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MicroRNA-184 acts as a potential diagnostic and prognostic marker in epithelial ovarian cancer and regulates cell proliferation, apoptosis and inflammation

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MicroRNA-184 (miR-184) is found to be significantly deregulated in human cancers associated with tumorigenesis and progression. In this study, we aimed to investigate the role and mechanism of miR-184 expression in epithelial ovarian cancer (EOC). Relative expression of miR-184 was measured by quantificational real-time polymerase chain reaction assay (qRT-PCR) in 80 EOC patients. Kaplan-Meier curve and the log-rank test were conducted to detect the prognostic value of miR-184. Function assays including cell proliferation, apoptosis and inflammation were further explored *in vitro*. We found that miR-184 was down-regulated in EOC tissues and cell lines compared with paired non-cancerous tissues and IOSE, respectively. Moreover, miR-184 was expressed at significantly lower levels in late-stage (III/IV) EOC tissues. Cox regression multivariate analysis indicated that miR-184 and FIGO stage were independent prognostic indicators for EOC patients. Patients with high miR-184 level achieved significantly a higher 5-year survival rate compared with low level group ($P < 0.001$). Functional assays showed that miR-184 over-expression could suppress EOC cell proliferation as well as inflammation and induce apoptosis *in vitro*. Altogether, our results suggest that miR-184 together with pathologic diagnosis is critical for prognosis determination in EOC patients and help select treatment strategy.

1. Introduction

Epithelial ovarian cancer (EOC) is one of the most lethal gynecological cancers in the world. The patients at advanced stages (FIGO III/IV), two thirds of whom were diagnosed at these stages, have a 5-year survival rate of around 10% (Duffy et al. 2005). That is largely due to lack of effective early diagnosis including biomarkers with high sensitivity and specificity for diagnosis and prognosis of ovarian cancer. Numerous publications reported that microRNAs could be used as tumor diagnostic and prognostic biomarkers (Davis-Dusenbery and Hata 2010, Krol, Loedige and Filipowicz 2010).

MicroRNAs (miRNAs) are small (19–25 nt), endogenous non-coding RNAs that constitute a novel class of gene regulators by binding to sites at the 3′ untranslated region (UTR) of their target mRNA transcripts (Pasquinelli 2012). MiRNAs regulate various biological processes, including cell differentiation, cell proliferation, apoptosis, and drug resistance (Ambros 2003; Brennecke et al. 2003; Xu et al. 2004; Chen et al. 2004; Cheng et al. 2005). Additionally it is suggested that abnormal miRNA expression is related to many human cancers (Zhang et al. 2007; Calin and Croce 2006), including epithelial ovarian cancer (Zhang et al. 2006; Iorio et al. 2007); hence, miRNAs could be predictive of ovarian cancer prognosis and maybe potential biomarkers and targets for cancer therapy (Merritt et al. 2008; Li et al. 2009).

Table 1: Clinical correlation of miR-184 expression in EOC patients.

Parameter	No. of cases	miR-184 expression		P-value
	n = 80	Low (%)	High (%)	
Age (years)				
<55	35	18 (51.4)	17 (48.6)	0.822
≥55	45	22 (48.9)	23 (51.1)	
Menopausal status				
Premenopausal	21	10 (47.6)	11 (52.4)	0.799
Postmenopausal	59	30 (50.8)	29 (49.2)	
FIGO stage				
early (I/II)	24	6 (25.0)	18 (75.0)	0.003*
late (III/IV)	56	34 (60.7)	22 (39.3)	
Histological grade				
low-grade (0/1)	20	12 (60.0)	8 (40.0)	0.302
high-grade(2/3)	60	28 (46.7)	32 (53.3)	

* $P < 0.05$ was considered to be a statistically significant difference

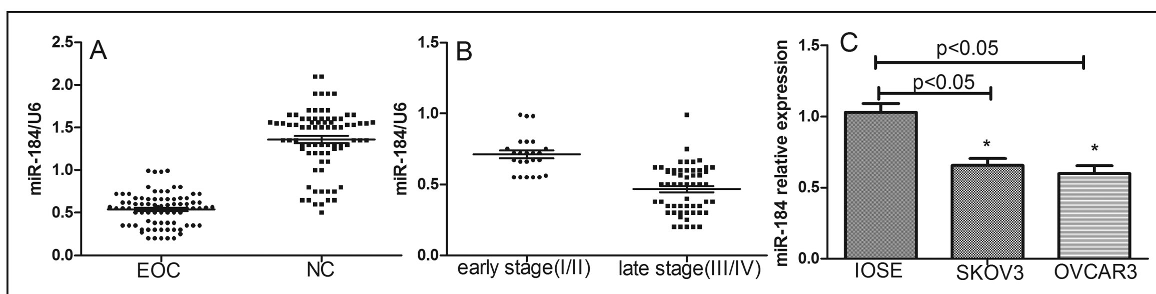

Fig. 1: MiR-184 expression is decreased in EOC tissues as well as cell lines and associated with FIGO stage and lymph node metastasis. (A) The level of miR-184 was determined in EOC and corresponding non-cancerous tissues (NC) by qRT-PCR and normalized against U6 RNA. (B) The level of miR-184 in FIGO stages of EOC. (C) The down-regulation of miR-184 in EOC cell lines (SKOV3 and OVCAR3) compared with IOSE.

MiR-184 was reported to be widely dysregulated in human tumors (Sun et al. 2011). Yuan et al. (2014) revealed that the relative quantity of miR-184 was significantly up-regulated in human glioma cells by enhancing protein levels of HIF-1α. A high-throughput microarray study revealed that miR-184 were down-regulated in EOC compared with immortalized ovarian surface epithelium (IOSE) cell lines (Zhang et al. 2008). A number of inflammation related cytokines, including interleukin-6 (IL-6), IL-8 and tumor necrosis factor alpha (TNFα), have been found at increased levels in EOC (Maccio and Madeddu 2013; Dobrzycka et al. 2009). However, the roles and mechanisms of miR-184 in EOC proliferation, apoptosis and inflammation remain unknown.

In this study, we demonstrated the expression of miR-184 in clinical EOC tissues as well as EOC cell lines. The correlation between miR-184 level and clinical characteristics was also analyzed. Moreover, further *in vitro* studies of miR-184 function were performed to compare the proliferation, apoptosis and inflmmation difference in EOC cell lines.

2. Investigations and results

2.1. MiR-184 is down-regulated in EOC tissues and cell lines

We firstly tested the expression of miR-184 in 80 pairs of EOC tissues and matched adjacent non-cancerous tissues by qRT-PCR. The expression level of miR-184 in EOC tissues was significantly lower than that in the matched adjacent non-cancerous tissues ($P < 0.05$, Fig. 1A). Furthermore, we analyzed miRNA expression differences between the early-stage (I/II) and late-stage (III/IV) EOC according to FIGO stage and found that miR-184 levels were obviously reduced in late-stage EOC tissues compared with those in early-stage ($P < 0.05$, Table 1 and Fig. 1B). In addition, we demonstrated that the expression of miR-184 was significantly down-regulated in EOC cell lines (SKOV3 and OVCAR3) compared with IOSE ($p < 0.05$, Fig. 1C). Thus, down-regulation of miR-184 level was correlated with progression of EOC.

2.2. Prognostic performance of miR-184 levels in EOC

Kaplan–Meier survival analysis was used to analyze clinical survival information of patients. EOC patients with low expression level of miR-184 had worse overall survival rates than those with high level ($P < 0.001$, Fig. 2). MiR-184 (HR 0.221; 95% CI 0.114-0.429; $P < 0.001$) and FIGO stage (HR 4.292; 95% CI 1.160-15.87; $P < 0.05$) were independent prognostic indicators for EOC overall survival rates through Cox regression multivariate analysis (Table 2). These data indicate miR-184 as a potent biomarker for predicting prognosis in EOC patients.

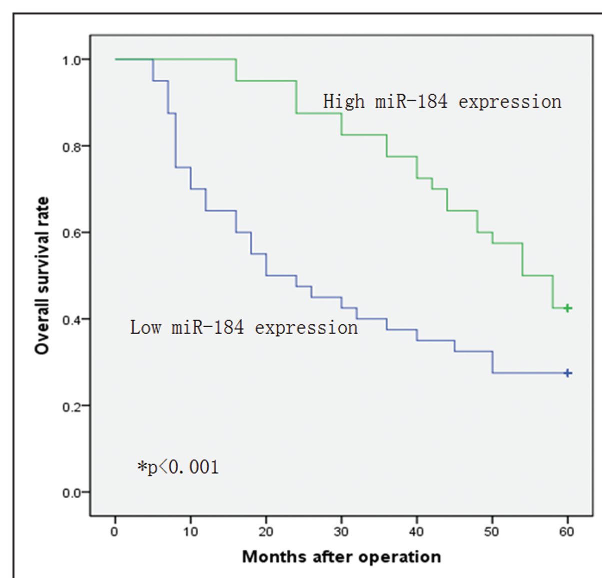

Fig. 2: Kaplan–Meier curve for overall survival in 80 EOC patients according to miR-184 expression.

Table 2: Multivariate analysis of overall survival in EOC patients.

Variables	Multivariate analysis		
	HR	95% CI	P
Age	1.043	0.994–1.093	0.088
Menopausal status	1.792	0.700-4.587	0.224
FIGO stage	4.292	1.160-15.87	0.029*
Histological grade	2.232	1.050-4.762	0.037*
miR-184 expression	0.221	0.114-0.429	<0.001*

* $P < 0.05$ was considered to be a statistically significant difference.

2.3. Effect of miR-184 over-expression on cell proliferation

To determine whether down-regulation of miR-184 affects cell proliferation in EOC cell lines, MTS assay was performed. After transfection with or miR-negative control mimics, the absorbance value of SKOV3 and OVCAR3 was significantly decreased in miR-184 mimics compared with their controls ($p < 0.05$, Fig. 3A, B). The data suggested that miR-184 could inhibit the proliferation of EOC cell lines.

2.4. MiR-184 regulates inflammation cytokines

To investigate impact of miR-184 on inflammation cytokines, transfection of mimics and NC mimic to EOC cell lines was con-

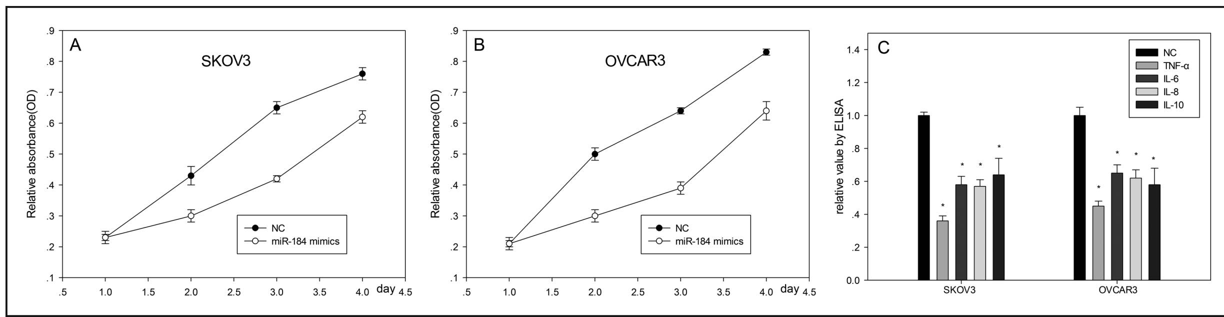

Fig. 3: Regulation of EOC cell proliferation and inflammation by miR-184. Growth curves of miR-184 mimics and NC mimic-transfected EOC cells were conducted by MTS assay in SKOV3 cell (A) and OVCAR3 cell (B). (C) ELISA assay detected the relative levels of inflammation markers including TNF-α, IL-6, IL-8 and IL-10. All experiments were carried out in triplicate. NC represents negative control. $^{*}p<0.05$ was considered as significant.

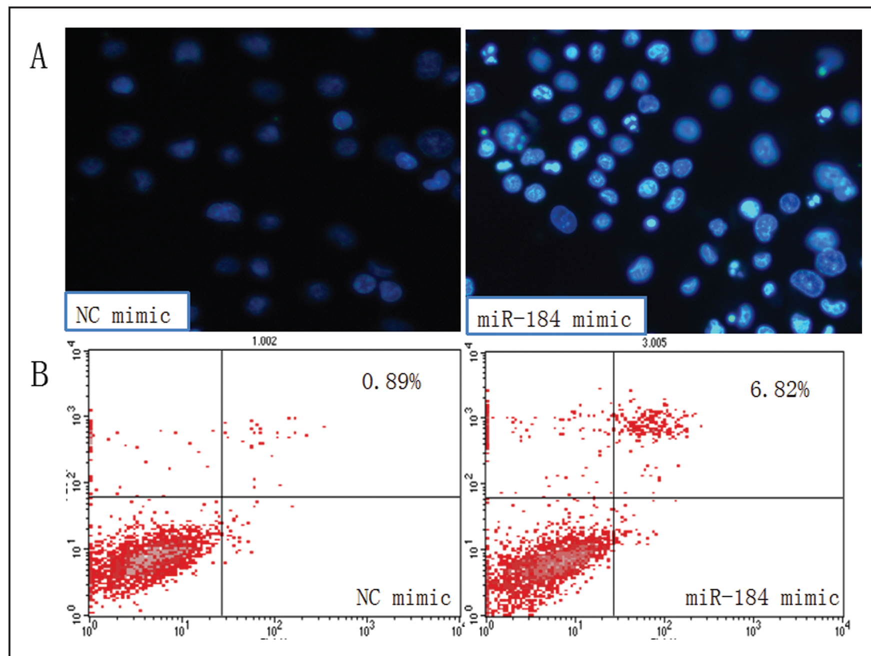

Fig. 4: MiR-184 induces EOC cell apoptosis in vitro. (A) Fluoresent photomicrographs of EOC cells with Hoechst 33342 staining. (B) Flow cytometry dot plot figures of apoptotic cells.

ducted. We examined the levels of TNF-α, IL-6, IL-8, and IL-10 in the culture medium using ELISA. As shown in Fig. 3B, levels of TNF-α, IL-6, IL-8 and IL-10 in miR-184 mimic groups were significantly lower than those in NC mimic groups ($p<0.05$, Fig. 3C).

2.5. *MiR-184 mediates EOC cell apoptosis*

Hoechst 33342 staining showed that most nuclei in the EOC cell lines control group (SKOV3) displayed uniform blue chromatin with organized structure (Fig. 4A). However, miR-184 mimic group resulted in apoptotic morphological changes (Fig. 4A). To further confirm miR-184 mimic on cell apoptosis, flow cytometry analysis with Annexin-V/PI double staining was performed. The representative images of flow cytometry are presented in Fig. 4B. MiR-184 mimic increased the apoptosis rate from 0.89% to 6.82% (SKOV3) compared with respective controls ($P<0.05$).
To clarify the role of miR-184 in EOC tumorigenesis, we also investigated the expression or activity of apoptosis-associated genes including Bcl-2, Bax, and caspase-3. MiR-184 mimic decreased the expression of Bcl-2 but increased the expression of Bax and caspase-3 activity in EOC cells (Fig. 5). These results demonstrated that miR-184 induced apoptosis through a change in the expression of apoptosis-associated genes that may control epithelial ovarian tumor growth.

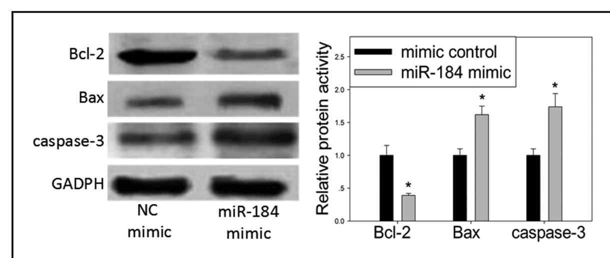

Fig. 5: MiR-184 induces apoptosis-related protein expression analyzed by Western blotting and quantified by densitometry. The values (mean ± SD from three independent experiments) are relative to control. $^{*}p<0.05$ was considered as significant.

3. Discussion

Ovarian cancer is the most lethal gynecologic neoplasm and common subtype of ovarian cancer (Siegel et al. 2012). The 5-year survival of the ovarian cancer patients diagnosed has been estimated to be 90 % for FIGO I, and less than 30 % for III or IV (Duffy et al. 2005; Badgwell and Bast 2007). Due to the high

mortality of epithelial ovarian cancer, identifying the molecular mechanisms of EOC will help identify novel diagnostic and/or prognostic markers and effective treatment of EOC. For instance, some studies have suggested miR-184 as a potential onco-miRNA (Walter et al. 2013). However, miR-184 also has tumor-suppressive role properties (Foley et al. 2010). In our study, we firstly detected the expression level of miR-184 in 80 EOC tissues and paired adjacent non-cancerous tissues and found that miR-184 was down-regulated in human EOC tissues (especially in late stage) and cell lines compared with paired non-cancerous tissues and IOSE, respectively. Importantly, our results showed that reduced miR-184 expression was associated with poor prognostic features for predicting overall survival in EOC. Further studies showed that miR-184 over-expression could suppress EOC cell proliferation, induce apoptosis and inhibit inflammation *in vitro*.

An interesting result of miRNA expression widely down-regulated in human tumors was extracted from high-throughput studies which found global expression of miRNAs is deregulated in most cancer types (Lu et al. 2005; Calin et al. 2005; Yanaihara et al. 2006; Volinia et al. 2006; Cummins et al. 2006). A high-throughput microarray study compared EOC cell lines with IOSE to investigate the miRNA alterations associated with OSE malignant transformation, and revealed that down-regulated miRNome is associated with IOSE malignant transformation and EOC progression (Zhang et al. 2008). This might reflect the fact that certain miRNAs may function as tumor suppressor genes (Lu et al. 2005).

MiR-184 is a single-copy gene and evolutionarily conserved at the nucleotide level from flies to humans (Liu et al. 2011). Although the function of miR-184 remains unknown, it is predicted to target several hundred genes. For instance, Weitzel et al. (2009) found that miR-184 regulated NFAT1 expression in umbilical cord blood CD4 + T cells. However, there were contradictory studies investigating the level of expression of miR-184 as indicator for cancer. Yu et al. (2008) observed that miR-184 appears to have a tumor suppressive effect in SCC cell lines, while Wong et al. (2008) found miR-184 to play an important role in the anti-apoptotic and proliferative processes of tongue SCC. Zhang et al. (2008) demonstrated that miR-184 expression was reduced in human EOC. Our data showed similar results: miR-184 level was significantly lower in EOC tissues, which was consistent with previous studies. In addition, FIGO stages are important factors in the EOC development and treatment strategy. We also investigated the impact of miR-184 expression on overall survival in EOC patients to further explore their potential prognostic role. Cox regression multivariate analysis indicated that miR-184 and FIGO stage were independent prognostic indicators for EOC patients. Altogether, our results suggest that the status of miR-184 together with pathologic diagnosis is critical for prognosis determination in EOC patients and help select treatment strategy.

Inflammation is considered as one of the pathogenesis factors of many cancers, including EOC (Wu et al. 2009; Toriola et al. 2011). Previous studies demonstrated that levels of IL-6, IL-8, IL-10, and TNFα increased in EOC patients (Aune et al. 2012; Wertel et al. 2011; Gadducci et al. 1995). This may be useful in the immunopathogenesis of EOC (Block et al. 2015). The results of the present study revealed that increased inflammation markers were down-regulated by miR-184, which could further contribute to understanding of the immunopathogenesis of EOC.

Previous studies suggest that miR-184 may be critical for regulating the proliferation and apoptosis of cancer cells. Chen and Stallings (2007) reported that miR-184 caused a decrease in cell proliferation and an increase in apoptosis in neuroblastoma cell lines. Foley et al. (2010) found that miR-184 inhibits neuroblastoma cell survival through targeting AKT2. MiR-184 was proved to play an important role in the anti-apoptotic and proliferative processes of tongue SCC (Wong et al. 2008). Our data demonstrated that miR-184 could inhibit cancer cell proliferation in the MTS assay, induce apoptosis and inhibit inflammation after transfected with miR-184 mimics in EOC cell lines. Further study is required to identify targets of miR-184 and thus deeply advance mechanism of miR-184 mediated EOC tumorigenesis. In this study, we found that miR-184 was down-regulated in EOC tissues and cell lines, especially in late-stage (III/IV) EOC tissues. Moreover, a low expression of miR-184 was correlated with poor prognostic features and short survival in EOC patients. Results also showed that miR-184 inhibited cell proliferation and induced cell apoptosis by regulating apoptosis-related genes in EOC tumorigenesis, and suppressed inflammation. Taken together, we consider that miR-184 may potentially act as a predictive miRNA and may also be a therapeutic target in EOC progression.

4. Experimental

4.1. Patients and tissue samples

The study was approved by the Ethics Committee of the Department of Clinical Pharmacology, Xiangya Hospital, Central South University and all patients signed a written informed consent prior to surgery. There were 80 patients with diagnosed epithelial ovarian cancer in the Xiangya Hospital recruited between August 2008 and January 2010. We also collected clinical information of the ovarian cancer patients. Clinical staging was evaluated by the International Federation of Gynecology and Obstetrics (FIGO) staging system (Seidman et al. 2004). All the ovarian cancer patients had been diagnosed histopathologically and had not received chemotherapy or radiotherapy prior to participation in this study. EOC tissue and paired adjacent non-cancerous tissue samples were immediately frozen in liquid nitrogen and stored at − 80 °C until use.

4.2. Cell culture and transfection

Two human ovarian cancer cell lines (SKOV3 and OVCAR3) were purchased from the American Type Culture Collection (Rockville, MD, USA), and the immortalized ovarian surface epithelium cell line (IOSE) was given as a gift from Professor Lai Hung-Cheng (Chou et al. 2010). SKOV3 and OVCAR3 cells were cultured in RPMI 1640 medium containing10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin. IOSE cells were grown in mixed medium 199 and MCDB105 (1:1) supplemented with 10% FBS.

About the transfection, microRNA mimics and negative control (NC) mimics were purchased from RIBOBIO Inc. Approximately 24 h before cell transfection, 2×10^5 cells were seeded into a 6 well plate, then transiently transfected with miR-184 mimics or negative control mimics (RiboBio, Guangzhou, China) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

4.3. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA according to the manufacturer's instructions. Reverse transcription was performed by PrimeScript RT reagent Kit With gDNA Eraser (Perfect Real Time) kit, and the SYBR Premix DimerEraser (Perfect Real Time) assay kits were used for the PCR amplification. The real-time PCR was performed using the Roche LC480 PCR System. The relative expression of miR-184 was shown as fold difference relative to small nuclear U6 RNA.

4.4. Western blotting

Total protein was isolated from cells in ice RIPA lysis buffer, and protein concentration was detected using a Bradford protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Total proteins were separated by 10% SDS-PAGE, and then transferred to nitrocellulose membranes. After incubation in blocking solution, membranes were incubated with a 1:500 dilution primary antibody for overnight at 4 °C. Membranes were washed, incubated with 1:2000 dilution of second antibody for 1 h, and then detected with Odyssey Infrared Imaging System. The following primary antibodies were used: Bcl-2 (Santa Cruz Biotechnology Inc, Dallas, USA), caspase-3 (Santa Cruz Biotechnology Inc, Dallas, USA), Bax (Abcam, Cambridge, Massachusetts, UK) and GAPDH (Abcam, Cambridge, Massachusetts, UK).

Second antibodies as loading controls were provided by Santa Cruz Biotechnology Inc.

4.5. Cell viability assay

The cell viability was detected by the Cell Titer 96 Aqueous-One-Solution Cell Proliferation Assay kit (MTS) in living cells. Briefly, cells were seeded at a density of 2×10^4 cells/well in 96-well plates in 100 μL medium and incubated until desired confluence. Another 24 h were needed for starvation medium incubation. MiR-184 mimics or control mimics were transfected into cells. At the time point of 24, 48, 72 or 96 h, 10 μL MTS solutions was added to each well and incubated for 2 h. The absorbance value was measured using a microplate reader spectrophotometrically at 490 nm according to the manufacturer's instructions.

4.6. Enzyme-linked immunosorbent assays (ELISA)

For the determination of cytokine levels, EOC cells were seeded in 24 well plates and collected the cell culture medium. Cell-free supernatant was diluted with assay diluents in accordance with the manufacturer's instructions. Inflammatory biomarkers including TNF-α, IL-6, IL-8, and IL-10 in cell culture medium were examined by commercial ELISA kits (eBioscience, CA, USA; R&D systems, MN, USA). All measurements were made in triplicate.

4.7. Apoptosis assay

Hoechst 33342 staining was used to detect the nuclear chromatin morphological changes of apoptotic cells. The cells were seeded in 96-well plates and transfected with miRNA mimics or NC control. After washing the plates twice with PBS, cells were fixed using 4% paraformaldehyde for 15 min. Then, cells were incubated in 50 ml of Hoechst 33342 solution for 20 min in the dark after twice washes with PBS. Fluorescence microscopy was applied to examine the nuclear DNA staining. Non-apoptotic nuclei appeared uniform blue chromatin with organized structure; while apoptotic changes showed bright blue fluorescent condensed nuclei.
Flow cytometry was performed to measure the percentage of apoptotic cells using Annexin V-FITC kit (Invitrogen, Carlsbad, CA, USA), as described in the manufacturer's instructions. Briefly, cells were pooled, washed with cold PBS, resuspended in binding buffer, and followed by the incubation with annexinV-FITC and propidium iodide (PI) for 15 min at room temperature in the dark. The cell apoptotic rates were then analyzed and quantified by flow cytometry.

4.8. Statistical analysis

The statistical analysis was performed with the SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA, USA), using Mann–Whitney U test, Pearson chi-squared test, Kaplan–Meier plot, log-rank test, or Student's t-test when appropriate. The Cox regression model of multivariate analysis was used to estimate the prognostic factors. Difference were considered statistically significant when $P < 0.05$.

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Conflict of interest: The authors declare no conflict of interest.

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