Tropical Journal of Pharmaceutical Research November 2019; 18 (11): 2299-2304
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i11.10

Original Research Article

MiR-646 targets PDK1 to recede aerobic glycolysis and cell proliferation in nasopharyngeal carcinoma

Kaiquan Zhu[1], Renyu Lin[1], Aidan Xia[2]*
[1]Department of Otolaryngology, The First Affiliated Hospital of Wenzhou Medical University, [2]Department of Hematology and Oncology, Wenzhou People's Hospital, Wenzhou City, Zhejiang Province 325000, China

For correspondence: **Email: AidanXiadyu@163.com; **Tel:** +86-577-88306139*

Sent for review: 4 September 2019 *Revised accepted: 30 October 2019*

Abstract

Purpose: *To investigate the effect and mechanism of miR-646 on aerobic glycolysis and cell proliferation in nasopharyngeal carcinoma.*
Methods: *MiR-646 expression in human nasopharyngeal carcinoma cell lines was determined by quantitative real-time polymerase chain reaction) (qRT-PCR). Cell counting kit-8 (CCK8) was used to evaluate cell viability, and colony formation assay was also performed. The target of miR-646 was determined by luciferase activity assay. The effect of miR-646 on aerobic glycolysis was assessed via glucose uptake, and lactate and ATP production. Western blot analysis was conducted to unravel the underlying mechanism involved in the regulation of miR-646 in nasopharyngeal carcinoma.*
Results: *MiR-646 was downregulated in human nasopharyngeal carcinoma cell lines. MiR-646 mimics decreased cell viability and inhibited cell proliferation, whereas miR-646 inhibitor increased cell viability and promoted cell proliferation. Pyruvate dehydrogenase kinase 1(PDK1) was identified as a target of miR-646, and its expression was negatively regulated by miR-646. MiR-646 probably inhibited aerobic glycolysis via regulation of PDK1, as shown by decreased glucose uptake and decreased lactate and ATP production. The inhibitory effect of miR-646 on nasopharyngeal carcinoma cell proliferation was partly via PDK1 regulation.*
Conclusion: *MiR-646 inhibits aerobic glycolysis in nasopharyngeal carcinoma and promotes cell proliferation via suppression of PDK1, suggesting miR-646 as a potential therapeutic target in nasopharyngeal carcinoma.*

Keywords: *MiR-646, Aerobic glycolysis, Proliferation, PDK1, Nasopharyngeal carcinoma*

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is widely known as a malignant tumor [1,2]. The onset of NPC is insidious and difficult to diagnose due to fast growth rate and metastasis [3]. The incidence of NPC increases with age, and the prognosis becomes worse [4]. Therefore, it will be of great clinical significance to investigate the occurrence and development of NPC at the molecular level in order to improve the early diagnosis rate, select the best treatment plan, predict the prognosis, and improve the survival rates of patients. Increasing evidences show that an imbalance in miRNA (microRNA) expression is related to the formation of NPC [5]. For

example, miR-26a inhibits NPC tumorigenesis [6], and miR-218 suppressed NPC development [7]. Recently, miR-646 was reported to inhibit cell proliferation and metastasis in gastric cancer [8], and to be associated with tumor metastasis in clear cell renal carcinoma [9]. The role of miR-646 in the pathogenesis of NPC remains to be reported.

Aerobic glycolysis, also called the Warburg effect, converts glucose to lactic acid for the growth of tumor cells, and is considered an important hallmark of tumor growth [10]. Several key rate-limiting glycolytic enzymes are dysregulated in aerobic glycolysis and contribute to cell proliferation and metastasis in NPC [11]. Pyruvate dehydrogenase kinase (PDK1), responsible for glucose metabolism via aerobic glycolysis in cancer cells [12], is an important therapeutic target in treatment of NPC [13].

The focus of this study is to investigate whether miR-646 binds to PDK1 to regulate aerobic glycolysis and cell proliferation in NPC.

EXPERIMENTAL

Cell culture

Human NPC cell lines (5-8F, SUNE-1, C666-1, 6-10B), human nasal epithelial cells (HNEpC), and human embryonic kidney 293 cells (HEK-293) were acquired from Sun Yat-Sen University Cancer Center (Guangzhou, China) and cultured in RPMI-1640 medium (Gibco, Waltham, MA, USA) at 37 °C.

Cell transfection

The miR-646 mimics, miR-646 inhibitor, and the corresponding controls were purchased from RiboBio (Guangzhou, China). The plasmid pcDNA3.1-PDK1 was obtained from AxyBio (Changsha, China). 5-8F cells (3×10^4) were seeded into 12-well plates overnight and then transfected with pcDNA3.1-PDK1, miR-646 mimics, miR-646 inhibitor or the negative controls via Lipofectamine 3000 (Thermo Fisher, Waltham, MA, USA). 48 h later, cells were used for the following analysis.

Cell viability

Cells (2×10^3 cells per well) were seeded into 96-well plates for 6 days and then incubated with 20 μL of CCK-8 solution (CK04, Dojindo, Kumamoto, Japan) for 2 h. The absorbance was determined at 450 nm using a microplate autoreader (Thermo Fisher) at 24-h intervals.

Colony formation assay

Cells (5×10^3 cells per well) were seeded into 6-well plates for 2 weeks, fixed in methanol stained (0.1% crystal violet), and finally photographed under a light microscope (Olympus, Tokyo, Japan).

Dual luciferase reporter assay

Sequences of wild-type or mutant 3' UTR of PDK1 were cloned into pmirGLO (GenePharma, Shanghai, China). HEK-293 cells overexpressing miR-646 or its negative control were transfected into pmirGLO-wt-PDK1 or pmirGLO-mut-PDK1. After 48 h, the luciferase activity was analyzed by Lucifer Reporter Assay System (E1910, Promega, Madison, Wisconsin, USA) and normalized to Renilla luciferase activity.

Glucose uptake and lactate and ATP production assays

For lactate production analysis, 5-8F cells were cultured and lysed, and the lysates were then analyzed (lactate assay kit, Cat. K627, BioVision, Milpitas, CA, USA). For glucose uptake analysis, 5-8F cells were firstly incubated with 2-NBDG (100 μM, 2-(N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) Amino)-2-Deoxyglucose, N13195, Thermo Fisher). Cells were then washed and analyzed cytofluorometrically by recording FL-1 fluorescence under flow cytometer (Attune, Life Technologies, Darmstadt, Germany). For ATP production analysis, 5-8F cells were lysed, and the lysates were then analyzed using an ATP assay kit (S0026, Beyotime, Beijing, China).

qRT-PCR

Trizol reagent (Thermo Fisher) was used for the isolation of total RNA, and miRcute miRNA isolation kit (Tiangen, Beijing, China) was used for miRNA extraction. RNAs were then reverse-transcribed into cDNA. LightCycler DNA Master SYBR Green I kit (Roche Diagnostics) was used to detect mRNA expression on a LightCycler system (Roche Diagnostics). The $2^{-\Delta\Delta CT}$ method was utilized. U6 or GAPDH expression was used as controls. The primer sequences were listed in Table 1.

Western blot assay

The isolated protein (30 μg per lane) was separated using 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA).

Table 1: Primer sequences

Gene	Sequence
miR-646	Forward: 5'-ACACTCCAGCTGGGAAGCAGCTGCCTC-3'
	Reverse: 5'-CTCAACTGTGCTGCATTAGTTAGCTCAGA-3'
PDK1	Forward: 5'-GTTTATCAGATGATCGTCGGCCTACCGCCATTC-3'
	Reverse: 5'-GAATGGCGGTAGGCCGACGATCATCTGATAAAC-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3'
	Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	Forward: 5'-TGCACCACCAACTGCTTAGC-3'
	Reverse: 5'-GGCATGGACTGTGGTCATGAG-3

The membrane was incubated with skimmed milk (5 %) at 37 °C for 2 h, and then with the primary antibodies: anti-PDK1 (1:1,000, ab90444, Abcam, Cambridge, MA, USA), anti-PCNA (1:1,500, ab29, Abcam), anti-p21 (1:1,500, ab227443, Abcam), and anti-GAPDH (1:2,000, ab9485, Abcam) overnight. The membranes were then incubated with secondary antibody (HRP goat anti-rabbit, Abcam, ab205718, 1:2,000) at 37 °C for 2 h. The chemiluminescence of the membranes was detected via ECL detection reagent.

Statistical analysis

The data were shown as mean ± standard deviation. One-way ANOVA with Tukey's test was applied to compare differences (> 2 groups). Student's t test was used to compare differences. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to be statistically different.

RESULTS

MiR-646 was downregulated in NPC cells and miR-646 overexpression inhibited NPC cell proliferation

The expression of miR-646 in human NPC cell lines (5-8F, SUNE-1, C666-1, 6-10B) was determined, and the results indicated that miR-646 expression in human nasal epithelial cells (HNEpC) was 2- to 5-fold higher than that in NPC cell lines (Figure 1 A), suggesting the potential role of miR-646 might in NPC. Increased or decreased miR-646 expression in 5-8F cells was observed upon transfection with miR-646 mimics or inhibitor (Figure 1 B), respectively. Moreover, CCK8 assay indicated that miR-646 mimics inhibited cell viability, whereas miR-646 inhibitor increased cell viability (Figure 1 C). The colony formation assay showed that miR-646 mimics inhibited cell proliferation and miR-646 inhibitor showed reversed results (Figure 1 D). These results revealed that miR-646 was downregulated in NPC cells and miR-646 overexpression inhibited cell proliferation.

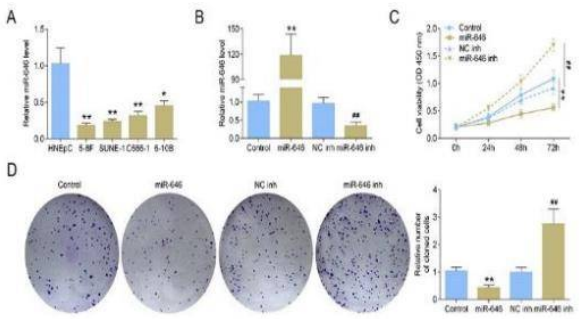

Figure 1: MiR-646 was downregulated in NPC cells and miR-646 overexpression inhibited cell proliferation. (A) Expression level of miR-646 in human NPC cell lines (5-8F, SUNE-1, C666-1, 6-10B) and human nasal epithelial cells (HNEpC). (B) Transfection efficiency of miR-646 mimics or inhibitor in 5-8F cells. ** $p < 0.01$ compared to control group; $^{\#\#}p < 0.01$ compared to NC inh. (C) MiR-646 mimics inhibited cell viability, and miR-646 inhibitor increased cell viability of 5-8F cells Cell viability was detected by CCK8 assay. (D) MiR-646 mimics inhibited cell proliferation, whereas miR-646 inhibitor increased cell proliferation of 5-8F cells. *$p < 0.05$ and ** $p < 0.01$compared to HNEpC or control group; $^{\#\#}p < 0.01$ compared to NC inh

PDK1 is a target of miR-646

To investigate the underlying mechanism, the putative target was predicted and determined. Figure 2 A showed that the 3' UTR region of PDK1 that binds to miR-646 (Figure 2 A). The luciferase activity of wild-type PDK1 reporter was suppressed by miR-646 mimics, whereas the activity of mutant PDK1 reporter displayed no significant change (Figure 2 B). Furthermore,

qRT-PCR analysis showed that PDK1 was decreased by miR-646 mimics, but increased by miR-646 inhibitor (Figure 2C) in 5-8F cells.

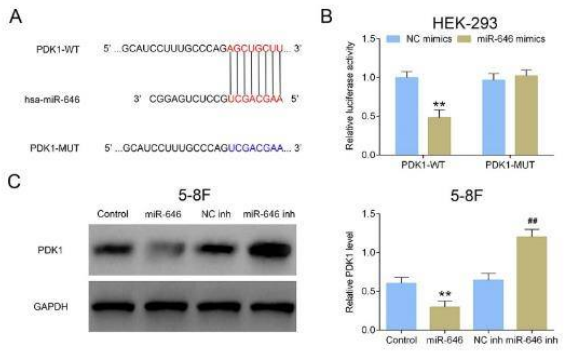

Figure 2: PDK1 is a target of miR-646. (A) Putative binding site of miR-646 in the 3' UTR of PDK1. (B) MiR-646 mimics suppressed luciferase activity of wild-type PDK1 reporter, whereas displayed no significant change to the activity of mutant PDK1 reporter Dual luciferase reporter assay was performed. **(C) MiR-646 mimics inhibited protein expression of PDK1, whereas miR-646 inhibitor increased expression in 5-8F cells. Western blotting was used; $^{**}p < 0.01$ compared to NC mimics or control group; $^{\#\#}p < 0.01$ compared to NC inh

MiR-646 inhibited aerobic glycolysis in NPC cells via regulation of PDK1

Since aerobic glycolysis is closely associated with tumor cell proliferation, the effect of miR-646 on aerobic glycolysis in 5-8F cells was then determined. The mRNA (Figure 3 A) and protein (Figure 3 B) expression levels of PDK1 were increased in cells transfected with pcNDA 3.1-PDK1. MiR-646 overexpression alone resulted in a decrease in lactate production, glucose uptake and ATP production (Figure 3 C- E) in 5-8F cells, suggesting the effect of miR-646 on suppressing aerobic glycolysis. However, 5-8F cells that co-transfected with miR-646 mimics and PDK1 vector exhibited restored lactate production, glucose uptake, and ATP production (Figure 3 C - E). These results suggested that miR-646 inhibited aerobic glycolysis via downregulation of PDK1 in NPC cells.

MiR-646 suppressed NPC cell proliferation via regulation of PDK1

The inhibition of cell viability caused by miR-646 mimics was abrogated after co-transfection of miR-646 mimics and pcDNA 3.1-PDK1 (Figure 4 A). Moreover, the inhibition of colony formation caused by miR-646 mimics was reversed by co-transfection of miR-646 mimics and pcDNA 3.1-PDK1 (Figure 4 B). The cell proliferation-related protein p21 was decreased and PCNA (proliferating cell nuclear antigen) was increased by miR-646 mimics (Figure 4 C) in 5-8F cells, whereas was reversed after co-transfection of miR-646 mimics and pcDNA 3.1-PDK1 (Figure 4C). Therefore, miR-646 could inhibit cell proliferation in NPC cells via downregulation of PDK1.

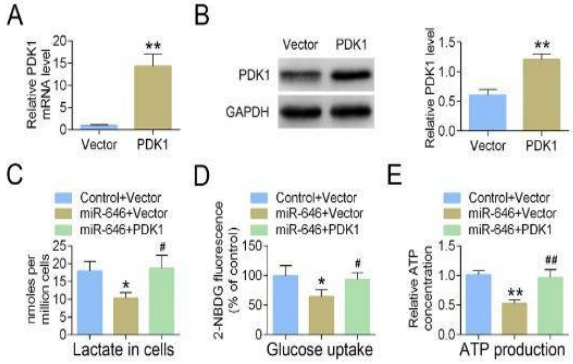

Figure 3: MiR-646/PDK1 regulated aerobic glycolysis in NPC cells. (A) Transfection efficiency of pcNDA 3.1-PDK1 in 5-8F cells detected by qRT-PCR. (B) Transfection efficiency of pcNDA 3.1-PDK1 in 5-8F cells detected by western blot. (C) MiR-646 mimics decreased lactate production in 5-8F cells, and PDK1 overexpression reversed it. (D) MiR-646 mimics decreased glucose uptake in 5-8F cells, and PDK1 overexpression reversed it. (E) MiR-646 mimics decreased AATP production in 5-8F cells, while co-transfection with miR-646 mimics and PDK1 reversed the ATP production. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to Control + Vector or Vector; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to miR-646 mimics + vector

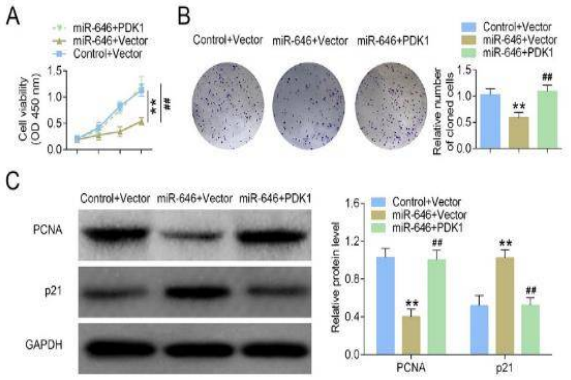

Figure 4: MiR-646/PDK1 regulated NPC cell proliferation. (A) MiR-646 mimics decreased cell viability of 5-8F cells, while co-transfection with miR-646 mimics and PDK1 reversed the cell viability. (B) MiR-646 mimics inhibited cell proliferation in 5-8F cells, while co-transfection with miR-646 mimics and PDK1 reversed the cell proliferation. (C) MiR-646 mimics decreased protein expression of PCNA and increased p21 in 5-8F cells, while co-transfection with miR-646 mimics and PDK1 reversed the protein expression. ** $p < 0.01$compared to vector vs. control + vector, $p < 0.01$; $^{\#\#}p < 0.01$ compared to miR-646 mimics + vector

DISCUSSION

It has been demonstrated that proliferation of tumor cells is greatly affected by dysregulation of miRNAs, especially in NPC [14]. This study showed that miR-646 was decreased in NPC cells and was associated with NPC carcinogenesis by regulating cell proliferation, and aerobic glycolysis.

MiR-646 overexpression significantly inhibited NPC cell proliferation, and miRNA-646 inhibitor enhanced NPC cell proliferation. Proliferating cell nuclear antigen (PCNA), important for DNA replication [15], is positively correlated with NPC occurrence [16] and poor prognosis in NPC [17]. Moreover, p21 functions, a cyclin-dependent kinase inhibitor, could regulate cell proliferation and cycle in NPC [18]. MiR-26a inhibited NPC cell growth via decreasing PCNA expression and increasing p21 expression [6]. Consistently, the results from this study indicated that miR-646 overexpression downregulated PCNA and upregulated p21, suggesting that miR-646 act as a tumor-suppressor in NPC cells.

Other than p21 and PCNA, aerobic glycolysis is the main approach for tumor cells to generate biomass for proliferation [19]. Moreover, miRNAs have been shown to directly regulate key enzymes associated with aerobic glycolysis in cancers. For example, miR-223 upregulates glucose uptake receptors (GLUTs) to regulate glucose uptake [20], and miR-200 directly targets phosphoglucose isomerase to mediate ATP production and the epithelial-mesenchymal transition in breast cancer [21]. Moreover, miR-29a, miR-29b, and miR-124 target MCT1 (monocarboxylate transporter 1) to regulate secretion of lactate in tumor cells [22].

Since miR-646 inhibits cell proliferation in NPC, this study hypothesized that the anti-tumor effect of miR-646 may be dependent on regulation of metabolic processes. Results in the present study revealed that PDK1 is a direct target of miR-646 and that miR-646 decreases PDK1 expression in NPC cells. Pyruvate dehydrogenase kinase 1 decreases the activity of pyruvate dehydrogenase to prevent the conversion of pyruvate to acetyl-CoA, thus switching carbon flow in the tricarboxylic acid cycle to generate lactate [23]. Therefore, PDK1 promotes liver cancer progression by facilitating metabolic adaptation to nutrient limitation [24].

This study found that miR-646 decreased lactate secretion, glucose uptake, and ATP production, thereby inhibiting aerobic glycolysis and exerting anti-tumor effects in NPC. Gain-of function assays further indicate that PDK1 overexpression reversed the effects of miR-646 on aerobic glycolysis and cell proliferation in NPC, suggesting that the ability of miR-646 to regulate NPC aerobic glycolysis and cell proliferation was dependent on PDK1.

Generally, hypoxia-inducible factor 1 (HIF1) functions as a transcription factor for the PER-ARNT-SIM (PAS) family [25] and regulates the gene expression involved in glucose uptake, glycolysis, and lactate production, thus blocking the entry of glucose into the TCA cycle via PDK1 [26]. The effect of miR-646 on HIF1 expression should be further investigated to uncover the underlying signaling pathway involved in the regulatory effect of miR-636 on aerobic glycolysis in NPC.

CONCLUSION

The findings of this current study revealed that miR-646 acted as a tumor promoter in NPC. Overexpression of miR-646 inhibited glucose uptake and decreased ATP and lactate production via downregulating PDK1 and inhibition of aerobic glycolysis, leading to decreased cell viability and proliferation in NPC. This study may provide a molecular target and potential treatment strategy for prevention of NPC.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Kaiquan Zhu designed all the experiments and revised the paper. Renyu Lin performed the lab experiements the experiments, and Aidan Xia wrote the paper.

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