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miR-16-5p/PDK4-Mediated Metabolic Reprogramming Is Involved in Chemoresistance of Cervical Cancer

Zhao Zhao,[1] Mei Ji,[1] Qianqing Wang,[2] Nannan He,[1] and Yue Li[1]

[1]Department of Gynecology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China; [2]Department of Gynaecological Oncology, Xinxiang Central Hospital, Xinxiang 453000, China

Cervical cancer is one of the most prevalent malignancies in women worldwide. Therefore, investigation about molecular pathogenesis and related therapy targets of cervical cancer is an emergency. The molecular mechanisms responsible for the chemoresistance of cervical cancer were investigated by the use of doxorubicin (Dox)-resistant HeLa/Dox and SiHa/Dox cells. Our data showed that chemoresistant cells exhibited significantly higher glucose consumption, lactate production rate, and ATP levels than that of their parental cells. Among metabolic and glycolytic related genes, the expression of PDK4 was upregulated in Dox-resistant cells. Knockdown of PDK4 can decrease glucose consumption, lactate production rate, and ATP levels and further sensitize resistant cervical cancer cells to Dox treatment. By screening microRNAs (miRNAs), which can regulate expression of PDK4, we found that miR-16-5p was downregulated in chemoresistant cells. Overexpression of miR-16-5p can decrease the expression of PDK4 and sensitize the resistant cells to Dox treatment. Xenograft models confirmed that knockdown of PDK4 can increase chemotherapy efficiency for *in vivo* tumor growth. Collectively, our data suggested that miR-16-5p/PDK4-mediated metabolic reprogramming is involved in chemoresistance of cervical cancer.

INTRODUCTION

Cervical cancer is the second most common type of cancer for women worldwide.[1] On a global scale, an annual global incidence of 530,000 new cases has been observed, of which approximately half will lead to death.[2] Radiotherapy and chemotherapy have been considered as the major treatment methods for cervical cancer.[3] The patients detected at early or preinvasive stages are often curable with local treatments. However, the resistance to chemotherapeutic agents, such as doxorubicin (Dox) and etoposide (VP-16), will significantly inhibit therapy efficiency.[4,5] For this reason, the investigation about mechanisms involved in chemotherapy resistance remains an important priority for cervical cancer.

It has been well known that cancer cells can alter metabolic pathways to acquire nutrients and produce building blocks or energy for survival.[6,7] The most common example is that cancer cells have an intracellular metabolic shift from oxidative phosphorylation to glycolysis, which is also called the Warburg effect.[8] In addition, fatty acids, glutamine, and oxidative phosphorylation (OXPHOS) can also regulate cancer progression.[9] Recently, it has been revealed that metabolic reprogramming is involved in chemoresistance of cancer cells.[10] For example, miR-214 can modulate cisplatin sensitivity of osteosarcoma cells through regulation of anaerobic glycolysis.[11] The oxido-metabolic driver ATF4 can enhance temozolamide chemoresistance in human gliomas.[12] However, the roles of metabolic shift and its related mechanisms in chemoresistance of cervical cancer are not investigated.

In the present study, we compared the metabolic characterization of chemoresistant cells and their corresponding parental cells of cervical cancer. Further, the roles of pyruvate dehydrogenase kinase 4 (PDK4) in chemoresistance and its regulated mechanisms were investigated. The results indicated that PDK4 might be a promising therapeutic target for overcome Dox resistance of cervical cancer.

RESULTS

Chemoresistant Cervical Cancer Cells Showed a Metabolic Shift of Active Glycolysis and OXPHOS

First, we checked the Dox sensitivity of resistant and parental cervical cancer cells. Our data showed that Dox sensitivity of HeLa/Dox and SiHa/Dox cells was much less than that of HeLa and SiHa cells, respectively. For HeLa cells, the IC_{50} values of Dox to parental and resistant cells were 2.9 μM and 13.7 μM, respectively (Figure 1A). For SiHa cells, the IC_{50} values of Dox to parental and resistant cells were 2.2 μM and 16.7 μM, respectively (Figure 1B). The data confirmed that both HeLa/Dox and SiHa/Dox cells are resistant to Dox treatment. We further tested the growth rates between Dox resistant and parental cells. Results showed that the growth rates of both HeLa/Dox and SiHa/Dox cells were significantly less than that of their corresponding parental cells (Figure S1).

We then checked the metabolic profiles of HeLa/Dox and SiHa/Dox cells. Our data showed that the HeLa/Dox and SiHa/Dox cells showed significant increased glucose consumption (Figure 1C) and lactate

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Correspondence: Mei Ji, Department of Gynecology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China.
E-mail: meijizzu@aliyun.com

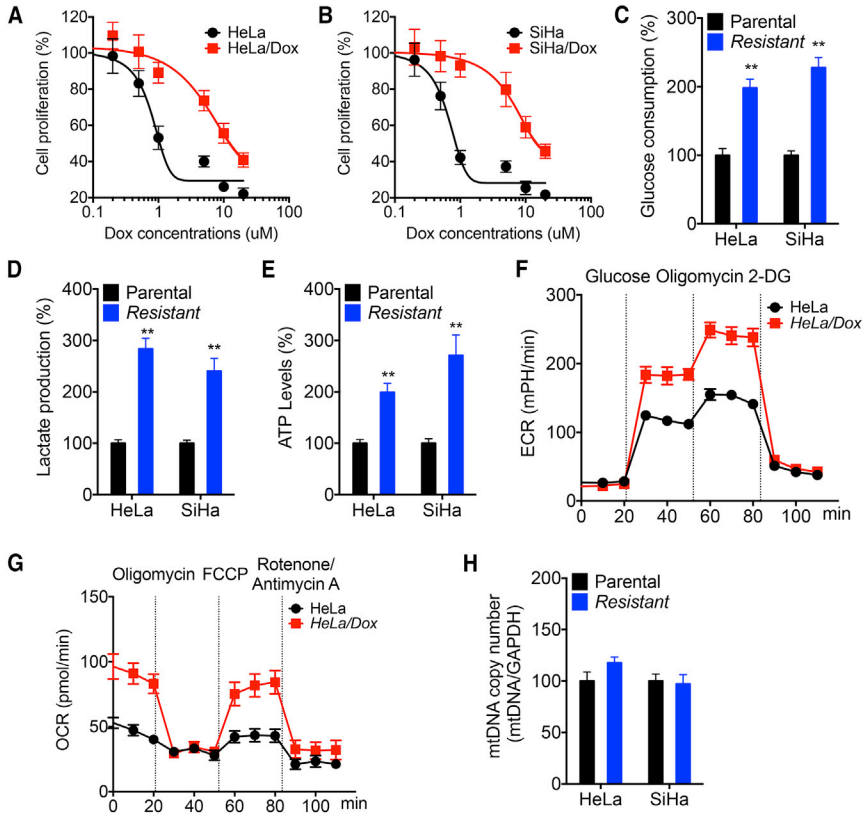


Figure 1. Chemoresistant Cervical Cancer Cells Showed a Metabolic Shift of Active Glycolysis and OXPHOS

(A and B) HeLa/Dox (A) and SiHa/Dox (B) or their corresponding sensitive cells were exposed to increasing concentrations of Dox for 24 h, and cell proliferation was tested. (C–E) The glucose consumption (C), lactate production (D), and ATP levels (E) in HeLa/Dox and SiHa/Dox or their corresponding sensitive cells. (F and G) The cellular ECAR (F) or OCR (G) of HeLa/Dox and HeLa cells was measured. (H) The relative levels of mitochondrial DNA (mtDNA) to GAPDH were measured in HeLa/Dox and SiHa/Dox or their corresponding parental cells. Data are presented as means ± SD of three independent experiments. **$p < 0.01$ compared with control.

production (Figure 1D) rates than that of their corresponding parental cells. Further, the extracellular ATP levels in HeLa/Dox and SiHa/Dox cells were significantly greater than that in their corresponding parental cells (Figure 1E). Seahorse analysis showed that HeLa/Dox cells showed increased extracellular acidification rate (ECAR), which reflects the overall glycolytic flux, than that of the parental cells (Figure 1F).

Cells produce ATP through glycolysis and mitochondrial OXPHOS. To determine which was involved in the chemoresistant cells, we measured the oxygen consumption rate (OCR) by a Seahorse Bioenergizer (Seahorse Bioscience), which is often an indicator of mitochondrial OXPHOS activity.[13] The results showed that HeLa/Dox cells exhibited a significantly elevated cellular OCR (Figure 1G), suggesting that the resistant cells may produce more ATP through upregulation of their mitochondrial OXPHOS.[14] We further checked the mitochondrial mass. Our data showed that the mitochondrial DNA content was comparable between HeLa/Dox and HeLa cells or between SiHa/Dox and SiHa cells (Figure 1H). All these data suggested that the chemoresistant cells showed increased ATP production, glycolysis, and OXPHOS than that of their parental cells.

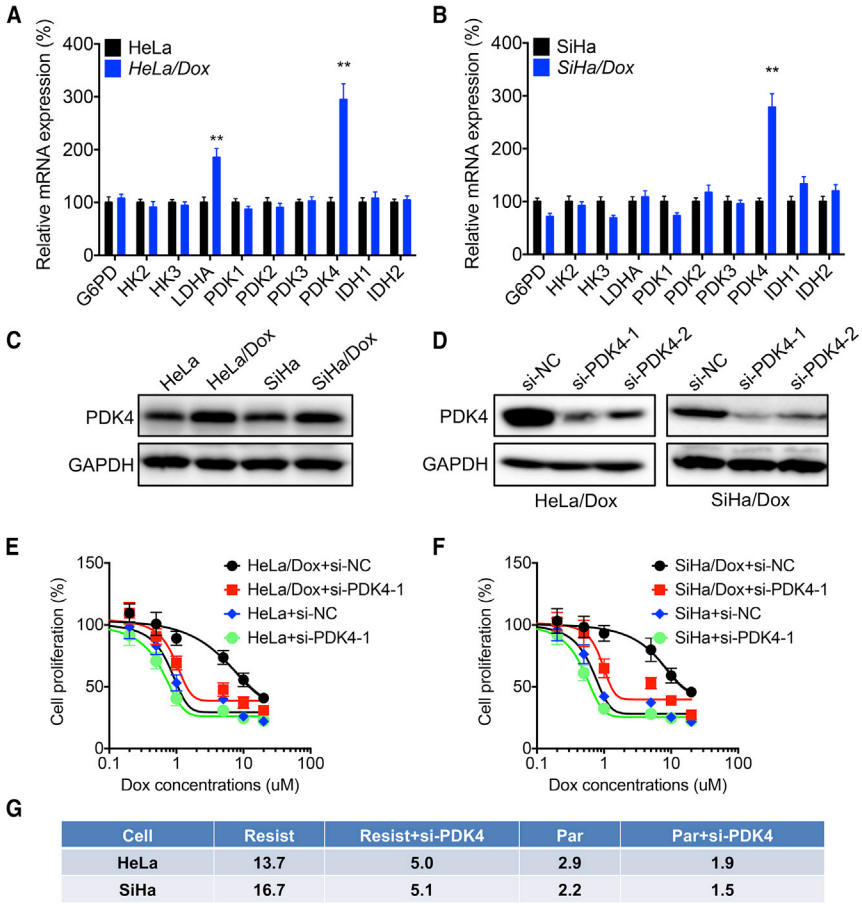
Upregulation of PDK4 Was Essential for Dox Resistance of Cervical Cancer Cells

In order to evaluate the mechanisms involved in the increased glycolysis of Dox-resistant cervical cancer cells, we checked the variation of key genes regulated the glycolysis including G6PD, HK2, HK3, LDHA, PDK1, PDK2, PDK3, PDK4, IDH1, and IDH2. Our data showed that PDK4 and LDHA were significantly increased in HeLa/Dox cells as compared with that in HeLa cells (Figure 2A). However, only PDK4, while not others, was increased in SiHa/Dox cells as compared with that in SiHa cells (Figure 2B). It indicated that the upregulation of LDHA might be cell line dependent and not essential for chemoresistance. Western blot analysis confirmed that PDK4 was upregulated in both HeLa/Dox and SiHa/Dox cells as compared to that in their corresponding parental cells (Figure 2C). Previous studies indicated that HIF-1α is an important regulator of chemoresistance.[14–17] Our data also showed that the mRNA and protein expression of HIF-1α in Dox resistant was significantly greater than that in parental cells (Figure S2).

To test whether PDK4 was involved in chemoresistance of cervical cancer cells, we knocked down the expression of PDK4 in HeLa/Dox and SiHa/Dox cells by use of its specific small interfering RNA (siRNA) (Figure 2D). Our data showed that si-PKD4 can significantly increase the Dox sensitivity of both HeLa/Dox (Figure 2E) and SiHa/Dox (Figure 2F) cells. Further, the IC_{50} values of Dox indicated that the response to knockdown of PDK4 in Dox-resistant cervical cancer cells was greater than that in parental cells (Figure 2G). However, knockdown of PDK4 had no effect on apoptosis of HeLa cells (Figure S3). All these results suggested that upregulation of PDK4 was essential for Dox resistance of cervical cancer cells.

PDK4 Regulated the Metabolic Shift of Chemoresistant Cervical Cancer Cells

We further evaluated whether PDK4 was involved in the metabolic shift of chemoresistant cervical cancer cells. Our data showed that si-PDK4 can attenuate the upregulation of glucose consumption (Figure 3A) and lactate production (Figure 3B) of HeLa/Dox cells as compared with that in HeLa cells. Consistently, si-PDK4 can also



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Cell	Resist	Resist+si-PDK4	Par	Par+si-PDK4
HeLa	13.7	5.0	2.9	1.9
SiHa	16.7	5.1	2.2	1.5

Figure 2. Upregulation of PDK4 Was Essential for Dox Resistance of Cervical Cancer Cells

(A and B) The mRNA expression of glycolysis related genes in HeLa/Dox (A) and SiHa/Dox (B) or their corresponding sensitive cells was checked by qRT-PCR. (C) The protein expression of PDK4 in HeLa/Dox and SiHa/Dox or their corresponding parental cells was checked by western blot analysis. (D) Cells were transfected with si-NC or si-PDK4-1/-2 for 24 h, the knockdown efficiency of PDK4 was checked. si-PDK4-1 was used for next studies. (E and F) HeLa/Dox (E) or SiHa/Dox (F) cells and their corresponding parental cells were transfected with si-negative control (si-NC) or si-PDK4-1 for 6 h and then further treated with increasing concentration of Dox for 24 h. (G) The IC_{50} values (μM) of all group cells treated with increasing concentration of Dox for 24 h. Data are presented as means ± SD of three independent experiments. **$p < 0.01$ compared with control.

reverse the upregulation of extracellular ATP levels of HeLa/Dox cells than that of HeLa cells (Figure 3C). Seahorse analysis showed that si-PDK4 can decrease the ECAR of HeLa/Dox cells (Figure 3D). However, the effects of si-PDK4 on metabolic characteristics of HeLa/Dox cells were attenuated in HeLa cells (Figures 3A–3D), which might be because chemoresistant cells were more reliable on glycolysis. These results suggested that PDK4 regulated the metabolic shift of chemoresistant cervical cancer cells.

The Upregulation of PDK4 in Chemoresistant Cells Was Due to Upregulation of mRNA Stability

We further investigated the mechanisms responsible for upregulation of PDK4 in chemoresistant cancer cells. First, we checked the promoter activity of PDK4 in cervical cancer cells by luciferase assay. Our data showed that the promoter activity of PDK4 in HeLa/Dox and SiHa/Dox cells was comparable with that in their corresponding parental cells (Figure 4A). Further, there is no significant difference between the precursor (Figure 4B) or nucleus export (Figure 4C) of PDK4 mRNA in HeLa/Dox and SiHa/Dox cells as compared with that in their corresponding parental cells. However, our data showed that the mRNA stability of PDK4 in HeLa/Dox (Figure 4D) and SiHa/Dox (Figure 4E) cells was significantly greater than that in their corresponding parental cells. Further, the protein stability of PDK4 in HeLa/Dox cells was comparable with that in HeLa cells (Figure 4F). Consistently, the protein stability of PDK4 in SiHa/Dox cells was comparable with that in SiHa cells (Figure 4G). All these data indicated that the upregulation of PDK4 in chemoresistant cells was due to the upregulation of mRNA stability.

miR-16-5p Regulated the Expression of PDK4 in Cervical Cancer Cells

Since miRNA can bind with the 3′ UTR of mRNA to regulate the stability of its target,[18] the expression of miR-182,[19] miR-15b-5p,[20] and miR-16-5p was measured in chemoresistant cells. Our data showed that the expression of miR-16-5p, while not the other two miRNAs, was significantly decreased in HeLa/Dox cells as compared to that in HeLa cells (Figure 5A). Consistently, only miR-16-5p was decreased in SiHa/Dox cells as compared to that in SiHa cells (Figure 5B). Further, the mimic of miR-16-5p can significantly decrease the protein (Figure 5C) and mRNA (Figure 5D) expression of PDK4 in both HeLa/Dox and SiHa/Dox cells. This might be because the miR-16-5p mimic can decrease the half-life of PDK4 mRNA in HeLa/Dox cells (Figure 5E). To ensure that PDK4 mRNA was the direct target of miR-16-5p, we cloned the 3′ UTR of PDK4 to generate pmirGLO-*PDK4*-3′ UTR and mutant (Mut) the bind sites between miR-16-5p to generate pmirGLO-*PDK4*-3′ UTR-Mut (Figure 5F). Our data showed that miR-16-5p can significantly decrease the luciferase activity of pmirGLO-*PDK4*-3′ UTR, while had limited effects on that of pmirGLO-*PDK4*-3′ UTR-Mut in both HeLa and SiHa cells (Figures 5G and 5H). It suggested that miR-16-5p regulated the expression of PDK4 in chemoresistant cervical cancer cells.

Further, we treated HeLa/Dox cells with dichloroacetate (DCA), a PDK inhibitor.[21,22] The results showed that DCA can also increase the Dox sensitivity of HeLa/Dox cells (Figure S4A). However, DCA

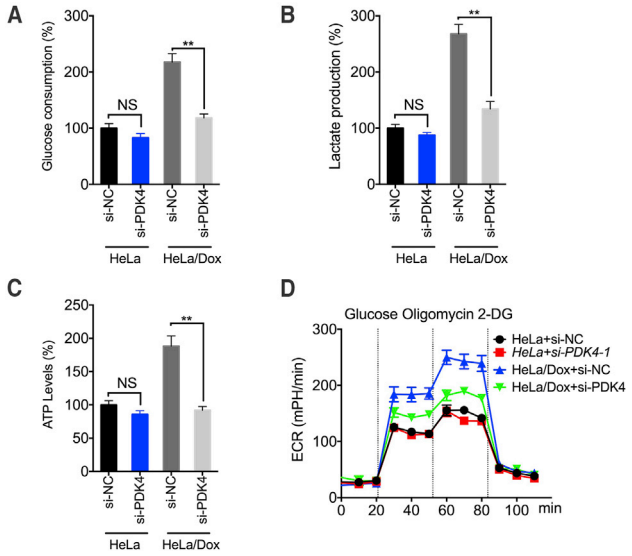

Figure 3. PDK4 Regulated the Metabolic Shift of Chemoresistant Cervical Cancer Cells

(A–D) HeLa and HeLa/Dox cells were transfected with si-NC or si-PDK4-1 for 24 h, the glucose consumption (A), lactate production (B), ATP levels (C), and cellular ECAR (D) were checked. Data are presented as means ± SD of three independent experiments. **$p < 0.01$ compared with control; NS, not significant.

had no effect on the expression of miR-16-5p in either HeLa/Dox or SiHa/Dox cells (Figure S4B). It suggested that miR-16-5p can decrease the expression of PDK4 while PDK4 had no effect on the expression of miR-16-5p.

miR-16-5p Regulated the Glycolysis and Proliferation of Cervical Cells via a PDK4-Dependent Manner

We then investigated the potential roles of miR-16-5p in the progression of cervical cancer. Our data showed that miR-16-5p mimic can sensitize both HeLa/Dox (Figure 6A) and SiHa/Dox (Figure 6B) cells to Dox treatment. Further, in HeLa/Dox cells transfected with PDK4, the miR-16-5p mimic-increased Dox sensitivity was attenuated (Figure 6C). HeLa/Dox cells transfected with PDK4 can attenuate miR-16-5p-suppressed colonization (Figure S5). In addition, we found that miR-16-5p mimic (Figure 6D) can decrease the glucose consumption (Figure 6E), lactate production (Figure 6F), and ATP levels (Figure 6G) in HeLa/Dox cells. However, overexpression of PDK4 can restore the miR-16-5p mimic-induced downregulation of glucose consumption, lactate production, and ATP generation (Figures 6E–6G). The data suggested that miR-16-5p regulated the glycolysis and proliferation of chemoresistant cervical cells via a PDK4-dependent manner.

miR-16-5p/PDK4 Axis Regulated Chemotherapy Efficiency and *In Vivo* Progression of Cervical Cancer

We then evaluated the potential effects of PDK4 on the chemotherapy efficiency of cervical cancer by use of xenograft models. As observed in the results, HeLa/Dox cell group showed significant less sensitivity to Dox treatment than that of HeLa groups (Figures 7A and 7B). However, sh-PDK4 in HeLa/Dox cells can obviously increase the Dox sensitivity (Figures 7A and 7B). The results of immunohistochemistry (IHC) (Figure 7C) and western blot analysis (Figure S6A) confirmed that PDK4 was increased in HeLa/Dox xenograft and sh-PDK4 worked effectively in the primary tumor xenografts. Further, we compared the levels of miR-16-5p in the three xenograft groups. The results showed that the expression of miR-16-5p in HeLa/Dox xenografts was significantly less than that in HeLa xenografts; however, there is no significant difference for the expression of miR-16-5p between HeLa/Dox and HeLa/Dox +sh-PDK4 groups (Figure S6B). It indicated that PDK4 regulated the chemotherapy efficiency of cervical cancer.

At this point, we asked whether there was a link between miR-16-5p/PDK4 axis and clinical cervical cancer development. Using the online Kaplan-Meier plotter bioinformatics tool, we found that cervical cancer patients with decreased miR-16-5p expression showed significantly reduced overall survival (OS; Figure 7D). Consistently, cervical cancer patients with increased PDK4 expression showed significantly reduced OS (Figure 7E). In cervical cancer tissues, the expression of PDK4 was significantly negative correlated with that of miR-16-5p (Figure 7F). These results suggested that miR-16-5p/PDK4 axis can regulate the clinical progression of cervical cancer.

DISCUSSION

The roles of metabolic shift and its mechanisms in chemoresistance of cancer cells are not well illustrated in cervical cancer. Our present study revealed that chemoresistant cervical cancer cells showed a metabolic signature of active glycolysis via upregulation of PDK4. Further, PDK4 can regulate the glycolysis and chemosensitivity of cervical cancer cells. Mechanistically, the upregulation of PDK4 in chemoresistant cancer cells was due to the increase of mRNA stability rather than transcription or protein stability. Further, the downregulation of miR-16-5p, which can directly bind to the 3′ UTR of PDK4, was responsible for the upregulation of PDK4 in chemoresistant cancer cells. The miR-16-5p/PDK4 axis can regulate the glycolysis, proliferation, and *in vivo* growth of cervical cancer cells and clinical progression of cervical cancer.

Aerobic glycolysis has important roles in sustaining cancer cell survival and proliferation.[23] Recently, increasing evidence suggested that enhanced glycolysis was also involved in therapy resistance of cancer cells.[10] Our data showed that Dox-resistant cells showed increased levels of glucose consumption, lactate production, and extracellular ATP levels. In addition, the levels of ECAR and cellular OCR were also increased in chemoresistant cells. Consistently, recent studies revealed that chemoresistant cells reprogram metabolic pathways via increasing ATP generation and OCR.[24,25] Transient elevation of glycolysis can enhance the radio-resistance by facilitating DNA repair in cancer cells.[26] Inhibition of glycolysis can sensitize HepG2 cells to Dox treatment.[27] Several mechanisms have been involved in this metabolic shift. For example, miR-186-3p/EREG axis can orchestrate tamoxifen resistance and aerobic glycolysis in

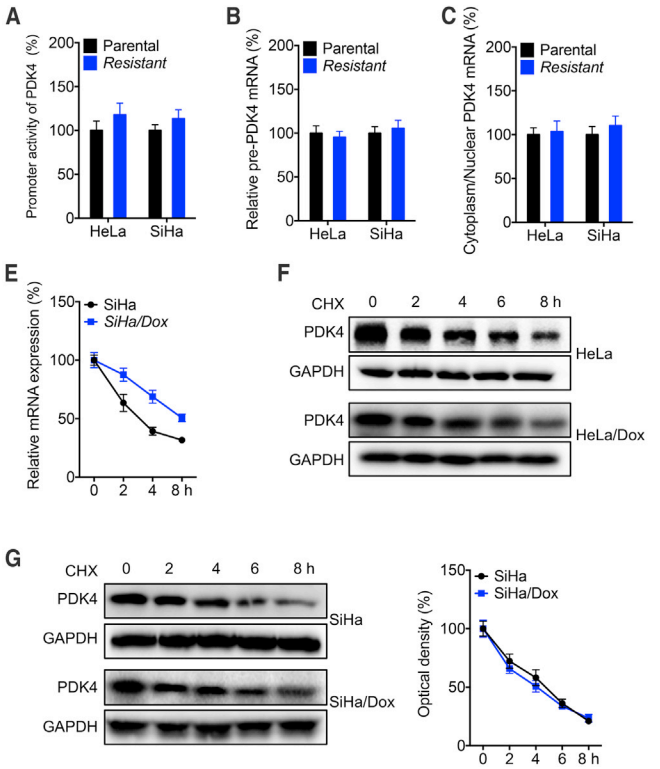

Figure 4. The Upregulation of PDK4 in Chemoresistant Cells Was Due to Upregulation of mRNA Stability

(A) The promoter activity of PDK4 in HeLa/Dox and SiHa/Dox or their corresponding sensitive cells. (B) The relative precursor mRNA of PDK4 in HeLa/Dox and SiHa/Dox or their corresponding sensitive cells. (C) The relative levels of PDK4 mRNA in cytosol/nucleus of HeLa/Dox and SiHa/Dox or their corresponding sensitive cells. (D and E) HeLa/Dox (D) and SiHa/Dox (E) or their corresponding sensitive cells were treated with Act-D for the indicated time periods, the mRNA of PDK4 was checked. (F) Both HeLa and HeLa/Dox cells were treated with CHX for the indicated time periods, the protein of PDK4 was checked and quantitatively analyzed. (G) Both SiHa and SiHa/Dox cells were treated with CHX for the indicated time periods, the protein of PDK4 was checked and quantitatively analyzed. Data are presented as means ± SD of three independent experiments.

breast cancer cells.[28] miR-214 can regulate the anaerobic glycolysis to confer cisplatin sensitivity of osteosarcoma cells,[11] while enolase1 can stimulate glycolysis to promote chemoresistance in gastric cancer.[29] These data were consistent with our present findings that increased glycolysis and ATP levels have been observed in Dox-resistant cervical cancer cells.

We found that PDK4 was essential for the increased glycolysis and chemoresistance of cervical cancer cells. PDK4 is the key enzyme involved in the Warburg effect, which can phosphorylate pyruvate dehydrogenase (PDH) and switch metabolic from mitochondrial respiration to cytoplasmic glycolysis.[30] PDK4 has been implicated in the progression of several cancers such as hepatocellular carcinoma, bladder cancer, and colon cancer.[21,31] For example, PDK4 was upregulated in endometrial cancer cells to trigger its malignancy.[32] Knockdown of PDK4 can inhibit the proliferation of lung cancer cells via decreasing PDH flux and *de novo* lipogenesis.[22] Our data showed that knockdown of PDK4 can sensitize cervical cancer cells to Dox treatment. This was consistent with recent studies that PDK4 was upregulated in antiestrogen resistance in human breast cancer cells.[33] and cisplatin resistance of bladder cancer cells.[31] Our results, together with the published data, confirmed that PDK4 is critical for the metabolic shift and chemoresistance of cancer cells.

PDK4 can be regulated by miRNAs in cancer cells.[19,34] We found that miR-16-5p was downregulated in chemoresistance of cervical cancer cells and responsible for the upregulation of PDK4. Further, miR-16-5p mimic can also regulate the glycolysis and chemoresistance of cervical cancer cells via PDK4. Consistently, previous studied indicated that miR-16-5p can inhibit the proliferation and migration of chordoma cells[35] and enhance radiosensitivity of prostate cancer cells through modulating Cyclin D1/E1 expression.[36] It has been reported that PDK4 could be regulated by several critical transcriptional activators such as peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1), forkhead box O1 (FOXO1), or Rb- E2F transcription factor 1 (E2F1).[34,37,38] Due to the transcription of PDK4 was not variated in chemoresistance cells, these factors might be not responsible for the upregulation of PDK4.

In summary, we showed that miR-16-5p/PDK4 can regulate the chemoresistance of cervical cancer cells via regulation of glycolysis. Our results provided strong interconnection between glycolysis and chemoresistance and reinforced the view that PDK4 might be a promising therapeutic target for cervical cancer patients.

MATERIALS AND METHODS

Cells and Cell Culture

HeLa, SiHa, HeLa/Dox, and SiHa/Dox cells were purchased from Procell Life Science and Technology (Wuhan, China) and cultured with DMEM medium supplementary with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. To maintain the drug resistance, we added 500 ng/mL Dox (Sigma-Aldrich) in the medium for Dox-resistant cell for cell culture. The Dox maintaining of resistant cells was withdrawn 3 days before experiment.

Cell Proliferation Assay

The cell proliferation was evaluated by use of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay according

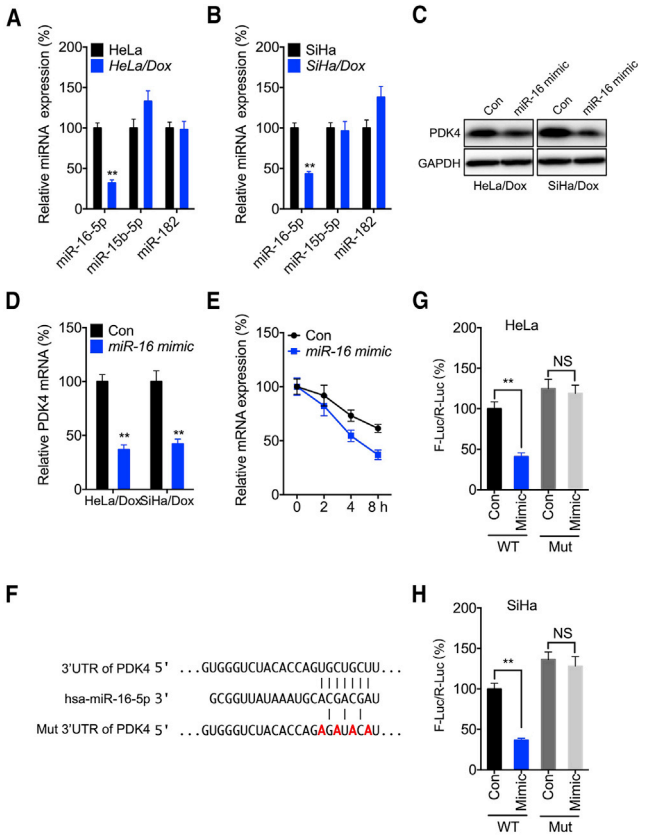

Figure 5. miR-16-5p Regulated the Expression of PDK4 in Cervical Cancer Cells

(A and B) The expression of miRNAs in HeLa/Dox (A) and SiHa/Dox (B) or their corresponding sensitive cells was checked. (C and D) After they were transfected with control or miR-16-5p mimic for 24 h, the protein (C) and mRNA (D) of PDK4 were measured. (E) After they were pre-transfected with control or miR-16-5p mimic for 24 h, cells were further treated with Act-D for the indicated times. (F) The wild-type and mutation of 3′ UTR and its bind sites with miR-16-5p. (G and H) HeLa (G) or SiHa (H) cells were transfected with PDK4 3′ UTR-wild-type or mutant (Mut) for 6 h and further transfected with control or miR-16-5p mimic. Data are presented as means ± SD of three independent experiments. **$p < 0.01$ compared with control; NS, not significant.

to the previous study.[39] Briefly, cells were seeded in 96-well plates at a density of 3.0×10^3 cells and cultured for 12 h before treatment with increasing concentrations of Dox. At the end of the experiment, cells were incubated with 10 μL MTT (a final concentration of 0.5 g/L) for 4 h and assessed by measuring absorbance at 570 nm using a microplate reader. The relative cell proliferation was calculated as the percentage of the values obtained for the controls.

Glucose Consumption, Lactate Production, and ATP Level Assays

The glucose consumption, lactate production, and ATP level assays were conducted according to the previous study.[40] After treatment, the culture medium was collected and measured by use of Lactate Assay kit (catalog number, K607-100, BioVision, CA, USA) and glucose assay kit (catalog number, CBA086, Sigma-Aldrich) for lactate production and glucose consumption according to the instructions of the manufacturers, respectively. The levels of ATP were assessed using a colorimetric ATP Assay Kit (catalog number, ab83355, Abcam, Cambridge, UK) according to the instructions of the manufacturer.

Assays of ECAR and OCR

Cells were seeded a 96-well plate with a density of 10,000 cells/well and treatment as indicated. Then, cells were treated with ECAR reagents according to the manufacturer's recommendations (ab197244, Abcam, UK). The ECAR signals were collected at 5 min intervals for about 120 min by micro-plate reader system (Victor,[3] PerkinElmer) using excitation and emission wavelengths of 380 and 615 nm, respectively. The OCR was measured as described in the present study[41] using XF24 Analyzer (Seahorse). Briefly, cells were equilibrated with bicarbonate-free DMEM medium supplemented with 25 mM glucose, 1 mM pyruvate, and 2 mM glutamine. The OCR was measured at baseline and after addition of each chemicals. All measurements were done in 5 wells per condition per experiment and repeated at least 3 times.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

After treatment, total RNAs were isolated from cells using the TRIzol reagent (catalog number, 15596018, Invitrogen) according to the manufacturer's protocol. The concentration of RNA was measured using the Ribogreen RNA quantification kit. For RNA measurement, cDNA was synthesized using PrimeScript RT Master Kit (catalog number, RR037B, Takara, Dalian, China) and analyzed by SYBR green methods as described previously[40] with the program of 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 45 s. The sequence of primers was listed in Table S1. The expression of target transcript was calculated by $2^{-\Delta\Delta CT}$ method. GADPH and U6 was used as the loading control for mRNA and miRNA, respectively.

Western Blot Analysis

Cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) buffer containing 1X protease inhibitor cocktail (catalog number, 11697498001, Roche) on ice for 30 min. Equal protein was separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane (catalog number, n0139, Millipore, Bedford, MA, USA) at 80 V for 2 h. The protein was probed with primary antibodies (all from Abcam) at 1:1,000 dilution overnight at 4°C and then incubated with horseradish peroxidase-labeled secondary antibodies at 1:5,000 dilution for 1 h at room temperature. The protein was visualized by enhanced chemiluminescence (ECL, catalog number, RPN2232, GE Healthcare). GAPDH was used as the loading internal control. The results of densitometric analyses of western blots, obtained using ImageJ software, were presented as the relative optical density (%) to the control (GAPDH).

Plasmid and siRNA Transfection

The pcDNA3.1 vector and pcDNA 3.1/PDK4 were purchased from Hanbio (Shanghai, China). The siRNA negative control, si-PDK4-1

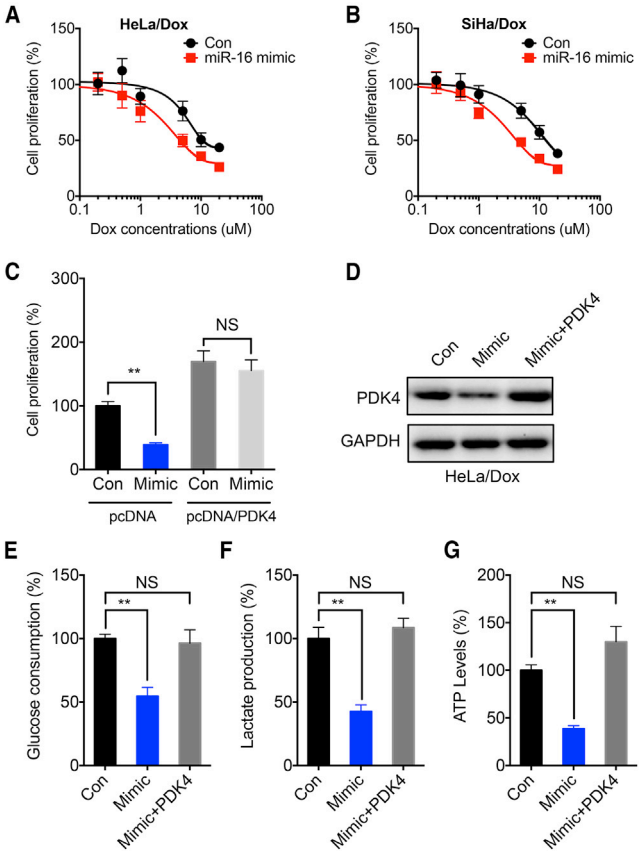

Figure 6. miR-16-5p Regulated the Glycolysis and Proliferation of Cervical Cells via a PDK4-Dependent Manner

(A and B) HeLa/Dox (A) and SiHa/Dox (B) were pre-transfected with control or miR-16-5p mimic for 12 h and then further treated with increased concentrations of Dox for 24 h. (C) HeLa/Dox cells were pre-transfected with control, miR-16-5p mimic, vector control, and pcDNA/PDK4 for 12 h and then further treated with 5 μM Dox for 24 h. (D–G) HeLa/Dox cells were treated transfected with control, miR-16-5p mimic, or miR-16-5p mimic/PDK4 construct for 24 h (D), the glucose consumption (E), lactate production (F), and ATP levels (G) were analyzed. Data are presented as means ± SD of three independent experiments. **$p < 0.01$ compared with control, NS, not significant.

(5′-GGACGTAAGAGATTCTCAT-3′), and si-PDK4-2 (5′-GGATTTGGTGGAGTTCCAT-3′) were purchased from Genepharm (Shanghai, China). The miR-16b-5p mimic (5′-GCGGUUAUAAAUGCACGACGAU-3′) and negative control (NC; 5′-AUUUGCCAGG UCGGA AUG-3′) were synthesized and purchased from Genepharm (Shanghai, China). The transfection was performed for cells (4×10^5 cells) seeded in 6-well plate using Lipofectamine 2000 reagent (catalog number, 11668019, Invitrogen) according to the manufacturer's instructions. For plasmid, 4 μg of plasmid was used for each well. For siRNA, the working concentration was 20 nM.

Promoter Activity and Luciferase Assay

The promoter (−1 kb) of wild-type PDK4 was amplified by PCR and cloned into the dual-luciferase reporter plasmid (Promega) pGL3-basic to yield pGL3-PDK4 reporter. The reporter was transfected into both parental and Dox-resistant cervical cancer cells seeded in 96-well plates at the density of 1.5×10^4 cells per well using the Attractene Transfection Reagent (catalog number, 301005, QIAGEN). The firefly luciferase activity was measured and normalized to that of Renilla luciferase. To analyze the effect of miR-16-5p on PDK4, we cloned the 3′ UTR of PDK4 into pmirGLO plasmid to generate pmirGLO-PDK4 plasmid. The effect of miR-16-5p on 3′ UTR of PDK4 was assessed by using the Attractene Transfection Reagent (QIAGEN).

Nuclear Export of PDK4 mRNA

The cytosol and nucleus fractions of cervical cancer cells were separated using the NE-PER-extraction kit (catalog number, 78833, Pierce) according to the manufacturer's instructions. Then RNAs were extracted by use of Trizol. The levels of PDK4 mRNA were checked by qRT-PCR.

mRNA and Protein Stability Assay

As to mRNA stability, cells were treated with 5 μg/mL actinomycin D (Act-D, catalog number A9415, Sigma, St. Louis, MO, USA) and incubated for the indicated time periods. The mRNA was checked by qPCR. As to protein stability, cells were treated with 100 μg/mL cycloheximide (CHX, catalog number 01810, Sigma) and incubated for the indicated time periods. The protein was checked by western blot analysis.

Animal Study

No human-related experiment was performed. The animal study was conducted according to the guidelines of the Animal Care and Use Committee of our hospital. Animals were maintained in accordance to the guidelines of the American Association of Laboratory Animal Care. The BALB/c nude mice (4 weeks old) were raised under pathogen-free conditions, and all animal experiments complied with the policy on the care and use of laboratory animals of our hospital. HeLa, HeLa/Dox, and HeLa/Dox-shPDK4 cells (2×10^6 per mouse) were diluted in 100 μL normal medium + 100 μL Matrigel (BD Biosciences) and subcutaneously injected into immunodeficient mice to investigate tumor growth. When the tumor was visible, a dose of 3.6 mg Dox/kg body weight (about 0.05 mL/28 g mouse) was injected into the lateral tail vein of the mice once each 3 days for five times. Tumor size was monitored every 3 days. At the end of experiments, mice were sacrificed and the tumors were removed and analyzed by use of IHC to measure the expression of PDK4 according to previous study.[42]

Database (DB) Analysis

The expression profiles of miR-16-5p and PDK4 in cervical cancer patients were downloaded from LinkedOmics (http://www.linkedomics.org) to analyze their correlation. LinkedOmics is a publicly available portal that includes multi-omics data from The Cancer Genome Atlas (TCGA) project.[32] The overall survival rate of cervical cancer patients was assessed by Kaplan Meier-plotter (KM plotter,

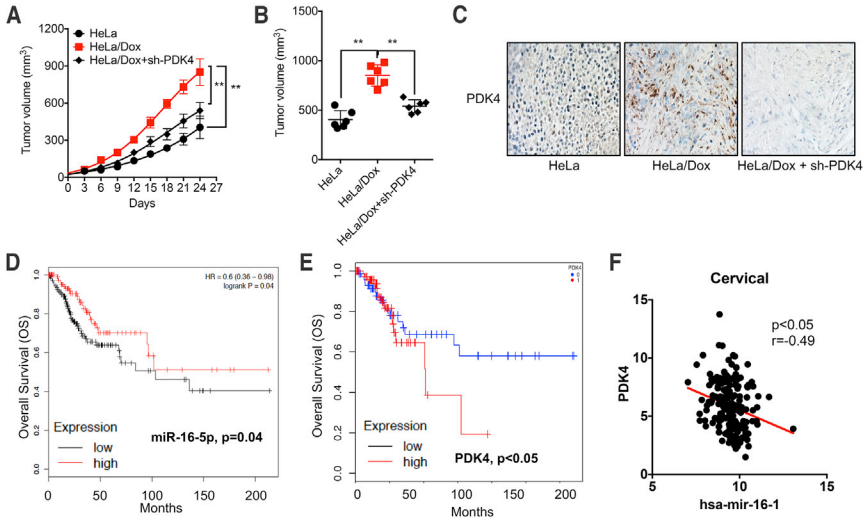

Figure 7. miR-16-5p/PDK4 Axis Regulated Chemotherapy Efficiency and *In Vivo* Progression of Cervical Cancer

(A) HeLa, HeLa/Dox, and HeLa/Dox+sh-PDK4 cells were used to generate xenograft models (n = 6 for each group). The tumor growth was measured every 3 days. (B) The tumor volume of each group at the end of the experiment. (C) The expression of PDK4 in each group was measured by immunohistochemistry (IHC). (D) OS of cervical cancer patients with high (n = 78) and low (n = 226) levels of miR-16-5p was plotted according to the Kaplan-Meier method. (E) OS of cervical cancer patients with high (n = 76) and low (n = 75) levels of PDK4 was plotted according to the Kaplan-Meier method. (F) The correlation between PDK4 and miR-16-5p in 169 cervical cancer patients with the data collected from TCGA. **p < 0.01 compared with control.

http://kmplot.com/analysis/)[43] based on GEO (Affymetrix microarrays only), EGA, and TCGA databases.

Statistical Analysis

The analysis was performed by use of SPSS software (SPSS, Chicago, IL, USA). Comparisons of two independent groups were analyzed using the two-tailed Student's t test. Comparisons of three or more groups were analyzed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.05.008.

AUTHOR CONTRIBUTIONS

Data collecting: Z.Z., M.J., and Y.L. Writing: Z.Z., M.J., and Q.W. Data analysis: Z.Z., N.H., and Y.L. Design: M.J., Q.W., N.H., and Y.L.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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