

Original Article

miR-338-3p targets pyruvate kinase M2 and affects cell proliferation and metabolism of ovarian cancer

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Abstract: MiR-338-3p is down-regulated in cancer, which inhibits cancer cell proliferation, metastasis, and increases chemosensitivity, but its functions in ovarian cancer remains unknown. The present study aims to identify the miR-338-3p targeted genes and to investigate the associated regulatory mechanisms in ovarian cancer cell proliferation and metabolism. Our results demonstrated miR-338-3p expression was down-regulated in most of ovarian cancer tissues and cell lines. Restoration of miR-338-3p expression in ovarian cancer cells could inhibit cell proliferation, lactate production and lactate production of ovarian cancer cells. PKM2 was verified as a target gene of miR-338-3p by luciferase assay. Further study indicated miR-338-3p controlled ovarian cancer cell metabolism by inhibiting PKM2 expression. It is summarized that the regulatory role of miR-338-3p on PKM2 expression in ovarian cancer may play important roles in cell metabolism.

Keywords: miR-338-3p, PKM2, ovarian cancer, metabolism, proliferation

Introduction

Ovarian cancer is one of the most common tumor from women. Every year, there are many cases diagnosed with ovarian cancer. In the past years, the overall survival rate of five years has been increased, however, there needs to further investigate the mechanism of ovarian cancer. Recently, studies elucidate some microRNAs (miRNAs) play important regulatory roles in ovarian cancer. MiRNAs are noncoding mRNA sequences containing around 22-nucleotides that act as important regulators of gene expression [1-3]. MiRNAs can silence their target genes by specifically binding and cleaving mRNAs or inhibiting their translation. Recently, there has been reported many miRNAs that involved in human ovarian cancer development. MiRNAs are aberrantly over-expressed or down-regulated during its progression, including miR-21, miR-15b, miR-16, miR-200c, miR-141, miR-101 and so on [1-4]. These miRNAs

play oncogenic or tumor-suppressive roles in the regulation of cell growth, migration and invasion by repressing their target genes.

Pyruvate kinase M2 (PKM2), which is expressed in fetal tissues, plays a critical role in glycolytic pathway as a rate-limiting enzyme, and catalyzes the PKM2 are upregulated in human cancer cells [7], PKM2 promotes glucose metabolism in cancer cells by stimulating transactivation of glycolytic genes [8]. However, it remains to be defined how PKM2 is regulated in cells. PKM2 emerges as an important regulator in glucose metabolism during cancer development and tumor growth [9-11]. Recent studies identified that PKM2 was regulated by AKT/mTOR signaling pathway in cell growth, survival, and metabolism. It was demonstrated that mTOR pathway upregulated glycolysis in hepatocellular carcinoma [12].

However, regulation of PKM2 in ovarian cancer is still not known. In this study, we plan to deter-

mine whether miR-338-3p regulates PKM2 in ovarian cancer and glycolytic activities.

Materials and methods

Cell culture

Normal ovarian epithelial cells (HUM-CELL-0088) were stored in our lab. Ovarian cancer cell lines HO-8910, A2780, AngIne, CoC1, CoC2, SKOV3 and Caov-3 cell lines were obtained from ATCC (Manassas, VA, USA). The cells were maintained in F-12K Medium (Invitrogen), or Eagle's Minimum Essential Medium (Invitrogen), or RPMI-1640 Medium (Invitrogen), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen).

RNA isolation and real-time RT-PCR

Total RNA, was isolated from the cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. After that, 2 µg of RNA was taken and treated with DNase to remove contaminating DNA prior to the reverse transcription to cDNA using SYBR® PCR Kit (Takara, Japan). To measure mRNA expression, real-time RT-PCR was performed using a sequence detector (ABI-Prism, Applied Biosystems). Primers were purchased from Invitrogen. The relative expression levels were calculated by comparing Ct values of the samples with those of the reference, all data normalized to the internal control GAPDH.

Cell proliferation assay

Cells were cultured in 24-well plates in a 5% CO₂ atmosphere at 37°C. Following the indicated treatments, 10 mg/mL methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added (50 µL/well), and the cells were incubated for an additional 2 h. The cells were then lysed with a lysis buffer (500 µL/well) containing 20% sodium dodecyl sulfate in dimethyl formamide/H₂O (1:1, v/v; pH 4.7) at 37°C for at least 6 h. The relative number of surviving cells in each group was determined by measuring the optical density (OD) of the cell lysates at an absorbance wavelength of 570 nm. The OD value of each treatment group was expressed as a percentage of the OD value of the untreated control cells [18].

Lentivirus mediated miR-338-3p transduction

Lentiviral vector mediated miR-338-3p was constructed according to the manual book from Invitrogen. The correct sequences and insertions were confirmed by DNA sequencing. Cells were transfected with either the miR-338-3p recombinant vector or its negative control (miR-control). Oligonucleotide transfection or lentivirus construction was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Lentivirus-mediated silencing of miR-338-3p was verified by qRT-PCR and western blot analysis.

Metabolism assays

Ovarian cancer cells were seeded into 24-well plate with 200 µl media each well. To determine the level lactate in the cells, the supernatants of cell culture media were collected and assayed for glucose and lactate levels by using lactate assay kit (BioVision, San Francisco, USA) according to the manufacturer's instructions.

Intracellular ATP assay

Briefly, 5 × 10⁵ cells were seeded into a well of a 6-well plate and treated with 20 nM cetuximab or left untreated in low-glucose, low-serum medium for 4 h. The cells were then harvested and resuspended in 1 mL of phosphate-buffered saline. An aliquot of 50 µL of the cell suspension was mixed with 100 µL of ATP-releasing reagent and 50 µL of distilled water in each well of a 96-well plate. The samples (100 µL) in each well were then transferred to a white opaque 96-well plate whose wells were each pre-filled with 100 µL of ATP assay mix. The amount of light emitted in each well was immediately measured using a FLUOstar Omega luminometer.

Western blot analysis

Protein from the cells was extracted and its concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA). Proteins were separated by SDS-PAGE and transferred to membranes (Millipore, Bedford, MA) at 80 V for 2 h at 4°C. After blocking in 5% nonfat dry milk in TBS, the membranes were incubated with primary antibodies overnight at 4°C, washed three times with TBST, and then

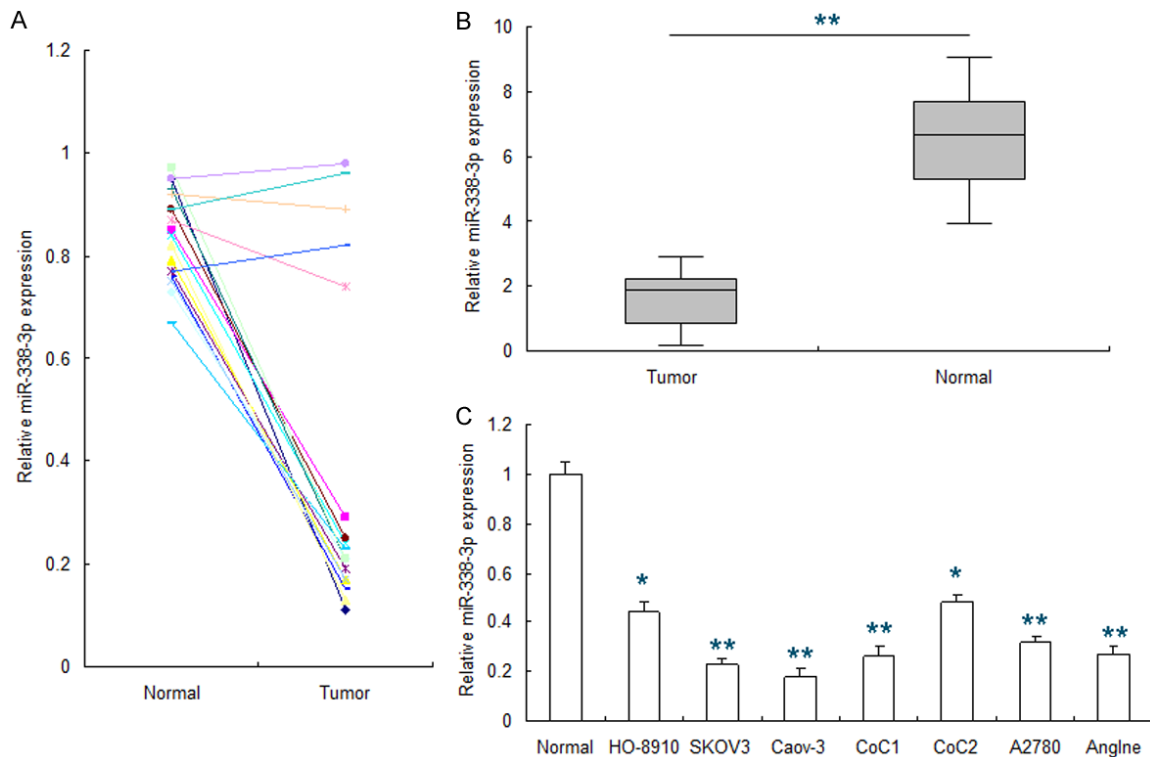


Figure 1. MiR-338-3p expression in ovarian cancer tissues and cell lines is reduced. (A) miR-338-3p expression in ovarian cancer tissues was examined by QRT-PCR. (B) Analyzed data from (A). (C) miR-338-3p expression in ovarian cancer cell lines was examined by QRT-PCR.

incubated with secondary antibodies conjugated with horseradish peroxidase in TBS for 2 h at room temperature. Membranes were washed again in TBST for three times at room temperature. Protein bands were visualized on X-ray film using ECL system.

Luciferase activity assay for 3'-UTR of PKM2

Plasmid vectors used in the luciferase reporter assays for miRNA post-transcriptional regulation were constructed as described previously (12). PKM2 3'-untranslated region (UTR) was amplified and the downstream of the luciferase gene in pGL4.13 vector (Promega, Madison, WI). All the constructs were verified by sequencing.

Statistical analysis

All experiments were performed at least three with similar results independently. SPSS 13.0 statistical software (SPSS, Chicago, IL, USA) was used to analyze the data. All the statistical tests were two-sided, and a *p* value less than

0.05 was considered as a significant difference.

Results

MiR-338-3p expression in ovarian cancer tissues and cell lines is down-regulated

Before we carried out the study of miR-338-3p on ovarian cancer cell metabolism, miR-338-3p expression was evaluated in ovarian cancer tissues and cells. Quantitative reverse-transcriptase PCR (QRT-PCR) analysis showed that miR-338-3p expression was significantly less in ovarian cancer tissues than in the compared adjacent tissues (Figure 1A and 1B). Next, we used ovarian cancer cells to verify the result and miR-338-3p expression levels was reduced much more in ovarian cancer cells than the normal epithelial ovarian cells (Figure 1C).

MiR-338-3p is related to ovarian cancer cell proliferation and metabolism

To evaluate the role of miR-338-3p in glucose metabolism, we carried out lactate production assay in SKOV3 and CAOV3 cells. The cells

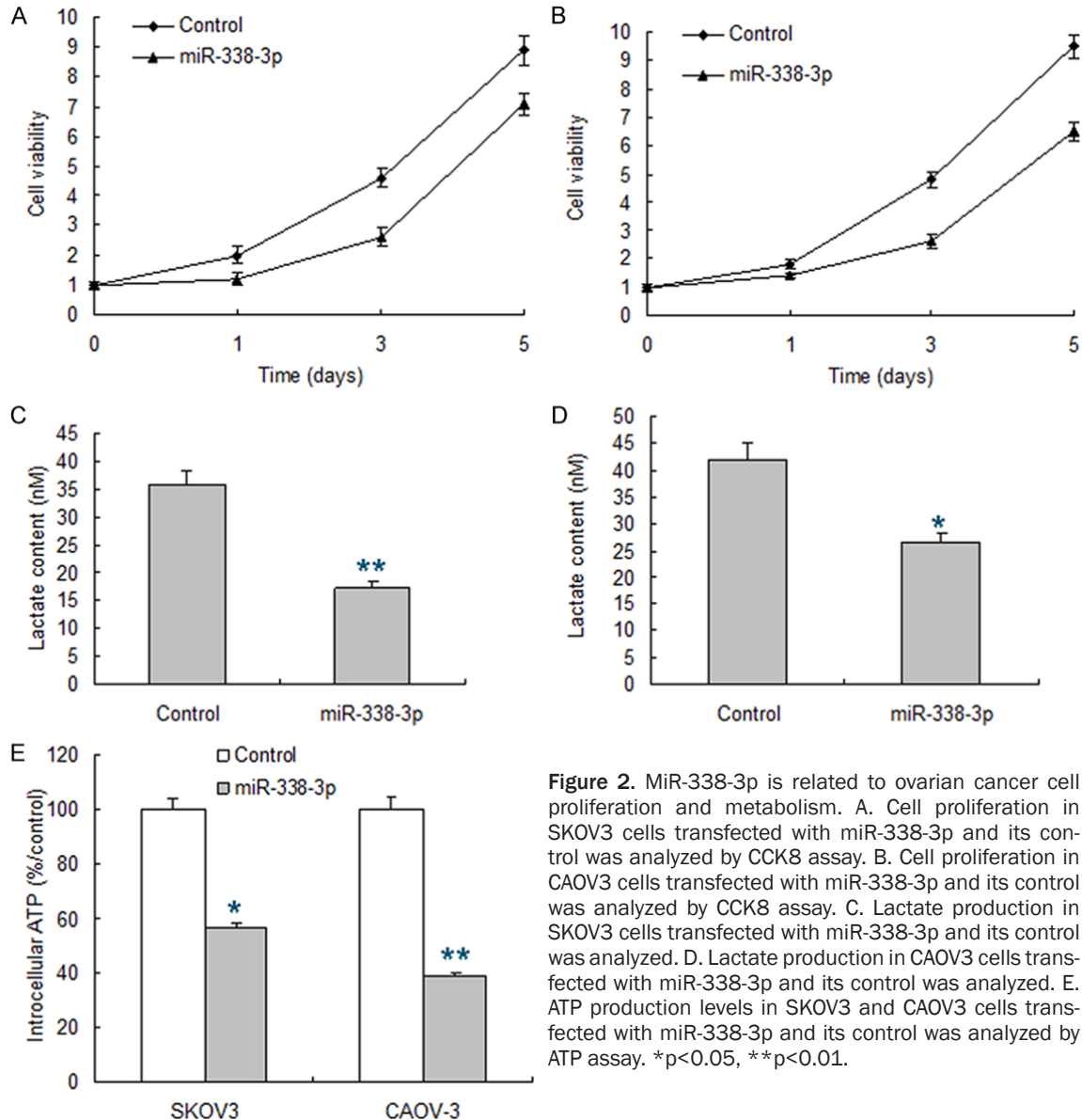


Figure 2. MiR-338-3p is related to ovarian cancer cell proliferation and metabolism. A. Cell proliferation in SKOV3 cells transfected with miR-338-3p and its control was analyzed by CCK8 assay. B. Cell proliferation in CAOV3 cells transfected with miR-338-3p and its control was analyzed by CCK8 assay. C. Lactate production in SKOV3 cells transfected with miR-338-3p and its control was analyzed. D. Lactate production in CAOV3 cells transfected with miR-338-3p and its control was analyzed. E. ATP production levels in SKOV3 and CAOV3 cells transfected with miR-338-3p and its control was analyzed by ATP assay. * $p < 0.05$, ** $p < 0.01$.

were transfected with miR-338-3p construct or vector control. QPCR was used to determine the transfection effect and miR-338-3p expression was restored in the two cell lines (**Figure 2A**). Cell proliferation was assayed by CCK8. When the ovarian cancer cells were transfected with miR-338-3p, the proliferation was suppressed (**Figure 2B** and **2C**). The lactate production was reduced in SKOV3 and CAOV3 cells with miR-338-3p restoration (**Figure 2D**). To find the difference of ATP production levels in ovarian cancer cells with miR-338-3p and its control, the ATP levels were assayed by ATP bioluminescent assay. The results showed that ATP in ovarian cancer cells with miR-338-3p decreased (**Figure 2E**).

MiR-338-3p targets PKM2 and regulates its expression

To validate PKM2 as a direct target of miR-338-3p, we transfected miR-338-3p expression vector into ovarian cancer cells, and found the PKM2 mRNA level was significantly reduced in the miR-338-3p cells, compared to the controls (**Figure 3A**). The endogenous protein level of PKM2 was also decreased upon ectopic expression of miR-338-3p (**Figure 3B**). In accordance with this observation, silencing miR-338-3p expression by using miR-338-3p inhibitor up-regulated mRNA levels of PKM2 in SKOV3 cells (**Figure 3C**). MiR-338-3p was predicted to bind the 3'UTR of PKM2 (**Figure 3D**). We found the

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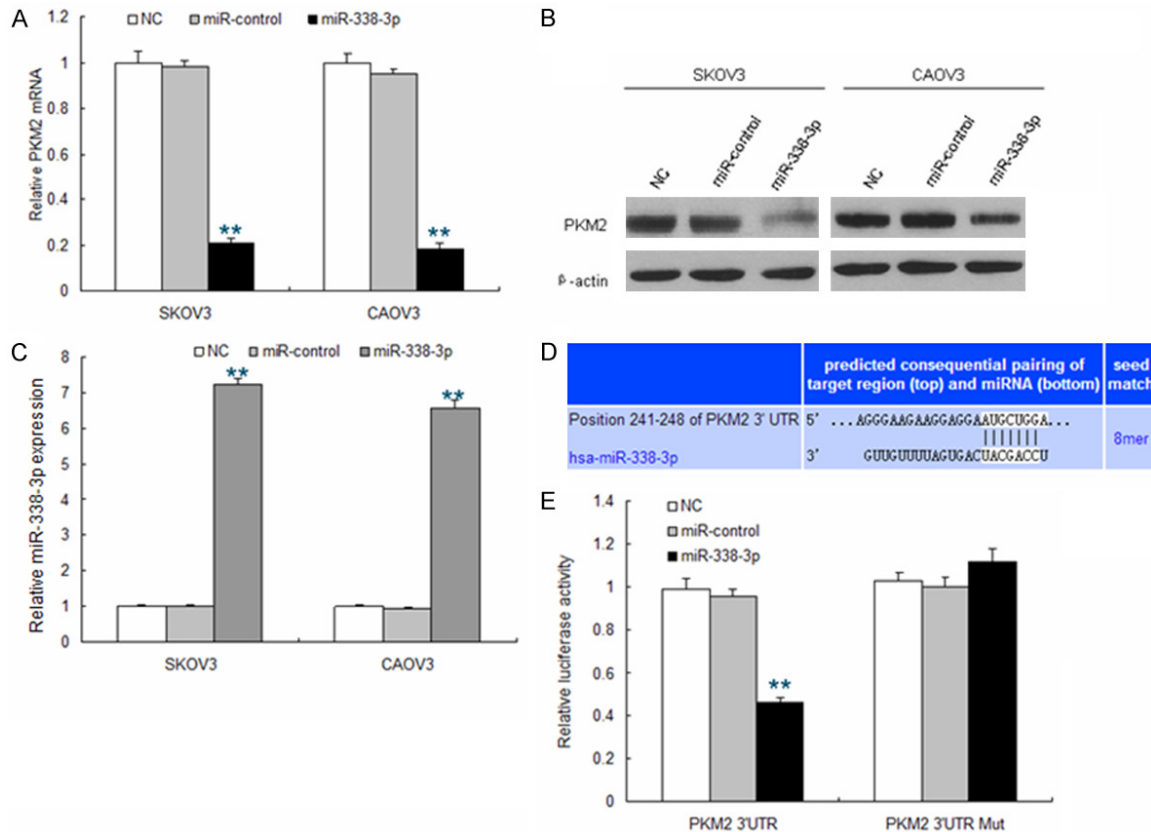


Figure 3. PKM2 is a direct target of miR-338-3p. A. Expression level of miR-338-3p levels in the ovarian cancer cells infected with lentivirus miR-338-3p and its control. B. Expression level of PKM2 levels in the ovarian cancer cells transfected with miR-338-3p and its control by western blotting. C. miR-338-3p levels in the ovarian cancer cells with transfection of miR-338-3p in SKOV3 and CAOV3 cells by QRT-PCR. D. Predicted binding interaction between 3'UTR of PKM2 and miR-338-3p. E. Luciferase activity in SKOV3 cells with co-transfection of miR-338-3p or vector with PKM2 3'UTR. Data were done for at least three independent experiments. ** $p < 0.01$.

activity of a luciferase construct bearing 3'UTR of PKM2 was significantly reduced upon expression of miR-338-3p (**Figure 3E**). Collectively, the data showed that PKM2 is a direct target of miR-338-3p.

miR-338-3p is negatively related to PKM2 in ovarian cancer cell lines and clinical samples

PKM2 expression was regulated by miR-338-3p in ovarian cancer cells. PKM2 was overexpressed in ovarian cancer tissues and cell lines (**Figure 4A** and **4B**). The analysis of relationship showed PKM2 was negatively associated with miR-338-3p in ovarian cancer tissues ($r = -0.61530$) (**Figure 4C**). There was the similar result in ovarian cancer cells (**Figure 4D**).

miR-338-3p regulates PKM2 mediated metabolism

To investigate whether miR-338-3p regulates metabolism of ovarian cancer cells by targeting

PKM2, we constructed PKM2 plasmid. SKOV3 cells were transfected with miR-338-3p and PKM2. We found that miR-338-3p could inhibit lactate production enhanced by PKM2 in SKOV3 cells (**Figure 5A**). Similar results were observed in CAOV3 cells (**Figure 5B**). Next, we transfected the two cell lines with either miR-338-3p or PKM2, and performed ATP assay at 48 hours. A significant reduction of ATP production was observed with PKM2 but not with the control (**Figure 5C** and **5D**). The data indicated that miR-338-3p and PKM2 regulatory relationship on cancer metabolism.

Discussion

Altered energy metabolism is a hallmark of cancer. Tumor metabolism is a hot area because targeting metabolism is one of the possible effective therapeutic method for cancer. There has been many studies about tumor metabolism and its epigenetics mechanisms in the

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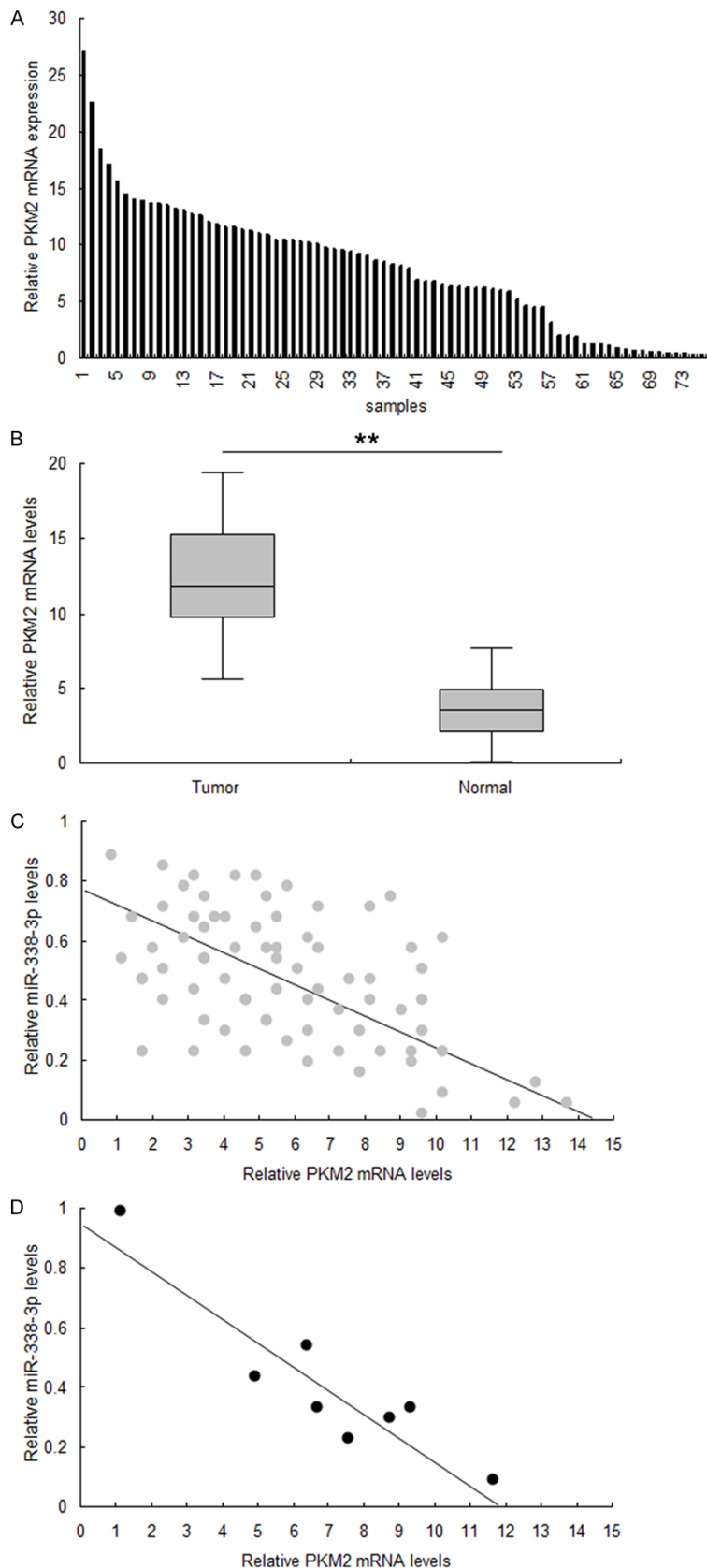


Figure 4. MiR-338-3p is negatively related to PKM2 in ovarian cancer cell lines and clinical samples. A. PKM2 expression was increased in ovarian

cancer tissues. B. PKM2 expression was increased in ovarian cancer cells. C. Expression correlation of PKM2 and miR-338-3p in ovarian cancer tissues. D. Correlation of PKM2 and miR-338-3p expression in ovarian cancer cells. ** $p < 0.01$.

pathogenesis or maintenance of tumors. In the present study, we analyzed the down-regulation expression of miR-338-3p in ovarian cancer tissues compared with patient-matched normal tissues. Ectopic expression of miR-338-3p inhibits proliferation, ATP and lactate production of ovarian cancer cells. The results also showed that miR-338-3p may function as a tumor suppressor by directly targeting PKM2. Thus, our results suggest that miR-338-3p is a potential diagnostic marker and therapeutic target of ovarian cancer.

MiR-338-3p is located at chromosome 17q25.3. In Previous reports showed that miR-338-3p was significantly down-regulated in HCC [13-14], non-small-cell lung carcinoma [15], neuroblastoma [16] and gastric cancer [17]. It play important roles in cell proliferation, increase sensitivity to sorafenib, invasion and migration by targeting genes includes FOXP4, hypoxia-induced factor 1 α , Ras-related protein 14, PREX2a, SSX2IP, CyclinD1 and other genes [13-21]. Our study data showed that the roles of miR-338-3p was significantly lower in most of ovarian cancer tissues and cell lines than the normal controls. Cellular function of miR-338-3p in ovarian cancer showed that miR-338-3p could suppress cell proliferation and glucose metabolism.

miR-338-3p inhibits ovarian cancer by down-regulation of PKM2

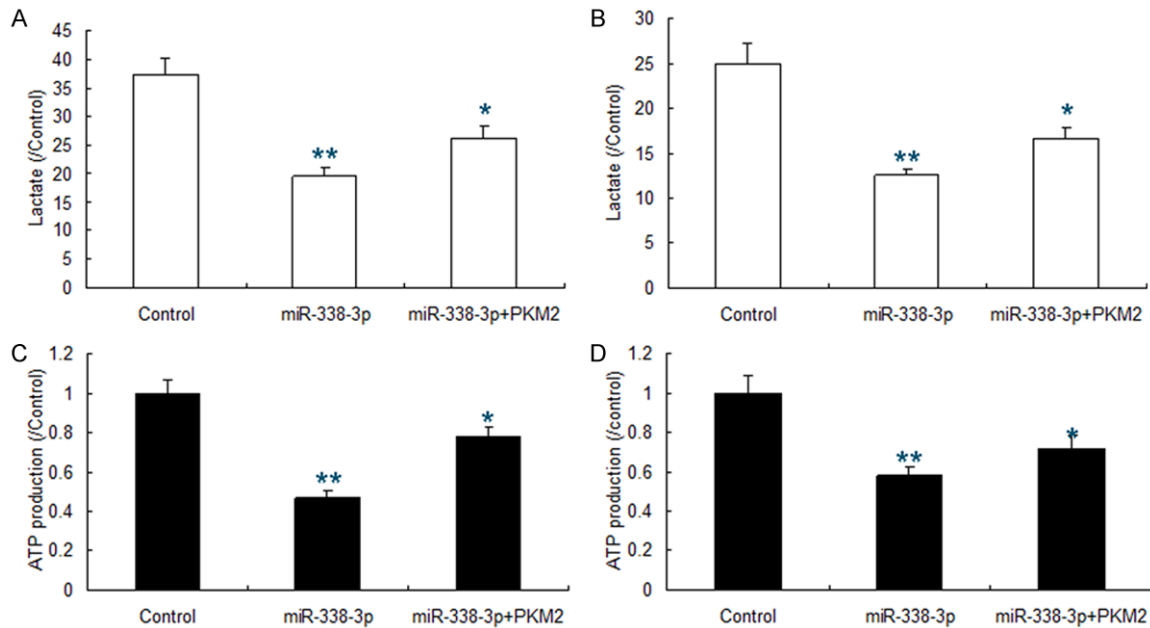


Figure 5. MiR-338-3p regulates PKM2 mediated metabolism. A, B. miR-338-3p inhibited lactate production in SKOV3 and CAOV3 cells by targeting PKM2. Cells were co-transfected with miR-338-3p and PKM2 for 48 h, and then performed for lactate assay. C, D. miR-338-3p inhibited ATP production in SKOV3 and CAOV3 cells by targeting PKM2. Cells were co-transfected with miR-338-3p and PKM2 for 48 h, and then performed for ATP assay. * $p < 0.05$, ** $p < 0.01$.

In contrast to normal differentiated cells that depend on mitochondrial oxidative phosphorylation for energy production, cancer cells have evolved to utilize aerobic glycolysis [Warburg's effect]. Glycolysis plays critical roles in ovarian cancer development. In the progress of glycolysis, there are a number of aberrant expression of glycolytic genes, which modulates their environment and promotes ovarian cancer cellular behaviors [22-23]. The PK family includes PKM1 and PKM2. PKM2 is the main isoform in cancer cells and PKM1 expression is very high in normal cells, which may explain that PKM2 may play a critical role in balancing growth and oxidative stress of cancer cells and promotes aerobic glycolysis and tumorigenesis in many tumor types, which plays a key role in the Warburg effect and therefore promotes tumorigenesis. The regulation of PKM2 expression is controlled by both oncogenes and tumor suppressors. Studies showed that cellular function of PKM2 is influenced by various modification [5]. Here, we showed PKM2 transcription is inhibited by miR-338-3p. The relationship between PKM2 and miR-338-3p in ovarian cancer tissues was analyzed and the data told us that miR-338-3p was negatively related to PKM2 expression.

In summary, the present study demonstrated a regulatory role of miR-338-3p in ovarian cancer metabolism, by targeting PKM2 that is involved in aerobic glycolysis supporting cancer cells survival and proliferation. Thus it is worth of investigating the due diligence of miR-338-3p therapeutic functions-by inhibiting cancer metabolisms, to benefit ovarian cancer patients.

Disclosure of conflict of interest

None.

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