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Circ_0007580 knockdown strengthens the radiosensitivity of non-small cell lung cancer via the miR-598-dependent regulation of THBS2

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Abstract

Background: Radioresistance is a common cause of treatment failure in many cancers, including non-small cell lung cancer (NSCLC). Circular RNA (circRNA) has been shown to be involved in the radiosensitivity of many cancers. However, the role and mechanism of circ_0007580 in the radiosensitivity of NSCLC remain unclear.

Methods: The expression levels of circ_0007580, miR-598 and thrombospondin 2 (THBS2) were estimated by quantitative real-time PCR. The radiosensitivity of cells was measured using colony formation assay. Cell proliferation and apoptosis were assessed by performing cell counting kit 8 assay, colony formation assay, flow cytometry, and by detecting caspase-3 and caspase-9 activities. Protein expression was determined using western blot analysis.

Results: Our data showed that circ_0007580 was highly expressed and miR-598 was lowly expressed in radioresistant NSCLC tissues. Functional experiments suggested that circ_0007580 silencing could improve the radiosensitivity of cells by suppressing cell proliferation and increasing apoptosis. MiR-598 was confirmed to be a target of circ_0007580, and its inhibitor could reverse the regulation of circ_0007580 on the radiosensitivity of NSCLC cells. MiR-598 was found to target THBS2. The suppressive effect of miR-598 on the radiosensitivity of cells could be reversed by THBS2 overexpression. Additionally, circ_0007580 could sponge miR-598 to regulate THBS2. In vivo experiments showed that knockdown of circ_0007580 enhanced the radiosensitivity of NSCLC tumors.

Conclusions: Our results revealed that circ_0007580 might be a target for improving the radiosensitivity of NSCLC, which was mainly achieved by regulating the miR-598/THBS2 axis.

KEYWORDS
circ_0007580, miR-598, non-small cell lung cancer, THBS2

INTRODUCTION

Non-small cell lung cancer (NSCLC) refers to malignant tumors derived from bronchial mucosal epithelium or alveolar epithelium.[1,2] At present, there are no clear results for the cause of NSCLC in clinical practice, but it is known that environment, heredity, and bad living habits are high risk factors that cause this disease.[3,4] Radiotherapy is one of the local treatments for NSCLC, but the emergence of radioresistance greatly reduces the probability of successful radiotherapy.[5,6] Therefore, seeking effective targets to improve radiosensitivity is expected to offer a theoretical basis for improving the cure rate of NSCLC patients.

As a kind of non-coding RNA with circular structure, the key role of circular RNA (circRNA) in human diseases

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has been clarified in recent years.[7] There is considerable evidence that circRNA is involved in cancer development and can function as a potential biomarker for the prognosis and treatment of cancer.[8,9] The key point to note is that many circRNAs have been found to be related to the sensitivity of cancer to radiotherapy.[10,11] In NSCLC-related studies, circMTDH.4 is considered to promote cell radioresistance, and its silencing might enhance the radiosensitivity of NSCLC.[12] In addition, a recent study had shown that targeted inhibition of circ_0086720 could effectively promote NSCLC radiosensitivity.[13] However, the role of many circRNAs in radioresistance is not fully understood. The study by Chen et al. determined that upregulated circ_0007580 had a promoting effect on the progress of NSCLC.[14] Our study found that circ_0007580 was differentially expressed in radiosensitive and radioresistant NSCLC tissues, but whether circ_0007580 is involved in the regulation of radioresistance of NSCLC is still unclear.

There is evidence that one of the mechanisms of circRNA is to act as a sponge of microRNA (miRNA) to mediate downstream targeted mRNA expression.[15] In many studies, miR-598 has been identified as a tumor suppressor mediating the regulation of cancer progression, such as in ovarian cancer[16] and gastric cancer.[17] In NSCLC, miR-598 expression has been found to be significantly low and associated with the malignant progression of NSCLC.[18–20] Here, we uncovered that miR-598 was lowly expressed in radioresistant NSCLC tissues, and bioinformatic analysis revealed that it had complementary binding sites between circ_0007580 and miR-598.

Thrombospondin 2 (THBS2) is believed to be a cancer-related gene whose function varies greatly among different cancer types. For example, Wet et al. suggested that THBS2 was underexpressed in cervical cancer, and could negatively regulate lymph node metastasis.[21] On the contrary, high THBS2 expression was discovered to be associated with colorectal cancer patient prognosis, and silenced THBS2 could inhibit cancer progression.[22] Studies have confirmed that THBS2 is upregulated in the serum of NSCLC patients, which is considered as a diagnostic marker for early NSCLC.[23] Here, we found that there are complementary binding sites between THBS2 and miR-598, and THBS2 expression is regulated by circ_0007580. Therefore, we propose the hypothesis of circ_0007580/miR-598/THBS2 axis to reveal the molecular mechanism of circ_0007580 regulating NSCLC radioresistance.

METHODS

Sample collection

In this study, 30 patients with NSCLC were recruited from Nanyang Central Hospital, and their NSCLC tissues and adjacent normal tissues were collected and stored in liquid nitrogen. After radiation therapy, the patient tissues were classified as radioresistant ($n = 16$) and radiosensitive ($n = 14$) NSCLC tissues based on radiation response. Each patient signed their written informed consent. This study was approved by the Ethics Committee of Nanyang Central Hospital.

Cell culture

Human NSCLC cell lines (A549 and H1299) and a normal bronchial epithelial cell line (BEAS-2B) were purchased from ATCC (Manassas, VA, USA). A549 and H1299 cells were cultured in RPMI-1640 medium (Gibco) and BEAS-2B cells were grown in BEGM Bullet Kit (Lonza) at 37°C with 5% CO_2 incubator. All media were supplemented with 10% FBS (Gibco) and 1% penicillin–streptomycin liquid (Gibco).

Cell transfection and radiation treatment

The circ_0007580 small interfering RNA and lentivirus short hairpin RNA (si-circ_0007580 and sh-circ_0007580), miR-598 mimic and inhibitor, pcDNA THBS2 overexpression vector (pc-THBS2), and their negative controls (si-NC, sh-NC, miRNA NC, inhibitor NC and pc-NC) were constructed by Ribobio (Guangzhou, China). For cell transfection, lipofectamine 3000 (Invitrogen) was diluted with 250 μl serum-free medium. Also, the siRNA (50 nM), shRNA (4.0 μg), mimic (50 nM), inhibitor (50 nM) or vector (4.0 μg) was diluted with 250 μl serum-free medium. After incubation for 5 min, respectively, lipofectamine 3000 and the plasmid mixtures were combined (total volume = 500 μl) and then added to the NSCLC cells. After transfection for 24 h, NSCLC cells were exposed to X-ray radiation with different doses for 24 h.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted utilizing TRIzol reagent (Invitrogen), and cDNA was synthesized utilizing M-MLV reverse transcriptase (Invitrogen). QRT-PCR was performed with EXPRESS SYBR GreenER qPCR Supermix (Invitrogen) in PCR system (ABI 7500; Applied Biosystems). Relative expression was calculated through $2^{-\Delta\Delta Ct}$ method with GAPDH or U6 as internal reference. The primer sequences are as below: circ_0007580, F 5′-GTGCAAGGAACACA TGATGG-3′, R 5′-TCACTCGGTCAAGGTTGTTG-3′; PR KCA, F 5′-GTCCACAAGAGGTGCCATGAA-3′, R 5′-AA GGTGGGGCTTCCGTAAGT-3′; miR-598, F 5′-GCCG AGTACGTCATCGTTGTCA-3′, R 5′-CTCAACTGGTGTC GTGGA-3′; THBS2, F 5′-GACACGCTGGATCTCACCT AC-3′, R 5′-GAAGCTGTCTATGAGGTCGCA-3′; GAPDH, F 5′-CTCTGCTCCTCCTGTTCGAC-3′, R 5′-CGACCAA ATCCGTTGACTCC-3′; U6, F 5′-CGCTTCGGCAGCAC ATATACTA-3′, R 5′-CGCTTCACGAATTTGCGTGT CA-3′. For the RNase R assay, the extracted RNA from A549 and H1299 cells was treated with RNase R (Geneseed), and the RNA was then used for detecting circ_0007580 and PRKCA expression.

Colony formation assay

This assay was used to assess cell radiosensitivity by detecting survival fraction, and to evaluate cell proliferation by measuring the number of colonies. For cell radiosensitivity, A549 and H1299 cells were cultured in 6-well plates (1×10^5 cells/well), and then treated with graded doses (0, 2, 4 and 8 Gy) of radiation. After 14 days, the cells were fixed with 4% paraformaldehyde (Beyotime) and stained with crystal violet (Beyotime). Under a microscope (E100; Nikon), cell colony number (>50 cells) was counted to calculate cell survival fraction. For cell proliferation, A549 and H1299 cells were seeded in 6-well plates (200 cells/well) and cultured for 14 days. The number of colonies were counted after being fixed and stained.

Cell counting kit 8 (CCK8) assay

Cells were seeded into 96-well plates and cultured for 48 h. Then, 10 μl CCK-8 solution (Dojindo) was added to the cells and further hatched for 4 h. The absorbance at 450 nm was determined with a microplate reader (SpectraMax iD5; Molecular Devices) to evaluate cell viability.

Flow cytometry

This was performed according to the instructions of Annexin-V-FITC/PI apoptosis detection kit (Vazymem). A549 and H1299 cells (5×10^5 cells) were incubated with Annexin V-FITC and PI. Cell apoptosis rate (%) was analyzed by a flow cytometer (CytoFLEX).

Detection of caspase-3 and caspase-9 activities

The caspase-3 and caspase-9 activities were analyzed using caspase-3 and caspase-9 activity assay kits (Beyotime). Briefly, A549 and H1299 cells were lysed with lysis buffer. The reaction system was prepared according to the kit instructions. Cell absorbance was determined at 405 nm using a microplate reader (SpectraMax iD5; Molecular Devices), and relative activity was calculated according to standard curve.

Western blot (WB) analysis

Protein was extracted with RIPA lysis buffer (Elabscience Biotechnology). Protein samples were resolved by 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore). After being blocked with skimmed milk, the membranes were incubated with anti-γH2AX (1:1000, Abcam), and anti-THBS2 (1:1000, Boster) or anti-GAPDH (1:2000, Boster). After further incubatation with horseradish peroxidase (HRP) conjugated goat anti-rabbit/mouse IgG (H + L) (1:5000, Boster), the protein signal was detected by enhanced chemiluminescence (Millipore).

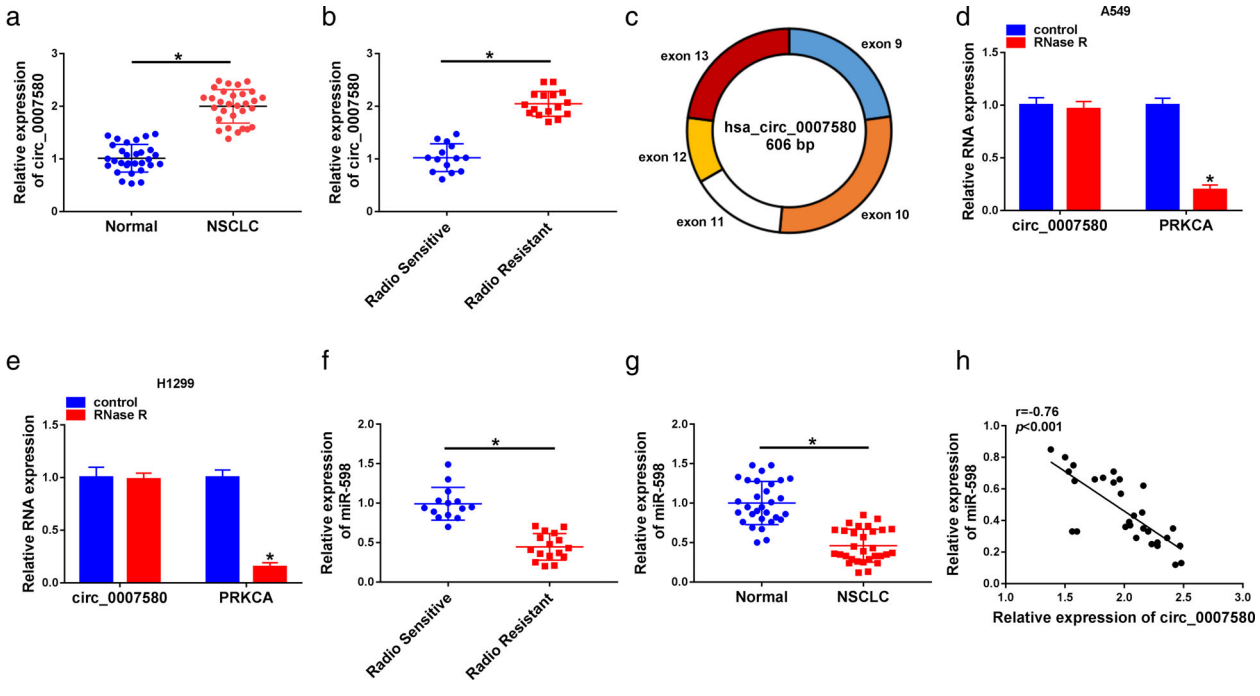

FIGURE 1 The expression of circ_0007580 and miR-598 in NSCLC tissues. (a) QRT-PCR was used to measure the expression of circ_0007580 in NSCLC tissues and adjacent normal tissues. (b) The expression of circ_0007580 in radioresistant and radiosensitive NSCLC tissues were determined by qRT-PCR. (c) The basic information of circ_0007580 was shown. (d,e) RNase R assay was used to measure the stability of circ_0007580 and PRKCA in A549 and H1299 cells. (f) The expression of miR-598 in NSCLC tissues and adjacent normal tissues was determined by qRT-PCR. (g) QRT-PCR was performed to assess the miR-598 expression in radioresistant and radiosensitive NSCLC tissues. (h) Pearson's correlation analysis was used to evaluate the correlation between circ_0007580 and miR-598. *$p < 0.05$

Dual-luciferase reporter assay

The wild-type (WT) and mutated-type (MUT) vectors of circ_0007580 or THBS2 3′UTR were constructed by inserting the putative binding sites and mutant sites of circ_0007580 or THBS2 3′UTR with miR-598 to the psiCHECK2 vector. A549 and H1299 cells were transfected with the above vectors and miR-598 mimic or miRNA NC. After culture for 48 h, relative luciferase activity was determined by dual-luciferase reporter assay kit (Beyotime).

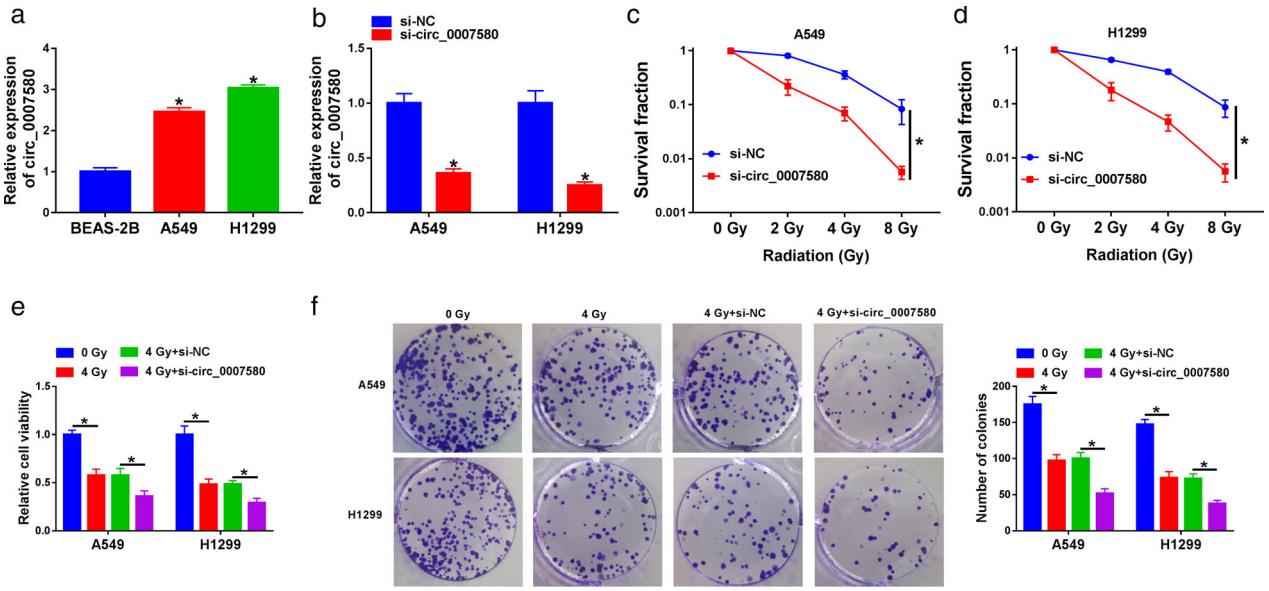

FIGURE 2 The regulation of circ_0007580 on the radiosensitivity and proliferation of NSCLC cells. (a) The circ_0007580 expression in NSCLC cells (A549 and H1299) and BEAS-2B cells was measured by qRT-PCR. (b) The transfection efficiency of si-circ_0007580 was assessed by detecting circ_0007580 expression using qRT-PCR. (c,d) Colony formation assay was used to analysis the survival fraction of cells to evaluate cell radiosensitivity. (e,f) A549 and H1299 cells were transfected with or without si-circ_0007580 and si-NC, and then treated with 0 Gy or 4 Gy radiation. CCK8 assay (e) and colony formation assay (f) were performed to assess the viability and the number of colonies of cells, respectively. $^*p < 0.05$

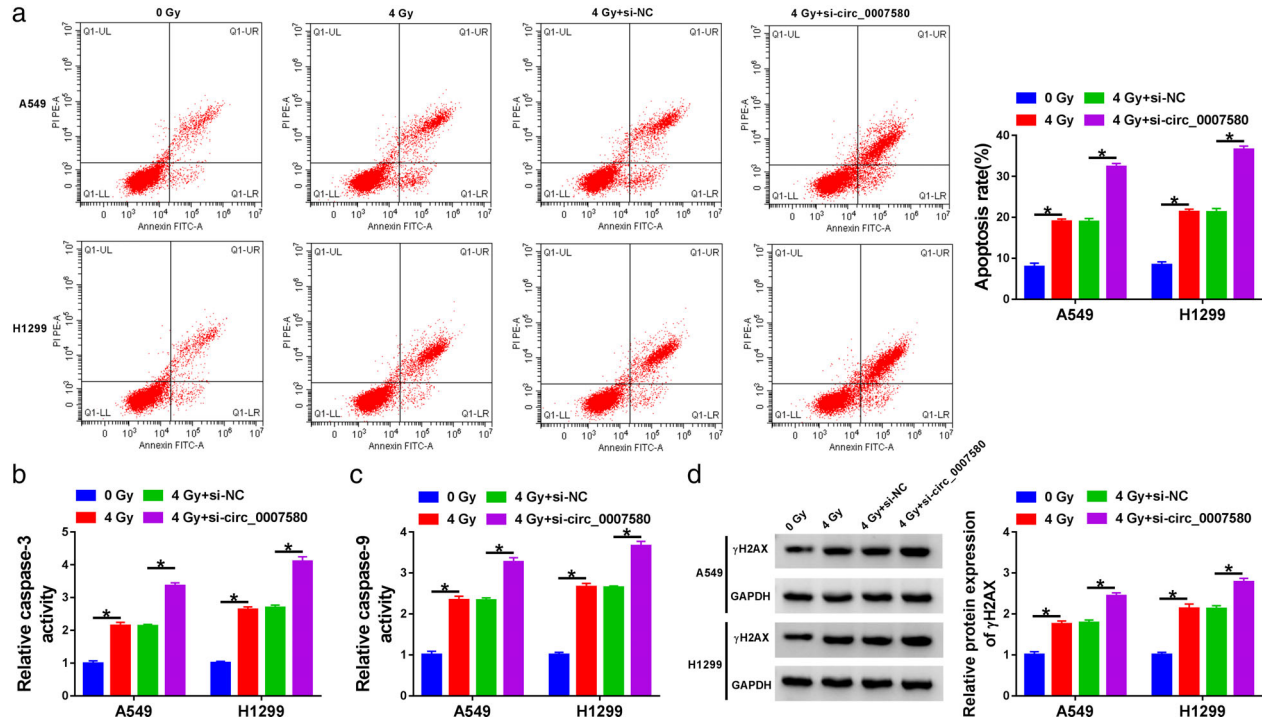

FIGURE 3 Effects of circ_0007580 knockdown on the apoptosis of NSCLC. A549 and H1299 cells were transfected with or without si-circ_0007580 and si-NC, and then treated with 0 or 4 Gy radiation. (a) The apoptosis rate of cells was measured by flow cytometry. (b,c) The caspase-3 and caspase-9 activities of cells were determined using caspase-3 and caspase-9 activity assay kits. (d) The protein expression of γH2AX was measured by WB analysis. $^*p < 0.05$

RIP assay

Based on the instructions of Magna RIP Kit (Millipore), A549 and H1299 cells were lysed using RIP buffer, and the cell lysates were then incubated with magnetic beads preadded with Ago2 antibody or IgG antibody at 4°C overnight. After purifying the RNA, the enrichment of circ_0007580 and miR-598 was assessed by qRT-PCR.

Xenograft tumors

Animal experiments were approved by the Animal Ethics Committee of Nanyang Central Hospital. BALB/c nude mice (Vital River) were divided into four groups ($n = 3$/group). H1299 cells were stably transfected with sh-circ_0007580 or sh-NC using lipofectamine 3000. After 48 h, cells were collected and resuspended with PBS. The transfected or nontransfected H1299 cells (2×10^6 cells/0.2 ml PBS) were then injected into the left flank of nude mice. After one week, one group of mice injected with untransfected cells served as control (0 Gy), while the other groups received 4 Gy radiation. Tumor volume was measured weekly. Four weeks later, all the mice were sacrificed, and the tumors were removed for weighing.

Statistical analysis

All experiments were performed in triplicate. All the data are expressed as mean ± standard deviation and were processed using Graphpad Prism software (Graphpad). Data analysis was performed with Student's *t*-test (two groups) and one-way analyses of variance followed by Tukey's post hoc test (multi groups). $p < 0.05$ was considered statistically significant.

RESULTS

Circ_0007580 was upregulated and miR-598 was downregulated in NSCLC tissues

Compared to adjacent normal tissues, we discovered that circ_0007580 was highly expressed in NSCLC

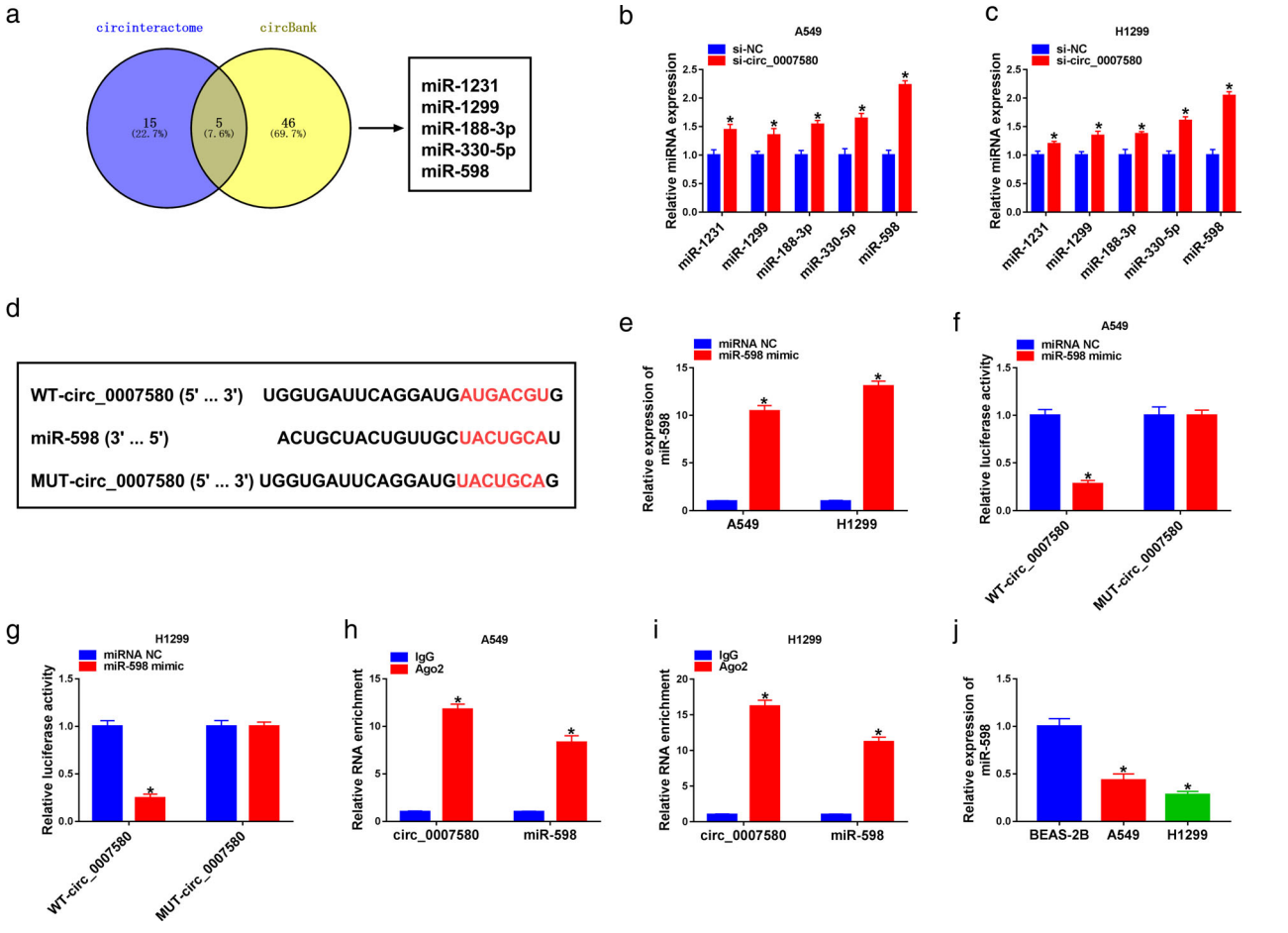

FIGURE 4 MiR-598 was sponged by circ_0007580. (a) The Venn diagram shows that miRNAs that can interact with circ_0007580 predicted by circinteractome software and circBank software. (b,c) A549 and H1299 cells were transfected with or without si-circ_0007580 and si-NC. The expression of 5 candidate miRNA (miR-1231, miR-1299, miR-188-3p, miR-330-5p and miR-598) was measured by qRT-PCR. (d) The sequences of WT-circ_0007580 and MUT-circ_0007580 were shown. (e) The transfection efficiency of miR-598 mimic was assessed by measuring miR-598 expression using qRT-PCR. Dual-luciferase reporter assay (f,g) and RIP assay (h,i) were used to verify the targeted binding relationship between miR-598 and circ_0007580. (j) The expression of miR-598 in NSCLC cells (A549 and H1299) and BEAS-2B cells was analyzed using qRT-PCR. *$p < 0.05$

tissues (Figure 1(a)). Moreover, circ_0007580 expression was markedly increased in radioresistant NSCLC tissues compared with that in radiosensitive NSCLC tissues (Figure 1(b)). Through analysis, circ_0007580 is formed by the back-splicing of the exon 9–13 of PRKCA gene (Figure 1(c)). Using the RNase R assay, we confirmed that circ_0007580 could resist the digestion of RNase R compared to its linear RNA PRKCA (Figure 1(d,e)). On the contrary, miR-598 expression was found to be lower in NSCLC tissues than that in adjacent normal tissues (Figure 1(f)). In addition, miR-598 expression was also significantly decreased in radioresistant NSCLC tissues compared to radiosensitive NSCLC tissues (Figure 1(g)). Correlation analysis showed that circ_0007580 expression was negatively correlated with miR-598 expression in NSCLC tissues (Figure 1(h)). Based on these results, we hypothesized that circ_0007580 and miR-598 might be related to the radioresistance of NSCLC.

Silenced circ_0007580 enhanced the radiosensitivity of NSCLC cells by decreasing cell proliferation and promoting apoptosis

Compared to normal bronchial epithelium cells (BEAS-2B), circ_0007580 expression was discovered to be upregulated in both NSCLC cells (A549 and H1299) (Figure 2(a)). To explore the function of circ_0007580 in the radiosensitivity of NSCLC, we decreased circ_0007580 expression using the si-circ_0007580 (Figure 2(b)). Using colony formation assay, we assessed the radiosensitivity of cells and the results showed that the survival fractions of A549 and H1299 cells were markedly reduced after silencing circ_0007580 (Figure 2(c,d)), indicating that circ_0007580 knockdown promoted cell radiosensitivity. Moreover, we found that 4 Gy radiation could markedly inhibit the viability and number of colonies of A549 and H1299 cells, and circ_0007580 knockdown further motivated this inhibition (Figure 2(e,f)). In addition, the silencing of circ_0007580

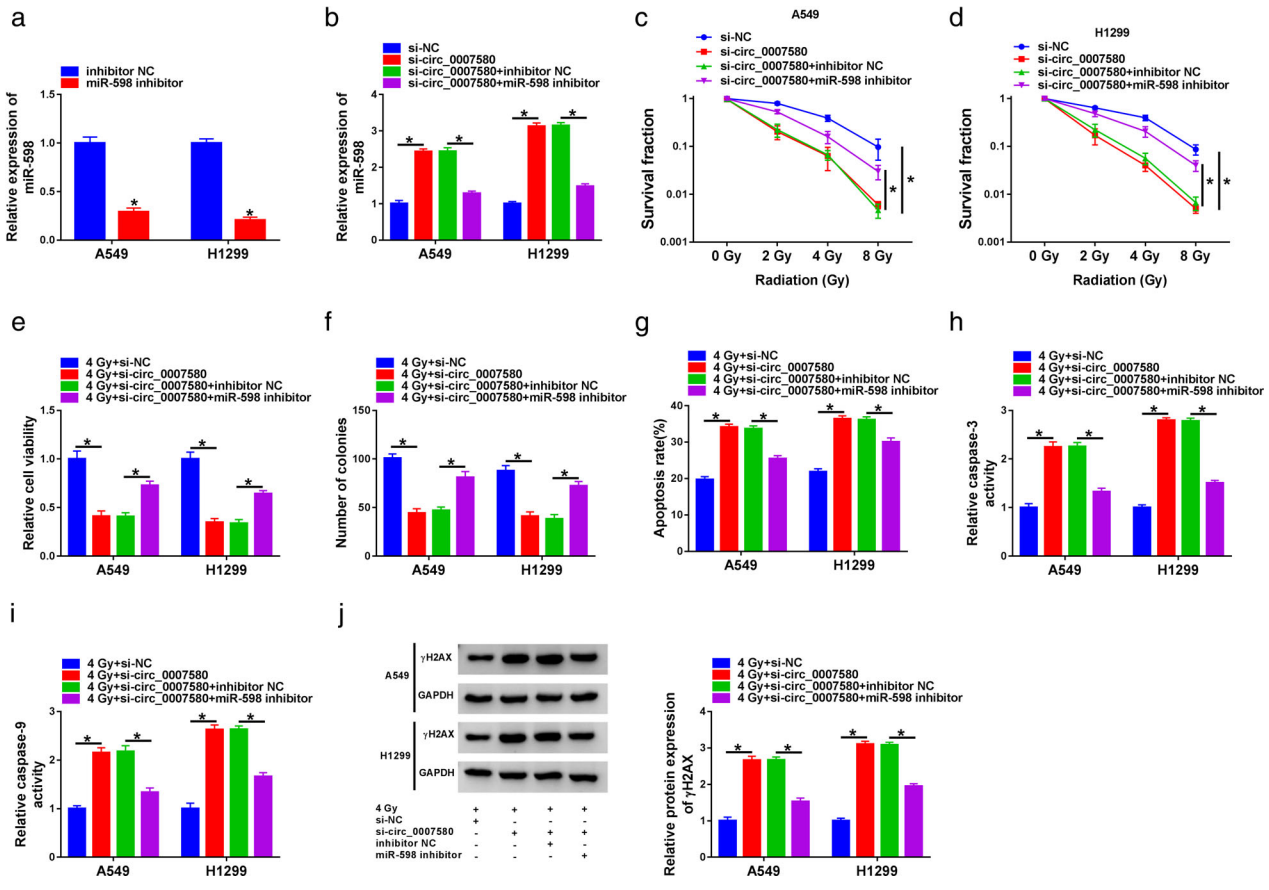

FIGURE 5 The regulation of circ_0007580 silencing and miR-598 inhibitor on the radiosensitivity of NSCLC cells. (a) QRT-PCR was used to detect miR-598 expression to assess the transfection efficiency of miR-598 inhibitor. (b–j) A549 and H1299 cells were transfected with si-NC, si-circ_0007580, si-circ_0007580 + inhibitor NC or si-circ_0007580 + miR-598 inhibitor. (B) The expression of miR-598 was measured by qRT-PCR. (c,d) The survival fraction of cells was measured by colony formation assay to analysis cell radiosensitivity. Under the treatment of 4 Gy radiation, CCK8 assay (e), colony formation assay (f) and flow cytometry (g) were used to determine the viability, the number of colonies of cells, and the apoptosis rate of cells. (h,i) Caspase-3 and caspase-9 activity assay kits were used to examine the caspase-3 and caspase-9 activities of cells. (j) WB analysis was employed to measure the protein expression of γH2AX. *$p < 0.05$

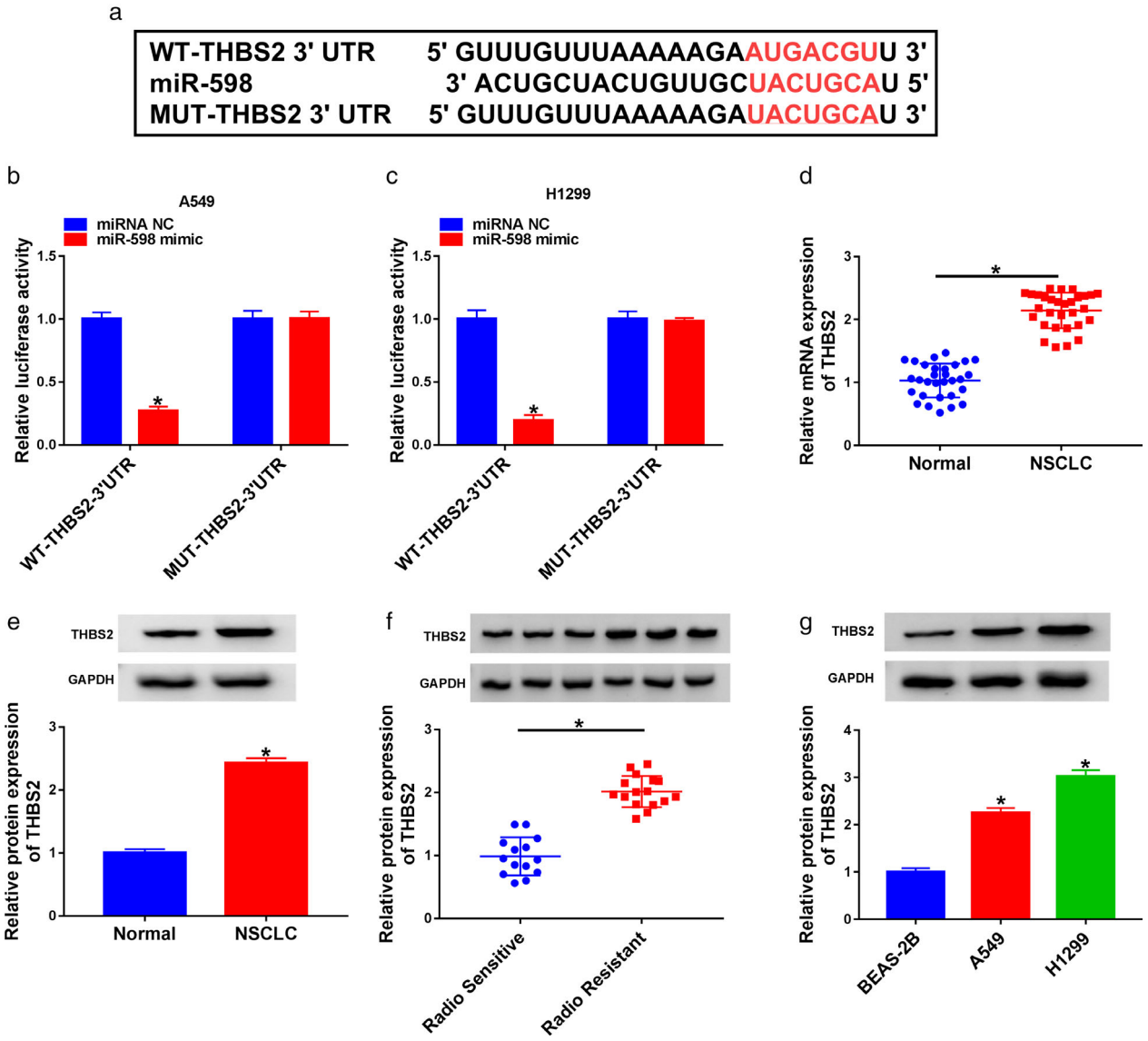

FIGURE 6 THBS2 was a target of miR-598. (a) The binding sites and mutated sites between miR-598 and THBS2 are shown. (b,c) Dual-luciferase reporter assay was performed to verify the interaction between THBS2 and miR-598. (d,e) The mRNA and protein expression levels of THBS2 in NSCLC tissues and adjacent normal tissues were determined using qRT-PCR and WB analysis. (f) WB analysis was used to detect THBS2 protein expression in radio-resistant and radio-sensitive NSCLC tissues (g) The THBS2 protein expression in NSCLC cells (A549 and H1299) and BEAS-2B cells was examined by WB analysis. *$p < 0.05$

could also aggravate the apoptosis rate and activities of caspase-3 and caspase-9 in A549 and H1299 cells under the treatment of 4 Gy radiation (Figure 3(a–c)). Additionally, we detected the expression of DNA damage marker gene γH2AX, and the results showed that 4 Gy radiation could significantly promote its expression, and the knockdown of circ_0007580 could aggravate this effect (Figure 3(d)). These data suggested that circ_0007580 regulated the proliferation and apoptosis of NSCLC to mediate the radiosensitivity of NSCLC cells.

MiR-598 was a targeted miRNA of circ_0007580

Using the circinteractome software and circBank software, we found that a total of 5 miRNA could bind with circ_0007580 (Figure 4(a)). After determining the candidate miRNA expression in A549 and H1299 cells transfected with si-circ_0007580, we discovered the increase multiple of miR-598 was the most significant (Figure 4(b,c)). Therefore, miR-598 was selected as circ_0007580 target for our research. The complementary binding sites between circ_0007580 and miR-598 are shown in Figure 4(d). We then constructed the miR-598 mimic and further confirmed that it could promote miR-598 expression in A549 and H1299 cells (Figure 4(e)). Subsequently, we performed a dual-luciferase reporter assay using the miR-598 mimic and the reporter vectors of circ_0007580. The results showed that miR-598 mimic could repress the luciferase activity of WT-circ_0007580 vector while it did not have any effect on that of the corresponding MUT vector (Figure 4(f,g)). Also, RIP assay results suggested

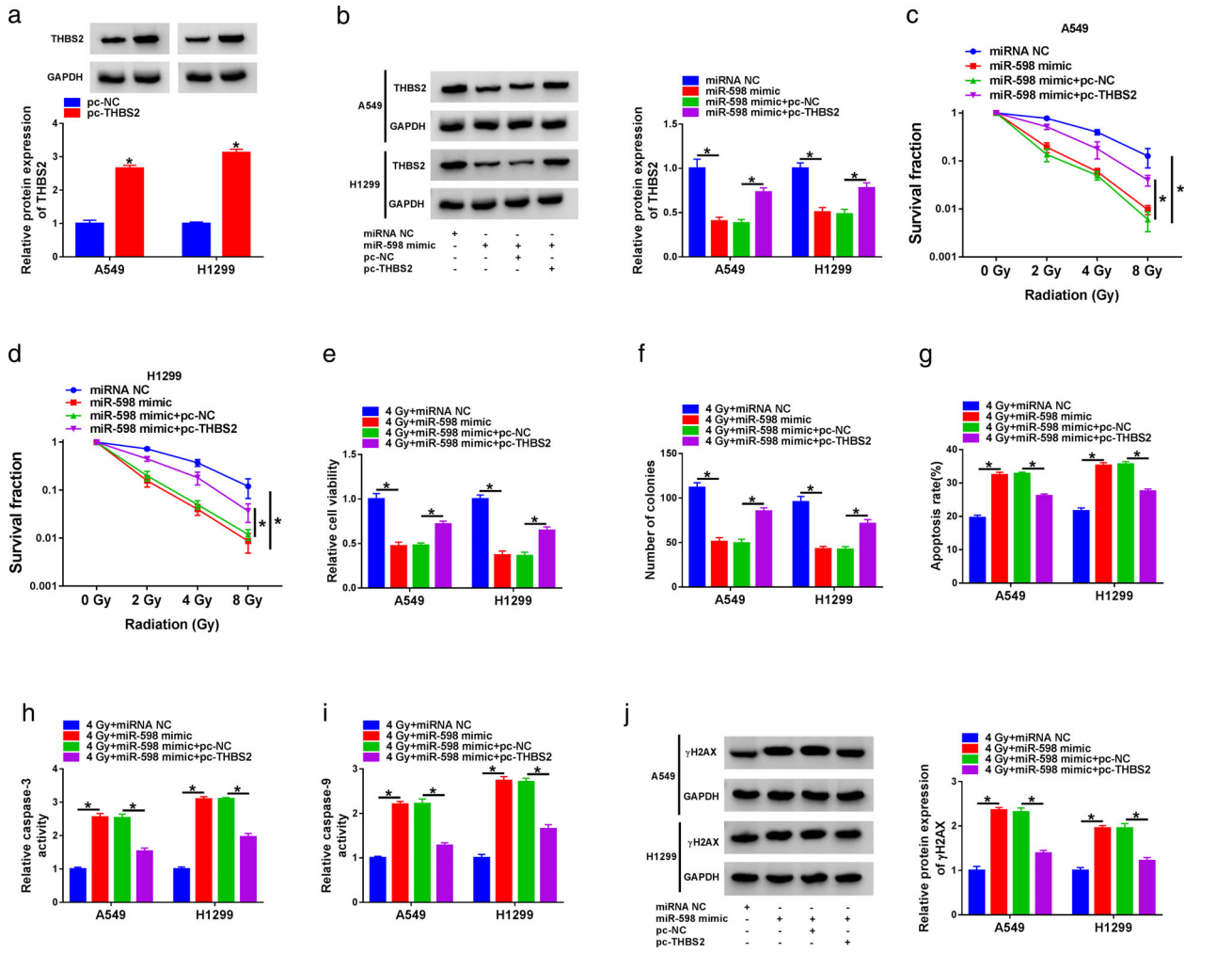

FIGURE 7 MiR-598 promoted the radiosensitivity of NSCLC cells by targeting THBS2. (a) WB analysis was performed to detect THBS2 protein expression to evaluate the transfection efficiency of pc-THBS2. (b–j) A549 and H1299 cells were transfected with miRNA NC, miR-598 mimic, miR-598 mimic + pc-NC or miR-598 mimic + pc-THBS2. (b) The protein expression of THBS2 was measured by WB analysis. (c,d) Colony formation assay was performed to determine the survival fraction of cells to assess cell radiosensitivity. Under the treatment of 4 Gy radiation, the viability, the number of colonies of cells, and the apoptosis rate of cells were detected using CCK8 assay (e), colony formation assay (f) and flow cytometry (g). (h,i) The caspase-3 and caspase-9 activities of cells were analyzed using caspase-3 and caspase-9 activity kits. (j) The protein expression of γH2AX was examined by WB analysis. $^*p < 0.05$

that the enrichments of circ_0007580 and miR-598 were increased in Ago2 compared to IgG (Figure 4(h,i)), further confirming the interaction between circ_0007580 and miR-598. Moreover, the lower expressed miR-598 also was found in both NSCLC cells compared to BEAS-2B cells (Figure 4(j)). All data showed that circ_0007580 could act as a sponge of miR-598.

MiR-598 inhibitor reversed the regulation circ_0007580 knockdown on the radiosensitivity of NSCLC cells

We then constructed the miR-598 inhibitor to reduce miR-598 expression in A549 and H1299 cells (Figure 5(a)). To investigate whether circ_0007580 regulated the radiosensitivity of NSCLC cells by sponging miR-598, we cotransfected si-circ_0007580 and miR-598 inhibitor into A549 and H1299 cells. We found that silenced circ_0007580 could markedly enhance miR-598 expression in A549 and H1299 cells, and this effect could be abolished by miR-598 inhibitor (Figure 5(b)). The detection results of survival fractions showed that miR-598 inhibitor could increase the survival fractions of NSCLC cells suppressed by circ_0007580 knockdown (Figure 5(c,d)). Subsequently, we measured the proliferation and apoptosis of NSCLC cells under the condition of 4 Gy radiation. Our data showed that the suppressive effect of circ_0007580 silencing on the viability and the number of colonies of A549 and H1299 cells could be abolished by miR-598 inhibitor (Figure 5(e,f)). Through detecting the apoptosis rate and the activities of caspase-3 and caspase-9, we discovered that miR-598 inhibitor reversed the promotion effect circ_0007580 knockdown on the apoptosis of A549 and

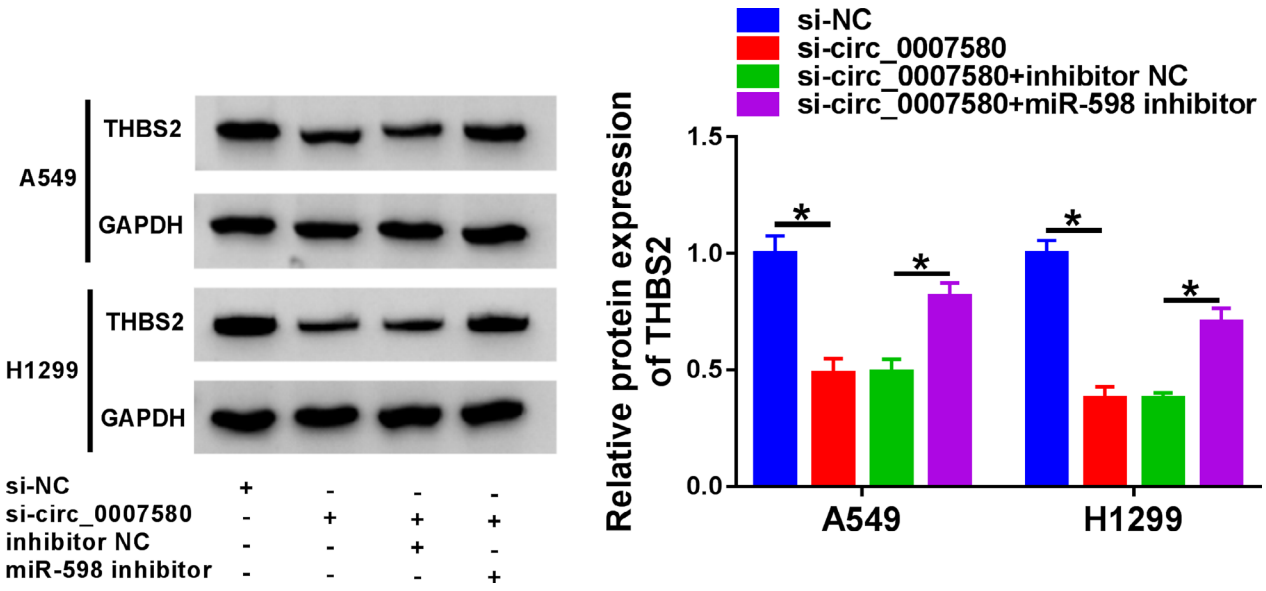

FIGURE 8 Circ_0007580 sponged miR-598 to regulate THBS2. A549 and H1299 cells were transfected with si-NC, si-circ_0007580, si-circ_0007580 + inhibitor NC or si-circ_0007580 + miR-598 inhibitor. The protein expression of THBS2 was measured by WB analysis. *$p < 0.05$

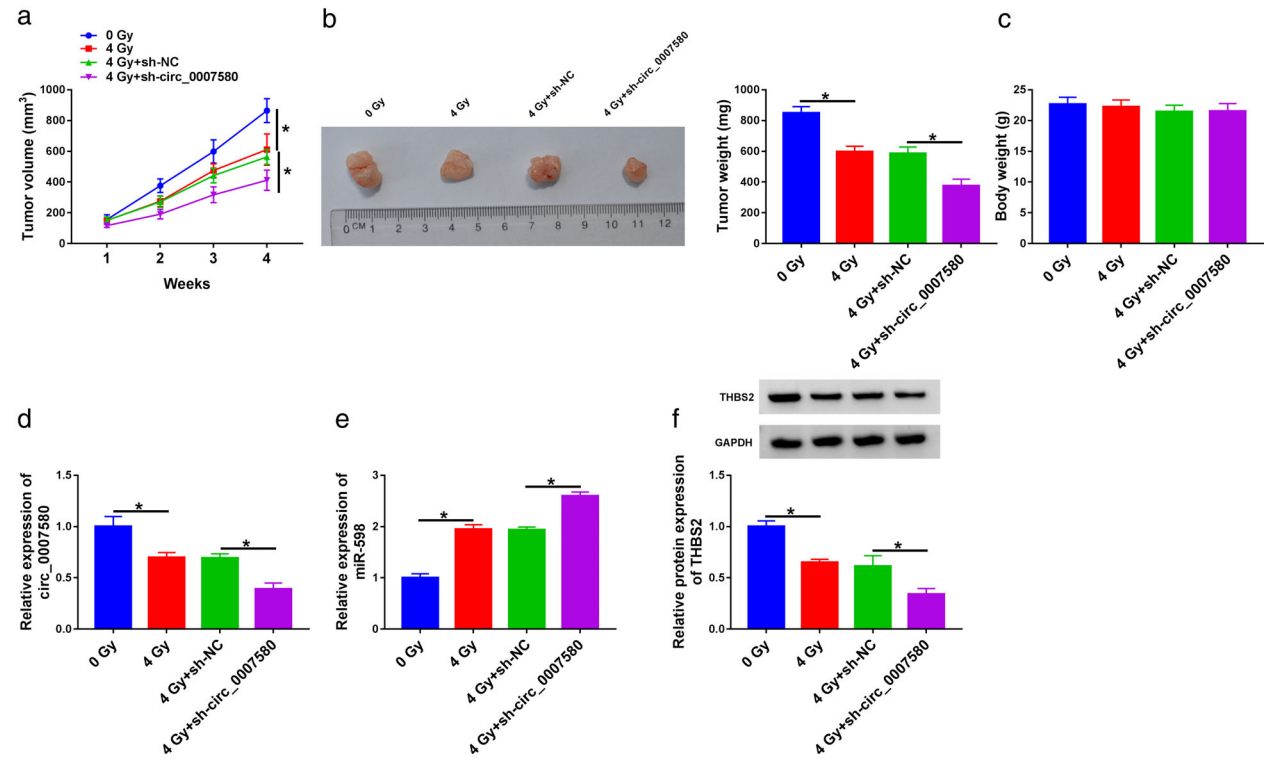

FIGURE 9 Circ_0007580 knockdown promoted the radiosensitivity of NSCLC tumor in vivo. H1299 cells were transfected with or without sh-NC or sh-circ_0007580, and then injected into nude mice followed by treatment with 0 or 4 Gy radiation. (a) Tumor volume was calculated every week. (b) Tumor weight was detected in each group. (c) The bodyweight of mice was measured in each group after tumor removal. (d, e) Circ_0007580 and miR-598 expression levels were detected by qRT-PCR. (f) The protein level of THBS2 was evaluated by WB analysis. *$p < 0.05$

H1299 cells (Figure 5(g–i)). Also, enhancing γH2AX protein expression regulated by circ_0007580 silencing was also reduced by miR-598 inhibitor (Figure 5(j)). These results revealed that circ_0007580 sponged miR-598 to regulate the radiosensitivity of NSCLC cells.

THBS2 was a target of miR-598

To confirm the downstream target of miR-598, we used the targetscan software. The 3′UTR of THBS2 was found to have binding sites with miR-598 (Figure 6(a)). The results of

dual-luciferase reporter assay indicated that the luciferase activity of WT-THBS2-3′UTR vector could be inhibited by miR-598 mimic, while the luciferase activity of MUT-THBS2-3′UTR vector did not change (Figure 6(b,c)). In NSCLC tissues, we found that THBS2 expression was markedly enhanced compared to adjacent normal tissues at the mRNA and protein levels (Figure 6(d,e)). Also, the THBS2 protein expression was higher in radioresistant NSCLC tissues than in radiosensitive NSCLC tissues (Figure 6(f)). Compared to BEAS-2B cells, a high THBS2 protein expression was also detected in A549 and H1299 cells (Figure 6(g)). Our data confirmed that miR-598 could target THBS2, and the expression of THBS2 in NSCLC tissues and cells was opposite to that of miR-598.

MiR-598 promoted the radiosensitivity of NSCLC cells by targeting THBS2

A THBS2 overexpression vector was constructed and its transfection efficiency was confirmed by detecting THBS2 protein expression (Figure 7(a)). To further analyze whether THBS2 was involved in the regulation of miR-598 on the radiosensitivity of NSCLC cells, we cotransfected with miR-598 mimic and pc-THBS2 into A549 and H1299 cells. By measuring THBS2 protein expression, we found that miR-598 could inhibit THBS2 expression, while this effect could be recovered by the addition of pc-THBS2 (Figure 7(b)). The survival fractions of A549 and H1299 cells were markedly reduced by miR-598 overexpression, and THBS2 overexpression could reverse this effect (Figure 7(c,d)). Under the condition of 4 Gy radiation, we discovered that miR-598 could suppress the viability and number of colonies, while promote the apoptosis rate and the activities of caspase-3 and caspase-9 in A549 and H1299 cells (Figure 7(e–i)). However, the inhibition effect of miR-598 on cell proliferation and the enhancing effect on cell apoptosis also could be reversed by overexpressing THBS2 (Figure 7(e–i)). Additionally, overexpressed THBS2 also abolished the increasing effect of miR-598 on γH2AX protein expression in A549 and H1299 cells (Figure 7(j)). Therefore, we confirmed that miR-598 could target THBS2 to accelerate the radiosensitivity of NSCLC cells.

Circ_0007580 sponged miR-598 to regulate THBS2

The above studies showed that circ_0007580 could sponge miR-598 and that miR-598 could target THBS2 to mediate the radiosensitivity of NSCLC. To determine the regulation of circ_0007580 on THBS2, we examined the expression of THBS2 in A549 and H1299 cells cotransfected with si-circ_0007580 and miR-598 inhibitor. Our data showed that circ_0007580 silencing remarkably decreased THBS2 protein expression in A549 and H1299 cells, while this effect could be reversed by miR-598 inhibitor (Figure 8). Hence, we propose that circ_0007580 can positively regulate THBS2 expression by sponging miR-598.

Circ_0007580 knockdown promoted the radiosensitivity of NSCLC tumor in vivo

To further determine that circ_0007580 could regulate the radiosensitivity of NSCLC, we performed the in vivo experiments by constructing the xenograft tumors. The results showed that 4 Gy radiation could significantly inhibit tumor volume and weight, which were lower in the circ_0007580 knockdown group than the sh-NC group under the condition of 4 Gy radiation (Figure 9(a,b)). After tumor removal, we found that there were no significant differences in bodyweight among the groups (Figure 9(c)). In addition, we discovered that 4 Gy radiation could reduce the circ_0007580 expression and THBS2 protein expression, while promoting miR-598 expression in tumor tissues, and these effects were more obvious after circ_0007580 knockdown (Figure 9(d–f)). All data confirmed that circ_0007580 regulated the radiosensitivity of NSCLC tumors through regulating the miR-598/THBS2 pathway.

DISCUSSION

The generation of radioresistance is a complex process of multigene interaction, which may be caused by the occurrence of tumor heterogeneity or the change of tumor microenvironment.[24,25] However, the specific mechanism of radioresistance in NSCLC is still unclear, and further exploration and research are urgently needed. Circ_0007580 has been found to be involved in NSCLC progression,[14] but whether it mediates NSCLC radioresistance is unclear. Our study showed that circ_0007580 had increased expression in radioresistant NSCLC tissues, suggesting that circ_0007580 might be involved in the occurrence of NSCLC radioresistance. Further loss-of-function experiments showed that downregulated circ_0007580 strengthened the radiosensitivity of NSCLC cells in vitro, mainly by inhibiting cell proliferation and promoting apoptosis. In addition, in vivo experiments results confirmed that knockdown of circ_0007580 could also improve the sensitivity of NSCLC tumor to radiotherapy, thus further reducing tumor growth. These results highlighted that circ_0007580 might be a key target for improving NSCLC radiosensitivity.

In previous studies, we found that miR-598, a tumor suppressor, was significantly underexpressed in radioresistant NSCLC tissues, which is contrary to the expression trend of circ_0007580. Through bioinformatic analysis and further experimental validation, we confirmed that circ_0007580 could interact with miR-598. In previous research, Tong et al. showed that miR-598 had negative

regulation on NSCLC proliferation and invasion, which was achieved by regulating ZEB2.[20] Also, miR-598 was discovered to target DERL1, thereby decreasing the metastasis of NSCLC.[18] Here, we found that miR-598 could reduce cell proliferation and increase cell apoptosis to enhance NSCLC radiosensitivity. It is worth noting that miR-598 inhibitor also reversed the promotion of circ_0007580 knockdown on the radiosensitivity of NSCLC by mediating cell proliferation and apoptosis. These results confirmed that circ_0007580 indeed regulated NSCLC radiosensitivity by targeting miR-598.

Most miRNAs cause gene silencing or degradation at the post-transcriptional level by pairing with complementary bases of target mRNAs.[26] Here, we discovered that miR-598 had binding sites with THBS2 3′UTR. As a member of the THBS family, THBS2 has a binding site for metal matrix transferase and can cooperate with other genes to mediate cell adhesion and migration.[27] A recent study proposed that circ_0020123 could upregulate THBS2 expression to accelerate NSCLC cell growth and tumorigenesis by sponging miR-590-5p.[28] In this study, a significantly high THBS2 expression was found in radioresistant NSCLC tissues. The rescue experiments suggested that the promoting of miR-598 on the radiosensitivity of NSCLC also was reversed by THBS2 overexpression. In addition, we confirmed the positive regulation of circ_0007580 on THBS2 expression, which perfected the existence of circ_0007580/miR-598/THBS2 network in NSCLC.

Overall, our study showed that circ_0007580 mediated the radiosensitivity of NSCLC by regulating proliferation and apoptosis. In terms of mechanism, we propose that circ_0007580 acts as miR-598 sponge to regulate THBS2 expression. Our study proposes a new target for regulating the radiosensitivity of NSCLC. According to our results, knockdown of circ_0007580 can also improve the sensitivity of NSCLC cells and tumors to radiotherapy, so we believe that targeted inhibition of circ_0007580 might be used to treat radioresistance in clinical practice.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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