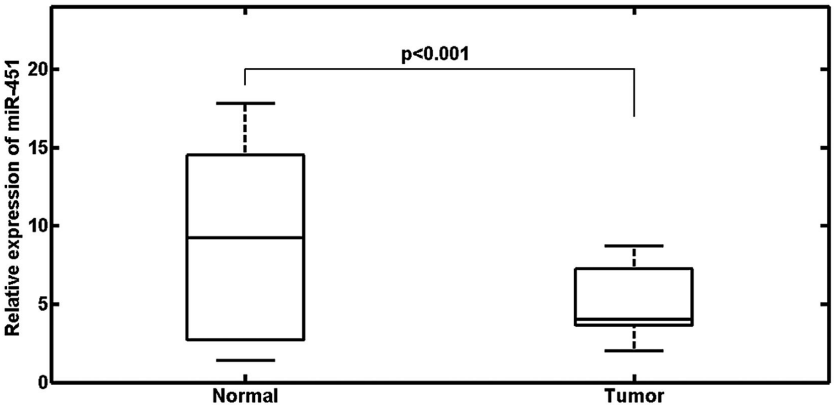

Figure 1. — miR-451 expression in 115 EOC and 34 normal ovarian tissues detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

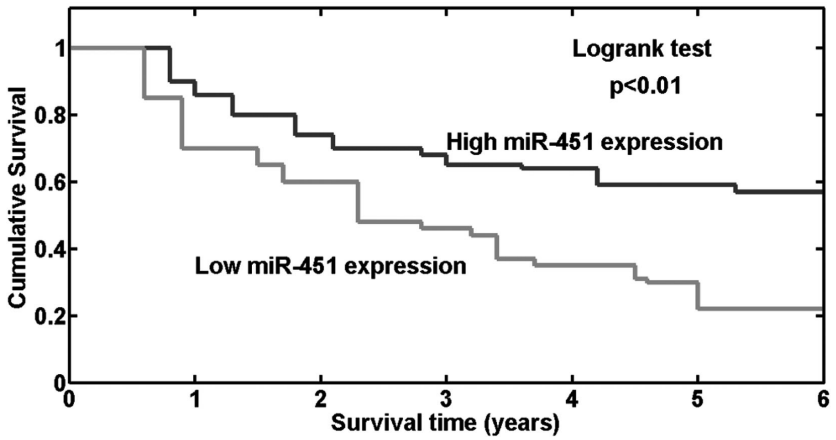

Figure 2. — Kaplan–Meier curves for overall survival time of patients with EOC, divided according to miR-451 expression levels.

Table 1. — *Association of miR-451 expression with clinicopathological variables of EOC patients.*

	Low miR-451 expression (n, %)	High miR-451 expression (n, %)	*p*-value
Age (years)			
≥ 50	38 (53.5%)	33 (46.5%)	
< 50	19 (43.2%)	25 (56.8%)	0.339
Histological type			
Serous	18 (56.2%)	14 (43.8%)	
Mucinous	23 (48.9%)	24 (51.1%)	0.256
Others	16 (44.4%)	20 (55.6%)	
Histological grade			
G1	27(49.1%)	28(50.9%)	
G2	15(53.6%)	13(46.4%)	0.323
G3	15(46.9%)	17(53.1%)	
FIGO stage			
I/II	18(34.0%)	35(66.0%)	0.005
III/VI	39(62.9%)	23(37.1%)	
Serum CA 125 level (U/L)			
< 5.0 x 10^5	20(35.7%)	36(62.3%)	0.005
≥ 5.0 x 10^5	37(62.7%)	22(37.3%)	
Ascites			
No	24(44.4%)	30(55.6%)	0.352
Yes	33(56.5%)	28(43.5%)	
Lymph node involvement			
No	20(31.7%)	43(68.3%)	0.002
Yes	37(71.2%)	15(28.8%)	

Results

miR-451 expression in EOC tissues and its association with clinicopathological characteristics

The expression levels of miR-451 in EOC and normal ovarian tissues were detected by qRT-PCR and normalized to U6 small nuclear RNA. For EOC tissues, the mean level of miR-451 expression was 3.5 (range, 1.4 - 9.2). For the normal ovarian tissues, the mean level of miR-451 expression was 8.7 (range, 2.9 - 17.8). The level of miR-451 was significantly lower in EOC tissues than in normal ovarian tissues ($p < 0.001$; Figure 1).

Next, the clinicopathological significance of miR-451 expression in human EOC was analyzed. The authors divided the patients into two groups according to their miR-451 expression levels using its median as a cutoff: high miR-451 expression group (n = 58) and low miR-451 expression group (n = 57). As shown in Table 1, downregulation of miR-451 was closely correlated with advanced FIGO stage ($p = 0.005$), higher serum CA125 expression level ($p = 0.005$), and lymph node metastasis ($p = 0.002$). However, there was no significant correlation between miR-451 expression and other clinicopathological variables including age, histological type, histological grade, and ascites.

Table 2. — *Univariate and multivariate analysis of prognostic variables by Cox regression analysis.*

Variables	Univariate analysis		Multivariate analysis	
	RR	P-value	RR	*p*-value
Age (years) (≥ 50 / <50)	1.43	0.218	1.55	0.095
Histological type (Serous/non-serious)	0.96	0.146	2.46	0.272
Histological grade (G1/G2+G3)	2.45	0.008	2.11	0.078
FIGO stage (III + IV / I + II)	3.12	0.006	1.79	0.003
Ascites (yes/no)	0.87	0.452	1.52	0.072
Serum CA 125 (≥ 5.0 ′ 10^5 / <5.0 ′ 10^5)	1.35	0.139	2.02	0.214
Lymph node involvement (yes/no)	2.16	0.012	3.04	0.016
Expression of miR-451 (low/high)	3.82	<0.001	2.32	0.008

Association of miR-451 expression with prognosis of EOC patients

The authors further evaluated whether miR-451 expression had prognostic potential for OS of EOC patients. The Kaplan-Meier curve is shown in Figure 2. They found patients with low miR-451 expression were more likely to have a shorter OS ($p < 0.001$), when compared to patients with high miR-451 expression. Aside from miR-451 expression ($p < 0.001$, RR = 3.82), univariate Cox proportional hazard regression analysis revealed that histological grade ($p = 0.008$, RR = 2.45), FIGO stage ($p = 0.006$, RR = 3.12), and lymph node status ($p = 0.012$, RR = 2.16) were also predictive factors for prognosis. Multivariate Cox proportional hazard regression analysis confirmed that low-level miR-451 expression ($p = 0.008$, RR = 2.32) was an unfavorable prognostic factor independent of other clinicopathological factors, including FIGO stage ($p = 0.003$, RR = 1.79) and lymph node metastasis ($p = 0.016$, RR = 3.04; Table 2).

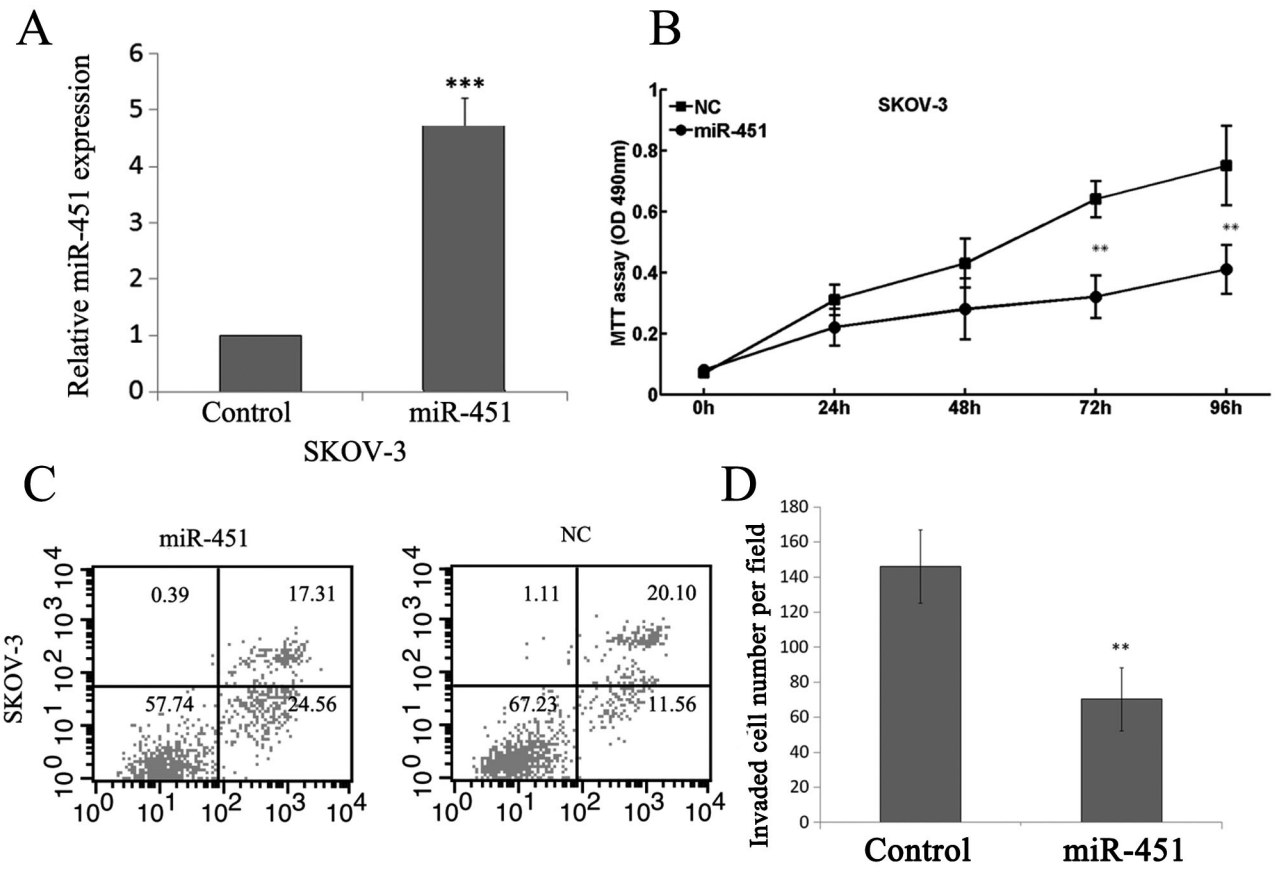

Figure 3. — Effects of miR-451 transfection on the proliferation, apoptosis, and invasion of EOC cell line SKOV-3. (A) miR-451 expression after treatment with miR-451 mimics or negative control in SKOV-3 cells assessed by quantitative RT-PCR. (B) MTT assay demonstrated reduced cell proliferation in SKOV-3 cells after transfection of miR-451 mimics. (C) transfection of miR-451 mimics promoted cell apoptosis in SKOV-3 cells. (D) miR-451 suppressed SKOV-3 cell invasion *in vitro*. The Matrigel invasion assay showed that the number of invaded cells was significantly lower in the miR-451-transfected cells than in the NC-transfected cells.

Effects of miR-451 expression on proliferation, apoptosis, and invasion of SKOV-3 Cells

The downregulation of miR-451 in EOC tissues prompted the authors to investigate its biological functions in carcinogenesis. SKOV-3 cells were transfected with miR-451 mimics or NC. As shown in Figure 3A, the expression level of miR-451 in miR-451 mimics transfected cells was significantly higher compared with NC transfected cells ($p < 0.001$). MTT assay demonstrated that transfection of miR-451 mimics reduced cell proliferation of SKOV-3 cells (Figure 3B; $p < 0.01$). The authors also observed promoted cell apoptosis in miR-451 mimics transfected cells (Figure 3C; $p < 0.05$). Furthermore, transwell invasion assay showed a significant decrease in invaded cell numbers after miR-451 transfection (Figure 3D, $p < 0.01$). These results indicate that miR-451 is involved in the negative regulation of EOC cell growth and invasion *in vitro*.

Discussion

Much work has been performed in an attempt to identify markers with diagnostic and prognostic implications for human EOC. Recently, many studies have shown that aberrant miRNAs are associated with tumorigenesis and progression in various human cancer types; however, their potential roles in EOC remain largely uncharacterized. In the current study, the authors firstly observed that miR-451 was downregulated in EOC tissues compared with normal ovarian tissues. Then, the downregulation of miR-451 in EOC tissues was significantly correlated with advanced FIGO stage, higher serum CA125 expression level, and lymph node metastasis. Furthermore, patients with low miR-451 expression showed poorer survival than those with high miR-451 expression. A multivariate analysis with the Cox proportional hazards confirmed that the status of miR-451 expression was an independent predictor of OS in EOC. Finally, transfection of miR-451 mimics in EOC cell line SKOV-3 was able to reduce cell proliferation, promote cell apoptosis, and inhibit cell invasion *in vitro*. To the authors' knowledge, this is the first report regarding the involvement of miR-451 in EOC.

Previous research has revealed dysregulated miR-451 expression in many human malignancies, and its functions as a tumor suppressor by targeting a number of oncogenic genes. For example, decreased miRNA-451 expression was confirmed in NSCLC tissues and correlated with advanced clinical stage, lymph node metastasis, and poor prognosis [25]. Upregulation of miR-451 significantly reduced the in vitro proliferation and enhanced apoptosis of NSCLC cell line SPC-A1 by targeting ras-related protein 14 (RAB14), and suppressed the development of tumors in nude mice. Tian *et al.* reported the anti-tumor function of miR-451 in glioma through targeting calcium binding protein 39 gene (CAB39) and inhibiting the PI3K/AKT pathway [21]. Bandres *et al.* identified a novel oncogene macrophage migration inhibitory factor (MIF) as a direct target of miR-451, and revealed the predictive effects of miR-451 on radiosensitivity and prognosis in gastric cancer patients [18]. In terms of the association between miR-451 and chemosensitivity, Bian *et al.*'s study indicated that ectopic overexpression of miR-451 could sensitize A549 NSCLC cells to cisplatin by increasing cisplatin-induced apoptosis [23]. Kovalchuk *et al.* reported that the enforced increase of miR-451 levels in MCF-7 breast cancer cells would downregulate expression of multidrug resistance 1 (MDR1) and increase sensitivity of the MCF-7 cells to DOX [19]. Taken together, miR-451 could not only be useful as a marker but also serve as a target for the development of novel therapeutic strategies to overcome tumor growth and drug resistance.

The mechanism by which miR-451 expression affects carcinogenesis, cancer progression and drug resistance is complex. Some useful targets have been identified during the past few years; however, there is no 'one-to-one' connection between miRNAs and target mRNAs. An average miRNA can have more than 100 targets [26]. Conversely, several miRNAs can converge on a single transcript target [27]. Thus, the potential regulatory circuitry afforded by miR-451 may be enormous, and its direct functional targets in EOC are not completely clear. Additionally, the current study was limited by its retrospective nature which led to the present results being considered exploratory, and the sample size was relatively smaller. Further prospective analysis containing a large number of tumor samples is worth performing.

In conclusion, the present findings suggested that miR-451 downregulation was closely correlated with tumor aggressive progression and poor prognosis of human EOC. Ectopic expression of miR-451 inhibited cell growth and invasion *in vitro.* Therefore, miRNA-451 may be a potential novel target for gene therapy of EOC.

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