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MicroRNA-23b is an independent prognostic marker and suppresses ovarian cancer progression by targeting runt-related transcription factor-2

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ABSTRACT

Our previous study found that runt-related transcription factor-2 (RUNX2) was upregulated in human epithelial ovarian cancer (EOC) tissues and may be involved in tumor progression and prognosis. The aim of this study was to investigate the mechanism by which RUNX2 is aberrantly expressed in EOC. We firstly confirmed that miRNA-23b directly targets RUNX2 in EOC. Then, ectopic expression of miR-23b significantly inhibited ovarian cancer cell proliferation and tumorigenicity by regulating the expression of RUNX2. Furthermore, the down-regulation of miR-23b was significantly correlated with tumor aggressiveness and poor prognosis of patients with EOC. Collectively, miR-23b may function as tumor suppressor through inhibiting the upregulation of RUNX2, and may be a potential prognostic marker for EOC.

1. Introduction

Human ovarian cancer, one of the most common gynecologic malignancies worldwide, has the highest mortality rate among malignant tumors in female reproductive system [1]. The morphological origin of ovarian cancer is complicated, including cancers of epithelial, germ cell, undifferentiated and differentiated mesenchymal origin [2]. Among them, epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women and the most lethal gynecologic malignancy in the world [3]. It includes serous adenocarcinoma, clear cell carcinoma and endometrial adenocarcinoma [4]. Because of the lack of specific early symptoms or effective tumor biomarkers, most patients with EOCs are diagnosed at the advanced stages and the prognosis of these patients is still poor, even though there has been great improvement on traditional treatments, such as surgery, supplemented with radiotherapy and chemotherapy. The 5-year survival rate for EOC patients is only 30–40% [5]. It has been demonstrated that EOC may be associated with multistep changes in various molecules. Although a large number of studies have shown a great potential for the use of molecular markers in diagnosis, prognosis, and therapy in EOC, their clinical values are limited due to lack of sensitivity, high costs and or inconvenience. Therefore, it is extremely necessary to identify novel and efficient biomarkers used in the diagnosis and as therapeutic targets for human EOC.

MicroRNAs (miRNAs), originally identified in *Caenorhabditis elegans*, are a class of single-stranded, small (17–25 nt), non-coding, evolutionarily conserved RNAs [6]. They regulate gene expression by binding to imperfect complementary sites in the 3′-untranslated region of their target messenger RNA transcripts, and exert their negative regulation either by degrading the target mRNA, when they bind with near-perfect complementarity, or by post-transcriptionally repressing target-gene expression when they bind with imperfect complementarity [7]. In recent years, increasing amounts of experimental evidence have suggested that miRNAs may play important roles in essential processes, such as differentiation, cell growth, stress response and cell death, and may be involved in several human diseases, including cancer [8]. They function as either tumor suppressors or oncogenes by repressing the expression of important cancer-related genes. Especially in human EOC, accumulating studies have found the aberrant expression of miRNAs which were associated with tumor

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clinicopathological characteristics and clinical outcomes. For example, Wu et al. [9] reported that miR-145 may modulate ovarian cancer growth and invasion by suppressing p70S6K1 and MUC1, functioning as a tumor suppressor; Wang et al. [10] indicated that miR-182 may promote cell growth, invasion, and chemoresistance by targeting PDCD4 in human EOC; Zhang et al. [11] showed that mR-124 may inhibit the migration and invasion of ovarian cancer cells by targeting SphK1. These findings suggest that miRNAs may be involved in ovarian tumorigenesis and cancer progression.

Mammalian Runt-related transcription factor (RUNX) family contains three members (RUNX1–3) which form the core binding factor (CBF) complex and bind DNA to either activate or repress gene transcription [12]. Accumulating studies have reported the oncogenic and tumor suppressive functions of the RUNX members [13]. In the previous study, our data suggest for the first time that RUNX2 overexpression may be associated with advanced tumor progression and poor clinical outcome of EOC patients, and RUNX2 might be a novel prognostic marker of EOC [14]. More recently, Wang et al. [15] further demonstrated that the inhibition of RUNX2 transcriptional activity may block the proliferation, migration and invasion of EOC cells, suggesting that RUNX2 might be a novel EOC therapeutic target. However, the mechanisms by which RUNX2 is aberrantly expressed in EOC have not been fully elucidated. Thus, we in the current study used miRNA target prediction program TargetScan6.2 to predict miR-23b as a candidate miRNA that targets RUNX2. Then, the regulatory function of miR-23b to RUNX2 was validated by transient transfection of the EOC cells in vitro with oligonucleotides that mimic miR-23b and the luciferase reporter assay. Moreover, the roles of miR-23b in tumorigenicity of EOC cells were also investigated in vitro system. Furthermore, we determined the clinical relevance of miR-23b in EOC.

2. Materials and methods

2.1. Patients and tissue samples

The study was approved by the Research Ethics Committee of General Hospital of PLA, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 116 EOC specimens and 5 normal ovarian tissues were snap-frozen in liquid nitrogen and stored at −80 °C following surgery for qRT-PCR and in situ hybridization assays. The normal ovarian tissues were obtained from women who underwent hysterectomies for benign disease. All operations were performed in the Department of Obstetrics and Gynecology at General Hospital of PLA from January 2005 to December 2006. All patients with only gynecology tumor were treated without preoperative radiotherapy, chemotherapy, or hormonal therapy. Surgical staging was established according to the International Federation of Gynecology and Obstetrics (FIGO) system. Debulking status was defined according to the size of the nodules left in the peritoneal cavity after surgery. The clinical features of 116 EOC patients were summarized in Table 1.

2.2. Cell culture

Two EOC cell lines SKOV3 and OVCAR3 were purchased from American Tissue Type Collection (Manassas, VA) and were maintained at 37 °C in a humidified chamber supplemented with 5% CO_2.

2.3. Target prediction

An online program Target-Scan (release 6.2) was used for predicting miRNAs that might target RUNX2.

Table 1
Association of microRNA-23b (miR-23b) expression with clinicopathological features of epithelial ovarian cancer tissues.

Features	No. of patients	miR-23b expression (*n*, %)		*P*
		Low	High	
Age				
<50	48	26 (54.17)	22 (45.83)	NS
⩾50	68	34 (50.00)	34 (50.00)	
Clinical stage				
I–II	26	4 (15.38)	22 (84.62)	0.02
III–IV	90	56 (62.22)	34 (37.78)	
Pathological grade				
1–2	33	17 (51.52)	16 (48.48)	NS
3	83	43 (51.81)	40 (48.19)	
Histological type				
Serous	88	45 (51.14)	43 (48.86)	NS
Non-serous	28	15 (53.57)	13 (46.43)	
Residual tumor after surgery		60	56	
<1 cm	66	35 (53.03)	31 (46.97)	NS
⩾1 cm	50	25 (50.00)	25 (50.00)	

Note: 'NS' refers to the difference without statistical significance.

2.4. RNA and miRNA extraction

For mRNA quantification, total RNAs from cell lines and tissues were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For miRNA quantification, total miRNA was extracted from cell lines and tissues using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

2.5. QRT-PCR

For mRNA and miRNA quantifications, stem-loop RT-PCR was performed to respectively detect the expression levels of miR-23b and RUNX2 in cell lines and tissues. In brief, 10 μg of small RNA and 20 μg of total RNA were reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI) with the RT primers which can fold into a stem-loop structure. The cDNA was used for the amplification of mature miR-23b, RUNX2 and the endogenous controls, U6 and β-actin, by PCR. The PCR conditions were: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s.

Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). Gene and miRNA expression in each sample were respectively normalized with the housekeeping genes (β-actin and RNU6B) expression. Relative quantification of target gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method.

2.6. EGFP-miR-23b expression vector

As the previous study [16], we got the commercial pGCMV/EGFP/Neo-Vector(GenePharma, China) with an over-expression of miR-23b, and the same negative-vector as control. EOC cells were transfected through Fugene transfecting agents (Roche) with hsa-miR-23b vector, negative control (NC) following the manufacturer's instructions.

2.7. Western blot analysis

Proteins in EOC cells were extracted at 24 h post-transfection and Western blot analysis was performed to detect the expression

levels of RUNX2 protein. Proteins (40 μg) were fractioned by SDS–PAGE and transferred onto Hybond nitrocellulose membranes (GE Healthcare). Filters were blocked in PBS-Tween skim milk and probed with anti-RUNX2 antibody (dilution 1:1000, Abnova Corporation, Taipei, Taiwan) or probed with anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, USA). β-Actin was used as equal protein loading control. The secondary antibody incubations were performed for 2 h at room temperature and protein bands

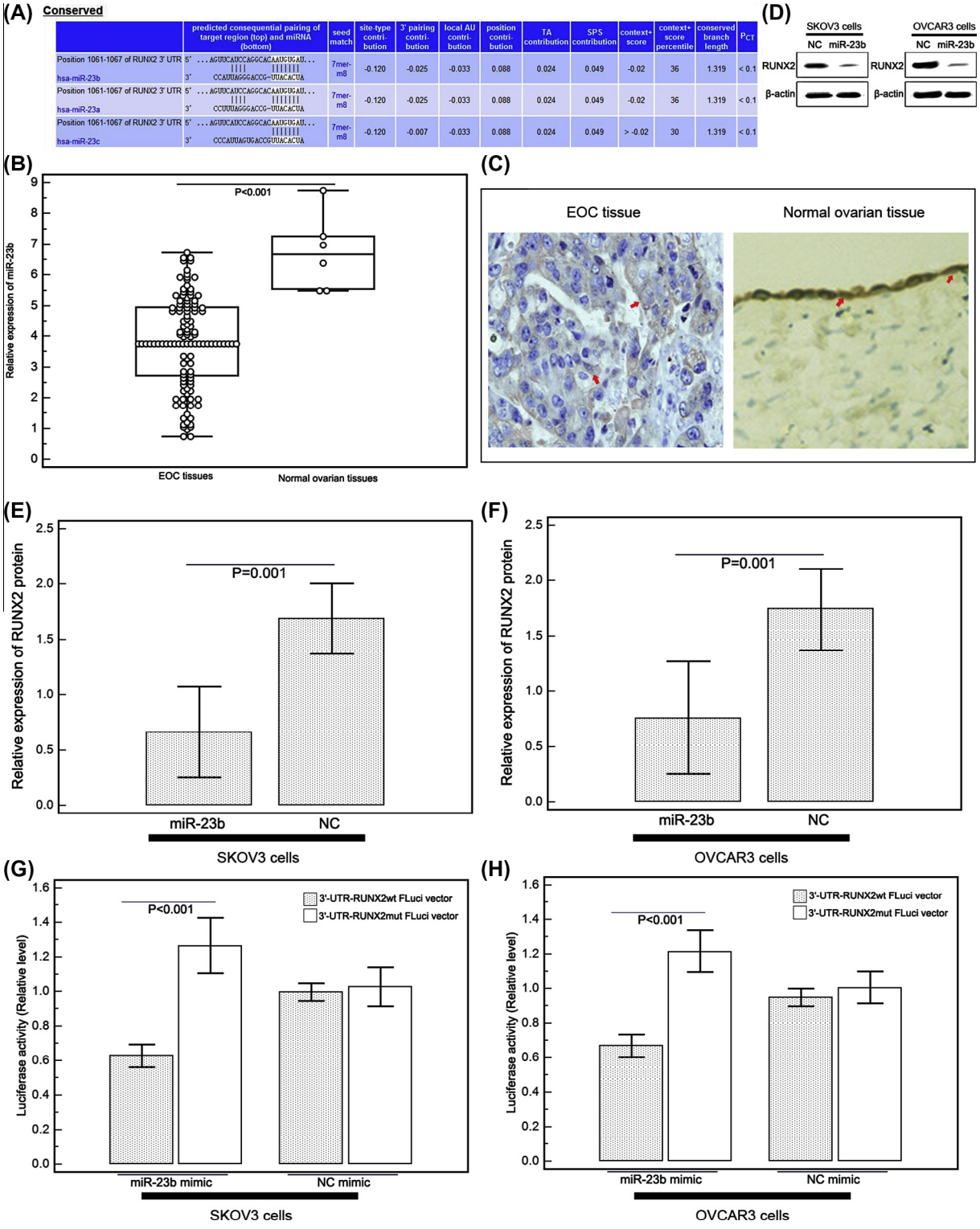

Fig. 1. MicroRNA-23b (miR-23b) targets runt-related transcription factor-2 (RUNX2) in epithelial ovarian cancer (EOC) tissues. (A) MiR-23b is predicted to target RUNX2; (B) MiR-23b is downregulated in human EOC tissues detected by qRT-PCR analysis; (C) Representative examples of miR-23b expression verified by in situ hybridization (Original magnification ×400). Red arrows point out the cells with positive expression of miR-23b. (D–F) The RUNX2 protein in SKOV3 and OVCAR3 cells by Western blot at 24 h post-transfection of miR-23b vector. β-Actin was used as an internal loading control. 'NC' refers to negative control vector. (G and H) Luciferase report assay was performed to confirm the miR-23b binding target. The luciferase activity was detected after co-transfection of FLuci vector (3′-UTR-RUNX2wt FLuci vector or 3′-UTR-RUNX2mut FLuci vector), miR-23b mimic or negative mimic into SKOV3 and OVCAR3 cells.

were visualized by SuperSignal West PICO chemiluminescent detection system (Pierce Biotechnology).

2.8. 3′-UTR luciferase reporter assay

The miR-23b-targeted gene was evaluated by using a luciferase reporter assay in EOC cells. The FLuci (3′-UTR of RUNX2 luciferase) vector was constructed using the pGL3 luciferase reporter vector (Promega, Madison, WI, USA) with a fragment of mRNA 3′-UTR of RUNX2, which carries a putative miR-23b complementary site (NM_006793.2; 3′-UTR: 460-529) or mutation sequence. EOC cells were cultivated in 24-well plates and were co-transfected using Fugene (Roche) with 100 ng of FLuci reporter construct, 10 ng miR-23b mimic or NC mimic, and 2 ng pRL-SV40 RLuci vector (Promega). After 48 h, EOC cells were harvested and luciferase activity was measured using the Dual- Luciferase Reporter Assay System (Promega). The Renilla luciferase activities were used as an internal control. The experiments were performed independently in triplicate.

2.9. Small interfering RNA (siRNA)-mediated RUNX2 knockdown in EOC cells

EOC cells were grown in complete 1640 medium in 10 ml cell culture plates until 50% confluence, and then transfected using LipofectamineTM 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The siRNA against RUNX2 and the control siRNA were synthesized by QIAGEN China Co., Shanghai, China. For every 105 cells, 0.5 μg RUNX2 siRNA (RUNX2 target sequence AATGGCAGCACGCTATTAAAT) or control siRNA (target sequence AATTCTCCGAACGTGTCACGT) was diluted and mixed with 3 μl transfection reagent. After mixing and incubation for 30 min, the transfection mixture was added to the cells. After 6 h, medium was changed to growth medium. EOC cells were used for functional assay 24 h after transfection.

2.10. In vitro invasion and migration assays

Cell invasion and migration were respectively analyzed by Matrigel coated and uncoated transwell cell culture chambers (8 μm pore size, Millipore, Billerica, MA, USA) as described previously [17]. In brief, 48 h after transfection, EOC cells were resuspended in 200 μl serum-free 1640 medium were placed into the upper chamber of the insert with or without Matrigel. Medium with 5% FBS was added into the lower chambers as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane were carefully removed. Cells that had migrated or invaded through the membrane were manually counted at 200× magnification from ten different fields of each filter. All experiments were done in triplicate. Mean normalized gene expression ± S.E. was calculated from independent experiments.

2.11. In vitro cell proliferation assay

The in vitro cell proliferation of EOC cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method following the protocol of the previous studies [17]. In brief, cells were seeded into 96-well plates and transfected. At 48 h after transfection, 20 μl MTT solution (Sigma, USA) was added into the culture medium for 4 h incubation. Then, the medium was replaced by 0.1 ml of DMSO (Sigma, St Louis, MO, USA) and plates were shaken at room temperature for 10 min. The absorbance at 490 nm was measured using a mQuant Universal Microplate Spectrophotometer (BioTek, Winooski, VT). All experiments were done in triplicate. Mean normalized gene expression ± S.E. was calculated from independent experiments.

2.12. In situ hybridization

Expression patterns and subcellular localizations of miR-23b in EOC and normal ovarian tissues were detected by in situ hybridization. The 5′ end biotinylated Locked nucleic acid-modified probes (Exiqon, Denmark), which is directed against full-length mature miR-23b sequence, were used for this experiment on frozen section of tissues. Following the incubation of substrate-chromogen solution with 0.04% DAB (DAKO, Denmark) and 0.05% H_2O_2 for 30 min, the tissue sections were detected using the in situ hybridization kit (Cat No: MK1030, Boster, China) according to manufacturer's instructions.

The in situ hybridization results were scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The in situ hybridization scores were calculated based on both the intensity of hybridization and the percentage of positive cells. The former was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The latter was as follows: 0 (0%), 1 (1–10%), 2 (11–50%) and 3 (>50%). A final score was obtained for each case by multiplying the intensity and the percentage scores.

2.13. Statistical analysis

The software of SPSS version13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables in Figs. 1B, E, F, 2B, 3 and 4 were expressed as $\overline{X} \pm s$. Categorical variables in Table 1 are presented as numbers and percentages. Comparisons between groups were performed using the Kruskal–Wallis test for continuous variables and the χ^2 test for categorical variables. The Kaplan–Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. A multivariate survival analysis was performed for all parameters that were significant in the univariate analyses using the Cox regression model. Differences were considered statistically significant when P was less than 0.05.

3. Results

3.1. MiRNA-23b targets RUNX2 in EOC tissues

In the previous study, we performed the immunohistochemistry analysis and found that the expression level of RUNX2 protein in EOC tissues was significantly higher than that in normal ovarian tissues ($P < 0.001$) and its overexpression was associated with advanced tumor progression and poor clinical outcome of patients with EOC [14]. In the current study, using miRNA target predicting program (TargetScan human 6.2), three miRNAs miR-23a, miR-23b and miR-23c were all predicted to target RUNX2 (Fig. 1A). According to our literature retrieval, there were no previous studies on the involvement of miR-23a and miR-23c in EOC. In contrast, Park et al. [18] indicated that the aberrant expression of miR-23b might be significantly implicated in chemoresistance and tumor progression in ovarian cancer. On this basis, we chose miR-23b as a candidate miRNA which targets RUNX2 during the tumorigenicity of EOC. To test our hypothesis, qRT-PCR, in situ hybridization, transient transfection of the two EOC cell lines with miR-23b vector plasmid, and luciferase reporter assay were performed.

According to the results of qRT-PCR analysis, miR-23b expression in EOC tissues was significantly lower than that in normal ovarian tissues (3.83 ± 1.49 vs. 6.73 ± 1.22, $P < 0.001$, Fig. 1B). We also validated the miR-23b expression by in situ hybridization.

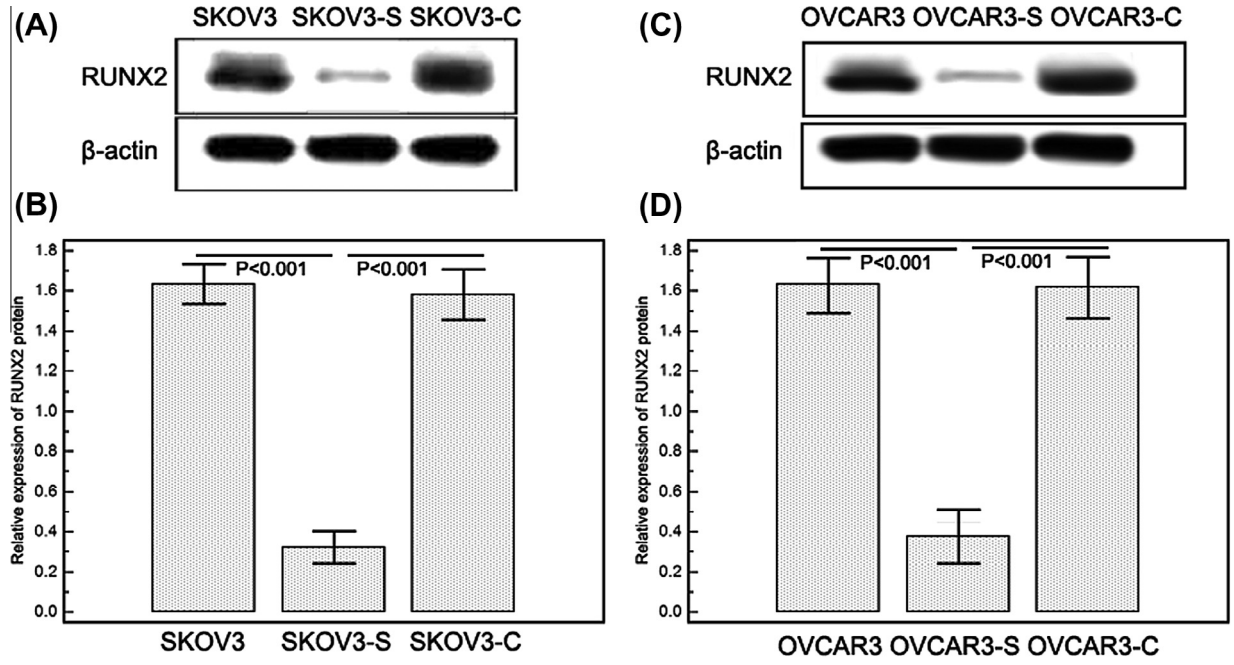

Fig. 2. Down-regulation of runt-related transcription factor-2 (RUNX2) by siRNA interference. (A∼D) Western blot analysis was performed to detect the expression levels of RUNX2 protein in RUNX2 siRNA-transfected (SKOV3-S and OVCAR3-S), control siRNA-transfected (SKOV3-C and OVCAR3-C) and non-transfected (SKOV3 and OVCAR3) cells. β-Actin was used as an internal loading control.

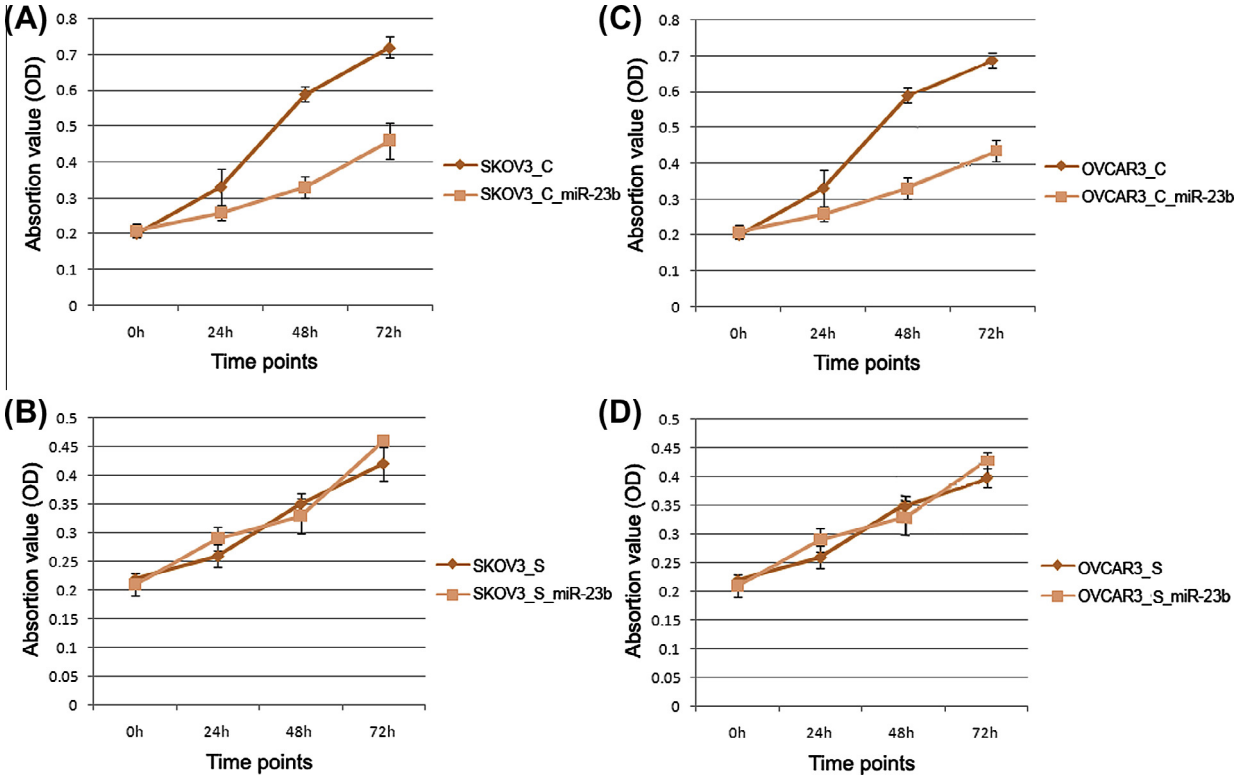

Fig. 3. MicroRNA-23b (miR-23b) inhibits cell proliferation of epithelial ovarian cancer (EOC) cells in vitro by targeting runt-related transcription factor-2 (RUNX2). (A and C) Growth curves of SKOV3 and OVCAR3 cells transfected with control siRNA (SKOV3-C and OVCAR3-C), and SKOV3 and OVCAR3 cells transfected with control siRNA and miR-23b (SKOV3-C-miR-23b and OVCAR3-C-miR-23b). (B and D) Growth curves of SKOV3 and OVCAR3 cells transfected with RUNX2 siRNA (SKOV3-S and OVCAR3-S), and SKOV3 and OVCAR3 cells transfected with RUNX2 siRNA and miR-23b (SKOV3-S-miR-23b and OVCAR3-S-miR-23b).

As shown in Fig. 1C, miR-23b was localized in the cytoplasm of tumor cells in EOC tissues, and its expression levels in EOC tissues were significantly lower than those in normal ovarian tissues (staining score: EOC = 3.62 ± 1.31 vs. Normal = 5.58 ± 1.69, $P < 0.001$), which was consistent with the results of qRT-PCR analysis.

In order to verify our predicted target of hsa-miR-23b, we transfected EOC cells with hsa-miR-23b vector, negative vector (NC),

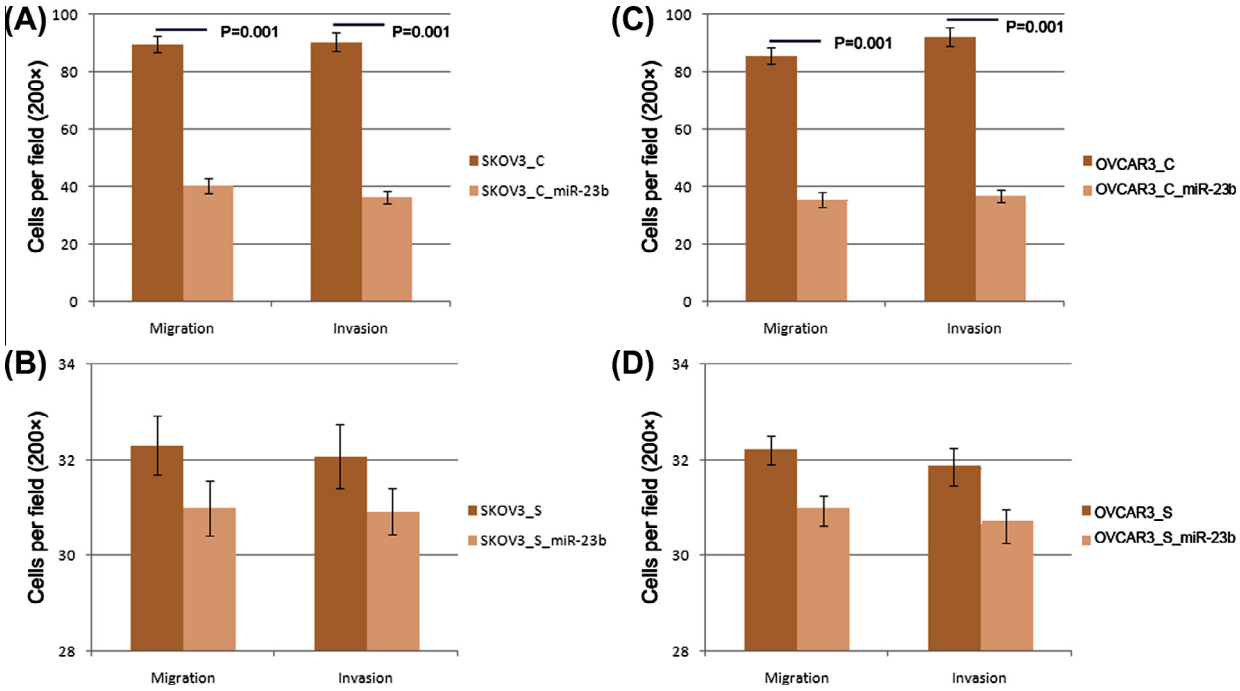

Fig. 4. MicroRNA-23b (miR-23b) inhibits epithelial ovarian cancer (EOC) cells migration and invasion in vitro by targeting runt-related transcription factor-2 (RUNX2). (A and C) Transwell migration assay and Matrigel invasion assay of EOC cells transfected with control siRNA (SKOV3-C and OVCAR3-C), and EOC cells transfected with control siRNA and miR-23b (SKOV3-C-miR-23b and OVCAR3-C-miR-23b). (B and D) Transwell migration assay and Matrigel invasion assay of EOC cells transfected with RUNX2 siRNA (SKOV3-S and OVCAR3-S), and EOC cells transfected with RUNX2 siRNA and miR-23b (SKOV3-S-miR-23b and OVCAR3-S-miR-23b). Enforced expression of miR-23b significantly inhibited the cell migration and invasion of EOC cells transfected with control siRNA (SKOV3-C-miR-23b vs. SKOV3-C and OVCAR3-C-miR-23b vs. OVCAR3-C) but failed to do so in EOC cells transfected with RUNX2 siRNA (SKOV3-S-miR-23b vs. SKOV3-S and OVCAR3-S-miR-23b vs. OVCAR3-S).

and blank control culture medium (mock), respectively. At 24 h post-transfection, Western blot analysis showed that the enforced expression of hsa-miR-23b resulted in a significant decrease of endogenous RUNX2 protein levels compared with cells transformed with NC or mock (SKOV3 and OVCAR3 cell groups: both $P = 0.001$, Fig. 1D–F). Moreover, the luciferase reporter assay was performed by co-transfection of miR-23b and a luciferase reporter plasmid containing the 3′UTR of human RUNX2. Luciferase activity was detected at 48 h after the co-transfection of FLuci vector (3′-UTR-RUNX2wt FLuci vector or 3′-UTR-RUNX2mut FLuci vector), miR-23b mimic or NC mimic, and RLuci vector in SKOV3 and OVCAR3 cells. As shown in Fig. 1G and H, the luciferase activities were respectively decreased to 0.63 and 0.68 in SKOV3 and OVCAR3 cells co-transfected with 3′-UTR-RUNX2wt FLuci vector and miR-23b mimic compared with 3′-UTR-RUNX2mut FLuci vector and NC mimic.

Based on the evidence mentioned above, we confirmed that miR-23b may directly target RUNX2.

3.2. MiR-23b inhibits cell proliferation of EOC cells in vitro by targeting RUNX2

To verify whether miR-23b inhibited cell proliferation of EOC cell lines SKOV3 and OVCAR3 by targeting RUNX2, we firstly knocked down the expression of RUNX2 by siRNA transfection. As shown in Fig. 2, the expression level of RUNX2 protein in SKOV3 and OVCAR3 cells transfected with RUNX2 siRNA was significantly lower than that in SKOV3 and OVCAR3 cells transfected with or without control siRNA (both $P < 0.001$). In addition, we found that the enforced expression of miR-23b significantly inhibited cell proliferation of SKOV3 and OVCAR3 cells transfected with control siRNA (both $P = 0.01$, Fig. 3A and C) but failed to do so in SKOV3 and OVCAR3 cells transfected with RUNX2 siRNA (both $P > 0.05$, Fig. 3B and D). Thus, miR-23b may inhibit the cell proliferation of EOC cell lines by targeting RUNX2.

3.3. MiR-23b inhibits EOC cells migration and invasion in vitro by targeting RUNX2

To verify whether miR-23b inhibited the cell migration and invasion of the two EOC cell lines by targeting RUNX2, we made use of SKOV3 and OVCAR3 cells in which RUNX2 was knocked down by RUNX2 siRNA transfection. As shown in Fig. 4, the enforced expression of miR-23b significantly inhibited the cell migration and invasion of SKOV3 and OVCAR3 cells transfected with control siRNA (both $P = 0.001$, Fig. 4A and C) but failed to do so in SKOV3 and OVCAR3 cells transfected with RUNX2 siRNA (both $P > 0.05$, Fig. 4B and D). Therefore, miR-23b may suppress EOC cell metastasis via negatively regulating the migratory and invasive abilities of EOC cells by targeting RUNX2.

3.4. Association of miRNA-23b expression with the clinicopathological characteristics of EOC

Table 1 summarized the association of miRNA-23b expression with various clinicopathological features of EOC tissues. The median value (3.75) of miR-23b expression in all EOC tissues detected by qRT-PCR were used as a cutoff point to classified 116 patients with EOC into miR-23b-low ($n = 60$) and miR-23b-high ($n = 56$) expression groups. As the results, the EOC tissues with advanced clinical stage (III–IV) more frequently showed low miR-23b expression than those with low clinical stage (I–II, $P = 0.02$, Table 1). However, miR-23b expression was not correlated with age, grade, histological type and residual tumor after surgery (all $P > 0.05$).

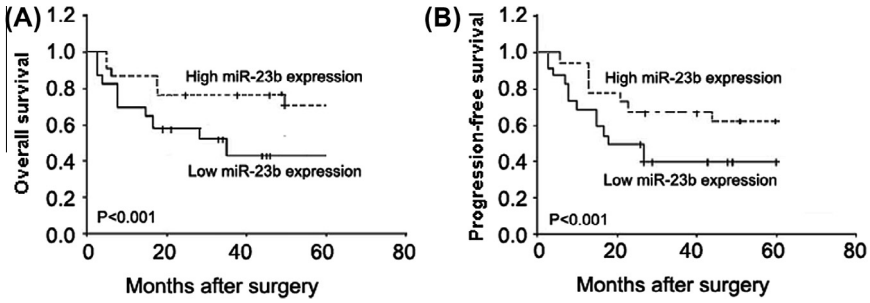

Fig. 5. Kaplan–Meier overall (A) and progression-free (B) survival curves for epithelial ovarian cancer patients with high and low miR-23b expression. Epithelial ovarian cancer patients with low miR-23b expression had significantly shorter overall ($P < 0.001$) and progression-free ($P < 0.001$) survival than those with high miR-23b expression did.

Table 2
Univariate analysis: factors predicting overall and progression-free survival.

Characteristic	Groups	Overall survival		Progression-free survival	
		P value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)
Age (years)	<50 vs. ⩾50	0.16	1.582 (0.832–3.168)	0.27	1.001 (0.739–2.802)
Clinical stage	I–II vs. III–IV	<0.001	10.859 (1.942–26.919)	0.008	7.736 (1.219–19.573)
Pathological grade	1–2 vs. 3	0.06	4.266 (1.031–10.533)	0.09	3.152 (1.006–8.131)
Histological type	Serous vs. non-serous	0.05	6.852 (1.062–11.781)	0.08	4.026 (1.022–9.781)
Residual tumor after surgery	<1 cm vs. ⩾1 cm	0.11	3.049 (1.001–7.103)	0.25	2.892 (0.903–6.098)
miR-23b expression	Low vs. High	<0.001	16.869 (1.859–39.262)	<0.001	17.188 (1.876–40.986)

Table 3
Multivariate analysis: factors predicting overall and progression-free survival.

Characteristic	Overall survival		Progression-free survival	
	P value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)
Clinical stage	0.01	8.236 (1.182–20.601)	0.03	6.968 (1.012–15.872)
miR-23b expression	0.01	8.889 (1.368–21.328)	0.01	8.121 (1.332–20.269)

3.5. Prognostic implications of miRNA-23b expression in EOC

In order to investigate the prognostic implications of miR-23b expression in overall survival and progression-free survival of EOC, the detail clinical information of all 116 EOC patients in high miR-23b expression and low miR-23b expression groups was reviewed. Median follow-up time was 66.8 months (range, 2.2–118.9 months; mean, 66.1 months). At last follow-up, 73 (62.9%) relapsed with a median time of 22.1 months (range, 2.8–85.2 months). As determined by the log-rank test, EOC patients with low miR-23b expression had significantly shorter overall ($P < 0.001$, Fig. 5A) and progression-free ($P < 0.001$, Fig. 5B) survival than those with high miR-23b expression did. Moreover, the univariate analysis revealed that both the advanced stage ($P < 0.001$ and $P = 0.008$, respectively) and low miR-23b expression (both $P < 0.001$) predicted poorer overall and progression-free survival of EOC patients (Table 2). Furthermore, the multivariate analyses identified clinical stage ($P = 0.01$ and $P = 0.03$, respectively) and miR-23b expression (both $P = 0.01$) in EOC cells as independent prognostic factors for overall survival and progression-free survival (Table 3).

4. Discussion

Despite great efforts in clinical and basic research, the high mortality of human EOC is due to late-stage diagnosis for more than 70% of cases, and is also due to the common resistance to the current chemotherapeutic regimens [19,20]. Elucidating the mechanisms underlying tumorigenesis and tumor progression will, thus, make significant contributions toward combating this disease. In recent years, miRNAs have been demonstrated to be important players in human cancer and their roles as therapeutic targets have been proposed. Accumulating studies have identified a number of miRNAs which are remarkably deregulated in EOC, suggesting that miRNAs are involved in the initiation and progression of this disease [9–11]. In the current study, the main findings are as following four points. Firstly, the miRNA target prediction program TargetScan was used to predict a miRNA–miR-23b as a candidate regulator for RUNX2 expression. Using the two EOC cell lines SKOV3 and OVCAR3 transfected with miR-23b overexpression vector, we validated that RUNX2 was down-regulated at protein level by the proposed target miRNA, miR-23b, which was also confirmed by the luciferase reporter assay, suggesting that miRNA-23b directly targets RUNX2 in EOC tissues. Secondly, the enforced expression of miR-23b inhibited the cell proliferation and tumorigenicity of the two EOC cell lines SKOV3 and OVCAR3 in vitro by targeting RUNX2. Thirdly, both qRT-PCR and in situ hybridization assays found that the expression level of miR-23b was decreased in clinical EOC tissues. Finally, the down-regulation of miR-23b in clinical EOC tissues was associated with the advanced tumor progression and poor prognosis of patients with this disease. These findings suggest that miR-23b may function as a tumor suppressor in EOC by targeting RUNX2.

MiR-23b, which is located in one genomic cluster (miR-23b/27b/24), has been demonstrated to be deregulated in several types of cancer, including prostate cancer, bladder cancer, colon cancer,

renal cancer, breast cancer and hepatocellular carcinoma [21–25]. Functionally, it acts as tumor promoter or tumor suppressor depending on specific cancer types. For example, miR-23b is highly upregulated in human breast cancer and its overexpression correlates with poor outcome in patients with this disease [21]. miR-23b has been reported to function as a renal cancer oncogene [22]. In contrast, miR-23b is significantly down-regulated in bladder cancer tissues and cell lines and that high expression level of miR-23b positively correlate with higher overall survival of patients after surgery, suggesting that it is a potential biomarker and tumor suppressor in bladder cancer [23]. miR-23b is a methylation silenced tumor suppressor in prostate cancer [24]. The overexpression of miR-23b leads to decreased migration and proliferation abilities of hepatocellular carcinoma cells [25]. In the present study, our data showed that miR-23b was functionally involved in suppressing EOC cell growth, migration and invasion, which was supported by both cell culture studies and clinical data. In cell culture experiments, over-expression of miR-23b led to the decrease of EOC cell proliferation and cell motility. More importantly, we confirmed that miR-23b may function as a tumor suppressor for EOC by targeting RUNX2. In clinical samples, miR-23b was dramatically down-regulated in EOC tissues with high clinical stage compared with EOC tissues with low clinical stage, and low miR-23b expression in tumors was associated with poor survival of patients with EOC. Notably, we have two different findings from the previous studies. Firstly, postoperative residual tumor size has been demonstrated to be an independent prognostic factor of PFS and OS in EOC [26–28]. However, the current data did not shown its prognostic value for patients with EOCs. Secondly, the OS in EOC is generally very poor. According to the previous study, the estimated 5-year OS of patients with EOCs is about 35% in stage III–IV tumors [29,30]. However, our data in Fig. 5 showed a 5-year OS of about 40% in the low miR-23b arm and approximately 70% in the high miR-23b arm. These differences may be caused by the heterogeneity of our cohort and the relative small sample size in the current study. To solve this problem, a randomized study investigating the association between miR-23b expression and prognosis should be conducted to confirm whether miR-23b could be used as a novel predictor of prognosis in patients with EOCs.

In conclusion, our data offer the convincing evidence for the first time that miR-23b may suppress tumor progression of EOC through down-regulating RUNX2 and may be a potential prognostic marker for this disease. Gene therapy using miR-23b mimics may be useful for the treatment of EOC.

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