



Downregulation of DNMT3A Attenuates the Warburg Effect, Proliferation, and Invasion via Promoting the Inhibition of miR-603 on HK2 in Ovarian Cancer

Technology in Cancer Research & Treatment
Volume 21: 1-13
© The Author(s) 2022
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/15330338221110668
journals.sagepub.com/home/tct


Jiaojiao Lu, PhD¹, Shuai Zhen, PhD², Xiaoqian Tuo, BD³, Shixue Chang, BD³, Xiling Yang, MD³, Yuanyuan Zhou, PhD⁴, Wei Chen, PhD³, Le Zhao, PhD³, and Xu Li, PhD³ 

Abstract

Background: Ovarian cancer is a highly malignant gynecological cancer. Aerobic glycolysis is one of the features of cancer cell metabolism. Studying the molecular modulation of the Warburg effect in ovarian cancer is significantly valuable for understanding the progression mechanism of ovarian cancer. **Materials and Methods:** The expression level and prognostic significance of DNMT3A were analyzed using public databases. DNMT3A was overexpressed by plasmid transfection, and DNMT3A was interfered with specific siRNAs transfection. miR-603 was overexpressed by mimic transfection or inhibited by inhibitor transfection. The expression of the molecules was detected by qPCR or western blotting. CCK-8 and transwell assays were used to determine the cell proliferation, migration, and invasion abilities of ovarian cancer. **Results:** We found that the DNMT3A protein level was higher in ovarian cancer tissues than in normal ovary tissues, but the mRNA level had no significant difference in ovarian cancer tissues and normal ovary tissues. The higher the RNA level of DNMT3A, the poorer prognosis of patients. DNMT3A knocking down impeded the Warburg effect, cell proliferation, migration, and invasion of ovarian cancer cells. Further investigations discovered that DNMT3A promoted ovarian cancer cell malignancy via silencing miR-603. **Conclusion:** We found that patients who overexpressed DNMT3A showed a poor prognosis. DNMT3A was found to promote the Warburg effect, cell proliferation, migration, and invasion of ovarian cancer by inhibiting the expression of miR-603. As a result, the research revealed that DNMT3A/miR-603/HK2 axis contributed to the Warburg effect of ovarian cancer and DNMT3A may be a potential therapeutic target for ovarian cancer.

Keywords

DNMT3A, ovarian cancer, miRNA, Warburg effect, proliferation, invasion

Abbreviations

SLC2A1, solute carrier family 2 member 1; GLUT1, glucose transporter 1; HK2, hexokinase-2; PFK, phosphofructokinase; PKM, pyruvate kinase M; PDK-1, pyruvate dehydrogenase kinase-1; LDHA, lactate dehydrogenase A; DNMT3A, DNA methyltransferase 3A; miRNAs, microRNAs; 3'-UTRs, 3'-untranslated regions; mRNAs, messenger-RNAs; HPA, Human Protein Atlas; K-M Plotter, Kaplan–Meier Plotter; qRT-PCR, quantitative real-time PCR; PFS, progression-free survival; OS, overall survival; SGI-110, guadecitabine

¹ Department of Radiology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China

² Center of Medical Genetics, The Northwest Women and Children's Hospital, Xi'an, China

³ Center for Translational Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, China

⁴ Department of Pathology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China

Corresponding Author:

Xu Li, Center for Translational Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China.

Email: lixu56@mail.xjtu.edu.cn



Received: January 19, 2022; Revised: May 20, 2022; Accepted: June 9, 2022.

Introduction

Ovarian cancer is the third most commonly diagnosed gynecological cancer and the second cause of death related to gynecological cancer.¹ Most of the ovarian cancer patients are diagnosed at an advanced stage and metastasis or chemotherapy resistance has already occurred.² Hence, there is still an urgent need to develop novel therapeutic options by finding new targets.

The Warburg effect, discovered by Otto Warburg and colleagues in the 1920s, is a metabolic reprogramming process to ferment glucose to lactate though in the presence of oxygen and is used by tumor cells to support the rapid production of ATP and high demand of macromolecular synthesis.³ The Warburg effect is significantly related to glycolysis-related genes including solute carrier family 2 member 1 (SLC2A1), also known as glucose transporter 1 (GLUT1), hexokinase-2 (HK2), phosphofructokinase (PFK), pyruvate kinase M (PKM), pyruvate dehydrogenase kinase-1 (PDK-1), and lactate dehydrogenase A (LDHA),⁴ among which HK2 catalyzes the essentially irreversible first step of the glycolytic reaction and is highly expressed in various cancers.⁵ For the Warburg effect, glucose is the substrate and lactate is the end product, thus, glucose consumption and lactate production could be used as parameters of aerobic glycolysis.^{6–8} The Warburg effect not only furnishes cancer cells with ATP and anabolic precursors for macromolecules but also provides an acidic environment which could promote local invasion and proliferation of cancer cells.^{9,10} Because of the important role of the Warburg effect in tumor growth and progression, hunting for the Warburg effect-related therapeutic targets has important value.

Study in the regulation of gene expression is of significant importance, and great attention has been paid to epigenetic modifications, among which DNA methylation is one of the most important epigenetic regulatory mechanisms. DNA methyltransferase 3A (DNMT3A) is considered to be one of the two de novo methyltransferases which could change the methylation status of unmethylated CpG sites and has been largely investigated in recent years.¹¹ In mammalian genomic DNA, almost 5% of cytosine exists in the form of 5-methylcytosine, and 70% of 5-methylcytosine is located within CpG sequences, which suggests that CpG methylation plays an important role in the regulation of gene expression.¹² Besides, the inactivation of tumor suppressor genes by aberrant methylation of CpG sites has been reported in many cancers, including ovarian cancer.^{12,13} The functions of DNMT3A in cancer progression need to be clarified.

MicroRNAs (miRNAs) are composed of approximately 22 nucleotides and function by binding to the 3'-untranslated regions (3'-UTRs) of the targeted messenger-RNAs (mRNAs) with imprecise or precise complementarity, giving rise to either decay or translational repression of the targeted

mRNAs.¹⁴ miRNAs have been reported as potential biomarkers of various cancers, affecting cancer cell energy metabolism, proliferation, and invasion.^{15,16} The expression level of miRNAs could be regulated by DNA methylation.¹⁷ Among the nearly 2000 miRNAs that have been listed on miRbase, miR-603 is recently discovered through miRNA CHIP microarray in thyroid cells, which targets the CCND1 and CCND2 genes.¹⁸ In addition, miR-603 is recognized as a tumor-suppressor miRNA that regulates a part of genes involved in the proliferation, invasion, and sensitivity to chemoradiation of cancer cells.^{19–21} As reported recently, liposomes encapsulating miR-603 complexes could enhance radiation sensitization in glioblastomas.²² However, the interaction between miR-603 and DNMT3A remains largely unexplored. Previously we found overexpression of DNMT3A increased the methylation level in the promoter region of the miR-603 precursor gene,²³ but the feedback between DNMT3A and miR-603 needs to be further studied.

In this study, we explored the promotive role of DNMT3A in the Warburg effect, proliferation, migration, and invasion of ovarian cancer cells via silencing miR-603 expression, promising that DNMT3A has the potential to be used as a therapeutic target in ovarian cancer patients.

Materials and Methods

Bioinformatic Mining Methods

The expression level of DNMT3A in ovarian cancer based on TCGA/GTEX data could be achieved by boxplots with the GEPIA2 database (<http://gepia2.cancer-pku.cn/>)²⁴. The cut-off value of \log_2FC was set as 1, and the *P*-value cutoff was set to 0.01. We obtained the immunohistochemical data from the Human Protein Atlas (HPA) (<https://www.proteinatlas.org/>) and the CPTAC database (<http://ualcan.path.uab.edu/>) to compare the protein levels of DNMT3A in ovarian cancer samples and normal tissue samples.^{24–26} GEPIA2 and cBioPortal for Cancer Genomics (<http://www.cbioportal.org>) were used to analyze the correlation between DNMT3A and glycolysis-related genes at the mRNA level.^{27–29} Next, we utilized Kaplan–Meier Plotter (K-M Plotter) database (<http://kmplot.com/analysis>) which is capable to perform survival analysis of genes in cancer patients to evaluate the prognostic values of DNMT3A in ovarian cancer patients.³⁰ Another dataset, GSE12418, included 54 stage-III ovarian cancer patients, in which 20 fresh frozen tumors were from 5-year survivors and 34 tumors from deceased patients in order to find the differed gene expression between the two groups,³¹ obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo>), was used to validate the predictive capability of DNMT3A in 5-year survival rate.

Cell Culture

The SKOV3 and A2780 cell lines were acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and the Shandong Academy of Medical Sciences (Jinan, China), respectively. The cell line accession number is as follows: the ATCC number of SKOV3 is HTB-77™ and the ECACC catalog number of A2780 is 93112519. Both cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA) with 10% newborn bovine serum (GIBCO, Grand Island, NY, USA) at 37°C in a 5% CO₂ incubator.

Transfection

Human ovarian cancer SKOV3 and A2780 cells (3×10^5) were plated into 6-well plates and were transfected with the DNMT3A siRNA (RiboBio Co., Ltd, Guangzhou, China), DNMT3A plasmid (Addgene plasmid #35521), miR-603 mimic (RiboBio Co., Ltd, Guangzhou, China), miR-603 inhibitor (RiboBio Co., Ltd, Guangzhou, China) or the negative control using X-treme GENE siRNA Transfection Reagent (Roche, Indianapolis, IN, USA) or X-treme GENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA). DNMT3A siRNA sequence is CATCCACTGTGAATGATAA. After transfection for 48 h or 72 h at 37 °C, cells were prepared for the following analysis.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from lysed cells by TRIzol reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions, and RNA concentration was determined by absorbance at 260 nm using a spectrophotometer. Total RNA (1 µg) was compounded to cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara; Dalian, China) according to the manufacturer's instructions. q-PCR was performed using SYBR Premix Ex Taq™ II kit (Takara; Dalian, China). DNMT3A amounts were normalized to β-actin and U6 snRNA for miR-603, respectively (Applied Biosystems). The levels of gene expression were automatically calculated with the $2^{-\Delta\Delta Ct}$ method. The primers for DNMT3A and β-actin were synthesized by Beijing Genomics Institute (Beijing, China), and the primer sequences were listed as follows: DNMT3A forward: 5'-TATTGATGAGCGCACAAGAGAGC-3'; DNMT3A reverse: 5'-GGGTGTTCCAGGGTAACATTGAG-3'; β-actin forward: 5'-TCCCTGGAGAAGAGCTACGA-3'; and β-actin reverse: 5'-AGCACTGTGTTGGCGTACAG-3'. Besides, primers for miR-603 and U6 were purchased from RiboBio Co., Ltd (Guangzhou, China).

Western Blotting

Protein expression levels were analyzed using western blotting assays. Ovarian cancer cells were lysed with RIPA buffer containing 1 mM PMSF and 1% complete protease inhibitor

(Roche Applied Science; Indianapolis, IN, USA) to extract the proteins. Protein samples were subjected to SDS-PAGE gel and transferred to nitrocellulose membranes (Pall Life Science, Port Washington, NY, USA) using the Bio-rad system at 320 mA for 100 min. The nitrocellulose membranes were then blocked in 5% skimmed milk on a shaker at room temperature for 1 h and incubated overnight at 4°C with anti-DNMT3A (1:500, Cell Signaling Technology, Beverly, MA, USA), anti-HK2 (1:1000, Cell Signaling Technology, Beverly, MA, USA) and anti-β actin (1:1000, Cell Signaling Technology; Beverly, MA, USA) primary antibodies. After washing three times with TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated (1:2000, Pierce; Rockford, IL, USA) corresponding goat-anti-rabbit or goat-anti-mouse secondary antibody for 1 h at room temperature, followed by washing three times. ECL system (Bio-Rad, Richmond, CA, USA) was used for the immunoreactive bands of target proteins detection with enhanced chemiluminescence reagents (Millipore, Billerica, MA, USA).

Cell Proliferation Assay

The transfected ovarian cancer cells were seeded in 96-well plates with 3000 cells per well. After 0 h, 24 h, 48 h, 72 h, and 96 h of culture respectively, CCK-8 (7 sea pharmtech Co., Ltd, Shanghai, China) was added to each well for 1 h of incubation according to the manufacturer's instructions. The absorbance in each well was measured with a microplate reader at 450 nm.

Cell Migration and Invasion Assays

Cell migration and invasion assays were performed by transwell chambers (Millipore Co., Bedford, MA, USA) without matrigel (for migration assay) or with matrigel (for invasion assay). In brief, 500 µl of 1640 medium supplemented with 20% newborn bovine serum was added to the bottom chamber, and either 1×10^5 cells (for migration assay) or 4×10^5 cells (for invasion assay) transfected ovarian cancer cells were suspended in 100 µL of serum-free 1640 medium and added to the upper chamber. After culturing for 24 h (for migration assay) or 48 h (for invasion assay), the cells on the top were removed with a cotton swab. The migrated and invaded cells were fixed in methanol and stained with 0.1% crystal violet. The migrated and invaded cells were counted under the inverted light microscope (original magnification $\times 200$). Each experiment was performed three times.

Statistical Analysis

GraphPad Prism 6.0 software (GraphPad Software, Inc.; La Jolla, CA, USA) and SPSS 21.0 software (IBM Corp. Armonk, NY, USA) were adopted for graphing and data analysis, respectively. Comparisons between two groups were performed using a two-tailed t-test, and Kaplan–Meier method was used to estimate the prognosis. Pearson's test was adopted to

analyze the correlation between DNMT3A and glycolysis-related genes. All experiments were carried out in triplicate and data were presented as means \pm SD. A *P*-value less than 0.05 was considered statistically significant.

Results

1. Overexpressed DNMT3A indicated poor survival in ovarian cancer

The GEPIA2 database analysis showed no significant difference in DNMT3A mRNA level in ovarian cancer tissues and normal ovary tissues (normal: $n=88$; tumor: $n=426$) (Figure 1A). The immunohistochemistry data of ovarian tissues (normal: $n=3$; tumor: $n=12$) in the HPA database showed the medium staining of DNMT3A (Antibody CAB009469) in normal ovarian tissues but high staining in ovarian cancer tissues (Figure 1B), and the protein levels of DNMT3A had a significant difference in ovarian cancer

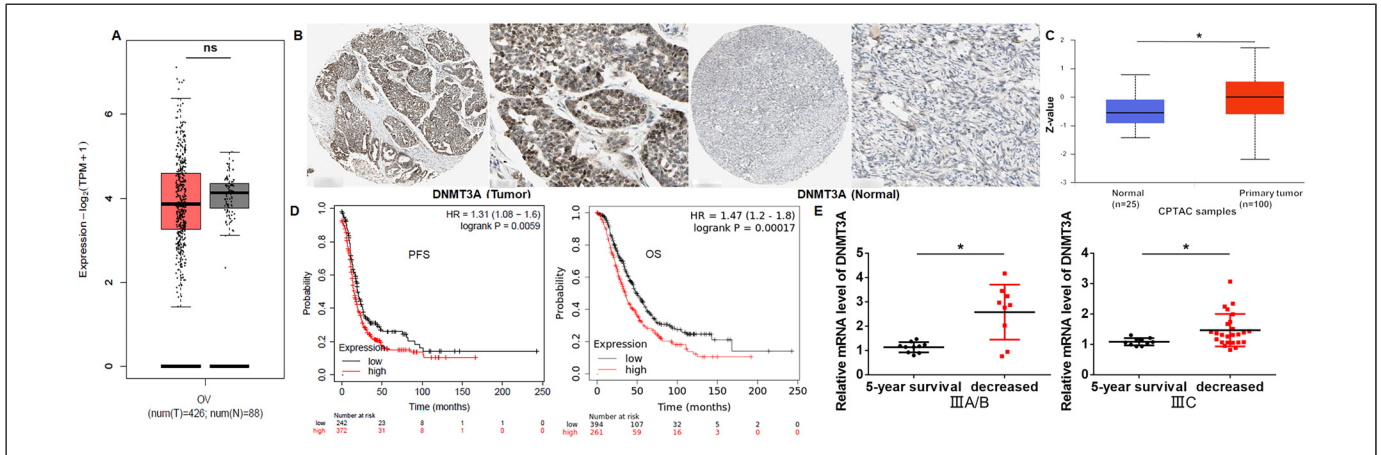


Figure 1. Expression and prognostic values of DNMT3A. (A) There was no significant difference in mRNA levels of DNMT3A in ovarian cancer and normal tissues using the GEPIA2 database. The red box indicated ovarian cancer and the gray box indicated normal ovarian tissues. ns = non-significant. (B) The immunohistochemical results of DNMT3A showed DNMT3A protein expression was higher in ovarian cancer than in normal tissues using the HPA database. (C) The protein levels of DNMT3A had a significant difference in ovarian cancer and normal tissues in the CPTAC database. (D) Survival curves of ovarian cancer patients in Kaplan–Meier plotter database indicated that the higher expression of DNMT3A, the worse PFS (HR = 1.31, $P = .0059$) or OS (HR = 1.47, $P = .00017$) in ovarian cancer. (E) The levels of DNMT3A in decreased patients with stage III A/B or III C ovarian cancer were higher than the 5-year survivors in the cohort GSE12418. * $P < .05$.

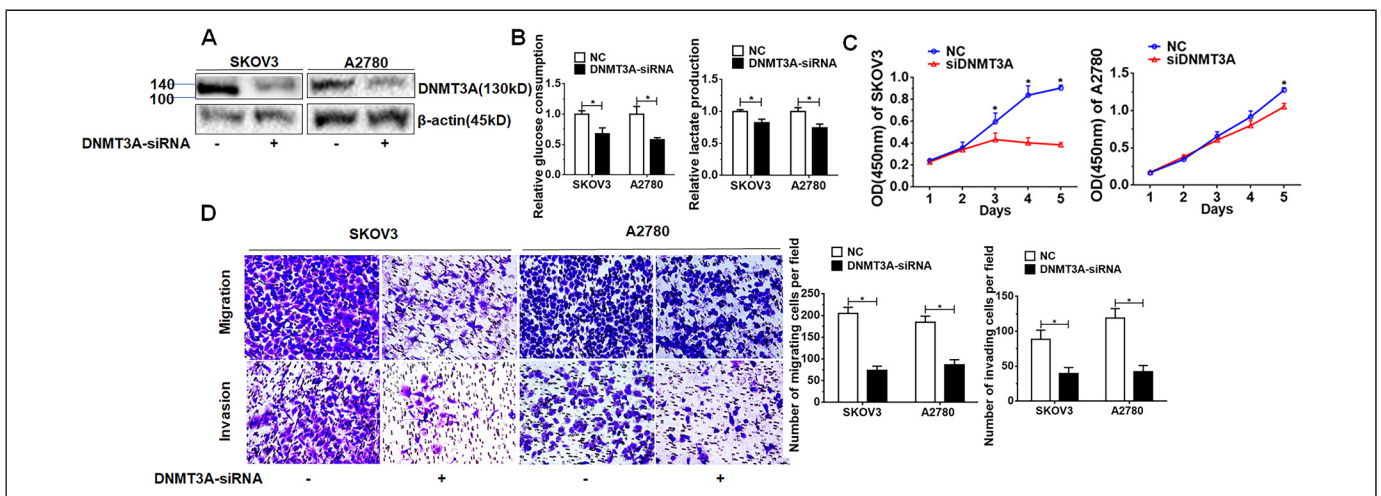


Figure 2. Effect of DNMT3A knockdown on the Warburg effect. (A) Western blotting results showed that DNMT3A was successfully interfered by siRNA transfection. (B) Transfection of DNMT3A-siRNA inhibited glucose consumption and lactate production. (C) Transfection of DNMT3A-siRNA retarded the growth of SKOV3 and A2780 cells. (D) Transfection of DNMT3A-siRNA significantly weakened the motility and invasion of SKOV3 and A2780 cells. All experiments were carried out in triplicate and data were presented as means \pm SD. Calculation of statistical significance was performed using a two-tailed t-test. * $P < .05$.

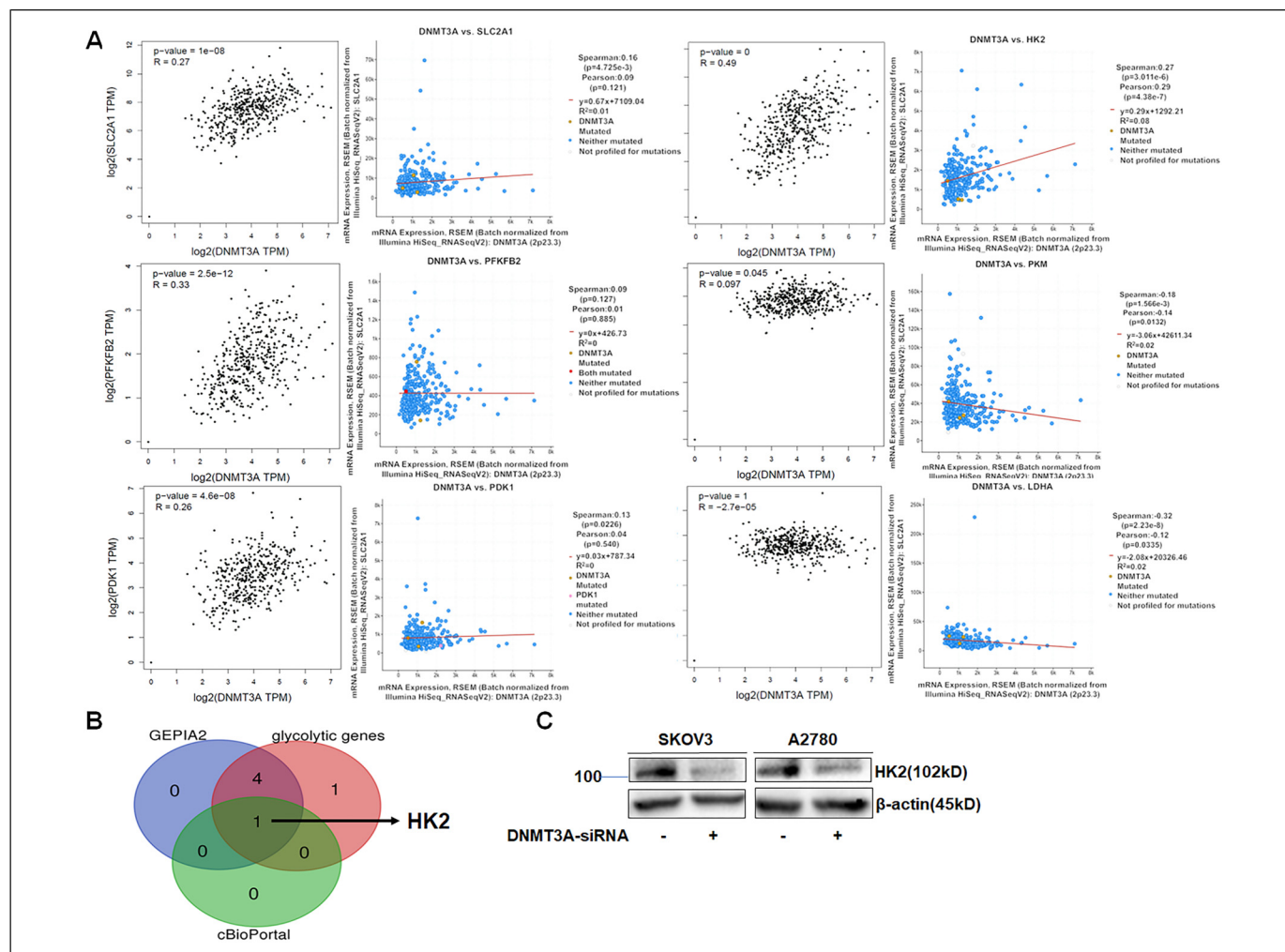


Figure 3. The regulatory mechanism of the Warburg effect is influenced by DNMT3A. (A) The relationship of DNMT3A to the expression of glycolysis-related genes was analyzed using GEPIA2 and cBioPortal for Cancer Genomics databases. DNMT3A positively correlated with SLC2A1, HK2, PFKFB2, PKM, and PDK1 while did not correlate with LDHA via GEPIA2. HK2 was positively correlated with DNMT3A through Spearman's and Pearson's correlation analysis via cBioPortal, however, PKM and LDHA were negatively relevant to DNMT3A. (B) HK2 was predicted by two algorithms that were significantly positively correlated with DNMT3A. (C) DNMT3A-siRNA transfection decreased the protein level of HK2.

tissues and normal ovary tissues in CPTAC database (Figure 1C), suggesting that the DNMT3A protein level was higher in ovarian cancer tissues than in normal ovary tissues. To estimate the prognostic significance of DNMT3A in ovarian cancer, the progression-free survival (PFS) and overall survival (OS) were acquired from K-M Plotter (high: $n=372$; low: $n=242$ and high: $n=261$; low: $n=394$ respectively) and 5-year-survival from GEO Database (III A/B survivors: $n=10$, deceased: $n=9$; III C survivors: $n=10$, deceased: $n=25$). The results showed that a higher level of DNMT3A is related to shorter PFS and OS (Figure 1D), and the DNMT3A level in deceased patients was higher than that in the 5-year survivors (Figure 1E). The data indicated that DNMT3A may serve as a potential biomarker and therapeutic target for ovarian cancer.

2. Knocking down DNMT3A attenuated the Warburg effect, proliferation, migration, and invasion of ovarian cancer cell

To determine the biological roles of DNMT3A in ovarian cancer, we specifically interfered DNMT3A expression in ovarian cancer cells with DNMT3A siRNA (Figure 2A). Since the Warburg effect plays an important role in tumor development, the indicators of the Warburg effect including glucose consumption and lactate production were examined to explore the relationship of DNMT3A to the Warburg effect. Knocking down of DNMT3A significantly reduced glucose consumption and lactate production (Figure 2B), suggesting DNMT3A promoted the Warburg effect in ovarian cancer cells. Besides, the CCK-8 assay found silencing DNMT3A significantly weakened ovarian cancer cell proliferation (Figure 2C). The transwell assay

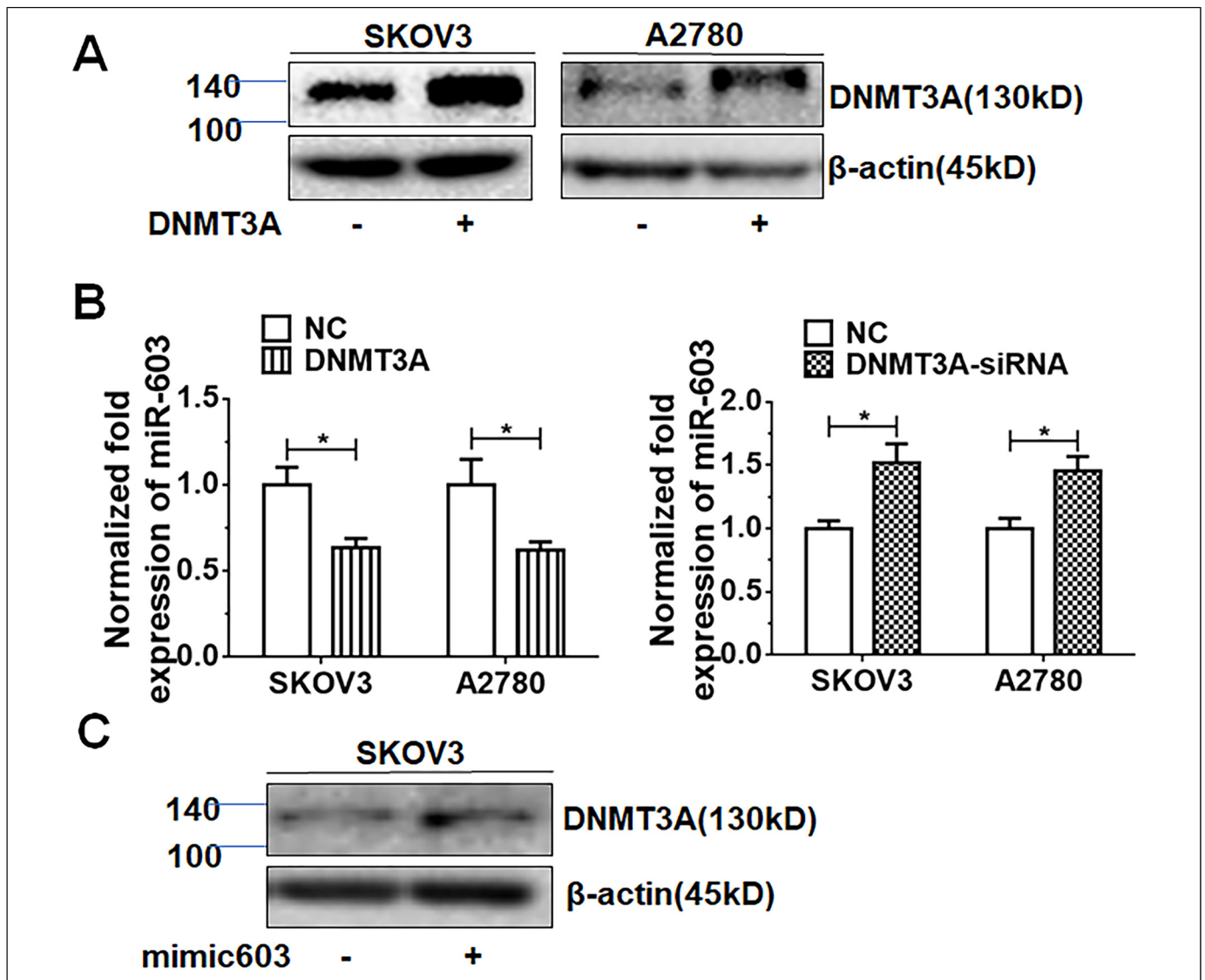


Figure 4. Regulation of miR-603 by DNMT3A in a unidirectional way. (A) DNMT3A was overexpressed in indicated ovarian cell lines with transfection of DNMT3A plasmid. (B) The overexpression of DNMT3A reduced the miR-603 level in SKOV3 and A2780 cells, and knocking down of DNMT3A promoted miR-603 expression. (C) miR-603 overexpression or knockdown did not change the protein level of DNMT3A in SKOV3 cells. All experiments were carried out in triplicate and data were presented as means \pm SD. Calculation of statistical significance was performed using a two-tailed t-test. * $P < .05$.

indicated that silencing DNMT3A statistically inhibited the migration and invasion ability of ovarian cancer (Figure 2D). GEPIA2 and cBioPortal for the Cancer Genomics database were used to illustrate the potential regulatory mechanism of DNMT3A in the Warburg effect. According to GEPIA2, SLC2A1, HK2, PFKFB2, PKM, and PDK1 were found to be positively correlated with DNMT3A, and via cBioPortal, HK2 was the only one which was significantly positively correlated with DNMT3A through Spearman's and Pearson's correlation analysis, however, PKM and LDHA were negatively relevant to DNMT3A (Figure 3A). As shown in Figure 3B, HK2 might be the downstream target of DNMT3A. Next, the expression level of HK2 was evidenced markedly repressed in

DNMT3A-siRNA transfected cells compared with that in the negative control cells (Figure 3C).

3. DNMT3A promoted tumor cell malignancy via methylation-mediated silencing of miR-603

Previous study²³ has showed DNMT3A negatively regulated miR-603 in ovarian cancer cells. In the present study, the association between DNMT3A and miR-603 was examined by rescue experiments. The results indicated that overexpression of DNMT3A (Figure 4A) reduced miR-603 expression in SKOV3 and A2780 cells, and knockdown of DNMT3A (Figure 2A) promoted miR-603 expression (Figure 4B). But no changes in DNMT3A expression were examined in miR-603

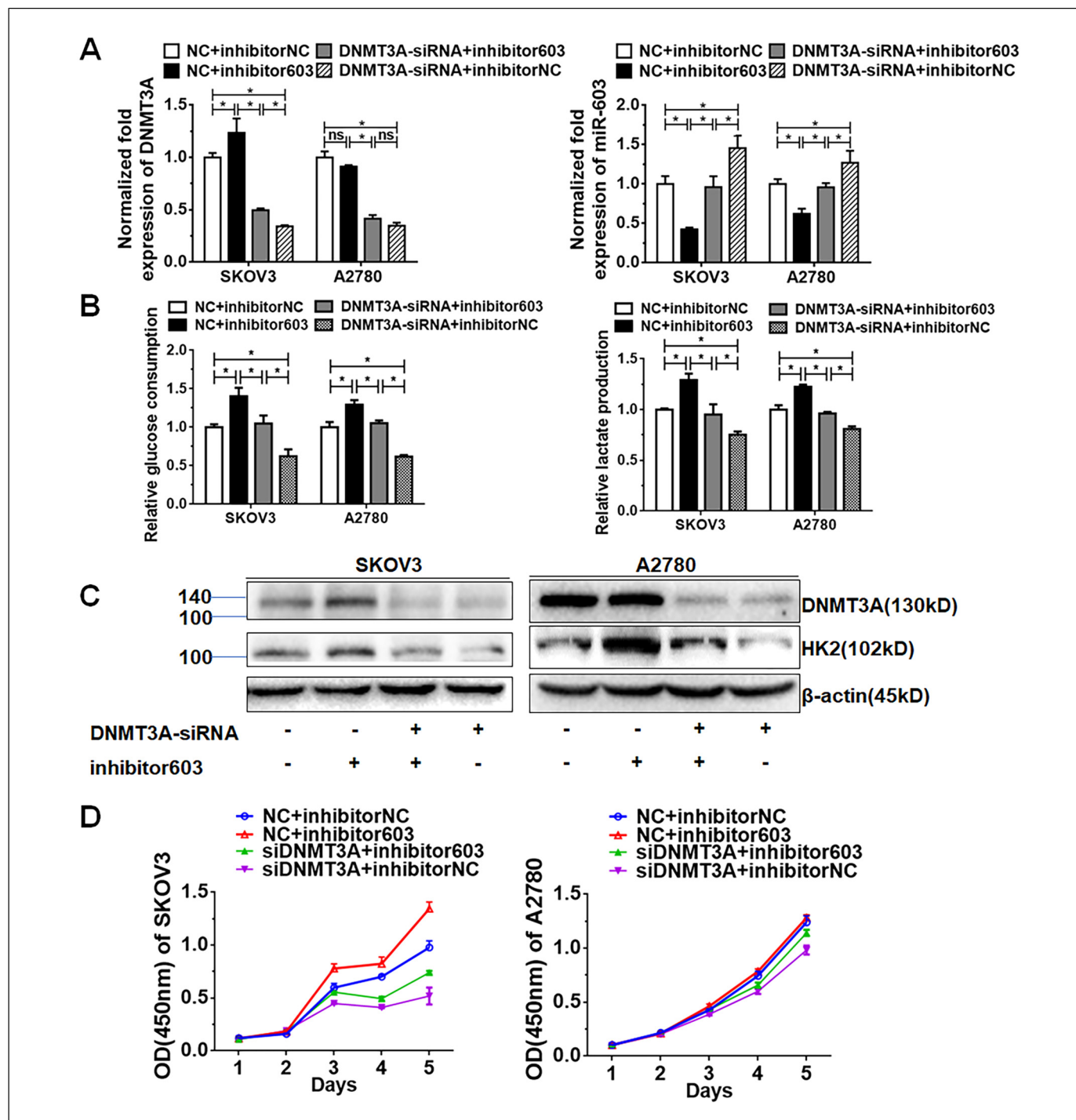


Figure 5. DNMT3A interference inhibited the Warburg effect and proliferation via regulating the miR-603/HK2 axis. (A) DNMT3A and miR-603 levels in DNMT3A-siRNA and miR-603 inhibitor cotransfected-SKOV3 and A2780 cells. (B) miR-603 inhibitor increased glucose consumption and lactate production and reversed the inhibitory effect of DNMT3A siRNA on glucose consumption and lactate production in SKOV3 and A2780 cells. (C) DNMT3A was increased in SKOV3 but unchanged in A2780 cells by inhibitor603. Inhibitor603 increased HK2 protein level, which was reversed by DNMT3A-siRNA in both SKOV3 and A2780 cells. (D) Cell proliferation analyzed by CCK-8 showed DNMT3A interference inhibited cell growth, which was reversed by inhibitor603. All experiments were carried out in triplicate and data were presented as means \pm SD. Calculation of statistical significance was performed using a two-tailed t-test. * $P < .05$.

overexpressed or knockdown SKOV3 cells (Figure 4C). These results indicated that DNMT3A negatively regulated miR-603 in a unidirectional way in ovarian cancer cells.

4.miR-603 inhibition reversed DNMT3A interference-attenuated ovarian cancer cell malignancy

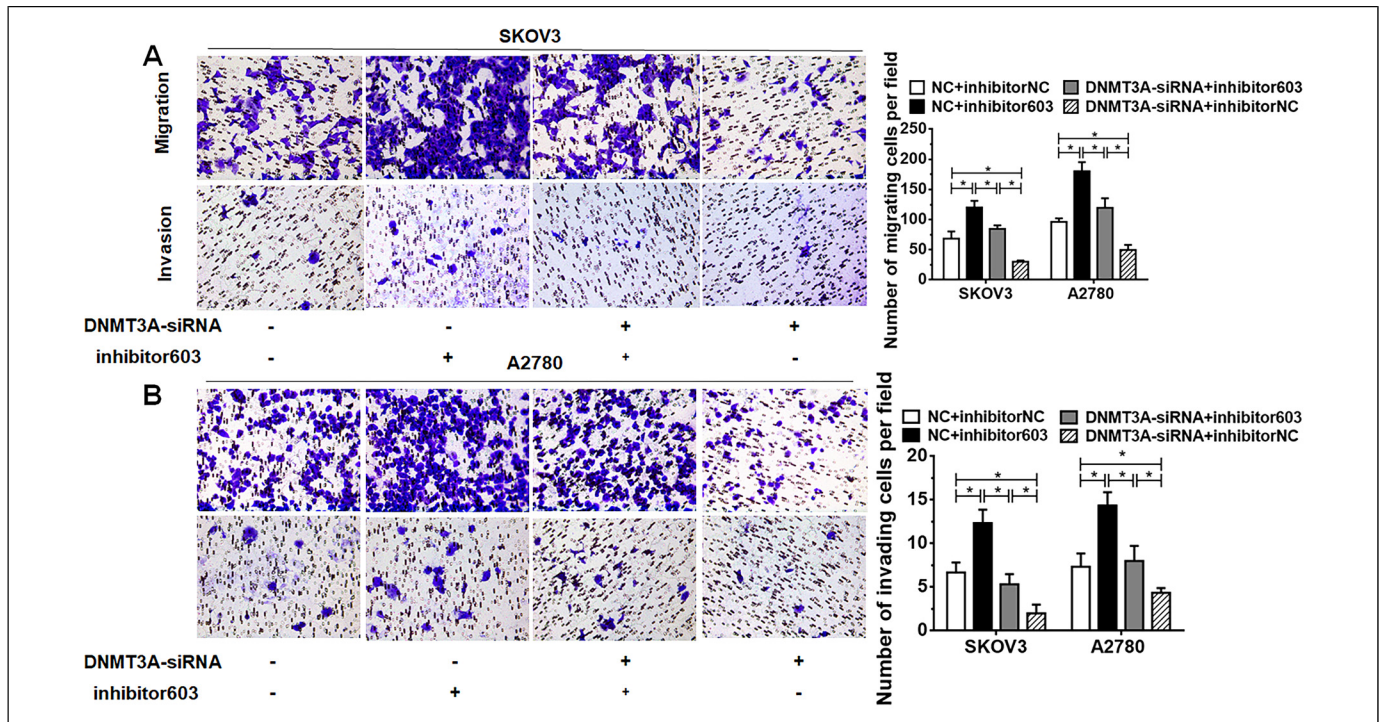


Figure 6. DNMT3A interference inhibited migration and invasion via regulating the miR-603/HK2 axis. DNMT3A interference repressed migration and invasion in SKOV3 (A) and A2780 (B) cells, and that was reversed by inhibitor603. All experiments were carried out in triplicate and data were presented as means \pm SD. Calculation of statistical significance was performed using a two-tailed t-test. * $P < .05$.

Considering that miR-603 directly targeted HK2 to inhibit the Warburg effect, proliferation, migration, and invasion of ovarian cancer cells and DNMT3A-siRNA markedly inhibited HK2, miR-603 might participate in the DNMT3A-triggered Warburg effect. To confirm the involvement of miR-603 in DNMT3A-promoted malignancy in ovarian cancer cells, DNMT3A and miR-603 were simultaneously inhibited. The results showed that DNMT3A-siRNA could effectively knock down DNMT3A in SKOV3 and A2780 cells (Figure 5A, C). However, miR-603 knockdown increased DNMT3A expression in SKOV3 cells, while did not influence DNMT3A in A2780 cells (Figure 5A, C). qPCR assay confirmed the inhibitory effect of inhibitor603 on miR-603 expression in ovarian cancer cells (Figure 5A). The expression of miR-603 was restored in cells treated with DNMT3A siRNA (Figure 5A). In parallel, inhibitor603 greatly counteracted the siDNMT3A-suppressed Warburg effect (Figure 5B), expression of HK2 (Figure 5C), cell proliferation (Figure 5D), migration, and invasion ability (Figure 6A, B) in both SKOV3 and A2780 cells. However, the rescue experiment of the CCK-8 assay in A2780 cells did not show a significant difference (Figure 5D). Altogether, the results suggested that knock-down of DNMT3A promoted miR-603 expression to suppress cell proliferation, migration, and invasion ability of ovarian cancer cells.

5. miR-603 overexpression reversed DNMT3A-induced ovarian cancer cell Warburg effect, proliferation, migration, and invasion

Gain-of-function studies of miR-603 and DNMT3A were conducted by transiently transfecting miR-603 mimics and DNMT3A plasmid into SKOV3 and A2780 cells. qPCR and western blotting assays confirmed the over-expressed effect of miR-603 mimics and DNMT3A plasmid (Figure 7A, C). DNMT3A was repressed by mimic603 in DNMT3A-plasmid and mimic603 cotransfected-SKOV3 and A2780 cells (Figure 7C). Ectopic expression of miR-603 partially attenuated DNMT3A-augmented glucose consumption, lactate production, and expression of HK2 (Figure 7B, C). In addition, miR-603 overexpression counteracted DNMT3A-induced promotion of cell proliferation (Figure 7D), migration, and invasion (Figure 8A, B) in both SKOV3 and A2780 cells. However, DNMT3A-plasmid solely had no significant influence on ovarian cancer cell proliferation (Figure 7D). Taken together, the results suggested that DNMT3A was able to promote the Warburg effect, proliferation, migration, and invasion ability of ovarian cancer cells by inhibiting miR-603 expression.

Discussion

Globally, ovarian cancer is one of the deadliest urogenital cancers in women. Thus, seeking out key genes that can be used as biomarkers and the therapeutic target is required for early diagnosis and prognosis. In this study, we provide strong evidence that aberrant expression of DNMT3A has a

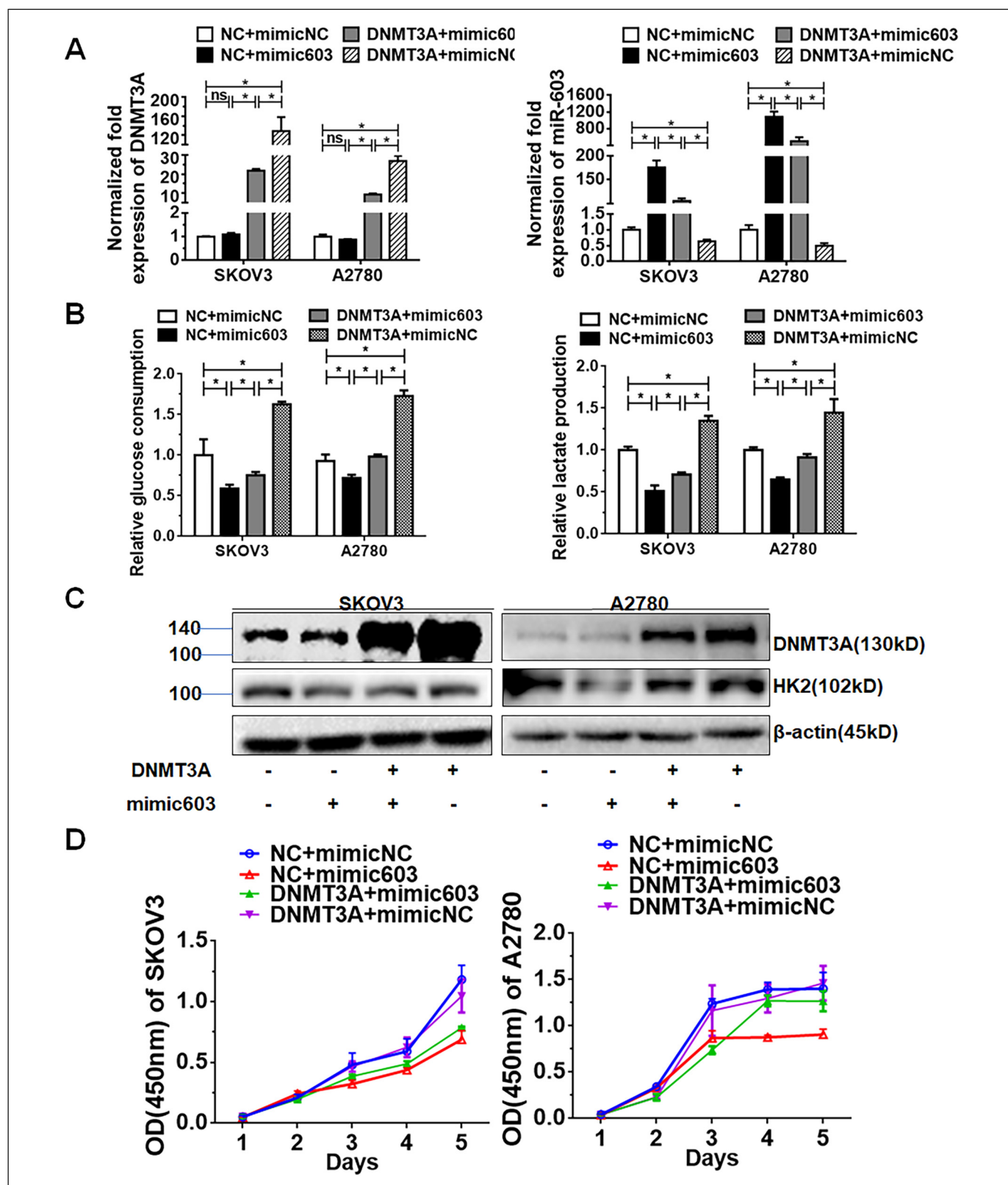


Figure 7. DNMT3A overexpression promoted the Warburg effect and proliferation via regulating the miR-603/HK2 axis. (A) DNMT3A and miR-603 levels in DNMT3A- plasmid and miR-603 mimic cotransfected-SKOV3 and A2780 cells. (B) miR-603 mimic inhibited glucose consumption and lactate production and reversed the promotion of DNMT3A plasmid on glucose consumption and lactate production in SKOV3 and A2780 cells. (C) Mimic603 inhibited HK2 protein level, which was reversed by DNMT3A-plasmid in both SKOV3 and A2780 cells. (D) Cell proliferation analyzed by CCK-8 showed miR-603 overexpression counteracted DNMT3A-induced promotion of cell growth in both SKOV3 and A2780 cells, but DNMT3A-plasmid solely had no significant influence on ovarian cancer cell proliferation. All experiments were carried out in triplicate and data were presented as means \pm SD. Calculation of statistical significance was performed using a two-tailed t-test. * $P < .05$.

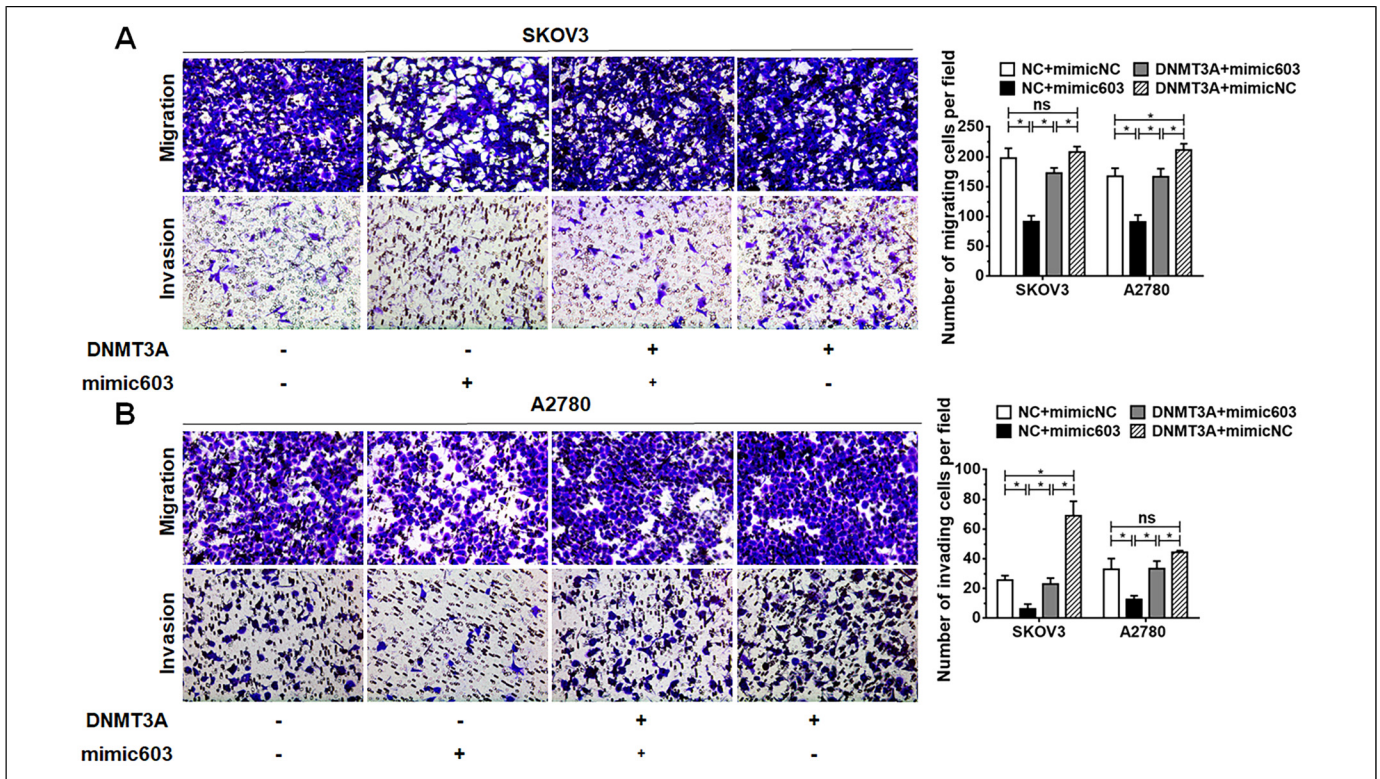


Figure 8. DNMT3A overexpression promoted migration and invasion via regulating the miR-603/HK2 axis. miR-603 mimic inhibited migration and invasion and reversed the promotion of DNMT3A plasmid on migration and invasion in SKOV3 (A) and A2780 (B) cells. All experiments were carried out in triplicate and data were presented as means \pm SD. Calculation of statistical significance was performed using a two-tailed t-test. * $P < .05$.

central role in the Warburg effect and progression of ovarian cancer via inhibiting the suppression of miR-603 on HK2.

Based on the GEPIA2 database, we demonstrated that mRNA levels of DNMT3A had no significant differences in ovarian cancer and normal tissues. However, the IHC data from the HPA database showed that compared to normal tissues, DNMT3A was highly expressed in ovarian cancer tissues. Gu and Chen *et al* have reported that DNMT3A mRNA levels were not different between cancer tissues and normal ovaries,^{32,33} which was consistent with ours, however, another report has shown that expression levels of DNMT3A mRNA were increased in ovarian cancer.³⁴ These differences may be caused by the limited sample size or different detection methods used by different studies. Additionally, several groups have also reported DNMT3A protein expression was higher in ovarian cancer than that in benign tumors or normal tissues.^{35,36} The difference between mRNA and protein levels of DNMT3A might be due to the inconsistent expression of DNMT3A at the transcription level and protein level, because regulation of post-transcription, translation, and protein degradation in the variation of protein concentrations is as important as transcription.³⁷

Considering that a higher expression level of DNMT3A correlated with a worse prognosis in ovarian cancer patients, it is of significant importance to illustrate the mechanism that DNMT3A involved in ovarian cancer. Since knockdown of

DNMT3A exhibited inhibition of the Warburg effect, abnormal expression of glycolysis-related genes seems to be a critical factor leading to the progression of ovarian cancer caused by DNMT3A. The results of the current study indicated that DNMT3A was significantly positively related to HK2. Subsequently, validation studies suggested that DNMT3A interference markedly suppressed HK2 protein levels. As a key enzyme that catalyzes the first committed step in glucose metabolism, HK2 promoted glycolysis, growth, and metastasis in many cancer cells, and a previous study indicated higher HK2 expression in ovarian cancer by immunohistochemistry compared with normal tissues, which was correlated with poor prognosis.³⁸ We previously reported that HK2 was one of the critical direct targets of miR-603,²³ and in recent years, the inhibition of tumor-suppressor miRNAs by abnormal DNA methylation has received increasing attention in various cancers.³⁹ Accordingly, we hypothesized that DNMT3A may be implicated with glycolysis regulation and malignant biological behaviors of ovarian cancer by miR-603/HK2 axis.

Further experiments were used to testify the hypothesis that DNMT3A plays an important role in miR-603 regulated expression of HK2. Herein, we verified that reduced DNMT3A expression led to increased miR-603 expression and decreased HK2 expression in ovarian cancer cells. Additionally, induction of DNMT3A substantially inhibited the expression of miR-603

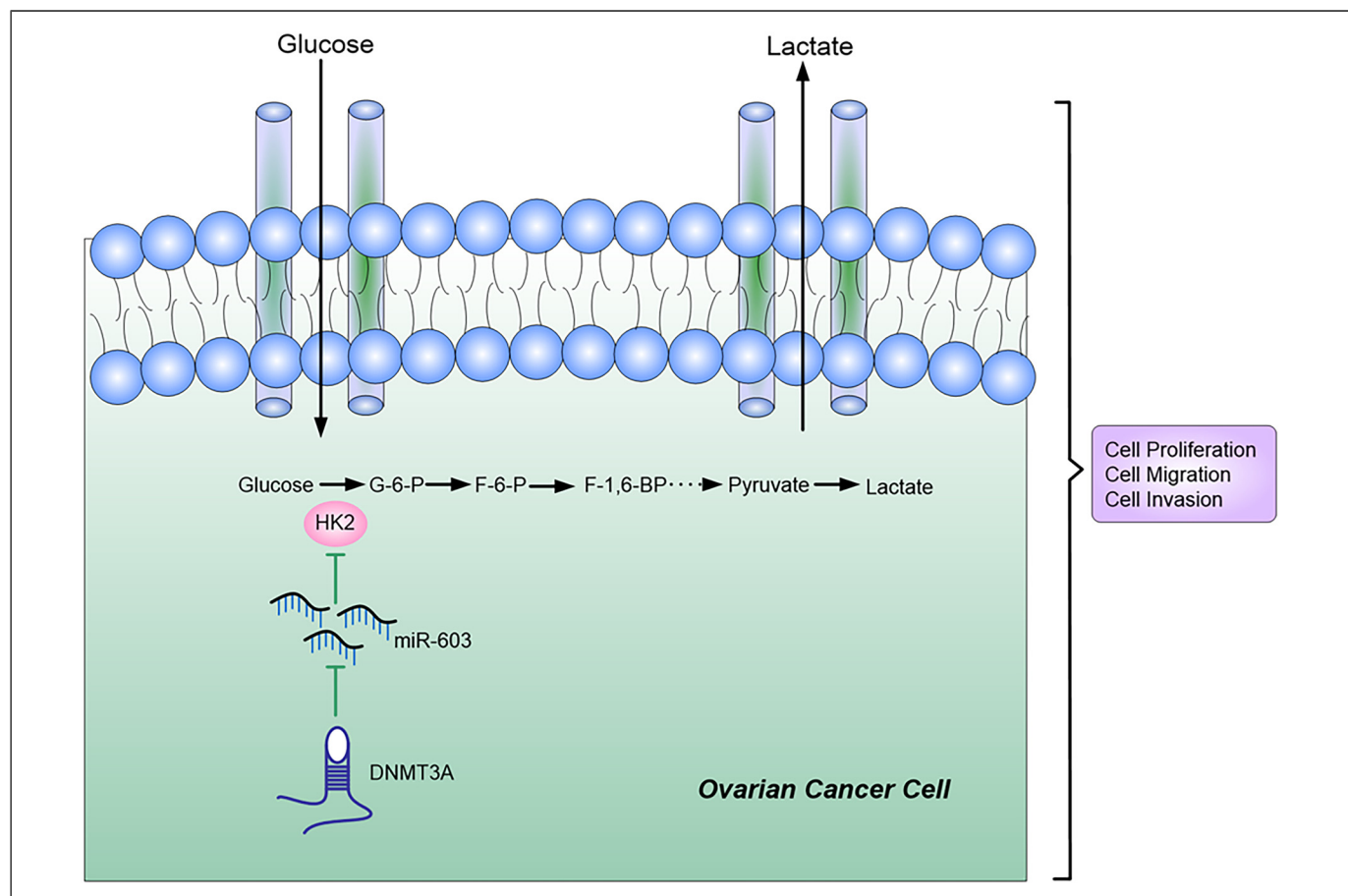


Figure 9. DNMT3A modulated the Warburg effect, proliferation, migration, and invasion of ovarian cancer cells by silencing miR-603 to induce the expression of HK2.

and made for concomitant accumulation of HK2. These results suggested that the knockdown of DNMT3A-mediated HK2 decay was at least in part due to the upregulation of miR-603.

We demonstrated that overexpression of DNMT3A-induced Warburg effect, migration, and invasion could be antagonized by overexpression of miR-603, and vice versa. However, we found overexpression of DNMT3A solely had no significant influence on ovarian cancer cell proliferation and that needs further experimental verification. Besides, miR-603 had no influence on DNMT3A expression in SKOV3 cells. These results indicated that DNMT3A mediated down-regulated expression of miR-603 in a unidirectional way and it could promote the Warburg effect, migration, and invasion in ovarian cancer. Since DNMT3A interference is associated with inhibition of ovarian cancer progression and promotes the inhibition of miR-603 on HK2, strategies targeting DNMT3A directly or indirectly by targeting its downstream genes such as miR-603 or HK2 may be a potential therapeutic approach against ovarian cancer.

In fact, DNMT3A inhibitors such as decitabine and guadecitabine (SGI-110) are commonly used to treat acute myeloid leukemia (AML) diseases.¹² Besides, Jueliger *et al* studied the efficacy of SGI-110 in preclinical models of hepatocellular carcinoma and revealed novel epigenetic anti-cancer effects of

SGI-110.⁴⁰ Overall, our results suggest that DNMT3A is involved in the Warburg effect, proliferation, migration, and invasion of ovarian cancer, which formed a DNMT3A/miR-603/HK2 axis to facilitate ovarian cancer tumorigenesis and development (Figure 9), indicating that understanding mechanism of DNMT3A regulation could provide the foundation for translation of DNMT3A inhibitors in ovarian cancer. Because of the conditions, the design of *in vivo* experiments will continue to be done in the future. In addition, we could not explain clearly why DNMT3A was influenced by miR-603 in co-transfected ovarian cancer cells and it should be further investigated in the future.


Conclusion

DNMT3A affects ovarian cancer progression via the miR-603/HK2 axis. DNMT3A are potential targets for anti-cancer therapy in ovarian cancer.

Ethics Statement

Our study did not require an ethical board approval because the data were released by the public database.

ORCID iD

Xu Li  <https://orcid.org/0000-0001-9551-8520>

Patient Consent for Publication

Not applicable.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article. This work was supported by the Foundation of the First Affiliated Hospital of Xi'an Jiaotong University (grant number 2019QN-07, 2020QN-27).

References

- Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi:10.3322/caac.21660.
- Nimmagadda S, Penet MF. Ovarian cancer targeted theranostics. *Front Oncol*. 2019;9:1537. doi:10.3389/fonc.2019.01537.
- Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol*. 1927;8(6):519-530. doi:10.1085/jgp.8.6.519.
- Potter M, Newport E, Morten KJ. The Warburg effect: 80 years on. *Biochem Soc Trans*. 2016;44(5):1499-1505. doi:10.1042/BST20160094.
- Garcia SN, Guedes RC, Marques MM. Unlocking the potential of HK2 in cancer metabolism and therapeutics. *Curr Med Chem*. 2020;26(41):7285-7322. doi:10.2174/0929867326666181213092652.
- Christofk HR, Vander Heiden MG, Harris MH, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature*. 2008;452(7184):230-233. doi:10.1038/nature06734.
- Guo W, Qiu Z, Wang Z, et al. MiR-199a-5p is negatively associated with malignancies and regulates glycolysis and lactate production by targeting hexokinase 2 in liver cancer. *Hepatology*. 2015;62(4):1132-1144. doi:10.1002/hep.27929.
- Sun L, Li J, Yan W, et al. H19 promotes aerobic glycolysis, proliferation, and immune escape of gastric cancer cells through the microRNA-519d-3p/lactate dehydrogenase A axis. *Cancer Sci*. 2021;112(6):2245-2259. doi:10.1111/cas.14896.
- Denisenko TV, Gorbunova AS, Zhivotovsky B. Mitochondrial involvement in migration, invasion and metastasis. *Front Cell Dev Biol*. 2019;7:355. doi:10.3389/fcell.2019.00355.
- Liberti MV, Locasale JW. The Warburg effect: how does it benefit cancer cells? *Trends Biochem Sci*. 2016;41(3):211-218. doi:10.1016/j.tibs.2015.12.001.
- Lyko F. The DNA methyltransferase family: a versatile toolkit for epigenetic regulation. *Nat Rev Genet*. 2018;19(2):81-92. doi:10.1038/nrg.2017.80.
- Zhang J, Yang C, Wu C, Cui W, Wang L. DNA methyltransferases in cancer: biology, paradox, aberrations, and targeted therapy. *Cancers (Basel)*. 2020;12(8), doi:10.3390/cancers12082123.
- Papakonstantinou E, Androutopoulos G, Logotheti S, Adonakis G, Maroulis I, Tzelepi V. DNA methylation in epithelial ovarian cancer: current data and future perspectives. *Curr Mol Pharmacol*. 2020. doi:10.2174/1874467213666200810141858.
- Iwakawa HO, Tomari Y. The functions of MicroRNAs: mRNA decay and translational repression. *Trends Cell Biol*. 2015;25(11):651-665. doi:10.1016/j.tcb.2015.07.011.
- Ye J, Zou M, Li P, Liu H. MicroRNA regulation of energy metabolism to induce chemoresistance in cancers. *Technol Cancer Res Treat*. 2018;17:1533033818805997. doi:10.1177/1533033818805997.
- Razavi ZS, Tajiknia V, Majidi S, et al. Gynecologic cancers and non-coding RNAs: epigenetic regulators with emerging roles. *Crit Rev Oncol Hematol*. 2021;157:103192. doi:10.1016/j.critrevonc.2020.103192.
- Shu J, Xiao L, Yan S, Fan B, Zou X, Yang J. Mechanism of MicroRNA-375 promoter methylation in promoting ovarian cancer cell malignancy. *Technol Cancer Res Treat*. 2021;20:1533033820980115. doi:10.1177/1533033820980115.
- Mussnich P, D'Angelo D, Leone V, Croce CM, Fusco A. The high mobility group A proteins contribute to thyroid cell transformation by regulating miR-603 and miR-10b expression. *Mol Oncol*. 2013;7(3):531-542. doi:10.1016/j.molonc.2013.01.002.
- Bayraktar R, Pichler M, Kanlikilicer P, et al. MicroRNA 603 acts as a tumor suppressor and inhibits triple-negative breast cancer tumorigenesis by targeting elongation factor 2 kinase. *Oncotarget*. 2017;8(7):11641-11658. doi:10.18632/oncotarget.14264.
- Ramakrishnan V, Xu B, Akers J, et al. Radiation-induced extracellular vesicle (EV) release of miR-603 promotes IGF1-mediated stem cell state in glioblastomas. *EBioMedicine*. 2020;55:102736. doi:10.1016/j.ebiom.2020.102736.
- Kushwaha D, Ramakrishnan V, Ng K, et al. A genome-wide miRNA screen revealed miR-603 as a MGMT-regulating miRNA in glioblastomas. *Oncotarget*. 2014;5(12):4026-4039. doi:10.18632/oncotarget.1974.
- Shabana AM, Xu B, Schneiderman Z, Ma J, Chen CC, Kokkoli E. Targeted liposomes encapsulating miR-603 complexes enhance radiation sensitivity of patient-derived glioblastoma stem-like cells. *Pharmaceutics*. 2021;13(8), doi:10.3390/pharmaceutics13081115.
- Lu J, Wang L, Chen W, et al. miR-603 targeted hexokinase-2 to inhibit the malignancy of ovarian cancer cells. *Arch Biochem Biophys*. 2019;661:1-9. doi:10.1016/j.abb.2018.10.014.
- Uhlen M, Fagerberg L, Hallstrom BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347(6220):1260419. doi:10.1126/science.1260419.
- Thul PJ, Akesson L, Wiking M, et al. A subcellular map of the human proteome. *Science*. 2017;356(6340). doi:10.1126/science.aal3321.
- Chandrashekar DS, Bashel B, Balasubramanya SAH, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia*. 2017;19(8):649-658. doi:10.1016/j.neo.2017.05.002.
- Tang Z, Kang B, Li C, Chen T, Zhang Z. GEPIA2: an enhanced web server for large-scale expression profiling and interactive

- analysis. *Nucleic Acids Res.* 2019;47(W1):W556-W560. doi:10.1093/nar/gkz430.
28. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal.* 2013;6(269):11. doi:10.1126/scisignal.2004088.
 29. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* 2012;2(5):401-404. doi:10.1158/2159-8290.CD-12-0095.
 30. Gyorffy B, Lanczky A, Szallasi Z. Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients. *Endocr Relat Cancer.* 2012;19(2):197-208. doi:10.1530/ERC-11-0329.
 31. Partheen K, Levan K, Osterberg L, Horvath G. Expression analysis of stage III serous ovarian adenocarcinoma distinguishes a subgroup of survivors. *Eur J Cancer.* 2006;42(16):2846-2854. doi:10.1016/j.ejca.2006.06.026.
 32. Gu Y, Yang P, Shao Q, et al. Investigation of the expression patterns and correlation of DNA methyltransferases and class I histone deacetylases in ovarian cancer tissues. *Oncol Lett.* 2013;5(2):452-458. doi:10.3892/ol.2012.1057.
 33. Chen CL, Yan X, Gao YN, Liao QP. Expression of DNA methyltransferase 1, 3A and 3B mRNA in the epithelial ovarian carcinoma. *Zhonghua Fu Chan Ke Za Zhi.* 2005;40(11):770-774.
 34. Zhang S, Pei M, Li Z, Li H, Liu Y, Li J. Double-negative feedback interaction between DNA methyltransferase 3A and microRNA-145 in the Warburg effect of ovarian cancer cells. *Cancer Sci.* 2018;109(9):2734-2745. doi:10.1111/cas.13734.
 35. Bai X, Song Z, Fu Y, et al. Clinicopathological significance and prognostic value of DNA methyltransferase 1, 3a, and 3b expressions in sporadic epithelial ovarian cancer. *PLoS One.* 2012;7(6):e40024. doi:10.1371/journal.pone.0040024.
 36. Lu W, Lu T, Wei X. Downregulation of DNMT3a expression increases miR-182-induced apoptosis of ovarian cancer through caspase-3 and caspase-9-mediated apoptosis and DNA damage response. *Oncol Rep.* 2016;36(6):3597-3604. doi:10.3892/or.2016.5134.
 37. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet.* 2012;13(4):227-232. doi:10.1038/nrg3185.
 38. Siu MKY, Jiang YX, Wang JJ, et al. Hexokinase 2 regulates ovarian cancer cell migration, invasion and stemness via FAK/ERK1/2/MMP9/NANOG/SOX9 signaling cascades. *Cancers (Basel).* 2019;11(6), doi:10.3390/cancers11060813.
 39. Taberlay PC, Jones PA. DNA Methylation and cancer. *Prog Drug Res.* 2011;67:1-23. doi:10.1007/978-3-7643-8989-5_1.
 40. Jueliger S, Lyons J, Cannito S, et al. Efficacy and epigenetic interactions of novel DNA hypomethylating agent guadecitabine (SGI-110) in preclinical models of hepatocellular carcinoma. *Epigenetics.* 2016;11(10):709-720. doi:10.1080/15592294.2016.1214781.