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ORIGINAL RESEARCH

Knockdown of lncRNA *PCAT6* Enhances Radiosensitivity in Triple-Negative Breast Cancer Cells by Regulating *miR-185-5p/TPD52* Axis

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Background: Long non-coding RNAs (lncRNAs) have been reported to play essential roles in regulating the radiosensitivity of cancers. Prostate cancer-associated transcript 6 (*PCAT6*) exerts oncogenic roles in several tumors. However, the roles of *PCAT6* and its underlying mechanism in regulating the radiosensitivity of triple-negative breast cancer (TNBC) have not been investigated.

Methods: The expression levels of *PCAT6, microRNA-185-5p* (*miR-185-5p*) and tumor protein D52 (*TPD52*) were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Cell viability, apoptosis and colony formation were assessed by Cell Counting Kit-8 (CCK-8) assay, flow cytometry and colony formation assay, respectively. The interaction between *miR-185-5p* and *PCAT6* or *TPD52* was predicted by bioinformatics analysis and verified by dual-luciferase reporter assay. Western blot was carried out to detect the protein level of TPD52.

Results: *PCAT6* and *TPD52* were highly expressed and *miR-185-5p* was lowly expressed in TNBC tissues and cells, which was associated with an aggressive tumor phenotype in patients, affecting lymph node metastasis and clinical stage. *PCAT6* or *TPD52* knockdown or *miR-185-5p* overexpression enhanced the radiosensitivity of TNBC cells via inhibiting proliferation and inducing apoptosis. *PCAT6* directly interacted with *miR-185-5p* and negatively regulated *miR-185-5p* expression. Moreover, *TPD52* was confirmed as a target of *miR-185-5p*. Besides, *PCAT6* regulated the radiosensitivity of TNBC cells through acting as a molecular sponge of *miR-185-5p* to modulate *TPD52* expression.

Conclusion: Knockdown of *PCAT6* promoted the radiosensitivity of TNBC cells through regulating *miR-185-5p*/*TPD52* axis, providing a vital theoretical basis to improve the radiotherapy efficiency of TNBC.

Keywords: triple-negative breast cancer, *PCAT6*, *miR-185-5p*, *TPD52*, radiosensitivity

Introduction

Breast cancer (BC) is one of the most lethal and prevalent cancers, and leading cause of cancer-related deaths among females. In 2012, it is estimated that there will be about 1.7 million cases and approximately 522,000 deaths.[1] Triple-negative breast cancer (TNBC) acts as the most invasive and aggressive among the breast cancer subtypes.[2] Despite outstanding advances have been made in the treatment of BC in the past few decades, the prognosis for TNBC is still poor.[3,4] Radiotherapy has become an effective strategy for TNBC treatment; however, tumor recurrence commonly occurs after the development of radioresistance.[5,6] Hence, better

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clarifying the underlying mechanism of radioresistance is especially important for developing more effective therapeutic strategies to treat TNBC.

Long non-coding RNAs (lncRNAs) are longer than 200 nucleotides and lack protein-coding ability, which are associated with radioresistance, metastasis, and other clinical outcomes in various cancers.[7,8] Previous studies demonstrated that lncRNAs are tightly linked to the progression and development of many tumor types, including TNBC.[9,10] For example, lncRNA *MALAT1* promoted proliferation and invasion in TNBC cells by targeting miR-129-5p.[11] Moreover, lncRNA *ANRIL* accelerated the progression of TNBC by sponging miR-199a.[12] Besides, lncRNA *CCAT1* has been suggested to be involved in the radiosensitivity of BC.[13] Prostate cancer-associated transcript 6 (*PCAT6*) has been identified to play oncogenic roles in diverse cancers, such as gastric cancer,[14] lung cancer,[15] and cervical cancer.[16] However, the functional roles of *PCAT6* and its underlying mechanism in the radiosensitivity of TNBC have not been reported.

More and more reports have suggested that lncRNAs can serve as a microRNA (miRNA) sponge to competitively suppress miRNAs.[17] MiRNAs are a class of non-coding RNAs with about 22 nucleotides and negatively modulate target genes expression through binding to the 3′-untranslated regions (3′UTR) of mRNA containing complementary sequence.[18] At present, emerging evidence revealed that miRNAs could affect cellular responses to radiation and modulate the radiosensitivity of many cancers.[19] *MiR-185-5p* has been suggested to be dysregulated in many kinds of cancers, such as prostatic cancer,[20] hepatocellular carcinoma,[21] clear cell renal cell carcinoma.[22] Moreover, previous study suggested that *miR-185-5p* was expressed at a low level in BC cells.[23] Nevertheless, the functional effects of *miR-185-5p* on regulating the radiosensitivity of TNBC remain largely unknown.

It is well known that miRNAs exert biological function through directly binding to target mRNAs.[24] Tumor protein D52 (*TPD52*), an oncogene, was isolated from the amplification region of human chromosome 8q21 and highly expressed in many cancers.[25,26] In addition, the expression of *TPD52* was also overexpressed in BC.[27] However, the interactions among *miR-185-5p, TPD52* and *PCAT6* in the radiosensitivity of TNBC have not been investigated.

In our study, the effects of *PCAT6, miR-185-5p* and *TPD52* on the radiosensitivity of TNBC cells were first measured. Additionally, we explored the *PCAT6/miR-185-5p/TPD52* regulatory network in TNBC cells or the cells under irradiation, providing novel insights into improving the radiotherapy efficiency of TNBC.

Materials and Methods

Tissue Collection

In our study, 70 pairs of TNBC tissues and adjacent normal tissues were provided by the patients who underwent surgery at Liaoning University of Traditional Chinese Medicine and were diagnosed with TNBC (stage I, II, and III) based on histopathological evaluation. In these patients, lymph node metastasis had occurred in 46 cases. These patients had never received chemotherapy or radiotherapy before surgery, and these tissues were promptly frozen in liquid nitrogen and kept in −80°C until experiments were carried out. Every patient provided written informed consent in this study. And the research was approved by the Research Ethics Committee of Liaoning University of Traditional Chinese Medicine.

Cell Culture and Transfection

TNBC cells (MDA-MB-468 and MDA-MB-231) and breast epithelial cells (MCF-10A) were bought from American Tissue Culture Collection (ATCC; Manassas, VA, USA). These cells were grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in an incubator with 5% CO_2 at 37°C.

The small interfering RNA against *PCAT6* or *TPD52* (si-*PCAT6* or si-*TPD52*) and their negative control (si-NC), *miR-185-5p* mimics (*miR-185-5p*) and its negative control (NC), *miR-185-5p* inhibitors (anti-*miR-185-5p*) and its negative control (anti-NC), pcDNA-*PCAT6* overexpression vector (pc-*PCAT6*) and its negative control (pc-NC) were provided by RiBoBio (Shanghai, China). TNBC cells (2×10^5 cells/well) were placed in six-well plates and transfected with the oligonucleotides or vector (siRNA 50 nM, mimics/inhibitors 50 nM, vector 4 μg) using Lipofectamine 3000 (Invitrogen).

Ionizing Radiation

Transfected cells were exposed to various doses of 6-MV X-ray (0 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy) using a linear accelerator (Varian 2300EX, Varian, Palo Alto, CA, USA) at a dose rate of 200 cGy/min.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from frozen tissues or cultured cells was extracted with TRIzol reagent (Invitrogen), and then first-strand complementary DNA (cDNA) synthesis was performed using the PrimeScript RT reagent Kit (Takara,

Tokyo, Japan). After that, qRT-PCR was carried out with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and TaqMan MicroRNA Assay Kit (Applied Biosystems) on the ABI 7500 Real-Time PCR System (Applied Biosystems), respectively. The relative expression of *PCAT6, TPD52*, and *miR-185-5p* was evaluated with $2^{-\Delta\Delta Ct}$ method, and the expression of *PCAT6* and *TPD52* was normalized by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *miR-185-5p* level was normalized by *U6*. In this study, the sequences of primers were listed as below: *PCAT6* (Forward, 5′-CAGGAACCCCCTCCTTACTC-3′; Reverse, 5′- CTAG GGATGTGTCCGAAGGA-3′), *miR-185-5p* (Forward, 5′-TCCGCTGGAGAGAAAGGC-3′; Reverse, 5′-ATGGA GGCTGAGGAGCACTG-3′), *TPD52* (Forward, 5′- AAC AGAACATTGCCAAAGGGTG-3′; Reverse, 5′-TGACT GAGCCAACAGACGAAA-3′), *GAPDH* (Forward, 5′-CG CTCTCTGCTCCTCCTGTTC-3′; Reverse, 5′- ATCCGT TGACTCCGACCTTCAC-3′), *U6* (Forward, 5′-CTCGC TTCGGCAGCACATATACT-3′; Reverse, 5′-ACGCTTC ACGAATTTGCGTGTC-3′).

Cell Viability Assay

Cell Counting Kit-8 (CCK-8; Sangon Biotech, Shanghai, China) was utilized to evaluate the cell viability. Briefly, TNBC cells (100 μL) were placed in 96-well plates and transfected with the indicated vectors, and then exposed to 4 Gy dose of X-ray. At 0 h, 24 h, 48 h, or 72 h after irradiation, CCK-8 (10 μL) reagent was added to the wells and placed in the incubator for 3 h. Finally, the absorbance of the wells was examined with a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Cell Apoptosis Assay

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Sangon Biotech) was utilized to detect cell apoptosis. TNBC cells were seeded in 6-cm dishes and then transfected with indicated vectors, and then exposed to 4 Gy dose of X-ray. After irradiation for 24 h, cells were collected and double-stained using the Annexin V- FITC (10 μL) and PI (5 μL) for 20 min in the darkness. Lastly, cell apoptosis was assessed using a flow cytometry (Guava Technologies, Hayward, CA, USA).

Colony Formation Assay

TNBC cells were placed in six-well plates overnight and then transfected with indicated vectors, and then exposed to various doses of X irradiation (0 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy). After irradiation for 24 h, the medium was changed with fresh medium and updated every 3 days. After 2 weeks, TNBC cells were carefully washed with pre-cold phosphate-buffered saline (PBS) and fixed with the paraformaldehyde (4%, 1mL) for 30 min at 4°C. Subsequently, the cells were washed with PBS and stained with 0.1% crystal violet for 1 h, and again washed with PBS until per well was clear. If a colony exceeded 50 cells, the colony was counted.

Dual-Luciferase Reporter Assay

Putative binding sites of *miR-185-5p* and *PCAT6* or *TPD52* were predicted by DIANA-LncBase v2 or TargetScan. The *PCAT6* or *TPD52* 3′UTR fragment containing putative or mutated *miR-185-5p* binding sites was synthesized and cloned into pmirGlO luciferase reporter vector (Promega, Madison, WI, USA), namely *PCAT6*-wt and *TPD52*-wt or *PCAT6*-mut and *TPD52*-mut. The *miR-185-5p* or NC and reporter plasmid were co-transfected into TNBC cells for 48 h. After that, dual-luciferase reporter assay system (Promega) was employed to assess the luciferase activity, followed by normalizing to Renilla luciferase activity.

Western Blot Assay

Transfected cells were lysed using RIPA lysis buffer (Thermo Fisher, Wilmington, DE, USA) containing protease inhibitors (Beyotime, Shanghai, China) to extract the total protein. After quantification by using bicinchoninic acid (BCA) protein assay kit (Tanon, Shanghai, China), protein samples (about 30 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the gels were transferred onto the polyvinylidene fluoride (PVDF; Beyotime) membranes. Then, 5% non-fat milk (Sangon Biotech) in PBS containing 0.1% Tween 20 (PBST) was used to block the membranes, and then the membranes were immunoblotted for 12 h at 4°C by primary antibodies against *TPD52* (1:5000, ab155296, Abcam, Cambridge, UK) or β-actin (1:2000, ab8227, Abcam). Following washing with PBST, membranes were incubated by secondary antibodies (1:4000, D110058, Sangon Biotech). Immunoreactive bands were examined by enhanced chemiluminescence (ECL) reagent (Tanon). Protein expression of *TPD52* was evaluated using ImageJ software and normalized to the level of β-actin.

Statistical Analysis

All data in this study were expressed as the mean ± standard deviation from at least independent experiments. Student's

t-test was employed to determine statistical differences between the two groups. Spearman rank correlation was applied to explore the correlation between *miR-185-5p* and *PCAT6* or *TPD52* in TNBC tissues. All statistical analyses were performed by GraphPad version 5.0 program (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered statistically significant.

Results

The Expression of *PCAT6* Was Upregulated in TNBC Tissues

To explore the potential roles of *PCAT6* in TNBC, the expression of *PCAT6* in 70 pairs of TNBC tissues and matched adjacent normal tissues was measured by qRT-PCR. Results displayed that the expression of *PCAT6* was markedly increased in TNBC tissues in contrast to matched adjacent normal tissues (Figure 1A). Additionally, patients with higher *PCAT6* level were more likely to have lymph node metastasis (Figure 1B). Moreover, *PCAT6* expression was higher in patients with II/III clinical stages than those with I clinical stage, especially in III clinical stages (Figure 1C). Furthermore, the relationship between the relative expression of *PCAT6* and clinical characteristics in patients with TNBC was analyzed. As shown in Supplementary Table 1, high expression level of *PCAT6* was significantly associated with lymph node metastasis and tumor stage, but not with age, menopause and tumor size. These results indicated that *PCAT6* might be involved in the progression of TNBC.

Knockdown of *PCAT6* Promoted Radiosensitivity of TNBC Cells

The expression of *PCAT6* was further explored in TNBC cells, and qRT-PCR analysis revealed that *PCAT6* level was evidently increased in TNBC cells (MDA-MB-468 and MDA-MB-231) compared with that in breast epithelial cells (MCF-10A) (Figure 2A). Bases on the upregulation of *PCAT6* in TNBC tissues and cells, we supposed that *PCAT6* might influence the radiosensitivity of TNBC cells. MDA-MB-468 and MDA-MB-231 cells transfected with si-*PCAT6* were used to assess the knockdown efficiency for *PCAT6*. The qRT-PCR analysis suggested that the abundance of *PCAT6* was decreased in MDA-MB-468 and MDA-MB-231 cells transfected with si-*PCAT6* (Figure 2B), indicating that successful introduction of si-*PCAT6* into MDA-MB-468 and MDA-MB-231 cells. Subsequently, MDA-MB-468 and MDA-MB-231 cells transfected with si-NC or si-*PCAT6* were exposed to 4Gy irradiation treatment. CCK-8 assay demonstrated that *PCAT6* deficiency or radiation therapy alone suppressed the viability of MDA-MB-468 and MDA-MB-231 cells, and *PCAT6* knockdown together with radiation therapy obviously repressed cell viability in MDA-MB-468 and MDA-MB-231 cells (Figure 2C). MDA-MB-468/C4 and MDA-MB-231/C4 are radioresistant TNBC cells surviving from MDA-MB-468/WT and MDA-MB-231/WT cells after fractionated radiation (2 Gy × 30). In addition, knockdown of *PCAT6* also inhibited cell viability of MDA-MB-468/C4 and MDA-MB-231/C4 cells (Supplementary Figure 1A and B). Moreover, interference of *PCAT6* or radiation therapy alone promoted apoptosis in MDA-MB-468 and MDA-MB-231 cells, and combination of si-*PCAT6* and radiation therapy conspicuously accelerated apoptosis of MDA-MB-468 and MDA-MB-231 cells (Figure 2D). Furthermore, we found that interference of *PCAT6* also facilitated apoptosis of MDA-MB-468/C4 and MDA-MB-231/C4 cells, but radiation therapy had no significant effect on apoptosis of MDA-MB-468/C4 and MDA-MB-231/C4 cells (Supplementary Figure 1C and D). Besides, colony formation analysis disclosed that *PCAT6* knockdown

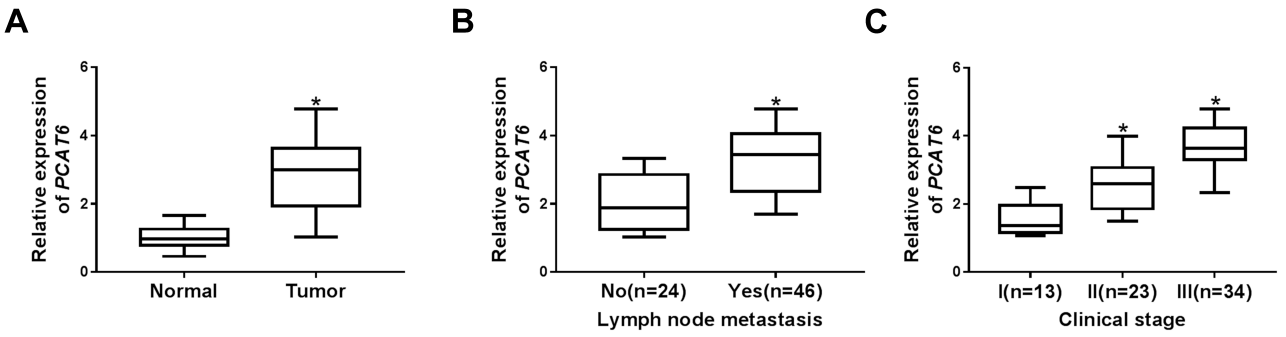

Figure 1 *PCAT6* was upregulated in TNBC tissues and associated with an aggressive tumor phenotype. (**A**) The level of *PCAT6* was detected by qRT-PCR in TNBC tissues (n=70) and matched adjacent normal tissues. (**B**) The expression of *PCAT6* was measured in tissues from TNBC patients with or without metastasis. (**C**) *PCAT6* abundance was analyzed in tissues from patients at different clinical stages. **P*<0.05.

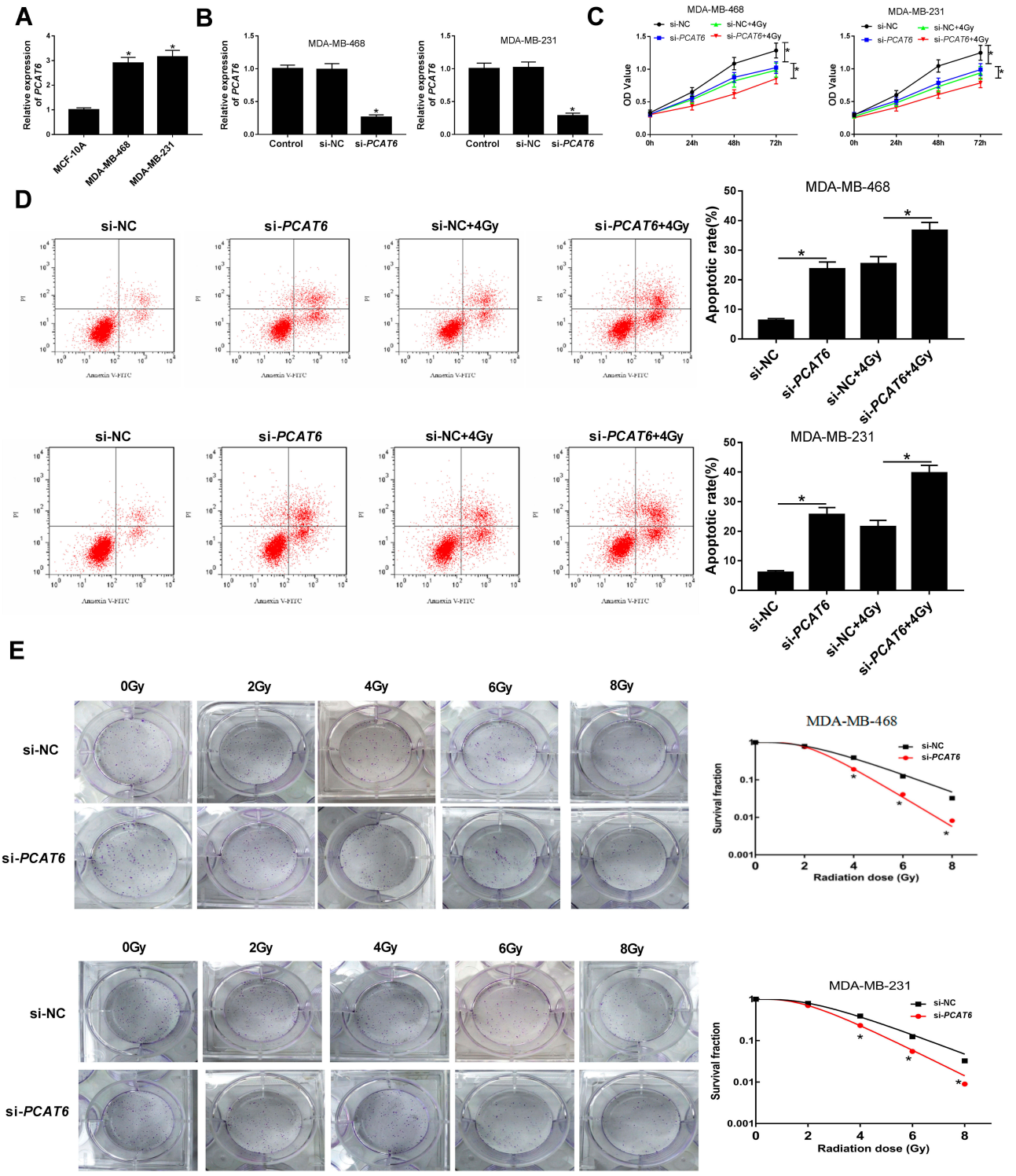

Figure 2 Effect of *PCAT6* on the radiosensitivity of TNBC cells. (**A**) The expression of *PCAT6* was determined in TNBC cells (MDA-MB-468 and MDA-MB-231) and breast epithelial cells (MCF-10A) using qRT-PCR. (**B**) The knockdown efficiency of *PCAT6* was examined in MDA-MB-468 and MDA-MB-231 cells by qRT-PCR. (**C**) Cell viability was evaluated by CCK-8 assay in MDA-MB-468 and MDA-MB-231 cells transfected with si-*PCAT6* or si-NC and/or treated with 4Gy irradiation. (**D**) Flow cytometry analysis was employed to determine the apoptosis rate of MDA-MB-468 and MDA-MB-231 cells transfected with si-*PCAT6* or si-NC and/or treated with 4Gy irradiation. (**E**) Colony formation assay was applied to detect colony survival rate 14 days after MDA-MB-468 and MDA-MB-231 cells transfected with si-*PCAT6* or si-NC and exposed to different doses of irradiation. **P*<0.05.

led to an obvious decrease of colony survival fraction in MDA-MB-468 and MDA-MB-231 cells treated with irradiation (Figure 2E). And we observed that *PCAT6* silence or radiation therapy alone increased the percentage of cells in G0/G1 phase but decreased the percentage of cells in S phase, combination of si-*PCAT6* and radiation therapy conspicuously

enhanced the percentage of cells in G0/G1 phase (Supplementary Figure 2A and B and Supplementary material and methods), suggesting *PCAT6* knockdown and radiation therapy blocked cell cycle progression at the G0/G1 phase. All these data revealed that downregulation of *PCAT6* elevated sensitivity of TNBC cells to ionizing radiation via inhibiting cell survival and promoting cell apoptosis.

PCAT6 Directly Interacted with *miR-185-5p* and Negatively Regulated *miR-185-5p* Expression in TNBC Cells

Previous report has demonstrated that lncRNAs commonly exhibit their biological roles by acting as sponges of miRNAs.[17] Through online bioinformatics database (DIANA-LncBase v2), *PCAT6* contained potential binding sites of *miR-185-5p* (Figure 3A). Subsequently, the prediction was validated by dual-luciferase reporter assay. Results showed that overexpression of *miR-185-5p* significantly suppressed the luciferase activity of *PCAT6*-wt, whereas luciferase activity of *PCAT6*-mut was not evidently affected after transfection with *miR-185-5p* in MDA-MB-468 and MDA-MB-231 cells (Figure 3B). However, knockdown of *miR-185-5p* presented an opposite effect on luciferase activity of *PCAT6*-wt in MDA-MB-468 and MDA-MB-231 cells (Figure 3C). Next, the effect of *PCAT6* on *miR-185-5p* expression was investigated in MDA-MB-468 and MDA-MB-231 cells. Results proved that overexpression of *PCAT6* led to a significant decrease of *miR-185-5p* expression and its knockdown had an opposite effect in MDA-MB-468 and MDA-MB-231 cells (Figure 3D). Our findings suggested that *PCAT6* acted as a molecular sponge of *miR-185-5p* and negatively modulated *miR-185-5p* expression in TNBC cells.

Overexpression of *miR-185-5p* Enhanced Radiosensitivity of TNBC Cells

To further investigate the roles of *miR-185-5p* in TNBC, the level of *miR-185-5p* in 70 pairs of TNBC tissues and matched adjacent normal tissues was determined by qRT-PCR. As presented in Figure 4A–C, the expression of *miR-185-5p* was reduced in TNBC tissues, patients with lymph node metastasis, and patients with advanced clinical stage. In addition, the association between the relative expression of *miR-185-5p* and clinical characteristics in patients with TNBC was assessed. As displayed in Supplementary Table 2, low expression level of *miR-185-5p* was significantly associated with lymph node metastasis and tumor stage, but not with age, menopause and tumor size. And we found that *miR-185-5p* expression was negatively correlated with *PCAT6* level in TNBC tissues (R^2=0.223, P<0.05) (Figure 4D). Besides, the abundance of *miR-185-5p* was also decreased in TNBC cells (MDA-MB-468 and MDA-MB-231) relative to breast epithelial cells (MCF-10A) (Figure 4E). QRT-PCR analysis showed that the expression of *miR-185-5p* was increased in MDA-MB-468 and MDA-MB-231 cells transfected with *miR-185-5p* mimics (Figure 4F), suggesting that successful introduction of *miR-185-5p* mimics into MDA-MB-468 and MDA-MB-231 cells. Subsequently, the effects of *miR-185-5p* on the radiosensitivity of TNBC cells were explored. CCK-8 assay showed that either *miR-185-5p* overexpression or irradiation exposure inhibited cell viability with respect to NC group, and combination of *miR-185-5p* restoration and radiation therapy strikingly suppressed cell viability compared with *miR-185-5p* group or 4Gy + NC group (Figure 4G). Moreover, MDA-MB-468 and MDA-MB-231 cells transfected with *miR-185-5p* or exposed to radiation therapy increased cell apoptosis, and apoptosis rate was remarkably enhanced in cells transfected with *miR-185-5p* and treated with radiation simultaneously (Figure 4H). Furthermore, *miR-185-5p* upregulation decreased colony formation rate in MDA-MB-468 and MDA-MB-231 cells exposed to irradiation (Figure 4I). Taken together, our results showed that *miR-185-5p* played an essential role in increasing the radiosensitivity of TNBC cells.

TPD52 Was a Direct Target of *miR-185-5p* in TNBC Cells

MiRNAs have been suggested to exert biological function via modulating their molecular targets.[24] Hence, TargetScan software online was used to predict target genes of *miR-185-5p*. As shown in Figure 5A, *TPD52* contained a putative target sequence for *miR-185-5p* in its 3′-UTR. To confirm whether *TPD52* was a downstream target of *miR-185-5p*, dual-luciferase reporter assay was performed. Results demonstrated that overexpression of *miR-185-5p* strongly suppressed the luciferase activity in *TPD52*-wt group, whereas the effect was little with respect to *TPD52*-mut group in MDA-MB-468 and MDA-MB-231 cells (Figure 5B). However, luciferase activity was prominently enhanced in MDA-MB-468 and MDA-MB-231 cells co-transfected with *TPD52*-wt and anti-*miR-185-5p*, while it was also not changed in *TPD52*-mut group (Figure 5C). Next, we observed the effect of *miR-185-5p* on TPD52 protein expression. Western blot assay proved that accumulation of *miR-185-5p* inhibited the protein expression of TPD52 and its knockdown presented an opposite effect

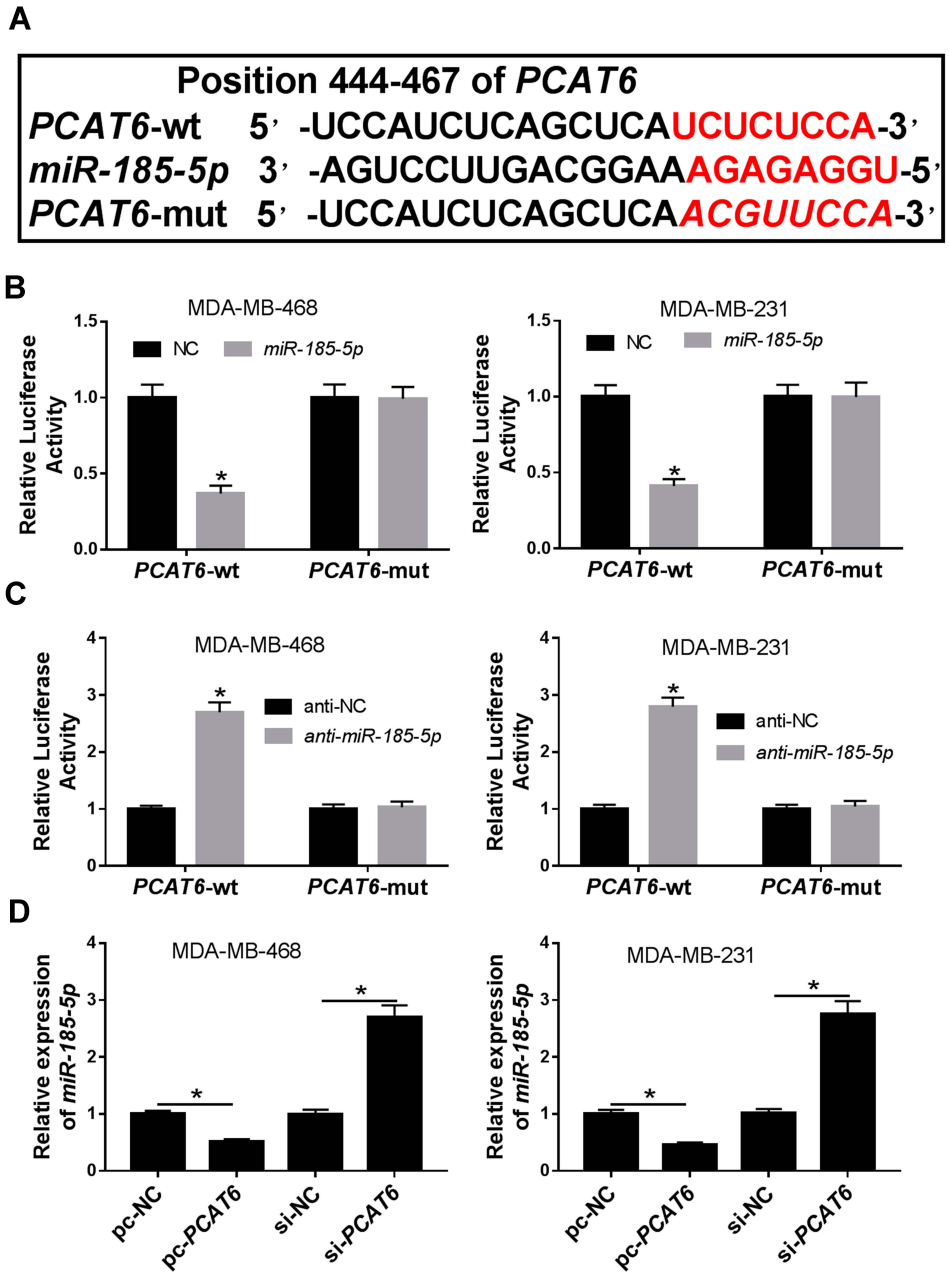

Figure 3 Regulator relationship between *PCAT6* and *miR-185-5p* in TNBC cells. (**A**) The putative binding sites between *PCAT6* and *miR-185-5p* were predicted by DIANA-LncBase v2. (**B**) Dual-luciferase luciferase reporter assay was utilized to analyze the luciferase activity in MDA-MB-468 and MDA-MB-231 cells co-transfected with *PCAT6*-wt or *PCAT6*-mut and NC or *miR-185-5p*. (**C**) Luciferase activity was measured in MDA-MB-468 and MDA-MB-231 cells co-transfected with *PCAT6*-wt or *PCAT6*-mut and anti-NC or anti-*miR-185-5p*. (**D**) The expression of *miR-185-5p* was determined by qRT-PCR in MDA-MB-468 and MDA-MB-231 cells transfected with pc-NC, pc-*PCAT6*, si-NC, or si-*PCAT6*. *P<0.05.

(Figure 5D). These results together indicated that *TPD52* was a direct target of *miR-185-5p* and was negatively regulated by *miR-185-5p* in TNBC cells.

Silencing *TPD52* Elevated Radiosensitivity of TNBC Cells

Next, *TPD52* expression was measured in TNBC tissues and cells by qRT-PCR. As expected, the results disclosed that the level of *TPD52* was increased in TNBC tissues, patients with lymph node metastasis, and patients with advanced clinical stage (Figure 6A–C). Likewise, the association between the relative expression of *TPD52* and clinical characteristics in patients with TNBC was also evaluated. As presented in Supplementary Table 3, high expression level of *TPD52* was significantly associated with lymph node metastasis and tumor stage, but not with age, menopause

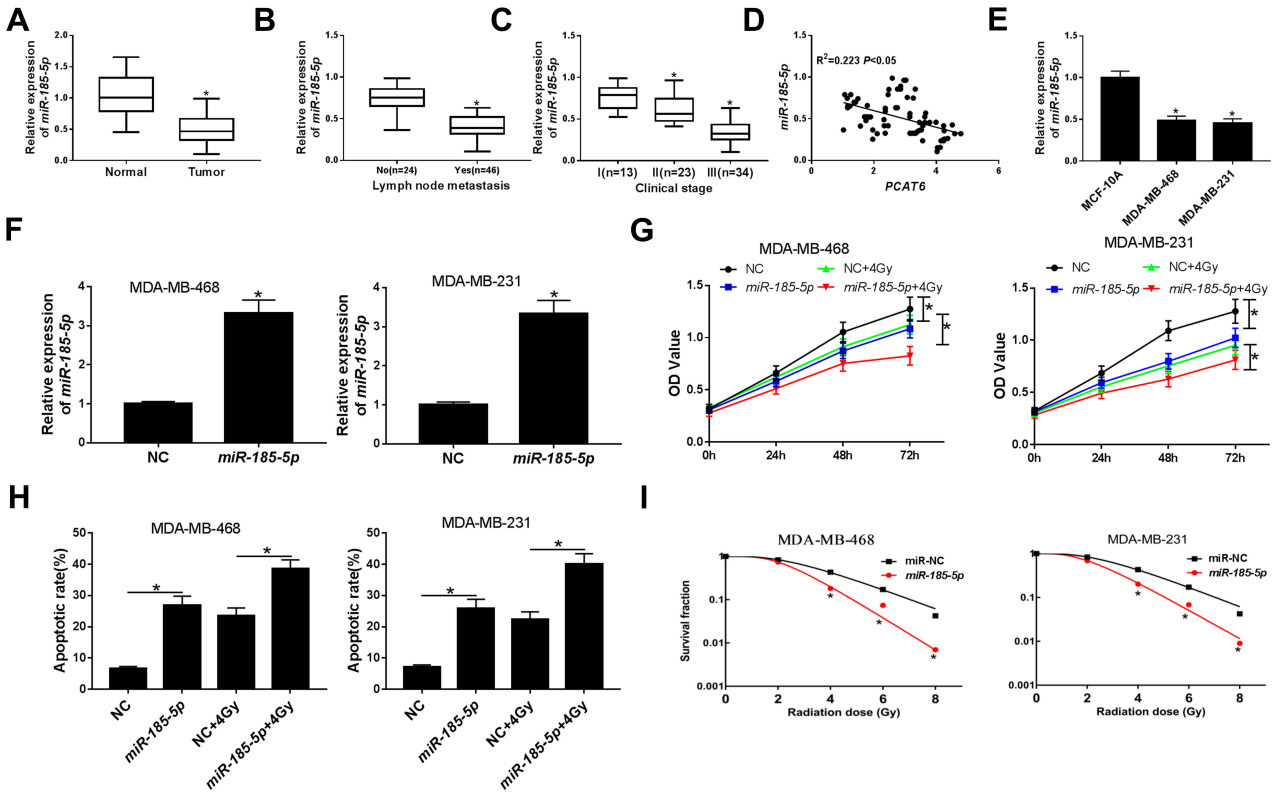

Figure 4 Effect of *miR-185-5p* on radiosensitivity of TNBC cells. (**A**) The expression of *miR-185-5p* was examined by qRT-PCR in TNBC tissues and matched adjacent normal tissues. (**B**) The level of *miR-185-5p* was assessed in tissues from TNBC patients with or without metastasis. (**C**) The level of *miR-185-5p* was analyzed in tissues from patients at different clinical stages. (**D**) The association between *PCAT6* level and *miR-185-5p* abundance was measured in TNBC tissues. (**E**) The level of *miR-185-5p* was analyzed in TNBC cells (MDA-MB-468 and MDA-MB-231) and breast epithelial cells (MCF-10A). (**F**) The transfection efficiency of *miR-185-5p* mimics was confirmed by qRT-PCR in MDA-MB-468 and MDA-MB-231 cells. (**G**) Cell viability was evaluated by CCK-8 assay in MDA-MB-468 and MDA-MB-231 cells transfected with NC or *miR-185-5p* and/or treated with 4Gy irradiation. (**H**) Flow cytometry analysis was applied to measure the cell apoptosis rate in MDA-MB-468 and MDA-MB-231 cells transfected with NC or *miR-185-5p* and/or treated with 4Gy irradiation. (**I**) Colony formation assay was used to determine colony survival fraction in MDA-MB-468 and MDA-MB-231 cells transfected NC or *miR-185-5p* and treated with different doses of irradiation. **P*<0.05.

and tumor size. Moreover, the *TPD52* abundance was negatively associated with *miR-185-5p* expression in TNBC tissues (R^2=0.123, *P*<0.05) (Figure 6D). Similarly, the mRNA and protein expression of TPD52 were also upregulated in TNBC cells (MDA-MB-468 and MDA-MB-231) compared with that in breast epithelial cells (MCF-10A) (Figure 6E). Western blot assay revealed that the protein level of TPD52 was reduced in MDA-MB-468 and MDA-MB-231 cells transfected with si-*TPD52*, suggesting that successful introduction of si-*TPD52* into MDA-MB-468 and MDA-MB-231 cells (Figure 6F). CCK-8 analysis showed that *TPD52* inhibition or treatment of 4Gy irradiation suppressed cell viability in MDA-MB-468 and MDA-MB-231 cells, and the cells transfected with si-*TPD52* and treated with 4Gy irradiation further inhibited cell viability compared with those cells transfected with si-*TPD52* or exposed to 4Gy irradiation (Figure 6G). Besides, *TPD52* deficiency or 4Gy irradiation exposure induced apoptosis of MDA-MB-468 and MDA-MB-231 cells, and combination of *TPD52* knockdown and irradiation significantly enhanced apoptosis rate in contrast to si-*TPD52* or 4Gy + si-NC group (Figure 6H). In a word, these data indicated that *TPD52* downregulation enhanced the radiosensitivity of TNBC cells by inhibiting cell viability and promoting apoptosis.

PCAT6 Regulated Radiosensitivity of TNBC Cells Through Acting as a Molecular Sponge of *miR-185-5p* to Modulate *TPD52* Expression

Further, we explored whether *PCAT6* regulated *miR-185-5p*/*TPD52* axis in TNBC cells. The protein expression of *TPD52* was investigated by Western blot in TNBC cells transfected with si-NC, si-*PCAT6*, si-*PCAT6* + anti-NC, or si-*PCAT6* + anti-*miR-185-5p*. Western blot assay proved that the protein expression of TPD52 was reduced in MDA-MB-468 and

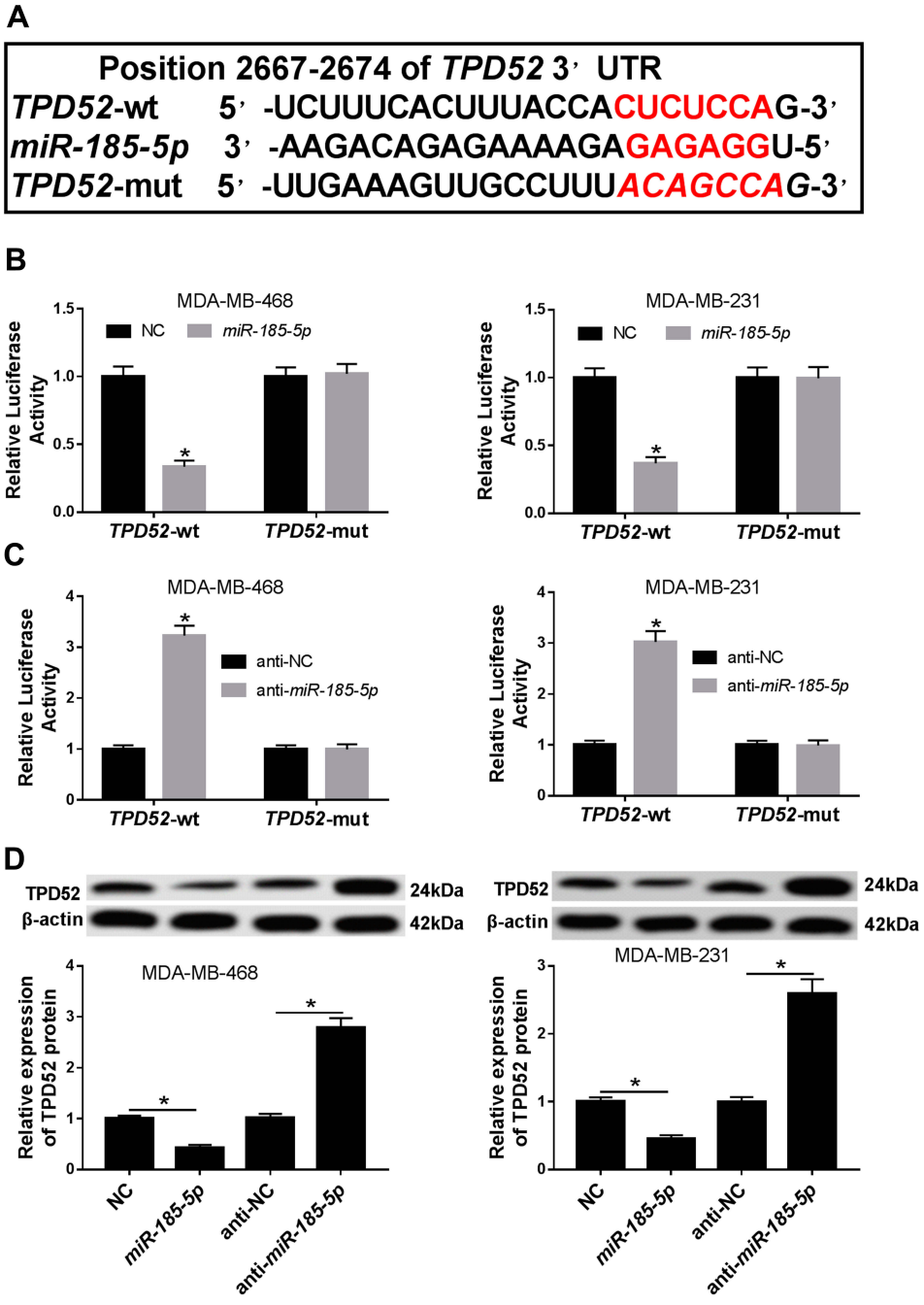

Figure 5 *TPD52* was a downstream target of *miR-185-5p* in TNBC cells. (**A**) The putative binding sites between *miR-185-5p* and *TPD52* were predicted by TargetScan. (**B**) Relative luciferase activity was determined in MDA-MB-468 and MDA-MB-231 cells co-transfected with *TPD52*-wt or *TPD52*-mut and NC or *miR-185-5p*. (**C**) Relative luciferase activity was detected in MDA-MB-468 and MDA-MB-231 cells co-transfected with *TPD52*-wt or *TPD52*-mut and anti-NC or anti-*miR-185-5p*. (**D**) Western blot was used to evaluate the protein level of TPD52 in MDA-MB-468 and MDA-MB-231 cells transfected with NC, *miR-185-5p*, anti-NC, or anti-*miR-185-5p*. *P<0.05.

MDA-MB-231 cells transfected with si-*PCAT6*, while the effect was partially reversed by knockdown of *miR-185-5p* (Figure 7A). Similarly, the mRNA and protein expression of TPD52 were also decreased in MDA-MB-468/C4 and MDA-MB-231/C4 cells after transfection of si-*PCAT6*, which was abolished by downregulating *miR-185-5p* (Supplementary Figure 3A–D). CCK-8 analysis showed that interference of *miR-185-5p* weakened the inhibitory effect of *PCAT6* knockdown on the viability of MDA-MB-468 and MDA-MB-231 cells treated with 4Gy irradiation as well as radioresistant TNBC cells (MDA-MB-468/C4 and MDA-MB-231/C4) (Figure 7B and Supplementary Figure 3E–F). Moreover, promotive effect of *PCAT6* deficiency on apoptosis was abolished by silencing *miR-185-5p* in MDA-MB-468 and

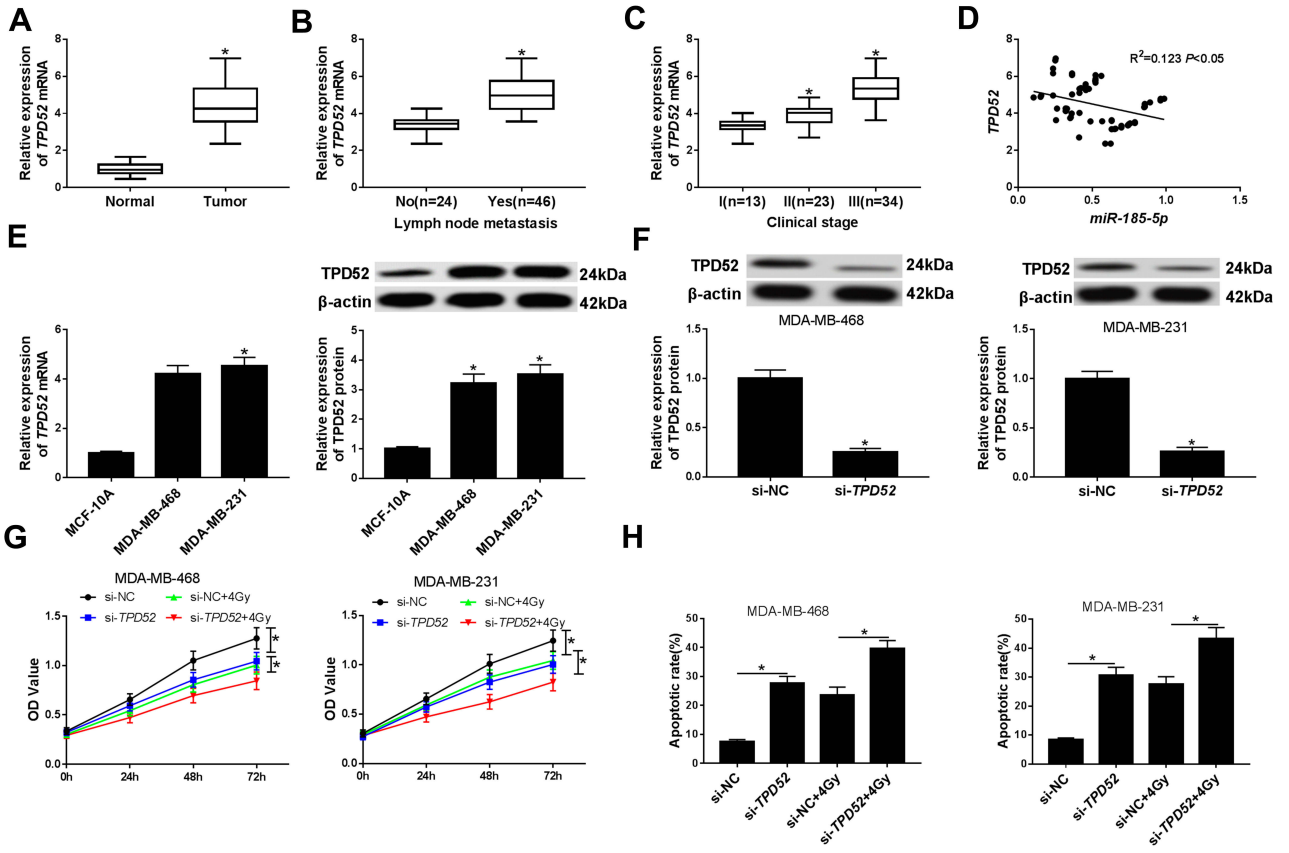

Figure 6 Effect of *TPD52* on radiosensitivity of TNBC cells. (**A**) The expression of *TPD52* was evaluated using qRT-PCR in TNBC tissues and matched adjacent non-tumor tissues. (**B**) The abundance of *TPD52* was determined in tissues from TNBC patients with or without metastasis. (**C**) The level of *TPD52* was examined in tissues from TNBC patients at different clinical stages. (**D**) The correlation between *TPD52* mRNA level and *miR-185-5p* expression was analyzed in TNBC tissues. (**E**) The mRNA and protein levels of TPD52 were analyzed in TNBC cells (MDA-MB-468 and MDA-MB-231) and breast epithelial cells (MCF-10A) by qRT-PCR and Western blot assays, respectively. (**F**) The transfection efficiency of si-*TPD52* was assessed using the Western blot analysis in MDA-MB-468 and MDA-MB-231 cells. (**G**) CCK-8 analysis was used to analyze the cell viability in MDA-MB-468 and MDA-MB-231 cells transfected with si-*TPD52* or si-TPD52*TPD52* and/or exposed to 4Gy irradiation. (**H**) Cell apoptosis was determined using the flow cytometry analysis in MDA-MB-468 and MDA-MB-231 cells transfected with si-*TPD52* or si-*TPD52* and/or treated with 4Gy irradiation. **P*<0.05.

MDA-MB-231 cells exposed to 4Gy irradiation (Figure 7C). Furthermore, knockdown of *miR-185-5p* attenuated the suppressive effect of *PCAT6* downregulation on colony formation in both DA-MB-468 and MDA-MB-231 cells treated with 4Gy irradiation (Figure 7D). All these data implied that *PCAT6* knockdown promoted the radiosensitivity of TNBC cells via sponging *miR-185-5p* to affect *TPD52* expression.

Discussion

Currently, radiotherapy is commonly considered a primary strategy for TNBC treatment. However, the development of radioresistance has become a main obstacle for treatment of cancer patients, resulting in tumor recurrence and repressing the effectiveness of radiation therapy.[28] Thus, identifying new therapeutic targets is so critical to improve radiosensitivity in patients with TNBC.

In recent years, increasing evidence suggested that lncRNAs played regulatory roles in cell behaviors, cancer occurrence and pathogenesis, chemotherapy and radiotherapy resistance.[29,30] Previous studies indicated that *PCAT6* acted as a tumor promoter in various cancers. For instance, Cui et al stated that *PCAT6* accelerated the progression of non-small cell lung cancer via regulating *miR-330-5p*.[31] Xu et al presented that *PCAT6* was overexpressed in gastric cancer tissues and promoted progression of gastric cancer via regulating *miR-30*.[14] Besides, Wu et al found that the *PCAT6* abundance was enhanced in colorectal cancer tissues and cell lines, and its knockdown alleviated colorectal cancer chemoresistance to 5-FU by regulating *miR-204/HMGA2/PI3K*.[32] In our study, results proved that the abundance of *PCAT6* was enhanced in TNBC tissues and cells, and we found that the level of *PCAT6* was positively associated with lymph node metastasis and clinical stage. Moreover, the data revealed that downregulation of *PCAT6* promoted the radiosensitivity of TNBC cells to irradiation through inhibiting cell growth and promoting apoptosis.

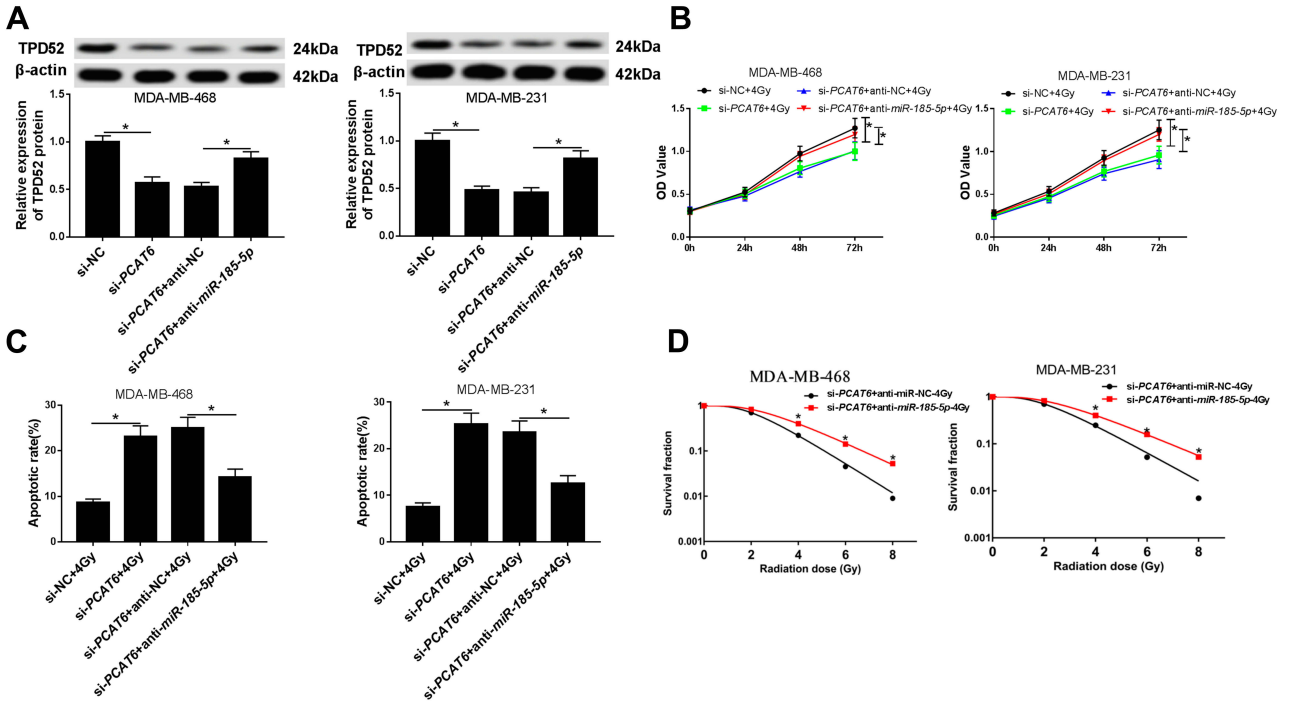

Figure 7 *PCAT6* downregulation promoted the radiosensitivity of TNBC cells via sponging *miR-185-5p* to downregulate *TPD52* expression. (**A**) The protein expression of TPD52 was explored through Western blot analysis in MB-468 and MDA-MB-231 cells transfected si-NC, si-*PCAT6*, si-*PCAT6* + anti-NC, or si-*PCAT6* + anti-*miR-185-5p*. (**B**) Cell viability was assessed by CCK-8 assay in MDA-MB-468 and MDA-MB-231 cells transfected with si-NC, si-*PCAT6*, si-*PCAT6* + anti-NC, or si-*PCAT6* + anti-*miR-185-5p* under irradiation. (**C**) Flow cytometry analysis was applied to detect the apoptosis rate in MDA-MB-468 and MDA-MB-231 cells transfected with si-NC, si-*PCAT6*, si-*PCAT6* + anti-NC, or si-*PCAT6* + anti-*miR-185-5p* and then treated with 4Gy irradiation. (**D**) Colony formation assay was performed to establish clonogenic survival curves in MDA-MB-468 and MDA-MB-231 cells transfected si-*PCAT6* + anti-NC or si-*PCAT6* + anti-*miR-185-5p* and then exposed to different doses of irradiation. *P<0.05.

Notably, accumulating evidence suggested that lncRNA stability and function could be potently regulated by miRNAs.[33] Here, DIANA-LncBase v2 was used to predict the potential target of *PCAT6*, and the data displayed that *miR-185-5p* and *PCAT6* had potential binding sites. Subsequently, we confirmed *miR-185-5p* was a downstream target of *PCAT6. MiR-185-5p* dysregulation was observed in some cancers. For example, Tian et alstated that the *miR-185-5p* was lowly expressed in prostate cancer tissues and cells.[34] Niu et al presented that *miR-185-5p* restoration impeded metastasis of hepatocellular carcinoma cells.[35] Pei et al pointed out that *miR-185-5p* accumulation elevated the sensitivity of lung cancer cells to cisplatin through inhibiting cell growth and promoting apoptosis.[36] Besides, Yin et al proved that *miR-185-5p* limited BC cell invasion and epithelial-mesenchymal transition (EMT).[23] However, there is no report on the function of *miR-185-5p* in the radiosensitivity of TNBC cells. In our research, *miR-185-5p* abundance was reduced in TNBC tissues, and the TNBC patients with low expression of *miR-185-5p* had an invasive tumor phenotype. Moreover, we found that *miR-185-5p* upregulation resulted in an obvious decrease of cell growth and increase of cell apoptosis after irradiation, implying that *miR-185-5p* overexpression enhanced the radiosensitivity of TNBC. To explore how *miR-185-5p* influenced the radiosensitivity of TNBC, potential target was predicted by the online tool. TargetScan software online showed that *TPD52* contained binding sites of *miR-185-5p*, which was then confirmed in TNBC cells through dual-luciferase reporter assay.

TPD52 is a tumor-promoting gene and is frequently overexpressed in multiple cancers, such as ovarian cancer,[37] prostate cancer,[38] colorectal cancer.[39] Due to the oncogenic role of *TPD52* in many cancers, *TPD52* should be further studied. Moreover, accumulating evidence revealed that *TPD52* was involved in cellular transformation, proliferation, apoptosis, and metastasis.[40,41] Yang et al demonstrated that knockdown of *TPD52L2* (a member of the *TPD52*) suppressed the proliferation and colony formation in BC cells.[42] Zhang et al found that *TPD52* expression was markedly increased in BC tissues and cells, and deficiency of miR-449 promoted proliferation and metastasis of BC cells through regulating *TPD52*.[27] In our research, results uncovered that *TPD52* expression was enhanced in TNBC tissues and cells. Besides, interference of *TPD52* inhibited cell viability and induced apoptosis in TNBC cells under irradiation, disclosing that *TPD52* interference enhanced the radiosensitivity of

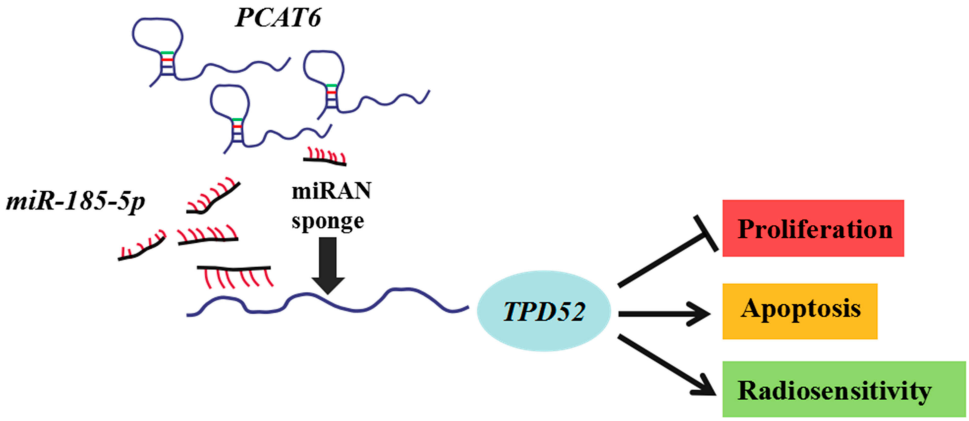

Figure 8 Mechanism diagram of *PCAT6/miR-185-5p/TPD52* axis in radiosensitivity of TNBC cells. *PCAT6* regulated cell proliferation and apoptosis to affect radiosensitivity by modulating *miR-185-5p/TPD52* axis in TNBC cells.

TNBC cells. It is widely reported that lncRNAs act as competing endogenous RNA (ceRNA) to affect genes expression by sponging miRNAs.[43] Our present study displayed that *TPD52* was positively regulated by *PCAT6* and negatively regulated by *miR-185-5p*. Moreover, our results proved that silencing *miR-185-5p* abated the effects of *PCAT6* downregulation on suppression of cell proliferation and promotion of apoptosis in TNBC cells under irradiation. These findings indicated *PCAT6* knockdown promoted the radiosensitivity of TNBC cells via sponging *miR-185-5p* to affect *TPD52* expression (Figure 8).

In conclusion, our research first explored the functional roles and underlying mechanism of *PCAT6* in the radiosensitivity of TNBC cells. These data revealed that silencing *PCAT6* promoted the radiosensitivity of TNBC cells via reducing cell proliferation and facilitating apoptosis by upregulating *miR-185-5p* expression and downregulating *TPD52* expression. These findings might provide a new therapeutic target to enhance radiotherapy efficiency for TNBC patients.

Disclosure

The authors report no funding and no conflicts of interest in this work.

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