

miR-182-5p enhances cisplatin resistance in epithelial ovarian cancer by downregulating GRB2

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Objective: Epithelial ovarian cancer (EOC) patients often experience cisplatin resistance. This study analysed the miR-182 level in EOC patients, and explored the association between miR-182 with chemoresistance in EOC. **Methods:** Using qRT-PCR, the miR-182-5p levels in EOC were determined. miR-182-5p and its associations with the clinicopathological characteristics and overall survival of EOC patients were analysed. A2780/DDP cells and A2780 cells were transfected with miR-182-5p mimics or inhibitor. Then, using a CCK8 assay, cell viabilities were assessed. To determine if GRB2 is a bona fide miR-182-5p target, a dual luciferase reporter assay was carried out. Then, the cell viability in A2780/DDP cells overexpressing GRB2 was determined. **Results:** Compared with adjacent normal tissues, the miR-182-5p was significantly upregulated in EOC tissues. High levels of miR-182-5p were associated with frequent platinum resistance and poor overall survival in EOC patients. Upregulation of miR-182-5p by its mimics significantly enhanced the cisplatin resistance of A2780 cells. Conversely, miR-182-5p inhibitors significantly enhanced the cisplatin sensitivity of A2780/DDP cells. GRB2 was confirmed as a bona fide miR-182-5p target in EOC. In GRB2-overexpressing EOC cells, cisplatin sensitivity was significantly enhanced. **Conclusions:** miR-182-5p enhanced the cisplatin resistance of EOC cells by downregulating GRB2, which is a novel target for predicting the prognosis and improving the treatment of EOC.

Keywords

Epithelial ovarian cancer; miR-182; GRB2; Cisplatin; Chemoresistance

1. Introduction

Epithelial ovarian cancer (EOC) is a malignant tumour, with great risk to threaten women's lives and health. The mortality rate of EOC ranks first among gynaecological tumours [1]. With the extensive development of cytoreductive surgery, the emergence of platinum-based drugs, the treatment of EOC and the quality of life of EOC patients has significantly improved in recent decades [2], but the EOC five-year survival rate remains < 50%, so platinum-based chemotherapy does not significantly improve overall survival [1]. One of the main reasons is the resistance of platinum-based drugs used in chemotherapy [3, 4]. Multiple mechanisms of tumour resistance have been demonstrated in literatures including oncogene activation and suppressor gene inactivation, inhibition of tumour cell apoptosis and decrease of intracellular

drug concentration and are the result of a comprehensive series of multi-gene, multi-factor, and multi-step actions [2]. Exploring the mechanism underlying the drug resistance of EOC and improving the chemotherapy sensitivity of EOC cells will effectively enhance the survival of EOC patients.

MicroRNAs (miRNAs) are regulated the gene posttranscription [5, 6]. They participate in important biological processes of organism development, cell differentiation and cell signal transduction [5, 7]. They also act as gene expression regulators and play a key role in the malignant tumours development, including EOC. It was demonstrated that some miRNAs are involved in chemoresistance and are significant indicators for predicting patient outcomes or disease progression after chemotherapy [8]. In breast cancer cells, the level of miR-182 is associated with resistance to trastuzumab [9]. miR-182 has an oncogenic role in human lung cancer, ovarian cancer and hepatocellular carcinoma, while miR-182 serves as a tumour suppressor in renal cancer [10]. miR-182 plays dual roles in prostate cancer progression. miR-182 is upregulated in localized prostate cancer, while it is downregulated in aggressive cancers [11]. In a relatively large cohort of serous ovarian cancer patients with platinum-based chemotherapy, miR-182 was downregulated, indicating that miR-182 might be associated with platinum resistance [12]. However, the associations between clinicopathological characteristics and miR-182-5p, cisplatin resistance in EOC patients are still unclear.

Here, we demonstrated the role of miR-182-5p in cisplatin resistance and survival in EOC. The increase of miR-182-5p significantly enhanced the cisplatin resistance in EOC cells. Furthermore, miR-182-5p inhibitors upregulated growth factor receptor bound protein 2 (GRB2), causing increases in the cisplatin sensitivity of EOC cells. Thus, this study reveals a novel mechanism underlying cisplatin resistance in EOC. miR-182-5p/GRB2 signalling might act as a therapeutic target for cisplatin-resistant EOC.

2. Materials and methods

2.1 EOC tissue specimens

A total of 20 normal ovarian epithelial tissues, and 89 EOC patients' tissues were collected at Zhejiang University

Mingzhou Hospital between 2009 and 2017. Tissues were immediately frozen in liquid nitrogen and then stored at -80 °C until the test. The included patients signed the informed consent. All enrolled patients underwent platinum-based therapy after surgery. If tumour relapse or progression was observed within 6 months of the last chemotherapy treatment, the tumour was defined as platinum resistance. If not, it was defined as platinum sensitive. To classify the EOC tissues, the mean value of miR-182-5p was set as the threshold to separate them into high and low miR-182-5p expression groups. Our study was approved by the ethics committee of Zhejiang University Mingzhou Hospital.

2.2 Cell culture and treatment

Human EOC cell lines A2780, A2780/DDP, SKOV3, and CaOV3 (American Type Culture Collection, USA), and immortalized ovarian epithelial cell line Moody were used. The cells were maintained with 5% CO₂ at 37 °C in DMEM (Gibco, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. GRB2 sequences were ligated into the pEX-2 vector or pEX-2 vector (NC) by GenePharma (China). Using Lipofectamine 3000 (Invitrogen, USA), the miR-182-5p mimics, inhibitor and NC (RiboBio, China) were transfected into cells.

2.3 qRT-PCR

Using TRIzol reagent (Invitrogen, USA), total RNA from cells or tissue samples were extracted. Then, using the PrimeScript RT reagent kit (Promega, USA), cDNA was synthesized. The primer sequences of miR-182-5p were described previously [13]. Using ABI 7500 real-time PCR system (Applied Biosystems, Germany), PCR was performed with SYBR Green PCR Kit (Takara, Japan). U6 was used as internal control. With 2^{-ΔΔCt} method, the relative levels were analysed.

2.4 Western blotting

Using RIPA (Beyotime, China), the proteins were extracted from cells and measured by BCA kit (Beyotime, China). After separation the extracted proteins by 10% SDS-PAGE, the blots were then electransferred to PVDF membranes (Millipore, USA). The membrane were incubated with 5% non-fat milk for blocking 1 h. Followed an overnight incubation at 4 °C with primary antibodies against GRB2 and GAPDH (Abcam, USA), The membranes were incubated with HRP-conjugate secondary antibody for 1 h. Using BioRad XRS imaging system (Bio-Rad, USA), the bands of proteins were visualized with Enhanced Chemiluminescence (ECL) Western Blotting Substrate (Abcam, USA) and quantified by ImageJ (NIH, USA).

2.5 Luciferase reporter assay

The sequence of the GRB2 3'-UTR containing the wild-type or mutated binding site for miR-182-5p was amplified and inserted into the psiCHECK-2 vector (Promega, USA). Transfection with miR-182-5p mimics and GRB2 reporter constructs was performed using Lipofectamine 3000 (Invitrogen, USA). The luciferase assay was performed in the

A2780/DDP cell line with the Dual-Luciferase Reporter Assay System (Promega).

2.6 Cell viability

Cells were transfected with miR-182-5p mimics, inhibitor or NC and then seeded in 96-well plates at 2 × 10⁴ and 1 × 10⁴ cells/well for A2780 cells and A2780/DDP cells, respectively. After 24 h. Cells were treated with cisplatin (Sigma, USA) for 48 h, and cell viability was determined by CCK-8 assay (Beyotime, China). The IC₅₀ values were calculated.

2.7 Statistical analysis

Data were obtained from 3 independent experiments. All results were presented as the means ± SD and analysed using SPSS 17.0 (SPSS, USA) with Student's *t*-test (for two groups) and ANOVA with Tukey's post hoc test. For survival analyses, kaplan-Meier analysis and the log-rank test was performed. The relationship between miR-182-5p level and EOC patients' pathological characteristics was analysed by X² test. *P* < 0.05 was considered statistically significant.

Table 1. The clinicopathological characteristics of EOC patients and their relationship with miR-182-5p level.

Variables	miR-182-5p (n = 89)		X ²	P
	High (n = 57)	Low (n = 32)		
Age (years)			0.002	0.964
≥ 50	27	15		
< 50	30	17		
FIGO stage			2.836	0.092
I-II	15	14		
III-IV	42	18		
Histology type			0.442	0.931
Serous	36	18		
Mucinous	10	7		
Clear cell	6	4		
Endometrioid	5	3		
Differentiation			1.312	0.519
G1	27	19		
G2	18	7		
G3	12	6		
Lymph node metastasis			5.237	0.022
Negative	34	11		
Positive	23	21		
Response to primary therapy			4.009	0.045
CR	39	28		
Non-CR	18	4		
Platinum status			4.298	0.038
Sensitive	30	24		
Resistant	27	8		

Note: To classify the EOC tissues, the mean value of miR-182-5p was set as the threshold to separate patients into high and low miR-182-5p expression groups. CR, complete response.

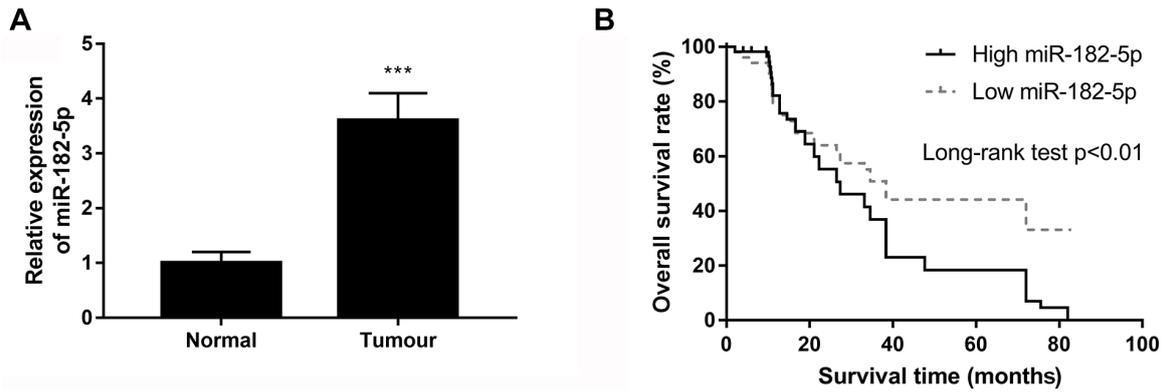


Fig. 1. miR-182-5p is upregulated in EOC tumour tissues and is positively associated with poor overall survival. (A) Relative expression of miR-182-5p in EOC tissues (n = 89) and fresh normal ovarian epithelial tissues (n = 20). (B) Kaplan-Meier analysis of the overall survival of EOC patients (n = 89) with low (n = 32) or high (n = 57) expression levels of miR-182-5p ($P < 0.01$). To classify the EOC tissues, the mean value of miR-182-5p was set as the threshold to separate them into high and low miR-182-5p expression groups. $***P < 0.001$.

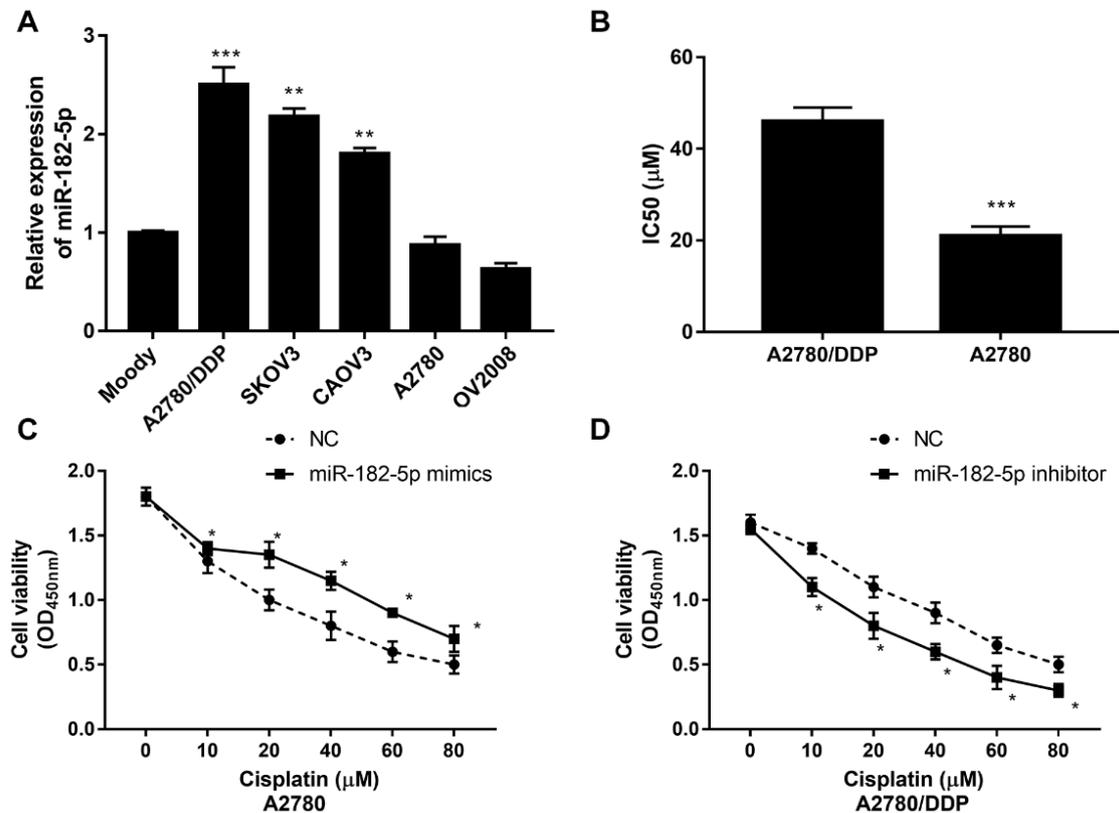


Fig. 2. Role of miR-182-5p in cisplatin resistance. (A) miR-182-5p expression levels in Moody and EOC cell lines. (B) A2780 and A2780/DDP cells were exposed to various concentrations of cisplatin for 48 h, and the IC50 of cisplatin was determined by the CCK-8 assay. (C,D) A2780 (C) and A2780/DDP (D) cells were transfected with miR-182-5p mimics and miR-182-5p inhibitor, or the corresponding NC. Cells were treated by cisplatin for 48 h. Cell viability was assessed by CCK-8 assay. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

3. Results

3.1 miR-182-5p is overexpressed in EOC

The expression of miR-182-5p in 30 pairs of EOC tissues and their respective adjacent normal controls were determined. Compared with normal controls, the miR-182-5p in EOC tissues were significantly upregulated ($P < 0.001$)

(Fig. 1A). The mean value of miR-182-5p level was taken as the threshold to separate the high and low levels of miR-182-5p. Moreover, the miR-182-5p levels in another 89 EOC patients were detected, and the associations between those expression levels and stage, age, differentiation, histology type, response to primary therapy, and platinum status were anal-

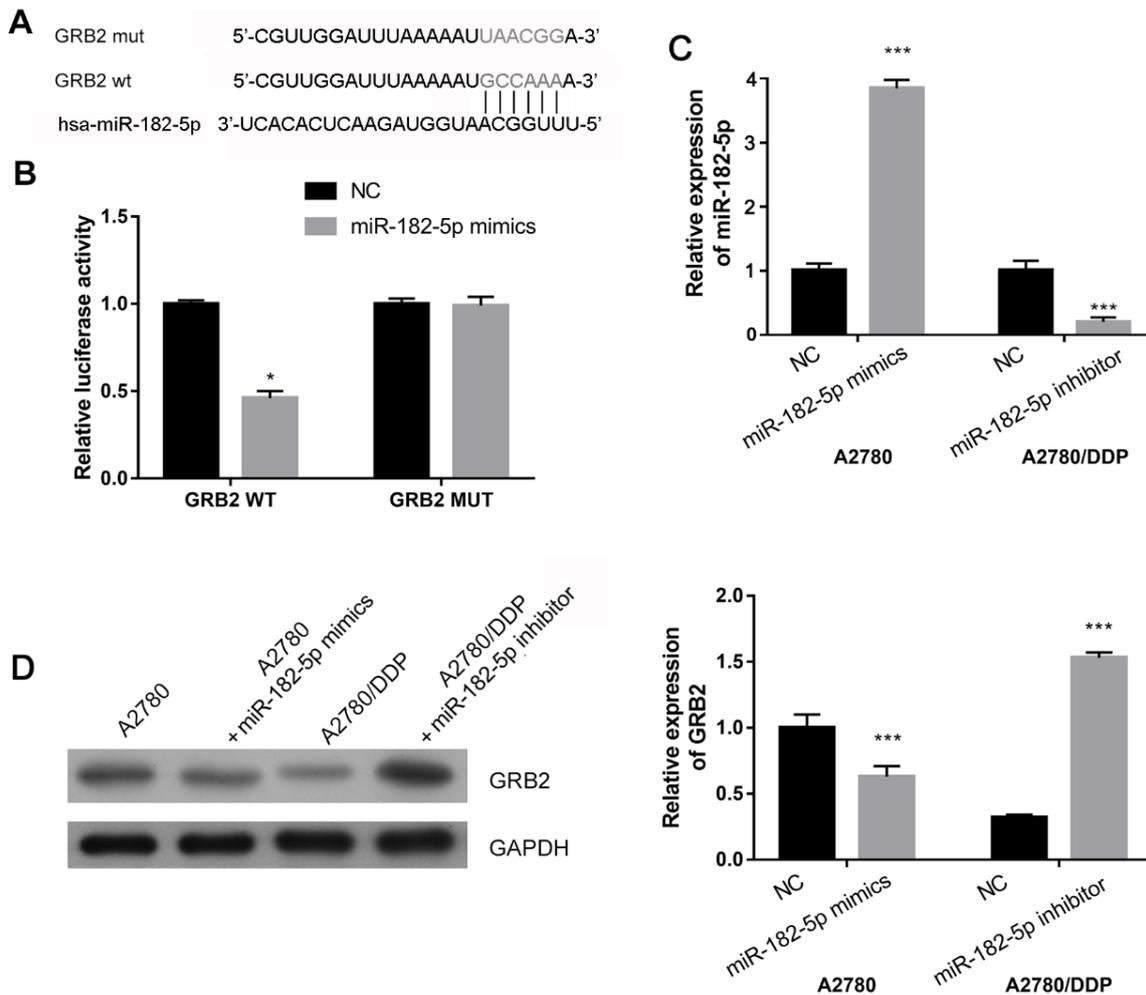


Fig. 3. GRB2 is a direct target of miR-182-5p in EOC cells. (A) Predicted binding sites of miR-182-5p on the 3'-UTR of GRB2 and mutant vector sequence. (B) Dual luciferase reporter assay for detection of the relationship between miR-182-5p and GRB2. (C,D) Expression of miR-182-5p (C) and GRB2 (D) in EOC cells after transfection with miR-182-5p mimics or inhibitors. * $P < 0.05$, *** $P < 0.001$.

used (Table 1). The miR-182-5p was not significantly correlated to age, histology type, differentiation or stage, but high level of miR-182-5p was correlated with complete response to primary therapy and high platinum resistance (Table 1). Furthermore, miR-182-5p was closely associated with EOC overall survival by Kaplan-Meier analysis (Fig. 1B).

3.2 miR-182-5p suppressed cisplatin-induced cytotoxicity

To study miR-182-5p in cisplatin-induced cytotoxicity, the miR-182-5p level in EOC cell lines were detected. Fig. 2 shows the miR-182-5p was upregulated in EOC A2780/DDP cell, SKOV3 and CAOV3 compared with Moody cells, but miR-182-5p was not significantly changed in A2780 EOC cells, compared with Moody cells (Fig. 2A). As miR-182-5p expression was upregulated in cisplatin-resistant EOC cell line A2780/DDP cells but not in cisplatin-sensitive parental A2780 cells, miR-182-5p might be responsible for EOC cell cisplatin resistance. The IC₅₀ of cisplatin in A2780 cells was markedly lower than that in A2780/DDP cells (Fig. 2B).

Then, transfection of miR-182-5p inhibitor downregu-

lated the miR-182-5p in A2780/DDP cells and A2780 cells, and transfection of miR-182-5p mimics overexpressed the miR-182-5p in those cells. The cell viability after 48 h was examined using CCK-8 kit. Overexpression of miR-182-5p by mimics significantly enhanced cisplatin resistance in A2780 EOC cells, while downregulation of miR-182-5p by inhibitors significantly enhanced cisplatin sensitivity in A2780/DDP cells, compared to that in control cells (Fig. 2C,D). Thus, miR-182-5p contributes to cisplatin resistance in EOC.

3.3 miR-182-5p directly targeted GRB2 in EOC cells

We predicted that GRB2 is a direct target of miR-182-5p by using TargetScan (http://www.targetscan.org/vert_71/) and miranda (mirorna.org) (Fig. 3A). Using dual luciferase reporter assay, we confirmed the relationship between GRB2 and miR-182-5p. After miR-182-5p mimics and GRB2 wild-type vector transfections, the luciferase activity was significantly inhibited compared with NC. However, after transfection with the GRB2 binding site mutation (mut) vector with

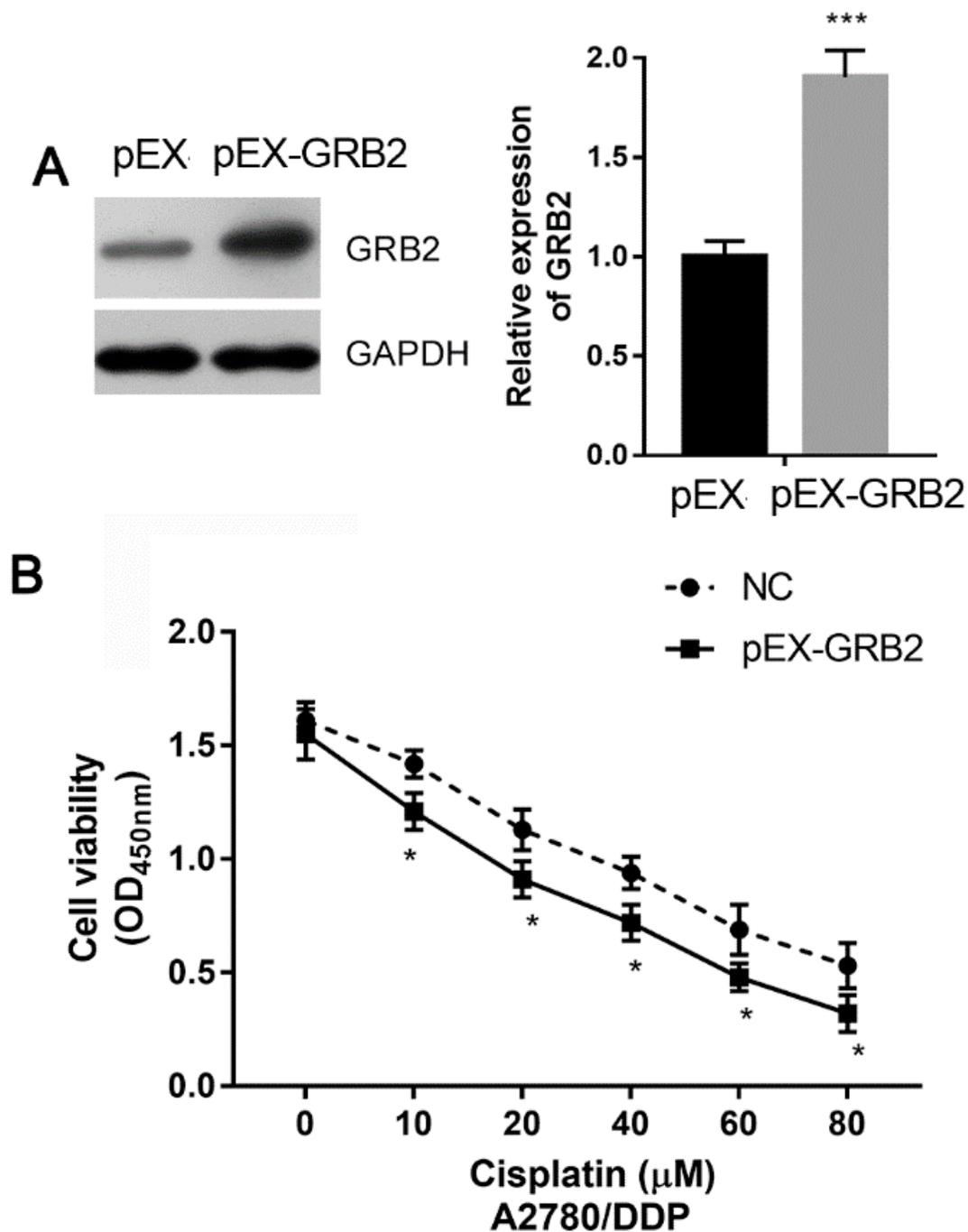


Fig. 4. Effect of GRB2 overexpression on the cisplatin sensitivity of EOC cells. Cells were transfected with GRB2-expressing vector, and then GRB2 expression (A) and cell viability (B) were determined by CCK-8 assay after cisplatin treatment at various concentrations for 48 h. * $P < 0.05$.

miR-182-5p mimics, the luciferase activity was not significantly changed, compared with control (Fig. 3B). GRB2 was significantly downregulated by miR-182-5p mimics in A2780 cells, while GRB2 was significantly upregulated by miR-182-5p inhibitor in A2780/DDP cells ($P < 0.001$, Fig. 3C,D). These data suggested that GRB2 serves as a bona fide miR-182-5p target in EOC.

3.4 Overexpression of GRB2 enhances EOC cell cisplatin sensitivity

To detect the role of GRB2 in EOC cisplatin resistance, GRB2 was overexpressed in A2780/DDP cells. GRB2 up-regulation (Fig. 4A) significantly enhanced the EOC cisplatin sensitivity (Fig. 4B), suggesting that miR-182-5p downregulation enhances cisplatin sensitivity of EOC cells by upregulating GRB2.

4. Discussion

Cisplatin is the chemotherapy drug for EOC. Acquired drug resistance frequently occurs following treatment with chemotherapeutics including cisplatin, which is believed to be the main cause of mortality in EOC [14]. Many EOC patients is nagged by the failure of chemotherapy. One of the important reasons for the failure of chemotherapy is the emergence of chemotherapeutic drug resistance. It is frustrating that there is still no effective way to prevent and overcome chemotherapy resistance. We show miR-182-5p up-regulation had a complete response to primary therapy and with high platinum resistance. A significant relationship existed between miR-182-5p expression and EOC overall survival. Our study also explored the role of miR-182-5p in platinum resistance and the underlying mechanism.

It is well-known that changes in specific miRNAs of tumour cells will lead to chemotherapy resistance. miRNAs dysregulation will contributed to multiple cell functions including cell proliferation, and cell apoptosis. In recent, increased evidence have shown that miRNAs also contribute to tumour occurrence, invasion and metastasis [15, 16]. The inhibition of miR-182 in trastuzumab-resistant breast cancer cell was mediated by phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) signalling pathway activation [9]. miR-182-5p inhibitor suppressed the cisplatin resistance of acute myeloid leukaemia cells by upregulating the expression of BCL2L12 and BCL2 [17]. miR-182 was upregulated in irradiated lung cancers [18]. miR-182-5p could downregulate GRB2, and the overexpression of miR-182-5p by mimics enhanced the resistance of EOC cells to cisplatin. GRB2 is a growth factor receptor binding protein 2, also known as Ash protein, which is encoded by the GRB2 gene in humans. GRB2 is widely distributed in cells and is mainly involved in the tyrosine kinase receptor signalling pathway [19]. It can bind directly to activated EGF receptor phosphorylated tyrosine, participate in EGF receptor-mediated signal transduction, and indirectly participate in signal transduction mediated by insulin receptor via binding to Shc phosphorylated tyrosine. GRB2 can bind with Shc and Sos to activate Sos [20]. Current studies shown GRB2 is involved in the occurrence and development of various malignant tumours [19, 21]. GRB2 can promote cell proliferation, invasion, metastasis and angiogenesis [22], which are important features of cancer cells [23, 24]. The level of GRB2 expression is closely correlated with the prognosis and survival of gastric cancer, colorectal cancer, and so on [25–28], suggesting GRB2 was closed associated with tumour progression. In our previous study, we demonstrated that GRB2 downregulation suppressed SKOV3 OC cell behaviours and chemosensitivity [29]. GRB2 was lower expressed in A280 cells than in A2780/DDP cells. Overexpression of GRB2 significantly enhanced the cisplatin sensitivity of EOC cells, suggesting that miR-182-5p downregulation enhances cisplatin sensitivity in EOC cells by upregulating GRB2, which might be related to the activation of the

tyrosine kinase receptor signalling pathway. It has been reported that tyrosine kinase receptors mediate platinum resistance [30]. However, the role of GRB2 in EOC patients with chemotherapeutic resistance and metastatic spreading should be confirmed in the future. Additionally, this study is focused on acquired cisplatin resistance. We will investigate intrinsic cisplatin resistance using cells inherently less sensitive to cisplatin, such as SKOV3, in the future.

Summary, miR-182-5p in EOC specimens is overexpressed, which enhanced cisplatin chemoresistance through a novel GRB2 pathway. These results present GRB2 as a novel potential target for predicting EOC prognosis and combating chemoresistance.

Author contributions

CYZ, CYC and PPH were designed and the acquired data, and all authors performed experiments and performed data analysis. All authors revised the manuscript.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Zhejiang University Mingzhou Hospital (approval number: ZJUMH2008102).

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Conflict of interest

The authors declare no conflict of interest.

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