Research Article

miR-375 Inhibits the Proliferation and Invasion of Nasopharyngeal Carcinoma Cells by Suppressing PDK1

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Purpose. In patients with nasopharyngeal carcinoma (NPC), the expression of PDK1 is remarkably improved in NPC tissue and correlated with the clinicopathological severity of NPC. We expressed miR-375 in NPC cells to study the effects on PDK1 gene expression. We also investigated the mechanism by which miR-375 affects the biological behavior of NPC cells through effects on PDK1.

Methods. qRT-PCR was carried out to analyze miR-375 and PDK1 levels in NPC cells. NPC cells were transfected with miR-375 inhibitor or miR-375 mimic. CCK-8 testing, colony formation testing, transwell testing, and flow cytometry analysis were carried out to quantify the cells' biological behavior. Rescue experiments demonstrated that the recovery of PDK1 expression was able to reverse the influence of miR-375 inhibition on NPC diffusion and intrusion. The interaction between miR-375 and PDK1 was verified by dual-luciferase reporter gene testing.

Results. The results revealed that miR-375 has a negative regulatory effect on PDK1 expression in NPC cells. Furthermore, PDK1 is a target gene for miR-375. The empirical results obtained demonstrated a negative correlation between tumor development and the level of miR-375 expression in NPC tissues. The excessive expression of miR-375 and the downregulation of PDK1 facilitated the diffusion and invasion of NPC cells.

Conclusion. The diffusion and incursion of NPC cells may be inhibited by direct targeting of PDK1 and decreasing the expression of miR-375. Our study highlights efforts to target PDK1 and miR-375 as potential therapeutic strategies for use in the treatment of NPC.

1. Introduction

Nasopharyngeal carcinoma (NPC) is the most common head and neck cancer in southeast Asia, with an extremely high incidence in southern China [1]. By analyzing the data from 72 local, population-based cancer registries (2009-2011) and from 22 other registries (2000-2011) for trend analyses, we were able to estimate that approximately 60,600 new cases of NPC and 34,100 deaths attributable to NPC occurred in China in 2015 [2].

Patients with NPC often appeared with advanced-stage disease at the time of the initial diagnosis. The 5-year survival rate is approximately 50–60%, because of locoregional relapses and high rates of metastasis to distant organs [3]. Radiotherapy is currently recognized as the mainstay of treatment for NPC [4]. The combination of radiotherapy and chemotherapy significantly improves the 5-year overall survival rate among patients with NPC (84.7–87.4%) [5]. It is therefore very important to figure out the molecular basis of NPC development, so as to facilitate efforts to identify a specific and effective molecular therapeutic target for the management of NPC.

Our early stage research revealed that expression levels of the gene PDK1 have significant clinical and prognostic value in the management of NPC. Multivariate analysis identified that PDK1 levels are an independent prognostic factor for primary nasopharyngeal carcinoma (pNPC). Patients with higher PDK1 expression had significantly lower rates of overall survival (OS), disease-free survival (DFS), locoregional relapse-free survival (LRRFS), and distant metastasis-free survival (DMFS) [6].

MicroRNAs (miRNAs) are widely involved in various critical cellular processes [7]. miRNAs exert their effects in numerous types of tumor cells by degrading target gene transcripts and by suppressing the translation of mRNA [8]. Increasing evidence indicates that miR-375 and PDK1 are
linked, signifying that miR-375 may be regarded as a molecular target in efforts to interfere with PDK1 expression. miR-375 expression was recently found to be dysregulated in many cancer types, including hepatocellular carcinoma, gastric carcinoma [9, 10], head and neck squamous cell carcinomas [11], and esophageal cancer [12]. Therefore, an in-depth study of the role of miR-375 in NPC cells will elucidate the interaction between miR-375 and PDK1, thus establishing a diagnostic biomarker and possible therapeutic target.

Unfortunately, the biological mechanism connecting miR-375 and PDK1 in nasopharyngeal carcinoma cells remains to be characterized fully. In our research, we sought to demonstrate experimentally that miR-375 inhibits the survival, proliferation, and invasion of nasopharyngeal carcinoma cells.

2. Materials and Methods

2.1. Clinical Samples. During the period from September 2015 to November 2016, 23 normal nasopharyngeal epithelial tissue samples and 38 NPC tissue samples were collected from patients who had not undergone chemotherapy or radiotherapy. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Wenzhou Medical University. All participants in the study provided their signed informed consent.

2.2. Cell Culture. The CNE-2, CNE-1, C666-1, and HONE1 human NPC cell lines, as well as the NP69 normal nasopharyngeal epithelial cell line (Cancer Research Institute of Guangdong Medical University), were maintained in Dulbecco’s Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (FBS) without serum media. All experiments were conducted on cells exhibiting logarithmic growth.

2.3. Transfection. miR-375 mimic, miR-375 suppressor, corresponding scramble controls (scramble), and PDK1 overexpression plasmids (pcDNA3.0-PDK1) were acquired from GenePharma Co. Ltd. (Shanghai, China). After CNE-1 and CNE-2 cells were added to 12-well plates, cells were transfected with miR-375 mimic, miR-375 NC, pcDNA3.0-PDK1, or empty vector using Lipofectamine 2000 (Invitrogen), on the basis of the manufacturer’s instructions.

2.4. qRT-PCR. TRIzol reagent (Invitrogen, New York, USA) was used to extract total RNA from samples. Extracted RNA was reverse transcribed into cDNA using an RT kit (Maryland Fermentation Agent). Expression levels of miRNA and mRNA was reverse transcribed into cDNA using an RT kit. Extracted RNA was used to extract total RNA from samples. Extracted RNA was reverse transcribed into cDNA using an RT kit was used to prepare total RNA. Expression levels of miRNA and mRNA were measured on the basis of the manufacturer’s instructions. The 2−ΔΔCt method was carried out to measure levels of miR-375. U6 and GAPDH were used as internal controls for miR-375 and PDK1.

2.5. Western Blot. RIPA lysis buffer was used to prepare total protein. Protein concentrations were quantified with the Bradford method. Samples (30 μg) of extracted protein were loaded on SDS-PAGE gels and diverted onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with rabbit anti-human PDK1 polyclonal antibody (1:1000, Abcam, Cambridge, MA, USA) or with mouse anti-human β-actin monoclonal antibody (1:10000, Abcam) overnight at 4°C, followed by incubation with a secondary antibody (Abcam) combined with horseradish peroxidase (HRP) for 1 hour at room temperature. The membranes were then developed with an enhanced ECL solution. The results were normalized to those obtained for β-actin.

2.6. MTT Assay. CNE-1 cells and CNE-2 cells were transfected with miR-375 mimic, miR-375 inhibitor, pcDNA3.0-PDK1, or empty vector for 36 h. During the period of logarithmic growth, CNE-1 and CNE-2 cells were added to 96-well plates at a density of 1000 cells per well. Each experiment was performed with 7 duplicates. Cell survival was measured at 24 h, 48 h, 72 h, and 96 h after seeding. MTT solution was added to each well (20 μl, 5 mg/ml). Samples were incubated for another 4 h. After 150 μl, DMSO was added to each well and absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.7. Colony Formation Assay. After incubation for 36 h, CNE-1 and CNE-2 cells transfected with a miR-375 inhibitor, pcDNA3.0-PDK1, or empty vector were plated into 6-well plates at a density of 2000 cells per well. Nonadherent cells were removed after incubation for 24 h. The remaining cells were incubated for 1 week without changing the medium. After the colonies were fixed with 4% paraformaldehyde and stained with 1% crystal violet, colony number and size were determined by microscopy. To be counted, colonies had to contain at least 50 cells.

2.8. Transwell Assay. Cell migration and invasion were measured with transwell inserts (pore size 8 mm; BD Bioscience, CA, USA). After 24 hours of starvation, the cells were cultured with serum-free media and digested with 0.25% trypsin. The upper chambers coated with Matrigel, 500 μl of cell suspension (50,000 cells) was added. To the lower chambers, 600 μl of complete media was added. After incubation for 24 hours, the cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet (Sinopharm Chemical Reagent Co., Shanghai, China). Cells that had moved through the membrane to its lower surface were viewed under the microscope. Colony number and size were recorded.

2.9. Apoptosis. We transfected CNE-1 and CNE-2 cells with miR-375 mimic, miR-375 suppressor, pcDNA3.0-PDK1, or empty vector for 36 h. The cells were then harvested and fixed twice with 70% ice-cold PBS prior to analysis with the Annexin V-FITC/PI apoptosis testing kit (Sigma-Aldrich), according to the manufacturer’s instructions. Cell cycle distribution was measured with a FACSCalibur Flow Cytometer (BD Bioscience).

2.10. Luciferase Assay. The pGL4.13 vector (Promega, Madison, WI, USA) was applied to create pGL4.13-PDK1 3′UTR (wt-3′UTR) and pGL4.13-PDK1 3′UTR-mut (mut-3′UTR) plasmids. CNE-1 and CNE-2 cells were cotransfected with wt-3′UTR or mut-3′UTR vectors using Lipofectamine 2000. Measurements of luciferase bioactivity were completed after 48 h transfection, which was performed using the dual-luciferase reporter system, in accordance with the manufacturer’s instructions.
2.11. Statistical Analysis. Statistical analyses were accomplished with GraphPad Prism 6.0 software (GraphPad Software, CA, USA). The two-tailed Student’s t-test was performed to analyze discrepancies between groups. Multiple comparisons were carried out by one-way analysis of square deviation. Data are presented as mean ± SD. Differences with $P < 0.005$ were regarded as statistically significant.

3. Results

3.1. Downregulated miR-375 and Upregulated PDK1 in NPC Samples and Cell Lines. qRT-PCR was carried out to measure the expression of miR-375 in 38 NPC samples and 23 samples of normal nasopharyngeal epithelial tissue (NP69). Expression levels of miR-375 were significantly lower in NPC tissue, compared with normal tissue (Figure 1(a)). Expression levels of PDK1 were higher in NPC tissue, compared with normal tissue (Figure 1(b)). These trends were observed in all four lines of NPC cells examined (CNE-1, CNE-2, C666-1, and HONE1; Figures 1(c) and 1(d)). We elected to use the CNE-2 and CNE-1 cell lines for subsequent experiments, because they had the lowest expression of miR-375 and the highest expression of PDK1.

3.2. miR-375 Overexpression Inhibited NPC Cell Viability, Proliferation, Migration, and Invasion. The results of qRT-PCR revealed that transfection with the miR-375 analog remarkably enhanced miR-375 expression and that transfection with the miR-375 inhibitor decreased the expression of miR-375 ($P < 0.01$, respectively; Figure 2(a)). To clarify the effect of miR-375 on NPC tumor cell proliferation, migration, differentiation, and apoptosis, we carried out CCK-8, colony formation, and transwell assays. The results of the CCK-8 assay suggested that cell proliferation was remarkably suppressed in the miR-mimic group ($P < 0.01$, Figure 2(b)). Colony formation was greater in the normal control group, compared with the miR-mimic group ($P < 0.05$, $P < 0.01$, **$P < 0.001$, Figure 2(c)). The results of the transwell assay indicate that downregulation of miR-375 fosters cell migration and, in patients with NPC, may indicate increased risk of local invasion or metastasis or both. ($P < 0.01$, Figure 2(d)). This effect of miR-375 on cell growth was confirmed by our finding that decreased expression of miR-375 increased cell proliferation in CNE-1 and CNE-2 cells. These results indicate that NPC cell progression through the cell cycle is inhibited by endogenous miR-375 expression.

3.3. miR-375 Arrests NPC Cells at Stage G0/G1 and Promotes Apoptosis. As G0/G1 is a key stage in the cell cycle, with
Figure 2: The progression of NPC cells was suppressed by the upregulation of miR-375. (a) The expression of miR-375 in transfected CNE-1 and CNE-2 cells was evaluated by qRT-PCR. (b) The MTT assay was performed to determine the viability of transfecting CNE-1 and CNE-2 cells with a miR-375 mimic or inhibitor. (c) Measured colony formation in cells that had already been transfected with miR-375 mimic or suppressor. Cell behavior was evaluated by transwell assay in cells that had completed the transfection process.
documented effects on tumor progression, we sought to confirm whether the positive influence of miR-375 on apoptosis was mediated by arresting NPC cells at the G0/G1 stage. The results of flow cytometry demonstrated that cell transfection with the miR-375 mimic increased the duration of the G0/G1 stage but had no effect on the duration of the G2 or S stages. Transfection with the miR-375 inhibitor decreased the duration of the G0/G1 phase and increased the duration of the S-
phase ($P < 0.05$, respectively; Figure 3(a)). Annexin V-FITC/PI staining showed that miR-375 promotes apoptosis ($P < 0.01$) by prolonging the G0/G1 phase of the cell cycle ($P < 0.05$, Figure 3(b)). These findings support our hypothesis that miR-375 promotes apoptosis by arresting cells at the G0/G1 stage.

3.4. miR-375 Binding to the 3′ UTR Region of PDK1 Downregulates PDK1 Expression. According to the TargetScan database, miR-375 acts as a tumor inhibitor in numerous types of cancer. We therefore selected this miRNA to regulate the expression of PDK1 (Figure 4(a)). The dual-luciferase reporter assay was carried out to demonstrate
that the downregulation of miR-375 promoted luciferase activity (Figure 3(b)). Neither the increase in luciferase activity in cells transfected with miR-375 inhibitor nor the decrease in luciferase activity in cells transfected with miR-375 mimic was observed in cells with a mutated version of PDK1 that disrupted the putative binding site for miR-375 (Figure 3(b)). This effect is related to a decrease in the luciferase bioactivity of luciferase reporter-containing wild-type PDK13′-UTR, indicating that mutation of this binding site mediated the observed downregulation (Figure 3(b)). To further clarify the mechanism underlying the regulation of PDK1 expression, we performed qRT-PCR and Western blot analysis to investigate the extent of miR-375 involvement. The results suggested that miR-375 negatively regulated the PDK1 expression in NPC cells (Figures 4(c) and 4(d)). These findings show that miR-375 targets PDK1 expression in NPC cells.

3.5. Restoration of PDK1 Expression Reverses the Antitumor Effect of miR-375. The results of qRT-PCR and Western blot assays showed that transfection with the PDK1 expression vector was positively correlated with the restoration of PDK1 expression in miR-375 mimic-transfected cells. PDK1 protein and mRNA expression were restored in CNE-1 and CNE-2 cells transfected with the PDK1 expression vector and miR-375 mimic. In CNE-1 and CNE-2 cells, cell viability and colony formation were increased in cells of the PDK1 overexpression group, compared to the miR-375 group (Figure 5(a)). The upregulation of PDK1 expression abrogated the inhibitory effect of miR-375 overexpression on the proliferation of NPC cells (Figure 5(b)). The results of invasion and migration assays also demonstrated that PDK1 overexpression reversed the decrease in the miR-375 group (P < 0.05; Figure 5(c)). PDK1 thus appears to be an immediate target of miR-375.

3.6. miR-375/PDK1 Axis Controls P13K/AKT Signaling. Previous studies published by our laboratory demonstrated that P13K/AKT signaling plays a vital role in NPC progression. We measured protein levels of AKT, p-AKT, and P13K in CNE-1 and CNE-2 cells by Western blot to verify the hypothesis that miR-375/PDK1 acts by regulating P13K/AKT signaling. Compared to the corresponding control groups, CNE-1 and CNE-2 cells transfected with the miR-375 mimic had reduced levels of P13K and p-AKT. AKT levels remained unchanged (all P < 0.05; Figure 6). In cells transfected with
the miR-375 mimic and PDK1, PDK1 overexpression inhibited this decrease in levels of p-AKT and P13K (all $P < 0.05$). Levels of AKT remained constant. These data indicate that miR-375/PDK1 signaling may act through the P13K/AKT axis to mediate NPC progression.

4. Discussion

Previous studies have demonstrated the dysregulation of miRNA expression in NPC [13–15]. Nevertheless, the molecular mechanism underlying the effects of miR-375 expression in NPC remains unclear. Our statistical analysis revealed dramatic downregulation of miR-375 in NPC tissue, compared with nontumor NP69. The results of our in vitro studies demonstrate that overexpression of miR-375 may inhibit the proliferation and migration of NPC cells, suggesting that miR-375 contributes to the progression of NPC.

The research presented above shows that miR-375 arrests NPC cells at the G0/G1 stage of the cell cycle. The results of rescue experiments showed that overexpression of PDK1 increased the proliferation of NPC cells previously transfected with miR-375. This finding indicates that the suppression influence of miR-375 on the proliferation of NPC cells may be reversed with the recovery of the PDK1 expression, suggesting an oncogenic role for miR-375 in NPC.

The data from this study are consistent with those of previous studies. For instance, Sabarimurugan et al. demonstrated that miR-375 is closely related to lymphatic spread, differentiation, and TNM staging in esophageal squamous cell carcinoma [15]. Plasma levels of miR-375 have been identified as a promising prognostic marker for patients with advanced MTC, although this finding requires validation in a larger cohort [16]. Zhang et al. reported that miR-375 plays a tumor-suppressive role in ccRCC through the regulation of YWHAZ, highlighting its potential as an underlying therapeutic target in the treatment of patients with ccRCC [17]. Furthermore, miRNA-375 inhibits growth and enhances radiosensitivity in OSCC cells by targeting IGF-1R, suggesting that miR-375 may represent a therapeutic target in the treatment of OSCC [18]. This accumulation of evidence shows that miR-375 may inhibit tumor growth in NPC.

Increased expression of PDK1 offsets the effects of miR-375 expression, thus weakening the oncogene suppressor function of miR-375. This mechanism may underlie the tumor-targeting effects of miR-375 in NPC. The results of our bioinformatics analysis identified PDK1 as a target of miR-375, which was significantly upregulated in NPC cells. miR-375 levels were associated with expression levels of the PDK1 3′ UTR, which were measured with the luciferase reporter system. The inverse correlation between miR-375 and PDK1 alone does not prove direct interaction as much as activity...
in a common pathway. The abrogation of the negative effect of miR-375 on PDK1 observed when a mutated form of PDK1 is expressed may serve as an indication of a direct interaction. This could be the basis for further investigation. Few previous studies have investigated the molecular signaling mechanism by which the miR-375/PDK1 axis regulates NPC tumor progression. Mechanistic analyses revealed that exosomal miR-9 from NPC cells inhibited endothelial tube formation and migration by targeting MDK and regulating PDK/AKT signaling [19]. The recovery of PDK1 expression reverses the effects of miR-375 overexpression, activating NPC cells through P13K/AKT signaling. We therefore assumed that the inactivation of P13K/AKT signaling by the miR-375/PDK1 axis may facilitate the NPC cells’ biological behavior.

Previous studies have demonstrated high expression of PDK1 in NPC tissues. In NPC, levels of PDK1 are also positively correlated with clinical stage and lymph node involvement. NPC patients with high PDK1 levels have poor outcomes [20–22]. These findings may guide us in moving forward to confirm the effects of miR-375 and PDK1 on prognosis in patients with NPC.

5. Conclusions
In conclusion, we found that miR-375 inhibited the proliferation, migration, invasion, and apoptosis of NPC cells, which are mediated by the oncogene PDK1. Additional studies will be necessary to further elucidate the underlying mechanism.

Data Availability
The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of Interest
The authors declare that there are no conflicts of interest.

Authors’ Contributions
Jia-yuan Xu and Wei Song performed most of the investigation and data analysis and wrote the manuscript; Zhi-jian Dai, Long-he Cao, and Fang-fang Lu provided important background information; and Sen Lin participated in the design of this study. All of the authors have read and approved the manuscript.

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