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A new and important relationship between miRNA-147a and PDPK1 in radiotherapy

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Abstract

It was found that the expression level of miR-147a was significantly increased and the pathway of PI3K/AKT was dramatically inhibited after radiation. In view of the relationship between miRNA and target genes, we put forward the question, what is the relationship between PI3K/AKT and miR-147a? In order to find the answer to the question, we used bioinformatics techniques to analyze the relationship between miR- 147 (a or b) and PI3K/AKT signaling pathway. miR-147a overexpression plasmid and PDPK1 3′UTR luciferase reporter gene plasmid were constructed. Dual luciferase reporter gene system validation experiments were carried out on miR-147a and PDPK1 relationship. The verification experiments were also carried out. Bioinformatics analysis showed that there is a miR-147a binding site in the non-coding region (3′UTR) of PDPK1. In the experimental groups transfected with wild type PDPK1 gene of 3′UTR plasmid, the luciferase activity decreased (or increased) significantly in miR-147a (or inhibitor) group compared with miR-NC (or anti-miR-NC); There was no significant difference between the miR-147a group (or inhibitor) and the miR-NC group (or anti- miR-NC) in the transfection of PDPK1–3′UTR-Mut gene vector. PDPK1 was a target gene for direct regulation of miR-147a downstream. Verifying test results showed that the expression of PDPK1 mRNA and protein was reduced after overexpression of miR-147a, which was up-regulated after silencing miR-147a in TC, and V79 cells. These results suggest that miR-147a could be involved in the regulation of PDPK1 transcription by binding to the target site in PDPK1 mRNA 3′UTR, and then regulated AKT.

Keywords

miRNA-147a; PDPK1; plasmid; radiation; target

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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1 | INTRODUCTION

In the medical field, IR is usually an available therapy for cancer therapy, but it also damages normal cells, and tissues1,2 through generating intermediate ions and reactive free radicals. After exposure to IR, such as γ-or X-rays, which are the most common damage factors, organisms instantly stimulate a series of biochemical pathways to keep genetic integrity for promoting autologous cell survival.

MiRNA are a class of non-coding, small, endogenous RNA with 18–25 nucleotides (nt) in length which can regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated regions (UTR) of target miRNA.3–6 A miRNA can target up to 100–200 mRNAs.7 miRNA may play an important role in the regulation of life and activities, and is closely related to the development of animal and plant organs, cell differentiation and apoptosis, and fat metabolism.8,9 Recent studies have found that many miRNA have high or low expression in many models of ionizing radiation injury, which indicates that miRNA is closely related to the occurrence and development of radiation damage.10,11

AKT is the downstream target of phosphatidylinositol-3- kinase (PI3K). P13K contains a subunit with protein molecular weight of 110 kD, which could catalyze PIP2 phosphorylation to generate PIP3. PIP3 enhances the activity of 3-phosphoinositide-dependent protein kinase-l (PDPK1), further activating AKT.12 PDPK1 plays an important role in cell proliferation and survival.13,14 Gene knockout PDPK1 —/— mouse embryos died at 9.5 days. The PDPK1−/+ mice were 40–50% smaller than normal mice, and the inhibition of cell proliferation was associated with low expression of PDPK1.15 In PDPK1 gene knockout cells or mice, AKT kinases were inactivated, indicating that PDPK1 was an important upstream kinase of AKT, and the loss of AKT activity was related to restriction of phosphorylation of itself.15,16 MiR-147 significantly increased after radiation,17 but the PI3K/AKT signaling pathway was inhibited.18,19 MiR-147 inhibits the PI3K/AKT/mTOR signaling pathway to prevented proliferation and migration of breast cancer cells, but its direct target was not elucidated.20,21

In this study, we focuses on the establishment and validation of miR-147 and PDPK1 target relationship.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Thymus cells of KM mice and Chinese hamster lung fibroblasts V79 were cultured in suspension in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2.

2.2 | Prediction of miR-147a targets

Log on to the TargetScanHuman database (http://www.targetscan.org/vert_71/), entering the name miR-147a to get the target genes.

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2.3 | DNA, miRNA constructs

To generate luciferase reporter constructs, PDPK1 3'-UTR (or PDPK1 3′-UTRmut) was produced by chemical synthesis. It is loaded into the vector without expression to prepare plasmid. Then the Xbal/Xbal enzyme digestion reaction was used to obtain the target gene fragments which were cloned into the Dual-Luciferase Reporter Gene Expression vector of GV272 (SV40-Luc-MCS). To generate mir-147a constructs, the mir- 147a was produced by PCR using human genomic DNA library. Following primers were used: mir-147a (309 bp), forward primer: 5′- ACGGGCCCTCTAGACTCGAGATAA-CAGCAGCCCACCAAAAAGC- 3′-reverse primer5′-GTTTAAACTTAAGCTTGGTACCCAGGAAAACTTATTTTT GAATTATG- 3′.The resulting PCR products were cloned into the Vector of GV268 (CMV-MCS-SV40-Neomycin). miR- 147a inhibitor plasmid was obtained from Shanghai Jikai Genechem Co., Ltd. Shanghai, China.

2.4| PCR identification of the colony

Single colony was put into 20 μL identification system, which was mixed evenly, placed in a PCR apparatus for reaction, and then subjected to 1% agarose gel electrophoresis. Identification primer were as follows: GV272-PDPK1 3′-UTR Luc-C-F:GAGGAGTTGTGTTTGTGGAC and RVprimer4: GAC-GATAGTCATGCCCCGCG;GV268-miR-147a CMV-F: CGCAAATGGGCGGTAGGCGTG and pcDNA-SEQR: TTATTAGGAAAGGACAGTGGG.The cycling parameters were as follows: 94°C for 3 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, another cycle 72°C for 5 min using the ABI real-time PCR detection system.

2.5 | Luciferase assay

293T cells were transfected with the miR-147a (control miRNA) and PDPK1 3′UTR(mut) in conjunction with the luciferase reporter constructs. Forty-eight hours after they were transfected, the cells were lysed and subjected to luciferase assays using the Dual Luciferase Reporter Gene Assay System (Promega, Madison, WI) according to the manufacturer's protocol.

2.6| RNA isolation and real-time polymerase chain reaction (PCR)

2.6.1| PDPK1—Total RNAwas extracted using RNAiso Plus reagent (TAKARA BIO INC., Shiga, Japan). The extraction procedure was then followed by cDNA synthesis using ReverTra Ace qPCR RT MasterMixkit (ToyoboCo.,Ltd.Life ScienceDepartment ScienceDepartmentOsaka Japan).Quantitative PCR was performed using the ABI5500 RTPCR system (Strata-gene) with the SYBR® Green Real-time PCRMasterMix kit (Toyobo Co., Ltd. Life ScienceDepartment) with the following conditions: 95°C for 1min, 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 45 s. Primer sequence of various genes were designed as follows: PDPK1 forward primer 5′-GCACATCCAGATCACAGAT-3′ and reverse primer 5′ -TTACACGCCGACTTCTCT-3′; GAPDH forward prime: 5′-AGGTCGGTGTGAACGGATTTG- 3′ and reverse primer: 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

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2.6.2 | MiR-147a—Total RNA was extracted using RNAiso Plus reagent (TAKARA BIO INC.). The extraction procedure was then followed by cDNA synthesis using miRNA cDNA Synthesis Kit (Beijing ComWin Biotech Co., Ltd. Beijing, China). Quantitative PCR was performed using the ABI5500 RT-PCR system with miRNA qPCR Assay Kit (Beijing ComWin Biotech Co., Ltd. China) with the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. Primer sequences of various genes were designed as follows: mir147 forward primer: GTGTGCGGAAATGCTTCTGCTA and reverse primer: provided by the kit without sequence; U6 forward primer: CTCGCTTCGGCAGCACA and reverse primer AACGCTTCACGAATTTGCGT.

2.6.3 | Immunoblotting—Thymus cells and V79 were collected and RIPA lysis buffer and 1 mM phenyl methyl sulfonyl fluoride (PMSF) (99:1) were added according to the amount of cells. The mixed liquid induced complete lysis and was subsequently centrifuged (4°C, 12 000 r/min, 5 min). The protein concentration was determined using the bicinchoninic acid assay (BCA) assay. The resulting supernatant was preserved at —80°C. The blocked blots were incubated with primary antibodies overnight at 4°C using PDPK1 antibody dilutions (1:1500) recommended by the manufacturer (Wanleibio Co., Ltd. China) and GAPDH (1:2500 Abcam, Cambridge, UK). Subsequent incubation was performed with horseradish peroxidase (HRP)-conjugated Goat-anti-rabbit antibody (1:3000, Jackson ImmunoResearch, Inc., West Grove, PA). The signal was detected using enhanced chemiluminescence (ECL) reagents.

3 | RESULTS

3.1 | The expression of miR-147a and PDPK1 was opposite after irradiation

As shown in Figure 1A, the optimal detection time of miR-147a was 30min after radiation. As shown in Figure 1B-D, the expression level of miR-147a was significantly increased and the expression level of PDPK1 mRNA and protein was significantly decreased after radiation, compared with the normal group|.

3.2 | Bioinformatic prediction of binding sites between miR-147a and PDPK1

We use the miRNA database Targetscan to analyze and forecast the target genes of miR-147a. It is found that there were three miR-147a binding sites in the non-coding region (3′UTR) of PDPK1 (Figures 2A and 2B), And we chose the binding site with high score.

3.3 | Construction of over expression plasmid

Overexpression plasmid vector information was shown in Figure 3A.

3.3.1 | Acquisition of target gene fragments—The plasmid containing the gene sequence of PDPK1 3′UTR, was digested with XbaI/XbaI enzyme, and was treated with 1% agarose gel electrophoresis. As shown in Figure 3B, a fragment of the enzyme was shown near marker of 250 bp, consistent with the synthetic target gene PDPK1 of PDPK1 3′UTR, (miR-147a) size of 210 bp, the same as expected.

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The primers were designed containing paired exchange bases, restriction sites, and containing 5′gene sequence. The miR-147a gene was amplified by PCR, and 1% agarose gel electrophoresis was performed. As shown in Figure 3B, a fragment of the enzyme was expressed near 300 bp. The size was consistent with target gene of 309 bp amplified by PCR, in line with expectation.

3.3.2 | Identification of recombinant clones by PCR—The target gene fragment was inserted into the linearized expression vector after recovery. That is to say, PDPK1 3′UTR was inserted into GV272 to construct PDPK1 3′UTR target sequence-reporter gene fusion vector of PDPK1 3′UTR —GV272. The recombinant clones were identified by PCR. As shown in Figure 3C, there was a positive transformation band between 500 and 750 bp, consistent with the design of the amplified product 667 bp.

PCR amplification products were exchanged with the vector, MiR-147a was inserted into GV268 to construct the fusion vector of miR-147a-GV268, and PCR was used to identify the recombinant clone.

As shown in Figure 3C, there was a positive conversion band between 500 and 750 bp, consistent with the design of the amplification product of 558 bp, in line with expectation.

3.3.3 | Positive clone sequencing—The results showed that the sequence of the inserted fragment was the same as that of pre-miR-147a and PDPK1 3′UTR (miR-147a) reported on Genbank, as shown in Figure 3D.

3.4 | PDPK1 was the target gene of miR-147a verified by dual luciferase reporter gene system experiments

Luciferase activity showed that in the experimental groups transfected with wild type PDPK1 gene of 3′UTR plasmid, the luciferase activity decreased (or increased) significantly in miR-147a (or inhibitor) group compared with miR-NC (or anti-miR-NC); There was no significant difference between the miR-147a group (or inhibitor) and the miR-NC group (or anti-miR-NC) in the transfection of PDPK1–3′UTR-Mut gene vector (Figures 4A and 4B).

These results indicate that miR-147a acted on the 3′UTR region of PDPK1 gene and inhibit the activity of it, which leads to the conclusion that PDPK1 was a target gene for direct regulation of miR-147a downstream.

3.5 | MiR-147a negatively regulated the expression of PDPK1

QRT-PCR and Western blot were used to detect the expression of PDPK1 mRNA and protein after overexpression of miR-147a or silencing miR-147a in TC (thymus cells) and V79 cells. QRT-PCR results showed that in TC and V79 cells, the expression level of PDPK1 mRNA was down regulated in the group of miR-147a overexpression plasmid transfected by liposome 2000, (24 hole plate, each hole 0.5 μg), compared with miR-NC. However, the expression of PDPK1 in the TC and V79 cells was significantly higher than that of the control group after transfection with miR-147a inhibitor, and the difference was statistically significant, as shown in Figures 5A and 5B.

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The results of Western blot showed that the expression level of PDPK1 protein was down regulated after in miR-147a was overexpressed in TC and V79 cells. Conversely, after silencing miR-147a, the expression level of PDPK1 protein in the cells was up-regulated, as shown in Figures 5C and 5D. The activity of PDPK1 was reduced by miR-147a compared with miR-NC (Figure 5E).

These results further confirmed that PDPK1 was a direct target gene of miR-147a.

4.| DISCUSSION

Activation of PI3K/AKT signaling pathway can promote cell proliferation, inhibit apoptosis, promote cell cycle progression, and promote cell invasion and metastasis, which play an important role in radiation induced cancer cell resistance and normal cell injury. After irradiation, some cancer cells activated PI3K/AKT22-24 pathway, inducing radiation resistance to reduce the radiation sensitivity. But PI3K/AKT signaling pathway was inhibited in normal cells after radiation.18,19 PI3K/AKT inhibitor was given to thymus cells in advance, and the apoptosis rate of cells increased significantly after irradiation.25 With the AKT1 gene knockout by AKT1 shRNA, retinal pigment epithelial cells is very sensitive to ultraviolet radiation.26 The downstream effect of AKT activation is closely related to the inhibition of apoptosis pathway.27 Therefore, AKT is closely related to radiation damage.

Recent studies have found that many miRNA have high or low expression in many models of ionizing radiation injury, which indicates that miRNA is closely related to the occurrence, and development of radiation damage.10,11 The expression levels of let-7 family were reported by several studies to be significantly changed by IR.28–30 Moreover, the let-7 family is a tumor suppressor in regulating cell proliferation and differentiation. After IR exposure, miR-34a was up-regulated in diverse human cell lines and miR-21 was similarly up-regulated in various normal and cancer cell lines11,28,31 MiR-147 has been found to be involved in the regulation of differential gene expression in many pathological models. In the radiation injury model, the expression of MiR- 147 and PI3K/AKT was the opposite. MiR-147 significantly increased after radiation,17 but the PI3K/AKT signaling pathway was inhibited. MiR-147 inhibits the PI3K/AKT/ mTOR signaling pathway to prevented proliferation and migration of breast cancer cells, but its direct target was not elucidated. 20,21 Gene silencing AKT1/2 produced a similar biological effect with that ofmiR-147, and the cells phenotype of AKT knockout was consistent with that of miR-147.32

So, we put forward the question, what is the relationship between PI3K/PDPK1/AKT and miR-147?

In order to find the answer to the question, we used bioinformatics techniques to analyze the relationship between miR-147 (a or b) and PI3K/PDPK1/AKT signaling pathway. Through the TargetScan target gene docking analysis, results were obtained as follows: (i) The mature sequence of miR- 147a was 5'-GUGUGUGGAAAUGCUUCUGC- 3′; (ii) PDPK1 mRNA 3′UTR region contains 3 potential miR- 147a binding sites (no miR-147b binding sites). These results suggest that miR-147a could be involved in the regulation of PDPK1

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transcription by binding to the target site in PDPK1 mRNA 3′UTR, and then regulated AKT.

In this study, we focuses on the establishment and validation of mir147a and PDPK1 target relationships. It was found that the expression level of miR-147a was significantly increased and the expression level of PDPK1 mRNA and protein was significantly decreased after radiation in thymus cells. Bioinformatics analysis showed that there was a miR- 147a binding site in the non-coding region (3′ UTR) of PDPK1. In the experimental groups transfected with wild type PDPK1 gene of 3′ UTR plasmid, the luciferase activity decreased(or increased) significantly in miR-147a(or inhibitor) group compared with miR-NC (or anti-miR-NC); There was no significant difference between the miR-147a group(or inhibitor) and the miR-NC group(or anti-miR-NC) in the transfection of PDPK1–3′ UTR-Mut gene vector. So, it was concluded that PDPK1 was a target gene for direct regulation of miR-147a downstream. Verifying test results showed that the expression of PDPK1 mRNA and protein was reduced after overexpression of miR-147a, which was up-regulated after silencing miR-147a in TC and V79 cells.

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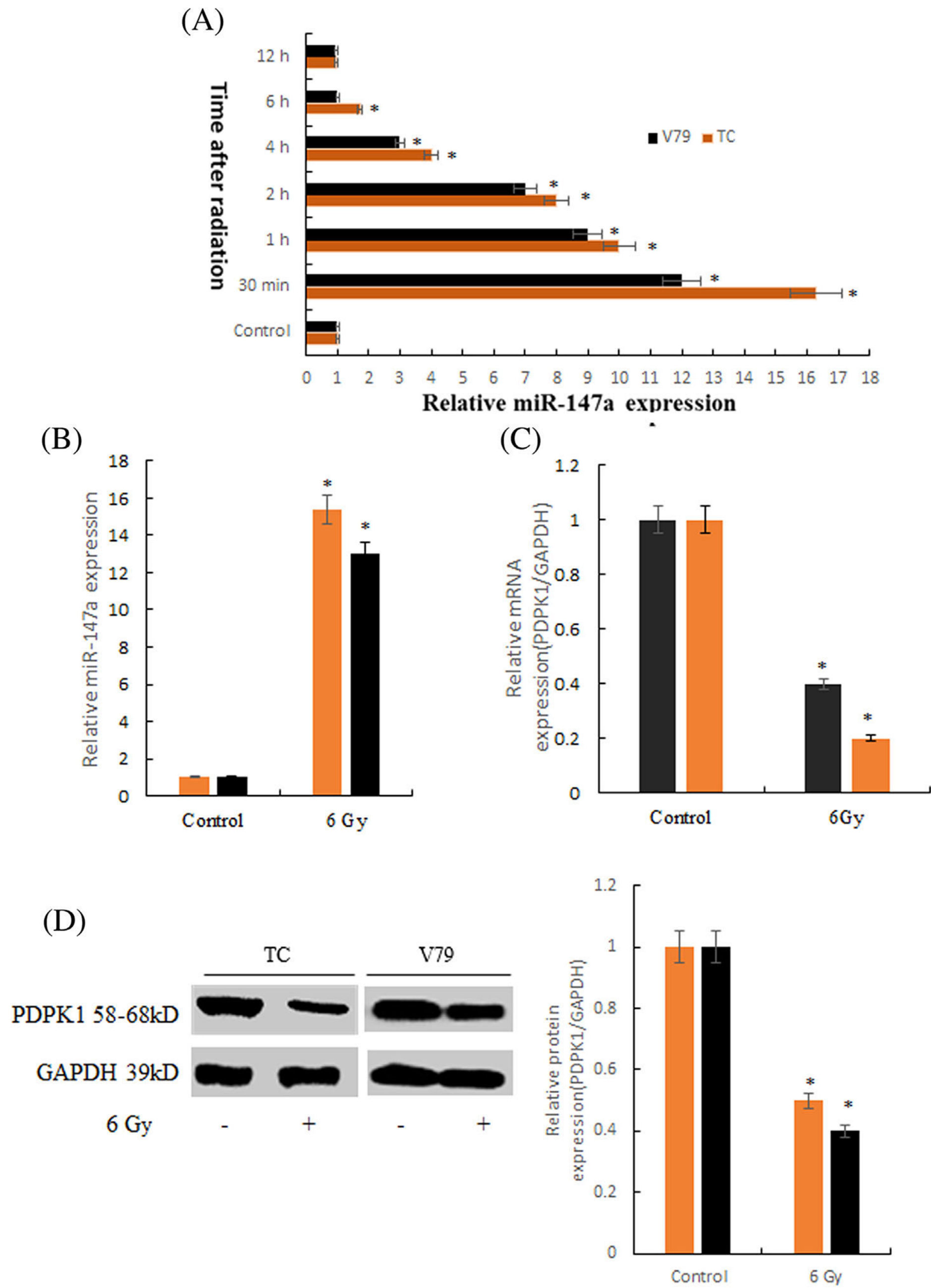

FIGURE 1.

The expressions of miR-147a and PDPK1 after irradiation in thymus cells. A, The optimal detection time of miR-147a was 30min after radiation (*P < 0.01 compared with Control). B, The expression level of miR-147a after radiation (*P < 0.01 compared with Control). C, The expression level of PDPK1 mRNA after radiation (*P < 0.01 compared with Control). D, The expression level of PDPK1 protein after radiation (*P < 0.01 compared with Control)

(A)

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 19-25 of PDPK1 3' UTR hsa-miR-147a	5' ...ACCCCAGCGGGAACGCACACAAG... 3' CGUCUUCGUAAAG—GUGUGUG	7mer-A1	-0.02	54	-0.01	0.073	N/A
Position 264-271 of PDPK1 3' UTR hsa-miR-147a	5' ...AAAAAAAACACCCAACCACACAA... 3' CGUCUUCGUAAAGGUGUGUG	8mer	-0.22	98	-0.21	0.413	N/A
Position 2631-2637 of PDPK1 3' UTR hsa-miR-147a	5' ...GGGCCAUGUGCCUCCCCACACAU... 3' CGUCUUCGUAAAGGUGUGUG	7mer-m8	-0.02	63	-0.02	0.012	N/A

Binding sites 264-271

Position 264-271 of PDPK1 3'UTR **5' ...AAAAAAAACACCCAACCACACAA... 3'**

has-miR-147a **3' CGUCUUCGUAAAGGUGUGUG 5'**

(B)

gga cgcttccaga ccacctgcca gccatcacaa ggggaacgca
gaggcggaaa ccttgcagca tttttattta aaagaaaaga agaaaaaaaa
cacccaacca cacaaagaac aaaaccagta acaaacacaa aggaattcag
ggtcgctttg cttgctctct gtgctccgtg gaggcctccg tgtgccctcg
ttgccgtggg gaccca

miR-147a binding sites 264-271

FIGURE 2.

Binding sites between miR-147a and PDPK1. A, Binding sites of miR-147a and PDPK1 3'UTR region. B, PDPK1 3′ UTR fragment marked with binding sites

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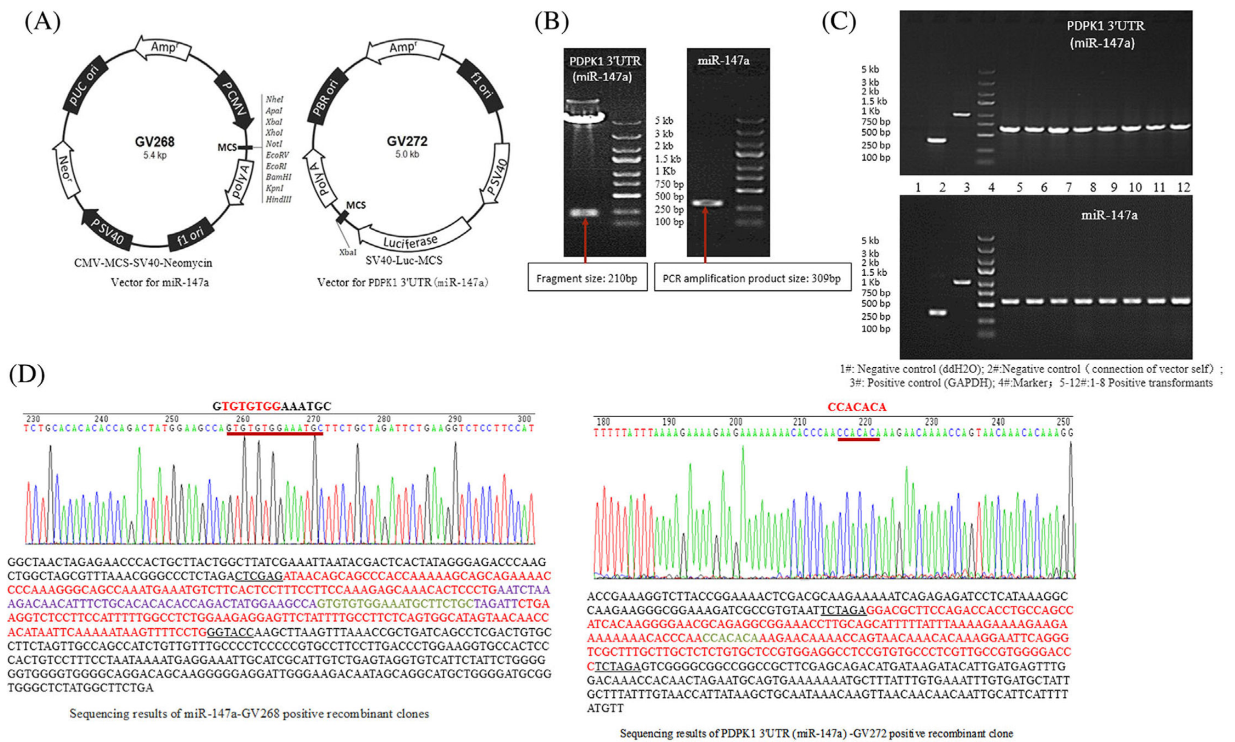

FIGURE 3.

Over expression plasmid construction. A, Vector information. B, Electrophoresis of the enzyme product containing PDPK1 3'UTR (miR-147a) gene sequence and electrophoresis of PCR amplified products of miR-147a. C, Identification of recombinant clones by PCR. D, Results of positive clone sequencing

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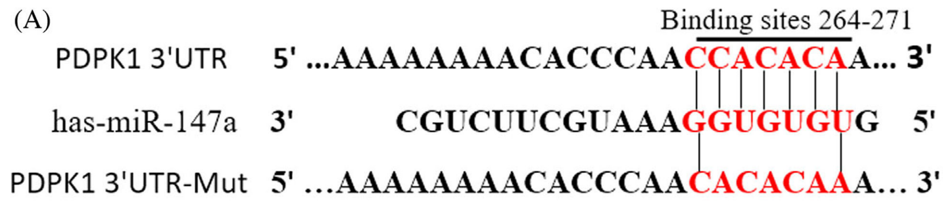

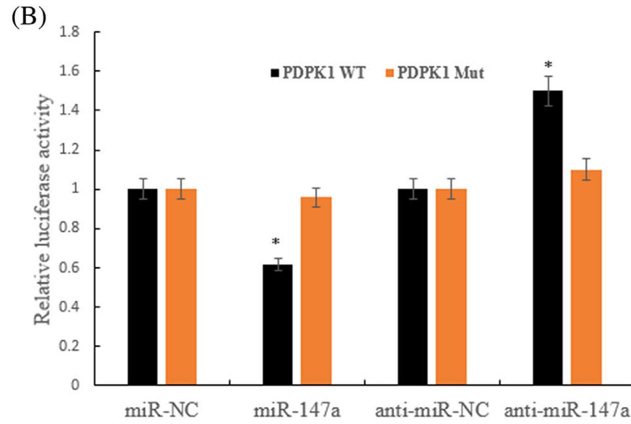

FIGURE 4.

Targeted binding of miR-147a and PDPK1. A, Wild type and mutant sequences of PDPK1–3′UTR and miR-147a binding sites; B, Overexpression or silencing of miR-147a (anti-miR-147a) effect on the activity of PDPK1–3′UTR verified by Dual-Luciferase Reporter Gene Assay (*P < 0.01 compared with NC)

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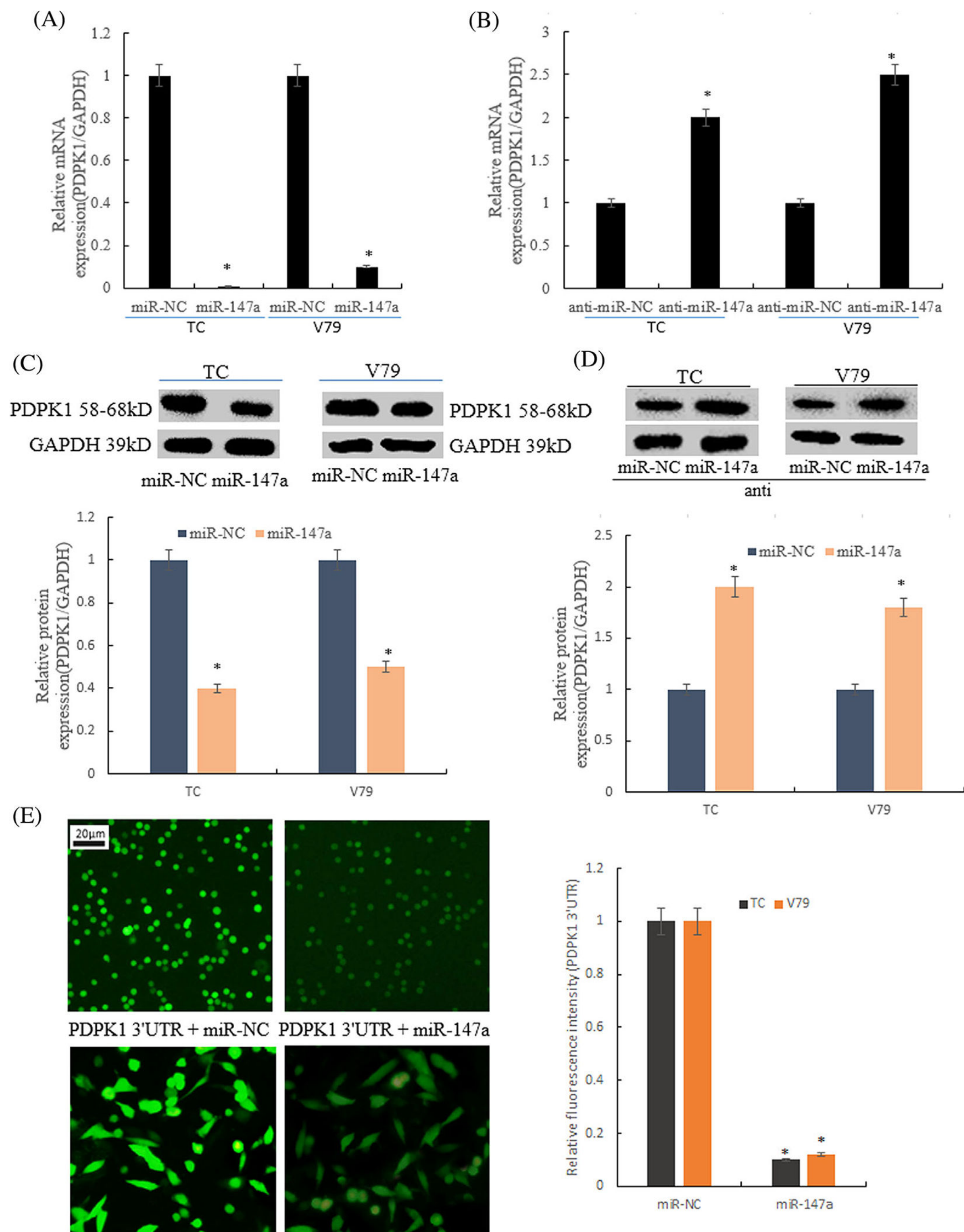

FIGURE 5.

Expression of PDPK1 negativly regulated by miR-147a. A, mRNA expression of PDPK1 was detected by qRT-PCR method after transfection of miR-147a overexpressing plasmid (*P < 0.01 compared with miR-NC); B, mRNA expression of PDPK1 was detected by qRT-PCR method after transfection of miR-147a inhibitor; C, Western blot method was used to detect the expression of PDPK1 in the cells transfected with miR-147a overexpression plasmid (*P < 0.01 compared with miR-NC); D, Western blot was used to detect the expression of PDPK1 protein in cells transfected with miR-147a inhibitor (*P < 0.01

compared with miR-NC); E, Fluorescence expression of PDPK1 3′UTR marked by GFP (*P < 0.01 compared with miR-NC)

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