

PVT1 Mediates Cell Proliferation, Apoptosis and Radioresistance in Nasopharyngeal Carcinoma Through Regulating miR-515-5p/PIK3CA Axis

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Background: Radioresistance greatly hinders the treatment of nasopharyngeal carcinoma (NPC). Long noncoding RNA (lncRNA) plasmacytoma variant translocation 1 (*PVT1*) has been corroborated to participate in diverse cancers, including NPC. Our aim was to investigate the underlying molecular mechanism of *PVT1* in NPC radioresistance.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to measure the expression levels of *PVT1*, microRNA (*miR*)-515-5p and phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) in NPC tissues and cells. Cell counting kit-8 (CCK8) assay, colony formation assay and flow cytometry assay were employed to detect cell proliferation, radiosensitivity and apoptosis, respectively. The protein levels of Cyclin D1, B-cell lymphoma 2 associated X (Bax), Cleaved-caspase-3, *PIK3CA*, protein kinase B (AKT) and phosphorylated AKT (p-AKT) in samples were measured by Western blot. The starBase was used to predict the binding sites between *miR*-515-5p and *PVT1* or *PIK3CA*. Dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay were performed to verify the interaction. Xenograft tumor model was established to investigate the biological role of *PVT1* in vivo.

Results: The levels of *PVT1* and *PIK3CA* were upregulated in NPC tissues and cells, opposite to the expression of *miR*-515-5p. Knockdown of *PVT1* inhibited cell proliferation, radioresistance and promoted cell apoptosis in NPC cells. Meanwhile, *PVT1* silencing downregulated Cyclin D1, and upregulated Bax and Cleaved-casp-3 in NPC cells after radiotherapy. Besides, *miR*-515-5p interacted with *PVT1* and targeted *PIK3CA* in NPC cells. Further studies indicated that *PVT1* regulated radioresistance via *miR*-515-5p/*PIK3CA* axis and modulated the AKT pathway by interacting with *miR*-515-5p. Moreover, knockdown of *PVT1* suppressed tumor growth in vivo.

Conclusion: Downregulation of *PVT1* inhibited proliferation, radioresistance and promoted apoptosis by downregulating *PIK3CA* via sponging *miR*-515-5p in NPC cells.

Keywords: NPC, *PVT1*, *miR*-515-5p, *PIK3CA*, radioresistance

Introduction

Nasopharyngeal carcinoma (NPC) is a cancer arising from the nasopharynx epithelium, and radiotherapy is the primary and only curative treatment for NPC.¹ However, radioresistance is a huge obstacle for the treatment of NPC.^{2,3} Hence, it is compulsory to figure out the hidden molecular mechanism of radioresistance in NPC.

Long noncoding RNAs (lncRNAs) are a kind of RNA molecules with the length exceeding 200 nucleotides and they cannot encode proteins.⁴ Growing studies showed that lncRNAs were associated with radioresistance in diverse human cancers,^{5,6}

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including NPC.^{7,8} Recently, lncRNA plasmacytoma variant translocation 1 (*PVT1*) was reported to induce radioresistance in NPC.⁹ However, the function of *PVT1* in mediating radioresistance in NPC is still poorly understood.

MicroRNAs (miRNAs) are small noncoding RNAs (nearly 22 nucleotides in length), which regulate gene expression by targeting the 3'-untranslated region (3'UTR) at the post-transcriptional level.¹⁰ Emerging reports have intensely deepened our comprehension of the crucial role of miRNAs in radioresistance.^{11–13} *MIR-515-5p* was reported to be correlated with breast cancer,¹⁴ gastric cancer¹⁵ and prostate cancer.¹⁶ Nevertheless, the role of *miR-515-5p* in radioresistance in NPC is little known and worth investigating.

Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) was involved in diverse human cancers. A previous study reported that *PIK3CA* acted as an oncogene in cervical cancer.¹⁷ *PIK3CA* mutations led to fulvestrant resistance in ER-positive breast cancer.¹⁸ Moreover, *PIK3CA* amplification was associated with the poor prognosis of NPC.¹⁹ Therefore, *PIK3CA* may be an appropriate drug target and novel regulators which modulate *PIK3CA* expression require to be defined.

In this study, the expression of *PVT1*, *miR-515-5p* and *PIK3CA* in NPC cells and tissues was measured. The potential regulatory mechanism of *PVT1* in radioresistance was further investigated by the subsequent experiments.

Materials and Methods

Samples and Cell Culture

Twenty-nine NPC tissues and the paired nearby non-cancerous tissues (within 3 cm around tumors) were collected from the First Affiliate Hospital of Xinjiang Medical University. The clinicopathologic features of these patients are presented in Table 1. The informed consent was acquired from every patient and this research was authorized by the Ethics Committee of the First Affiliate Hospital of Xinjiang Medical University. Human nasopharyngeal epithelial cell line (NP-69) and NPC cell lines (C666-1 and 5-8F) were purchased from Otwo Biotech (Shenzhen, China). McCoy's 5A medium (Sigma, St Louis, MO, USA) containing 5% CO₂ and 10% fetal bovine serum (FBS; Sigma) was utilized to culture cells.

Cell Transfection

Lentivirus harboring short hairpin RNA targeting *PVT1* (named as sh-*PVT1*) and its negative control (named as sh-

Table 1 The Clinicopathological Parameters of NPC Patients

Clinical Factor	Cases	PVT1 Level		P value
		Low	High	
	n=29	n=14	n=15	
Gender				0.586
Male	16	9	9	
Female	13	5	6	
Age (years)				0.635
<44	12	8	10	
≥44	17	6	5	
Clinical stage				0.032
I+II	12	7	4	
III+IV	17	7	11	
Lymphatic invasion				0.499
No	20	11	13	
Yes	9	3	2	
Distant metastasis				0.048
No	19	10	8	
Yes	10	4	7	

Control) were constructed by GeneCopoeia (Rockville, MD, USA). *MIR-515-5p* mimic (named as *miR-515-5p*), *miR-515-5p* inhibitor (named as Anti-*miR-515-5p*) and the corresponding controls (miR-Control and Anti-Control) were obtained from GenePharma (Shanghai, China). *PVT1* overexpression plasmid (named as *PVT1*), *PIK3CA* overexpression plasmid (named as *PIK3CA*) and the corresponding control (named as Vector) were acquired from RiboBio (Guangzhou, China). Cell transfection was executed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) referring to the given procedures.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Vazyme, Nanjing, China). Then, RNA was reversely transcribed to complementary DNA (cDNA) by PrimeScript™ RT Master Mix kit (Takara, Dalian, China). The qRT-PCR was performed by SYBR Green PCR Master Mix (Vazyme) and data were analyzed using 2^{-ΔΔCt} method. Beta-actin (*β-actin*) and *U6* were introduced as the inner references. Primers in this study were listed as follows: *PVT1* (forward 5'-TGCGGCAGTGGTTTTACCCTA TG-3', reverse 5'-CCAGTGCAGGGTCCGAGGT-3');

miR-515-5p (forward, 5'-TTCTCCAAAAGAAAGCACT TTCTG-3', reverse 5'-TGGTGTCGTGGAGTCG-3'); *PIK3CA* (forward, 5'-CCACGACCATCATCAGGTGAA-3', reverse 5'-CCTCACGGAGGCATTCTAAAGT-3'); *β-actin* (forward 5'-GCACCACACCTTCTACAATG-3', reverse, 5'-TGCTTGCTGATCCACATCTG-3'); *U6* (forward, 5'-TCCGGGTGATGCTTTTCCTAG-3', reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3').

Counting Kit-8 (CCK-8) Assay

After transfection, 3×10^4 C666-1 and 5-8F cells were seeded into 96-well plates and then incubated with 10 μ L CCK-8 solution (vazyme) for 2 h. Optical density values were examined at 450 nm wavelength under the microplate reader (Bio-Rad, Richmond, Virginia, USA).

Cell Radiosensitivity Analysis

The survival fractions of C666-1 and 5-8F cells under radiotherapy were checked by the colony formation assay. In brief, cells (1×10^3 cells/well) were seeded into 6-well plates and incubated overnight. Afterwards, cells were exposed to irradiation doses of 0, 2, 4, 6 and 8 Gy. After 12 d of culture, cell colonies were stained with 0.5% crystal violet (Solarbio, Beijing, China). The Image J software was utilized to quantify cell numbers.

Flow Cytometry

Annexin Apoptosis Detection Kit (Sigma) was hired to analyze cell apoptosis following the given procedures. Briefly, 5×10^4 cells were resuspended using the binding buffer and then 5 μ L Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and 5 μ L propidium iodide (PI) were added to the buffer to incubate for 5 min in the dark. The stained cells were analyzed by flow cytometry (Countstar, Shanghai, China).

Western Blot

Proteins were isolated using RIPA buffer (Vazyme) and segregated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto the polyvinylidene difluoride (PVDF) membranes (Vazyme) and the membranes were blocked with 5% skimmed milk (Vazyme). After being washed by phosphate-buffered saline (PBS), the membranes were incubated with the primary antibodies: Cyclin D1 (1:1000, ab16663, Abcam, Cambridge, United Kingdom), B-cell lymphoma 2 associated X (Bax) (1:3000, ab32503, Abcam), Cleaved-caspase-3 (Cleaved-casp-3) (1:1000,

ab2302, Abcam), phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) (1:2000, ab40776, Abcam), protein kinase B (AKT) (1:1000, ab8805, Abcam) and phosphorylated AKT (p-AKT) (1:1000, ab38449, Abcam) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2500, ab9485, Abcam) overnight. After being rewashed, the membranes were incubated with the secondary antibody (1:3000, ab205718, Abcam) for 3 h. The membranes were analyzed by the ChemiDoc™ MP Imaging System (Bio-Rad) after being treated with ECL kit (Vazyme).

Dual-Luciferase Reporter Assay

The potential complementary sequences of *miR-515-5p* and *PVT1* or *PIK3CA* were forecasted by starBase.²⁰ The luciferase activity was detected by dual-luciferase reporter assay as previously reported.²¹ The wild type sequence of *PVT1* or *PIK3CA* 3'UTR harboring the binding sites of *miR-515-5p* was inserted into the pGL3 vector (Promega, Madison, WI, USA) to construct the luciferase reporter vector *PVT1*-WT or *PIK3CA*-WT. Similarly, *PVT1*-MUT and *PIK3CA*-MUT reporter vectors were established by mutating the potential target sites of *miR-515-5p*. Then, the vectors with *miR-515-5p* or miR-Control were co-transfected into C666-1 and 5-8F cells using Lipofectamine 2000 (Invitrogen). The Dual-Glo® Luciferase Assay System kit (Promega) was utilized to measure luciferase activity.

RNA Immunoprecipitation (RIP) Assay

RIP was executed using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) referring to the provided protocols. Briefly, the harvested cells were lysed and incubated with magnetic beads conjugated with anti-Argonaute 2 (Anti-Ago2) antibody (Millipore) and anti-immunoglobulin G (Anti-IgG) antibody (Millipore) was used as a negative control. The protein was removed by Proteinase K. The immune precipitated RNA was purified and analyzed by qRT-PCR.²²

Xenograft Mice Model

Six-week-old BALB/c nude mice were acquired from Vital River Laboratory Animal Technology (Beijing, China). 2×10^6 C666-1 cells infected with sh-*PVT1* or sh-Control were subcutaneously injected into the flank of the nude mice. The tumor volume was calculated every 5 d according to the formula: $0.5 \times \text{length} \times \text{width}^2$. The tumor weight was measured after the mice were euthanized. The mRNA or protein levels of related genes in tumors were

checked by qRT-PCR or Western blot. The animal experiment was approved by the Animal Care and Use Committee of the First Affiliate Hospital of Xinjiang Medical University and performed following the instructions of the national animal protection and ethics institute.

Statistical Analysis

Experimental data were calculated by GraphPad Prism (GraphPad, La Jolla, CA, USA) and presented by mean \pm standard deviation (SD). Two independent groups were compared by using Student's *t*-test. For more than two groups, the one-way analysis of variance (ANOVA) was utilized to assess the difference. The Kaplan-Meier method was utilized to assess overall survival and the Log rank test was utilized to analyze the differences between survival curves. Every experiment was repeated at least three times independently. $P < 0.05$ represented statistical significance.

Results

PVT1 Was Highly Expressed in NPC and Was Associated with the Poor Prognosis

To explore the role of *PVT1* in NPC, we first measured its expression patterns. The qRT-PCR data showed that *PVT1* was significantly upregulated in NPC tissues compared with the paired non-tumorous tissues (Figure 1A). Afterwards, the correlation between *PVT1* expression and NPC progression was evaluated and the results showed that the level of *PVT1* was higher in advanced stages (III + IV) compared with early stages (I + II) (Figure 1B). Besides, *PVT1* was also upregulated in NPC cells (Figure 1C), and the higher level of *PVT1* resulted in the lower survival rate of NPC patients (Figure 1D). Altogether, these results demonstrated that *PVT1* might act as an oncogene in NPC and its high expression led to the poor prognosis.

Knockdown of PVT1 Inhibited Proliferation, Promoted Apoptosis and Suppressed Radioresistance in vitro

To study the function of *PVT1* in NPC, NPC cells were first infected with sh-*PVT1* or sh-Control and the knockdown efficiency was confirmed (Figure 2A). Then, CCK8 assay was performed and the result showed that downregulation of *PVT1* significantly inhibited proliferation of NPC cells (Figure 2B). Besides, colony formation assay indicated that *PVT1* silencing clearly promoted radiosensitivity in NPC cells (Figure 2C). Meanwhile, flow cytometry assay showed that downregulation of *PVT1* notably induced apoptosis in

NPC cells after radiotherapy (Figure 2D). Afterwards, the protein levels of Cyclin D1 (related to proliferation), Bax and Cleaved-casp-3 (related to apoptosis) were measured by Western blot and the data showed that silence of *PVT1* markedly decreased the level of Cyclin D1 and increased the expression of Bax and Cleaved-casp-3 in NPC cells after radiotherapy (Figure 2E and F). Collectively, these results illuminated that *PVT1* silencing handicapped proliferation and radioresistance and induced apoptosis in NPC cells in vitro.

PVT1 Targeted miR-515-5p and Negatively Regulated miR-515-5p in NPC Cells

The interaction between lncRNAs and miRNAs was documented in various human cancers.^{6,23} By using starBase, we found that *miR-515-5p* harbored the target sites of *PVT1* (Figure 3A). The dual-luciferase reporter assay was carried out to verify the interaction and the result showed that *miR-515-5p* significantly diminished the luciferase activity of *PVT1*-WT in NPC cells, rather than *PVT1*-MUT (Figure 3B). Also, RIP assay indicated that the relative enrichment of *PVT1* and *miR-515-5p* was higher in Anti-Ago2 group than in Anti-IgG group (Figure 3C). Thereafter, the expression of *miR-515-5p* was checked and the data disclosed that *miR-515-5p* was apparently declined in NPC tissues and cells (Figure 3D). To investigate the regulatory relationship between *PVT1* and *miR-515-5p*, *PVT1* or Vector was introduced to NPC cells and the overexpression efficiency was corroborated (Figure 3E). Our results showed that knockdown of *PVT1* markedly increased the level of *miR-515-5p*, while overexpression of *PVT1* clearly decreased the expression of *PVT1* in NPC cells (Figure 3F). All in all, these results illustrated that *miR-515-5p* was a target of *PVT1* and negatively modulated by *PVT1* in NPC cells in vitro.

Overexpression of PVT1 Reversed miR-515-5p-Mediated Effects on Proliferation, Apoptosis and Radioresistance in vitro

To further dissect the function of *PVT1* and *miR-515-5p* in NPC, NPC cells were transfected with *miR-515-5p*, *miR-515-5p* + *PVT1* or the matched controls. Cell proliferation was analyzed and the result indicated that *miR-515-5p* mimic significantly repressed the proliferation of NPC cells, whereas upregulation of *PVT1* reversed this effect (Figure 4A). Also, colony formation assay showed that overexpression of *PVT1* clearly inverted *miR-515-5p*-mediated inhibitory effect on

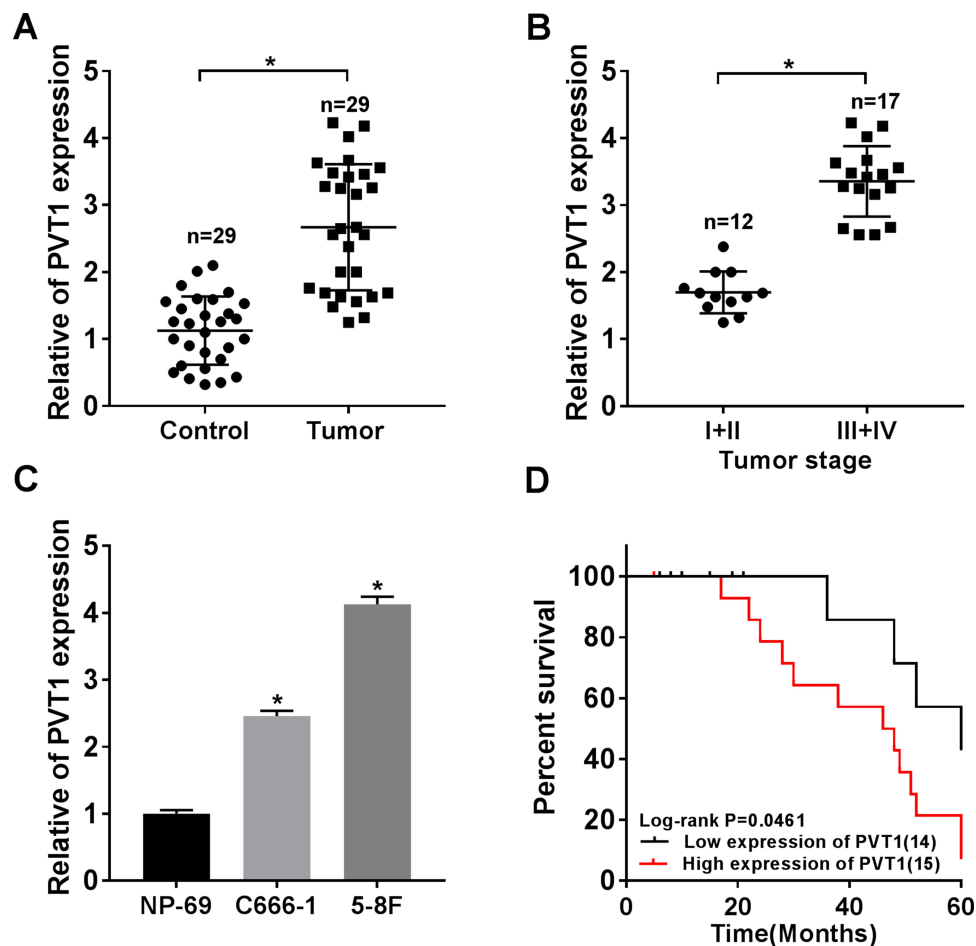


Figure 1 *PVT1* was highly expressed in NPC tissues and cells. **(A)** The expression level of *PVT1* was detected by qRT-PCR in NPC tissues (n = 29) and normal tissues (n = 29). **(B)** The level of *PVT1* in different TNM stages was checked by qRT-PCR. **(C)** The level of *PVT1* in normal cell line and NPC cell lines was measured by qRT-PCR. **(D)** The Kaplan–Meier method was utilized to estimate overall survival and the Log rank test was used to evaluate the differences between survival curves. Patients were divided into *PVT1* high (n = 15) and *PVT1* low (n = 14) groups by median value of *PVT1* expression. *P < 0.05.

radioresistance (Figure 4B). Simultaneously, flow cytometry assay indicated that the high apoptosis rate of NPC cells, treated with radiation and transfected with *miR-515-5p*, was transposed following the additional transfection with *PVT1* (Figure 4C and D). Analogously, the declined protein level of Cyclin D1 and increased level of Bax and Cleaved-casp-3 in *miR-515-5p* group were inverted following the infection with *PVT1* (Figure 4E and F). From these results, it could be concluded that *PVT1* mediated the proliferation, apoptosis and radioresistance in NPC cells by interacting with *miR-515-5p*.

miR-515-5p Bound to the 3'UTR of *PIK3CA* and Negatively Modulated *PIK3CA* in NPC Cells

To further probe the regulatory mechanism of *miR-515-5p*, we found its target gene *PIK3CA* using starBase

(Figure 5A). Subsequently, the dual-luciferase reporter assay and RIP assay were conducted to confirm the interaction. The dual-luciferase reporter assay showed that *miR-515-5p* significantly reduced the luciferase activity of *PIK3CA*-WT in NPC cells (Figure 5B). RIP assay indicated that *PIK3CA* and *miR-515-5p* were apparently enriched in Anti-Ago2 group (Figure 5C). Next, the mRNA and protein levels of *PIK3CA* were measured and the data indicated that *PIK3CA* was clearly increased in NPC tissues and cells (Figure 5D). We then checked the protein level of *PIK3CA* in NPC cells infected with *miR-515-5p*, *PIK3CA* or the matched controls, and the result indicated that *PIK3CA* was notably downregulated in *miR-515-5p* group (Figure 5E), while its expression was strikingly elevated in *PIK3CA* group (Figure 5F). Further analysis disclosed that the increased protein level of *PIK3CA* in *PIK3CA* group was reversed following the transfection with *miR-515-5p* mimic (Figure 5G). To sum up, these

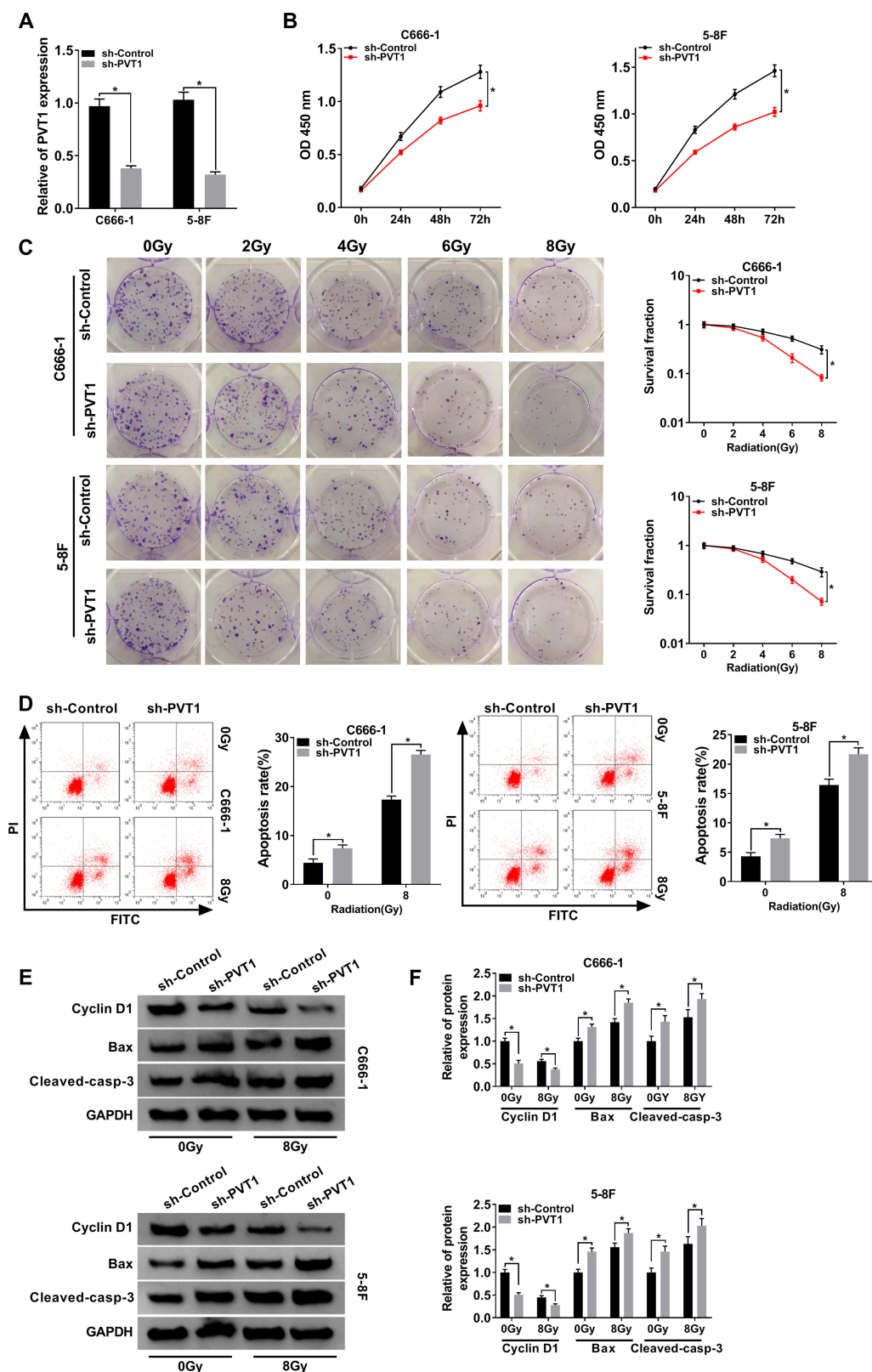


Figure 2 PVT1 regulated cell proliferation, apoptosis and radioresistance in NPC cells. **(A)** The expression level of PVT1 in NPC cells infected with sh-PVT1 or sh-Control was detected by qRT-PCR. **(B)** CCK8 assay was utilized to evaluate cell proliferation. **(C)** Colony formation assay was hired to analyze the survival fractions of C666-1 and 5-8F cells under different irradiation doses (0, 2, 4, 6 and 8 Gy). **(D)** Flow cytometry assay was performed to check apoptosis of infected NPC cells under different irradiation doses (0 and 8 Gy). **(E and F)** The protein levels of Cyclin D1, Bax and Cleaved-casp-3 in infected NPC cells under different irradiation doses (0 and 8 Gy) were measured by Western blot. *P < 0.05.

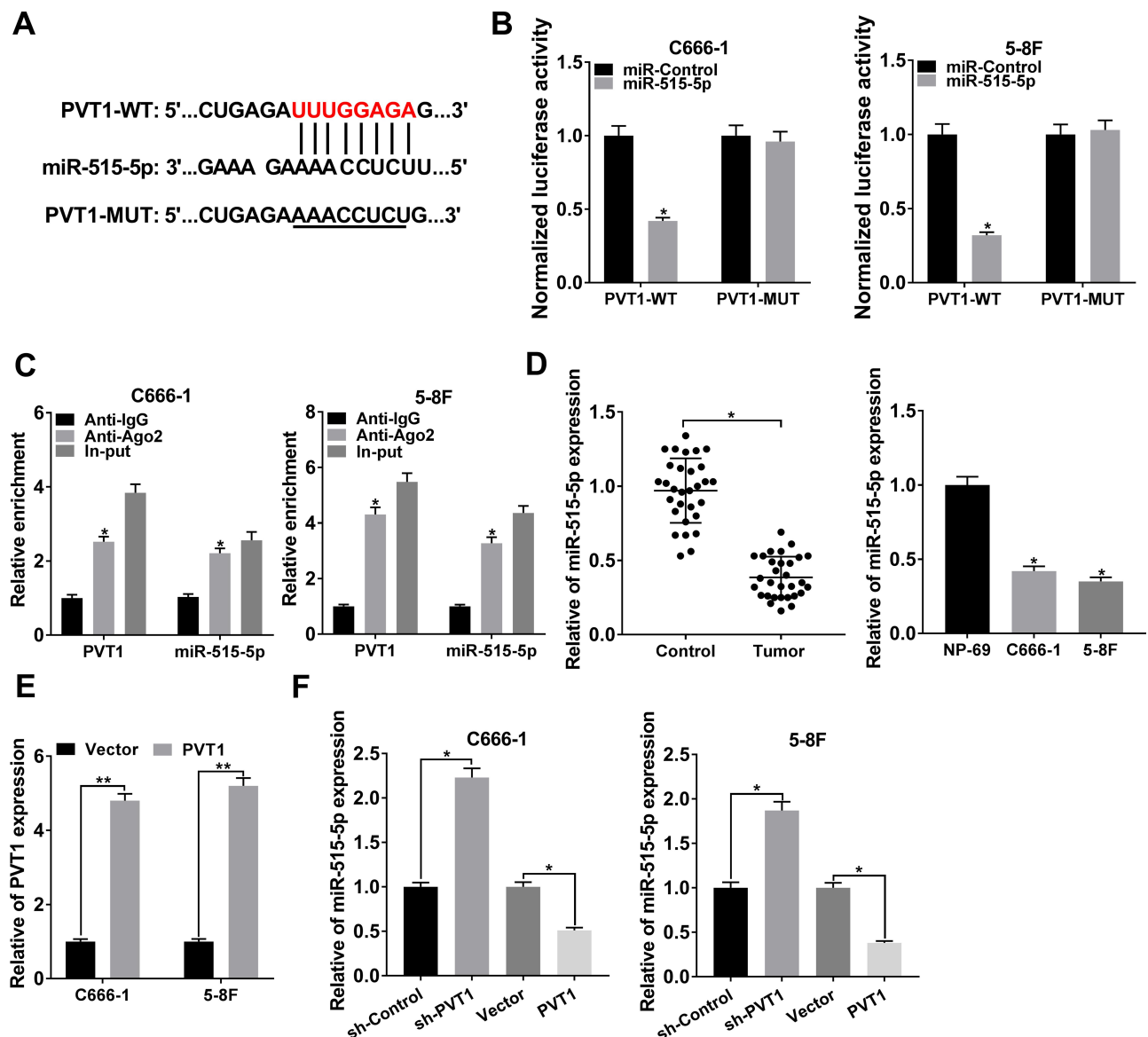


Figure 3 PVT1 interacted with miR-515-5p and negatively regulated the expression of miR-515-5p in NPC cells. (A) The putative binding sites between PVT1 and miR-515-5p were predicted by starBase. (B) The dual-luciferase reporter assay was used to check the luciferase activity of NPC cells cotransfected with the miR-515-5p and PVT1-WT or PVT1-MUT. (C) The RIP assay was conducted using Anti-Ago2 to investigate the relationship between PVT1 and miR-515-5p, and Anti-IgG was used as the control. (D) The level of miR-515-5p in NPC tissues and cells, as well as the matched controls was assessed by qRT-PCR. (E) The level of PVT1 in NPC cells infected with PVT1 or Vector was checked by qRT-PCR. (F) The expression of miR-515-5p in NPC cells infected with sh-PVT1 or PVT1, as well as the corresponding controls was detected by qRT-PCR. * $P < 0.05$.

results manifested that miR-515-5p targeted *PIK3CA* and negatively regulated *PIK3CA* expression in vitro.

Upregulation of miR-515-5p Inverted *PIK3CA*-Mediated Impacts on Proliferation, Apoptosis and Radioresistance in vitro

To further explore the roles of miR-515-5p and *PIK3CA* in NPC, the proliferation of NPC cells infected with *PIK3CA*, *PIK3CA* + miR-515-5p or corresponding controls was checked. The data showed that miR-515-5p mimic inverted

PIK3CA-mediated promoted effect on cell proliferation (Figure 6A). In addition, colony formation assay indicated that upregulation of miR-515-5p significantly rescued *PIK3CA*-mediated enhanced impact on radioresistance (Figure 6B). Also, the flow cytometry assay demonstrated that overexpression of *PIK3CA* inhibited the apoptosis of NPC cells treated with radiation, whereas the effect was abolished by miR-515-5p mimic (Figure 6C and D). Similarly, the increased protein level of Cyclin D1 and declined level of Bax and Cleaved-casp-3 in *PIK3CA* group were reversed following the transfection with miR-

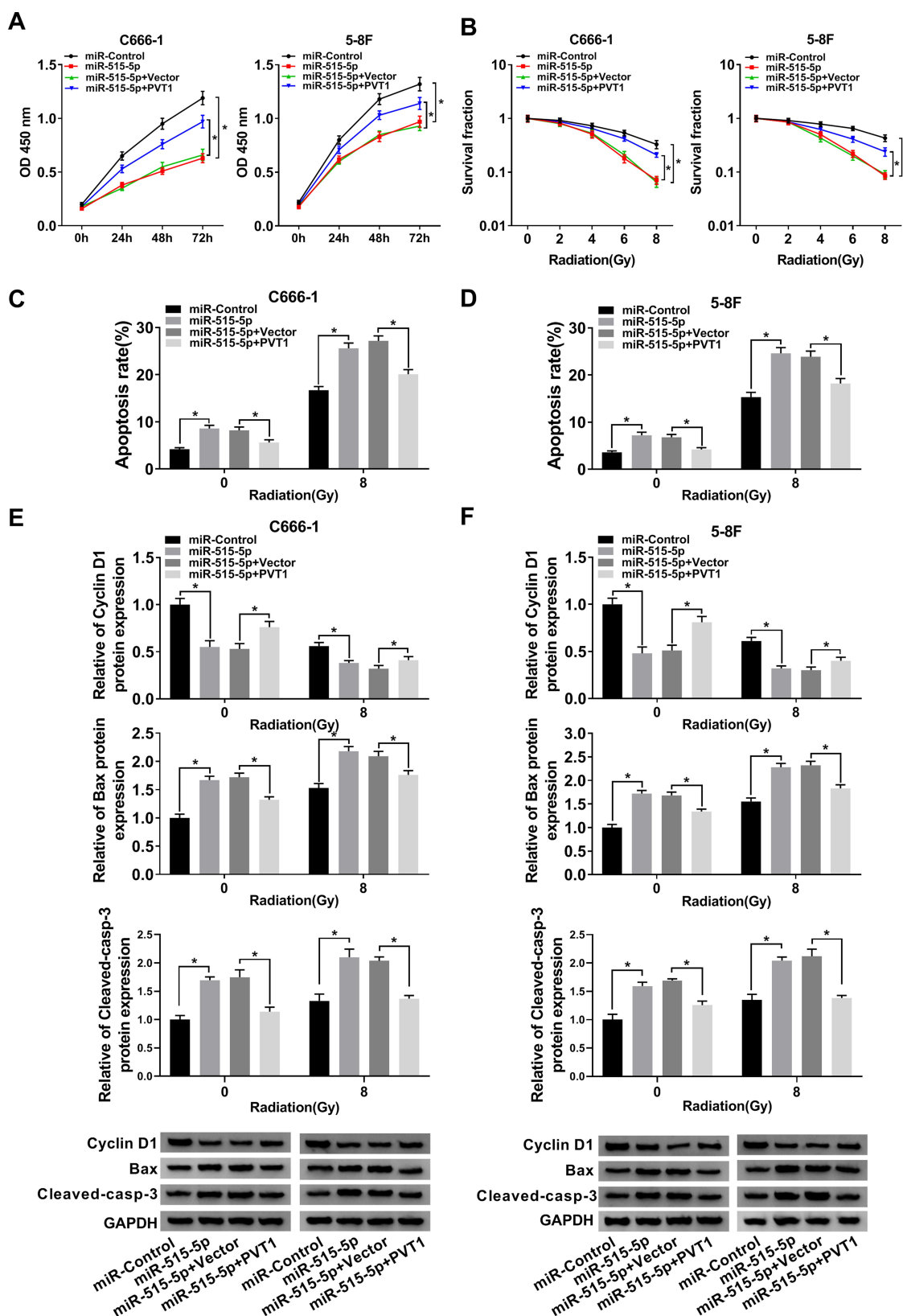


Figure 4 Upregulation of *PVT1* overturned *miR-515-5p*-mediated effects on proliferation, apoptosis and radioresistance in NPC cells. **(A)** CCK8 assay was employed to check the proliferation of NPC cells transfected with *miR-515-5p*, *miR-515-5p* + *PVT1* or their controls. **(B)** Colony formation assay was performed to analyze the survival fractions of C666-1 and 5-8F cells under different irradiation doses (0, 2, 4, 6 and 8 Gy). **(C)** and **(D)** Flow cytometry assay was executed to check apoptosis of transfected NPC cells under different irradiation doses (0 and 8 Gy). **(E)** and **(F)** The protein levels of Cyclin D1, Bax and Cleaved-casp-3 in transfected NPC cells under different irradiation doses (0 and 8 Gy) were measured by Western blot. **P* < 0.05.

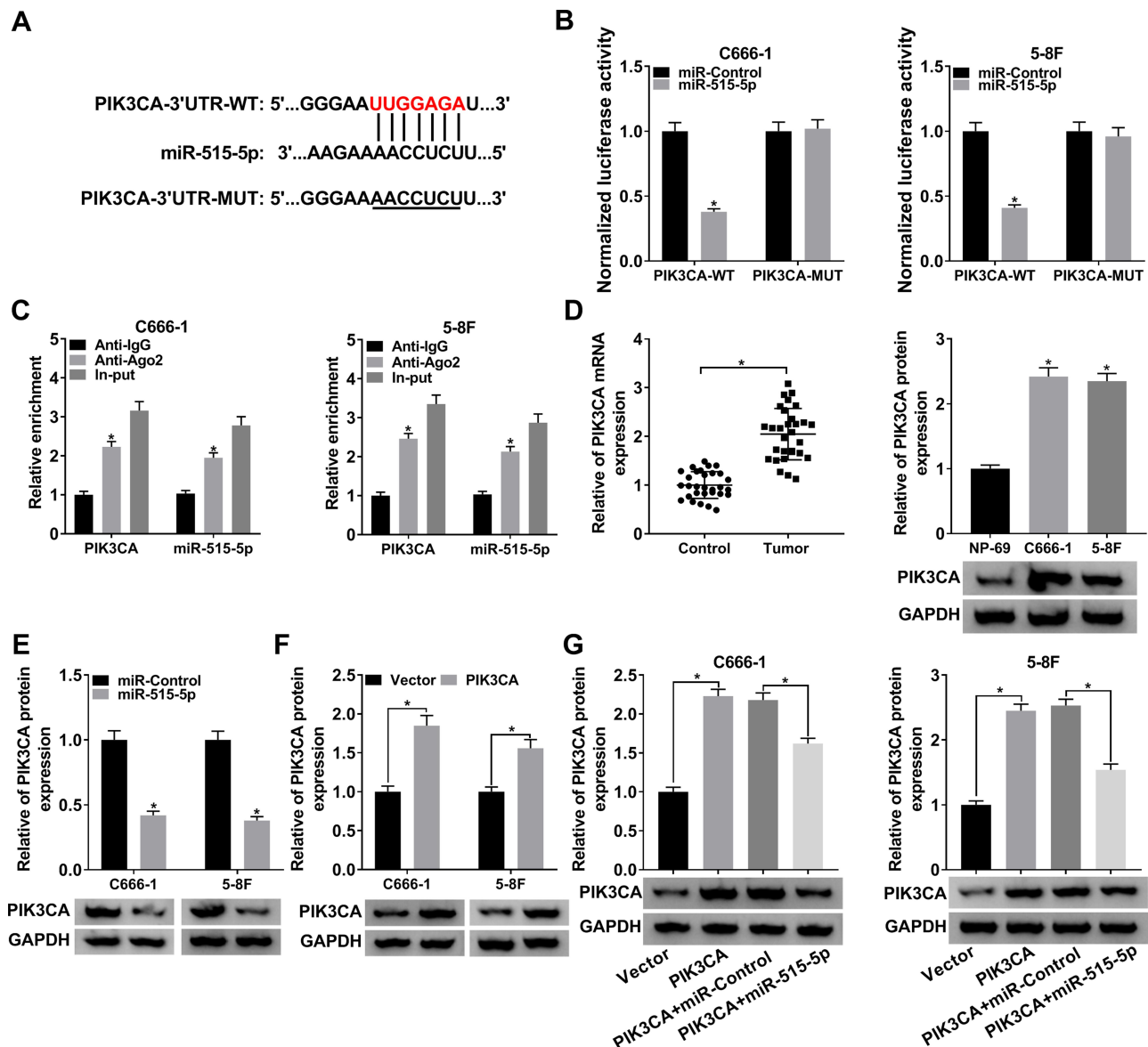


Figure 5 *PIK3CA* was a target of *miR-515-5p* and was negatively modulated by *miR-515-5p* in NPC cells. (A) The potential target sites between *miR-515-5p* and *PIK3CA* were forecasted by starBase. (B) The dual-luciferase reporter assay was used to verify the interaction between *miR-515-5p* and *PIK3CA*. (C) The RIP assay was carried out by using Anti-Ago2 to study the interaction between *miR-515-5p* and *PIK3CA*, and Anti-IgG was used as the control. (D) The mRNA and protein levels of *PIK3CA* in NPC cells were assessed by qRT-PCR and Western blot, respectively. (E) The protein level of *PIK3CA* in NPC cells infected with *miR-515-5p* or *miR-Control* was checked by Western blot. (F) The protein level of *PIK3CA* in NPC cells infected with *PIK3CA* or Vector was checked by Western blot. (G) The expression of *PIK3CA* in NPC cells infected with *PIK3CA* or *PIK3CA* + *miR-515-5p*, as well as the matched controls was checked by Western blot. * $P < 0.05$.

515-5p mimic (Figure 6E and F). In summary, these results suggested that *miR-515-5p* and *PIK3CA* played opposite roles in regulating proliferation, apoptosis and radioresistance in NPC cells.

PVT1/miR-515-5p Axis Regulated AKT Pathway in NPC Cells

A previous report indicated that AKT pathway activation was associated with *PIK3CA* mutations²⁴ and AKT signaling pathway functioned in radioresistance in many human

cancers.^{25,26} To figure out whether *PVT1* could affect AKT pathway, we checked the expression of *PIK3CA*, AKT, p-AKT in NPC cells infected with sh-*PVT1*, sh-*PVT1* + Anti-*miR-515-5p* or the matched controls. The results indicated that downregulation of *PVT1* markedly decreased the levels of *PIK3CA* and p-AKT, while *miR-515-5p* inhibitor transposed the effect (Figure 7A and B). Overall, these results manifested that *PVT1* mediated AKT pathway by interacting with *miR-515-5p* in NPC cells in vitro.

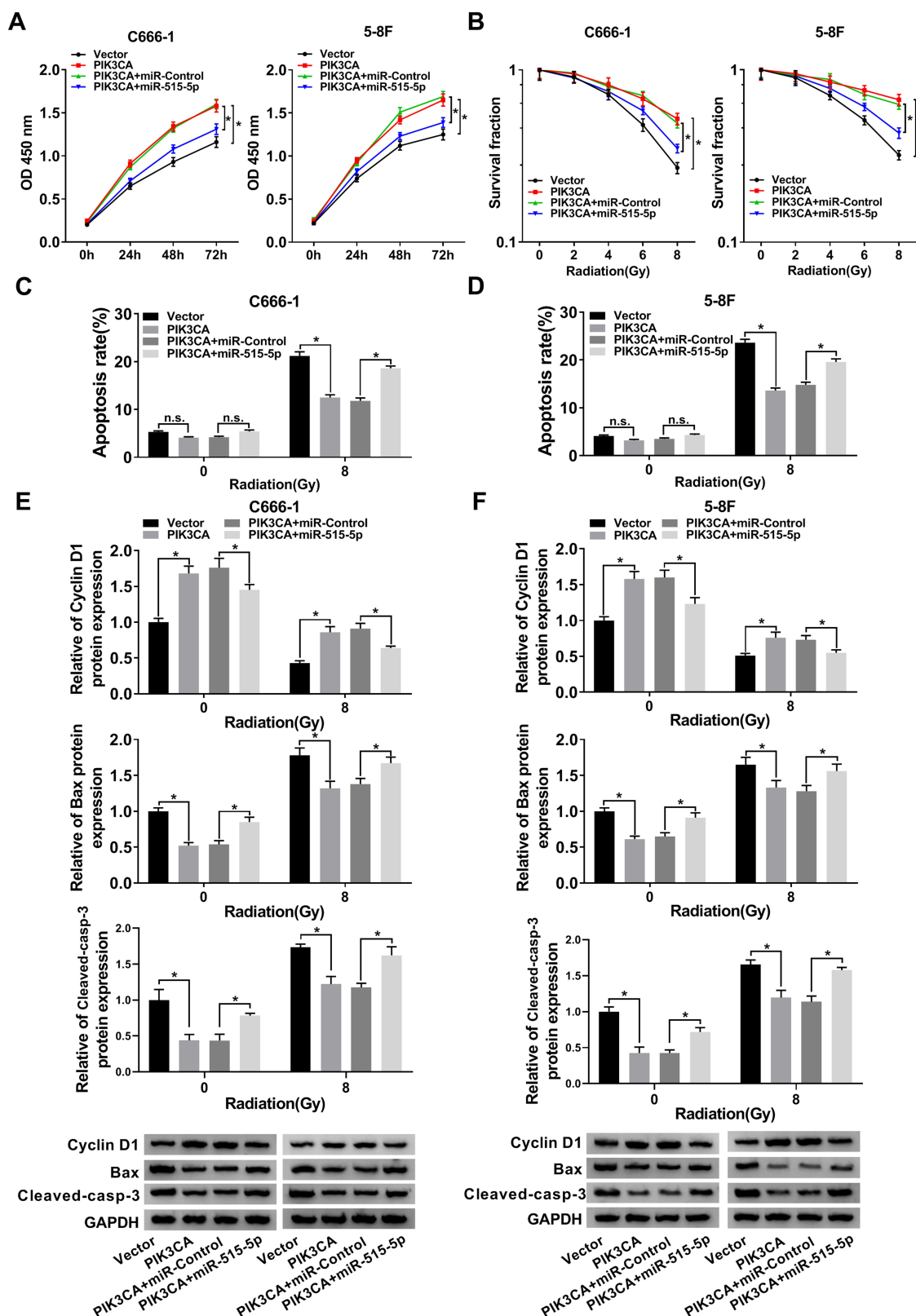


Figure 6 MiR-515-5p mimics reversed PIK3CA-mediated impacts on proliferation, apoptosis and radioresistance in NPC cells. (A) CCK8 assay was utilized to check proliferation of NPC cells transfected with PIK3CA, PIK3CA + miR-515-5p or the matched controls. (B) Colony formation assay was hired to analyze the survival fractions of NPC cells under different irradiation doses (0, 2, 4, 6 and 8 Gy). (C and D) Flow cytometry assay was utilized to analyze apoptosis of transfected NPC cells under different irradiation doses (0 and 8 Gy). (E and F) The protein levels of Cyclin D1, Bax and Cleaved-casp-3 in transfected NPC cells under different irradiation doses (0 and 8 Gy) were detected by Western blot. * $P < 0.05$.

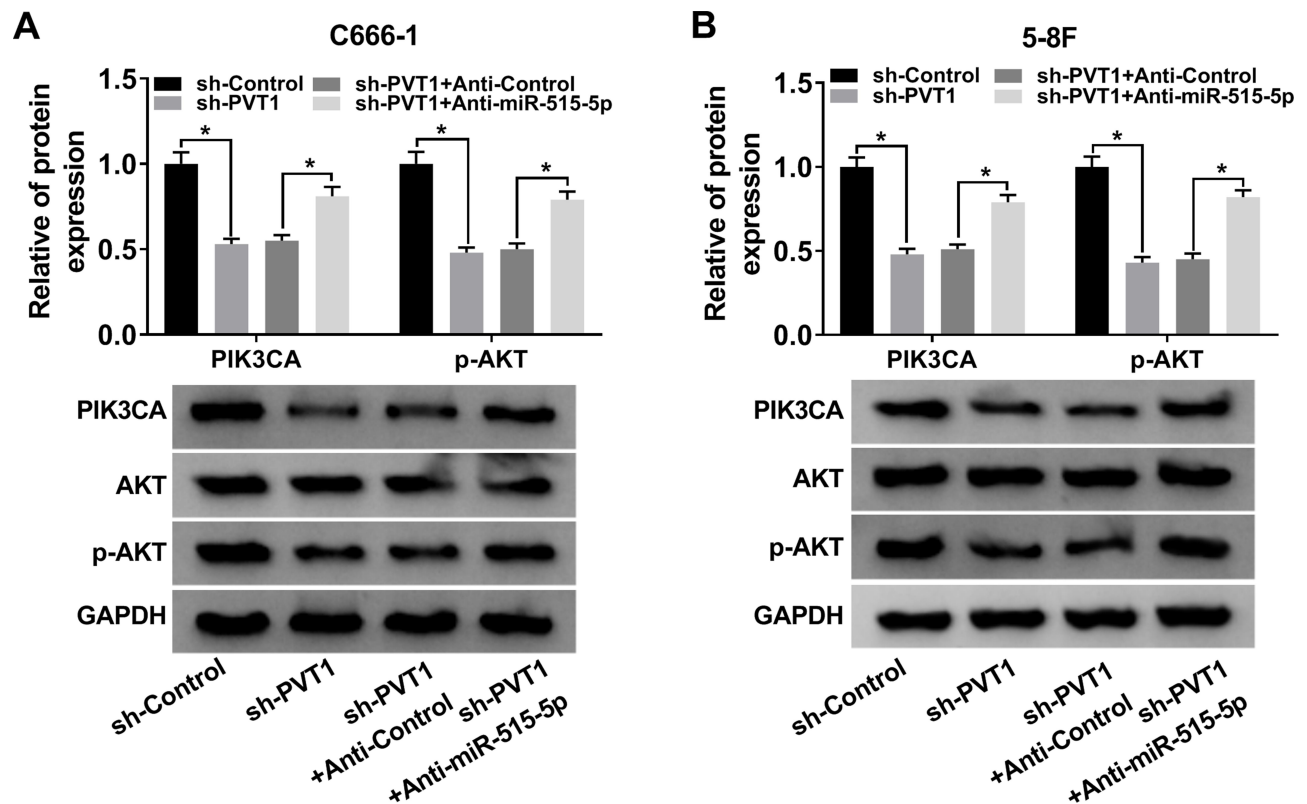


Figure 7 PVT1 regulated AKT pathway via sponging *miR-515-5p* in NPC cells. (A and B) The protein levels of PIK3CA, AKT and p-AKT in NPC cells transfected with sh-PVT1, sh-PVT1 + Anti-*miR-515-5p* or corresponding controls were evaluated by Western blot. * $P < 0.05$.

Knockdown of PVT1 Hampered Tumor Growth in vivo

To verify the function of *PVT1* in vivo, we established the xenograft mouse model using C666-1 cells transfected with sh-*PVT1* or sh-Control. The data showed that knockdown of *PVT1* contributed to the obvious shrink in tumor volume (Figure 8A) and decline in tumor weight (Figure 8B). Meanwhile, the levels of *PVT1* and *PIK3CA* were remarkably declined in sh-*PVT1* group (Figure 8C), opposite to the expression of *miR-515-5p*. Besides, knockdown of *PVT1* significantly reduced the expression of *PIK3CA* in tumors (Figure 8D). Taken together, these results suggested that *PVT1* silencing suppressed tumor growth in vivo.

Discussion

NPC is a growing threat to humans and radiotherapy is thought to be the only curative treatment.¹ Yet, radioresistance greatly discourages the therapeutic efficacy for NPC.^{2,3} Hence, it is essential to find the new molecular targets and investigate potential mechanisms. Lately, lncRNAs were reported to play roles in radioresistance in many human cancers. For instance, lncRNA *TP73-AS1*

silencing reduced radioresistance in hepatocellular carcinoma via the PTEN/Akt signaling pathway.⁵ LncRNA *LINC02582* promoted radioresistance in breast cancer cells.⁶ Moreover, knockdown of lncRNA *ANRIL* enhanced radiosensitivity in NPC cells.⁷ Also, a recent paper showed that lncRNA *PVT1* induced radioresistance in NPC cells.⁹ In order to investigate the role of *PVT1* in NPC, we checked its expression level and found that *PVT1* was strikingly upregulated in NPC tissues and cells and contributed to the poor prognosis, which was supported by a previous study.⁹ In-depth investigation indicated that *PVT1* silencing repressed proliferation and radioresistance and promoted apoptosis in NPC cells. In addition, in vivo experiment indicated that downregulation of *PVT1* inhibited tumor growth. Altogether, these results demonstrated that *PVT1* might function as an oncogene and could be a potential therapeutic target in NPC.

Emerging evidence has shed light on the fact that lncRNAs could regulate radioresistance by interacting with miRNAs in lots of human cancers.^{27,28} In our study, *miR-515-5p* was predicted to be a target of *PVT1* and this interaction was verified. We then checked its expression and found that *miR-515-5p* was clearly downregulated in

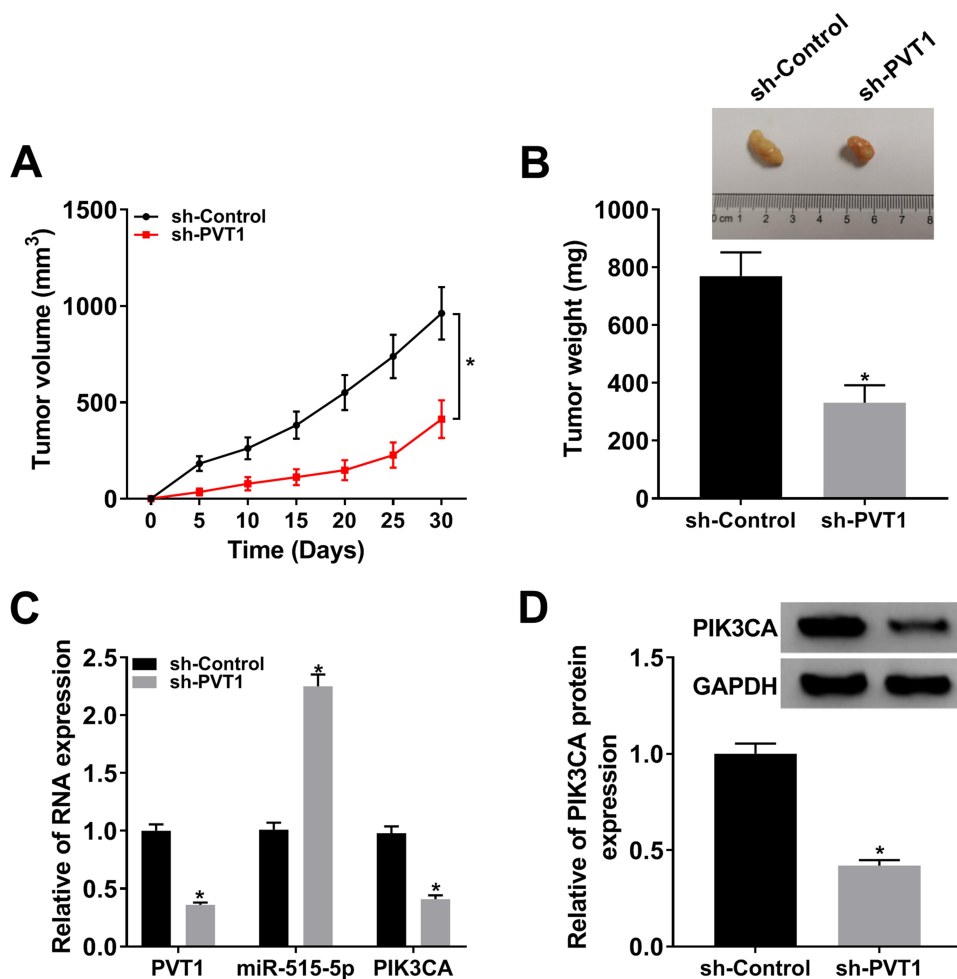


Figure 8 Downregulation of *PVT1* repressed tumor growth. **(A)** Tumor volume was measured every 5 d. **(B)** Weight of the resected tumor was examined after the mice were killed. **(C)** The mRNA levels of *PVT1*, *miR-515-5p* and *PIK3CA* in NPC cells infected with sh-*PVT1* or sh-Control were checked by qRT-PCR. **(D)** The protein level of *PIK3CA* in transfected NPC cells was measured by Western blot. * $P < 0.05$.

NPC tissues and cells. Besides, *PVT1* negatively modulated the expression of *miR-515-5p* in NPC cells in vitro. Further studies indicated that overexpression of *PVT1* reversed *miR-515-5p*-mediated repressive effects on cell proliferation and radioresistance, as well as the promotion impact on cell apoptosis in NPC cells. In addition, upregulation of *PVT1* inverted the decreased protein level of Cyclin D1 and the elevated expression of Bax in NPC cells treated with radiation. Our results suggested that *PVT1* targeted *miR-515-5p* to regulate cell proliferation, apoptosis and radioresistance in NPC cells in vitro.

To further figure out the regulatory mechanism of *miR-515-5p*, its target gene *PIK3CA* was predicated by bioinformatic analysis and the interaction was confirmed. In our research, the level of *PIK3CA* was apparently elevated in NPC tissues and cells, which was in line with a previous report.²⁹ Meanwhile, *PIK3CA* was negatively regulated by

miR-515-5p in NPC cells. Moreover, upregulation of *miR-515-5p* rescued *PIK3CA*-mediated promotion effects on cell proliferation and radioresistance, as well as the inhibitory effect on cell apoptosis in NPC cells in vitro. Recently, *PIK3CA* was reported to be involved in the activation of AKT pathway,²⁴ which played a pivotal role in regulating radioresistance in human cancers.^{25,26} In this research, we found that *PVT1* silencing notably decreased the expression of *PIK3CA* and p-AKT, whereas *miR-515-5p* depletion reversed this effect, indicating that *PVT1* might modulate cell radioresistance by affecting AKT pathway via interacting with *miR-515-5p* in NPC cells. Taken together, these results illustrated that *PVT1* mediated cell proliferation, apoptosis, radioresistance and AKT pathway via *miR-515-5p/PIK3CA* axis in NPC cells.

In conclusion, our research disclosed that *PVT1* was strikingly upregulated in NPC tissues and cells. Moreover,

knockdown of *PVT1* restrained cell proliferation, radio-resistance and induced cell apoptosis via *miR-515-5p/PIK3CA* axis in NPC cells. The novel mechanism may contribute to the improvement of radiotherapy for NPC.

Highlights

1. *PVT1* was upregulated in nasopharyngeal carcinoma tissues and cells.
2. *PVT1* silencing inhibited cell proliferation, radio-resistance and induced cell apoptosis in nasopharyngeal carcinoma.
3. *miR-515-5p* was a target of *PVT1* and targeted *PIK3CA* in nasopharyngeal carcinoma.
4. *PVT1* regulated nasopharyngeal carcinoma progression via *miR-515-5p/PIK3CA* axis.
5. Downregulation of *PVT1* inhibited tumor growth in vivo.

Abbreviations

NPC, nasopharyngeal carcinoma; *PVT1*, plasmacytoma variant translocation 1; qRT-PCR, quantitative real-time polymerase chain reaction; *PIK3CA*, phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha; CCK8, Cell counting kit-8; RIP, RNA immunoprecipitation.

Data Sharing Statement

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics Approval

The present study was approved by the ethical review committee of the First Affiliate Hospital of Xinjiang Medical University.

Author Contributions

All authors made substantial contribution to conception and design, acquisition of the data, or analysis and interpretation of the data; take part in drafting the article or revising it critically for important intellectual content; gave final approval of the revision to be published; and agree to be accountable for all aspect of the work.

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Disclosure

The authors declare that they have no competing interests for this work.

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