ABSTRACT

Objective: Recently, the role of long non-coding RNAs (lncRNAs) in hepatocellular carcinoma (HCC) has been assessed. Our research was determined to investigate the impacts of lncRNA TP73-AS1 on radioresistance of HCC by modulating PTEN/Akt signaling pathway.

Methods: Expression of TP73-AS1 in HCC tissues and cells was detected using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The HCC cells were conducted with different doses of irradiation, then the survival, colony formation and apoptosis were determined by a series of assays. The HCC cell line with a higher expression of TP73-AS1 was transfected with TP73-AS1-siRNA and X-rayed, the expression of TP73-AS1, cell survival, radiosensitivity, and apoptosis were evaluated. Subcutaneous tumorigenesis in nude mice was adopted to record the size of tumors before and after the radiation. RT-qPCR and Western blot analysis were used to clarify the activation of PTEN/Akt signaling pathway.

Results: TP73-AS1 was highly expressed in HCC tissues and cells. With the increasing dose of radiation, the relative proliferation activity and survival fraction (SF) of HCC cells was gradually reduced, while the total apoptosis rate was gradually elevated. TP73-AS1 knockdown promoted radiosensitivity and apoptosis, repressed cell proliferation, making it an inhibitor of tumor in HCC. Moreover, reduced TP73-AS1 was able to decline the phosphorylation of Akt and increase the expression of PTEN in HCC. Down-regulated TP73-AS1 could repress tumorigenesis by promoting radiosensitivity in nude mice with HCC.

Conclusion: Our study suggests that lncRNA TP73-AS1 was highly expressed in HCC and participated in radioresistance of HCC via the PTEN/Akt signaling pathway.

Abbreviations: lncRNAs: long non-coding RNAs; lncRNAs: HCC: hepatocellular carcinoma; RT-qPCR: reverse transcription quantitative polymerase chain reaction; survival fraction: SF; lncRNA TP73-AS1: LncRNA P73 antisense RNA 1T; PTEN: Phosphatase and tensin homologue; Akt: Protein kinase B; P13K: phosphatidylinositol 3-kinase; TNM: tumor, node and metastasis; ACJJ: American Joint Committee on Cancer; FBS: fetal bovine serum; EDTA: ethylene diamine tetraacetic acid; NC: negative control; DMEM: Dulbecco’s modified Eagle medium; OD: optical density; PE: Plating efficiency; FITC/PI: fluoresceine isothiocyanate/propidium iodide; PBS: phosphate buffered solution; GAPDH: Glyceraldehyde phosphate dehydrogenase; ANOVA: one-way analysis of variance; LSD-t: least significant difference test

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Introduction
Hepatocellular carcinoma (HCC) is one of the main malignancies in the world with a high incidence, and HCC also leads to a broad ratio of deaths [1]. When it comes to the risk factors of HCC, many studies have pointed out the factors such as obesity, diabetes, alcohol consumption and persistent hepatitis B or C virus infection were related with HCC [2,3]. Majority of the HCC patients missed the best time of surgery therapy due to the diagnosis at middle and advanced stages [4]. The current treatment for HCC remains surgical resection, radiotherapy, chemotherapy, and liver transplantation [5]. Among these methods, radiotherapy has performed critical effects on a variety of malignancies. As reported, factors such as cell viability, cell cycle distribution, DNA damage repairing, and cell apoptosis affect the radiosensitivity of tumor cells [6]. It has been uncovered that radioresistance-associated molecules such as mRNAs, microRNAs, and proteins could influence the radioresistance via modulating radioresistance-associated processes, including DNA repair capacity, apoptosis, cell cycle arrest, and protective autophagy [7].
Long noncoding RNAs (lncRNAs) are a group of mRNA-like transcripts that have been implicated in the processes of different cancers [7]. Several lncRNAs such as lncRNA-UCA1 [8], lncRNA HULC [9] and lncRNA HOTTIP [10] have been proved to be implicated in HCC. LncRNA P73 antisense RNA 1T (lncRNA TP73-AS1) is one of the lncRNAs, which performed an ectopic expression in cancers and was able to modulate apoptosis via p53-dependent anti-apoptotic genes [11]. According to the previous literatures, lncRNA TP73-AS1 was associated with some particular human diseases, such as clear cell renal cell carcinoma [12] and breast cancer [13]. Interestingly, lncRNA TP73-AS1 has also been studied by a recent research, in which lncRNA TP73-AS1 was verified to regulate the cell proliferation of HCC [14]. Phosphatase and tensin homolog (PTEN) has proved to be frequently disrupted in many kinds of tumors and targeted by germline mutations in cancer patients, which performed as a tumor inhibitor in some tissues [15]. It is well known that PTEN is an antioncogene that plays a key role in cell growth, apoptosis, adhesion, migration, and infiltration [16], and it has been reported that PTEN participated in the process of radiosensitivity [17,18]. Furthermore, protein kinase B (Akt) is a main downstream effector of phosphatidylinositol 3-kinase (PI3K), which was associated with drug resistance and sensitivity, and Akt has been clarified as a critical part of the insulin receptor intracellular signaling [19]. Moreover, a recent study has demonstrated that the PTEN/Akt pathway was involved in the sorafenib resistance in HCC cells [20]. However, there remains little known about the correlation among lncRNA TP73-AS1, the PTEN/Akt signaling pathway and HCC. Thus, this study was performed to determine the role of lncRNA TP73-AS1 in radioresistance of HCC via the PTEN/Akt signaling pathway, and we speculated that reduced lncRNA TP73-AS1 could play a regulative role in radioresistance of HCC through the modulation of the PTEN/Akt signaling pathway.

Materials and methods

Ethics statement

Written informed consents were gained from all patients before the study. The protocols of this study were approved by the Ethic Committee of The Affiliated Cancer Hospital of Zhengzhou University and based on the ethical principles for medical research implicating human subjects of the Helsinki Declaration. Animal experiments were strictly consistent with the Guide to the Management and Use of Laboratory Animals issued by the National Institutes of Health. The protocol of animal experiments was authorized by the Institutional Animal Care and Use Committee of The Affiliated Cancer Hospital of Zhengzhou University.

Study subjects

Seventy-two HCC tissues and 72 adjacent normal tissues (over 5 cm from cancer tissues) were harvested from patients with HCC who had undergone liver tumor resection in the Hepatobiliary Surgery of The Affiliated Cancer Hospital of Zhengzhou University from January 2014 to December 2017. All cases were confirmed as HCC by pathological diagnosis. Prior to the surgery, patients received no anti-tumor treatment. Among the patients, there were 54 cases ≥65 years old, 18 cases <65 years old, 50 males and 22 females. According to the tumor, node and metastasis (TNM) staging of HCC which was revised by American Joint Committee on Cancer (ACJJ) in 2010 [21], the samples were separated into two stages: 44 cases of I/II stage and 28 cases of III/IV stage, and 53 cases were moderately and highly differentiated, 19 cases were poorly differentiated or undifferentiated; 39 cases with lymphatic metastasis, and 33 cases without lymphatic metastasis.

Cell culture

Human normal liver cell strain HL-7702, human HCC cell line HepG2, Hep3B and SMCC-7721 were preserved and provided by general surgery laboratory of Union Hospital Affiliated with Tongji Medical College of Huazhong University of Science and Technology. Human HCC cells together with normal liver cells were cultured at 37°C and with 5% CO2 by RPMI-1640 medium containing 10% fetal bovine serum (FBS), trypsinized by 0.25% trypsin containing 0.03% ethylene
diamine tetraacetic acid (EDTA) when the cell confluence reached 70%-80%, which were generally passaged at 1: 3–5. All the cells were in the logarithmic growth phase, and the apoptosis rate was evaluated by trypan blue staining, cells with a death rate below 5% were allowed to the experiments.

**Ionizing radiation**

Human normal liver cell strain HL-7702, human HCC cell line HepG2, Hep3B, SMCC-7721 and HCC cell lines conducted with following TP73-AS1-siRNA grouping were X-rayed. HCC cell lines in the logarithmic growth phase were, respectively, trypsinized and counted after the cells were planked, then the cells were seeded into culture dishes or well plates and incubated for 12 h. The cells were conducted with 6 MV radiation of high energy X-ray by Varian 21 EX linac at 3 Gy/min. HCC cells were screened by vertical radiation at different doses (0, 2, 4, 6, 8 Gy), then Hep3B cells were selected for the radiosensitivity analysis, the cells were vertically radiated at 4 Gy, the irradiation distance was 100 cm and range was 10 cm × 10 cm. Then, the cells were cultured for 48 h for the subsequent experiments.

**Cell grouping and transfection**

With pcDNA3.1/Zeo (+) as the carrier, TP73-AS1-siRNA expression plasmids, overexpressed (oe)-TP73-AS1, and their relative negative control (NC) expression plasmids were synthetized by Shanghai GenePharma Co., Ltd. (Shanghai, China), related sequences were not provided because of some commercial factors. Twenty-four hours before the transfection, cells in the logarithmic growth phase were trypsinized and counted, the cell concentration was adjusted to 2 × 10⁵ cells/mL. The cell suspension (200 µL) was seeded onto six-well plates containing 2 mL medium, and the cells were cultured by Dulbecco’s modified Eagle medium (DMEM) without antibiotic (containing 10% FBS). The cells were separated into three groups: the blank group (transfected with empty vector); the si-NC group (transfected with NC-siRNA sequence); the TP73-AS1-siRNA (transfected with TP73-AS1-siRNA sequence). Cells in every group were transfected under the guide of the directions of Lipofectamine™ 2000 kits (Invitrogen, Carlsbad, CA, USA) after the cell confluence reached 50%-60%. The transfected cells were cultured at 37°C and with 5% CO₂ for 6 h, then observed and photographed under a confocal laser scanning microscope (Olympus, Tokyo, Japan).

**3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2-h-tetrazolium bromide (MTT) assay**

Cells have undergone ionizing radiation were seeded onto the 96-well plates at 5 × 10³ cells/mL and cultured for 48 h, cells that have not undergone ionizing radiation were taken as a control. Every well was supplemented with 20 µL MTT solution (5 mg/mL, GD-Y1317, Shguduo Biomart Co, Ltd., Shanghai, China) for the development. The medium was discarded after 4 h, each well was added with 150 µL dimethyl sulfoxide (DMSO, Amresco, Solon, OH, USA). The optical density (OD) value of each well was detected using a microplate reader (DeTie Experimental Equipment Co., Ltd., Nanjing, China) at 490 nm. The changes of cell viability in each group were determined, which were compared with the OD value. Relative cell viability (%) = OD value of the experimental group/OD value of the control group × 100%.

**Colony formation assay**

After made into cell suspension, cells undergone ionizing radiation were seeded into 60 mm dishes and cultured for 10–14 d, and cells that had not undergone ionizing radiation were taken as a control. The culture was stopped when the colonies were visible. The medium was discarded and the cells were fixed by 5 mL 95% ethanol for 15 min, the fixer was discarded, then stained by crystal violet solution for 10 min, rinsed by flow water and dried. The grid lines were marked, colonies with over 50 cells were counted and the number of colonies in each gridding was calculated. Plating efficiency (PE) = (the number of colonies/the number of seeded cells) × 100%; SF = (PE of the irradiation group/PE of the control group) × 100%.
Flow cytometry

Cell apoptosis was measured using Annexin V-fluoresceine isothiocyanate/propidium iodide (FITC/PI) staining. Cells undergone ionizing radiation were seeded onto 96-well plates at $5 \times 10^3$ cells per well and cultured for 48 h, cells that had not undergone ionizing radiation were taken as a control. The cells were trypsinized by 0.25% trypsin and the cell concentration was modulated to $1 \times 10^6$ cells/mL. Cells (1 mL) were centrifuged at 1500 r/min x 10 min with the supernatant discarded, then the cells were collected and per milliliter cells were supplemented with 1 mL phosphate-buffered solution (PBS), centrifuged and fixed by pre-cooled 70% ethanol solution at 4°C overnight. The fixed cells were washed by PBS for 2 times in the next day, then resuspended in 200 μL binding buffer, added with 10 μL Annexin V-FITC (K201-100, Biovision, Mountain View, CA, USA) and 5 μL PI, mixed up and reacted in the dark for 15 min, then the cells were appended with 300 μL binding buffer, and the apoptosis was detected using flow cytometry at the excitation wavelength of 488 nm.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The total RNA of tissues and cells in each group was extracted using RNA extraction kit (Invitrogen, Carlsbad, CA, USA). The primers (Table 1) were synthetized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The extracted RNAs were reversely transcribed according to the direction of the kit (Thermo Fisher Scientific, MA, USA). The reaction solution was conducted with RT-qPCR by TaqMan assay, the reaction system was based on the instruction of kit (Applied Biosystems Inc., Carlsbad, CA, USA). ABI 7500 RT-qPCR instrument (Applied Biosystems Inc., Carlsbad, CA, USA) was used for the RT-qPCR. Glyceraldehyde phosphate dehydrogenase (GAPDH) was taken as the internal reference of LncRNA TP73-AS1 and PTEN. The data were analyzed by $2^{-\Delta\Delta Ct}$ method [22].

Western blot analysis

Cells transfected for 48 h were collected and the total protein was extracted by radio-immunoprecipitation assay lysis buffer. The protein concentration was evaluated using bicinechonic acid assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the proteins, then the proteins were transferred onto the polyvinylidene fluoride membrane (Bio-Rad, Inc., CA, USA). After sealed by bovine serum albumin for 1 h, the proteins were added with rabbit anti-human primary antibodies Anti p-Akt (ab38449, 1: 500–1: 1000), Akt (ab0085, 1: 500), PTEN (ab32199, 1: 10,000) and GAPDH (ab181602, 1: 10,000) (all from Abcam Inc., Cambridge, MA, USA), then incubated at 4°C overnight. The proteins were rinsed by tris buffer solution with tween for 3 times and added with goat anti-rabbit secondary antibody labeled by horseradish peroxidase (ab6721, 1: 2000–1: 20,000), incubated for 1 h, then developed by enhanced chemiluminescent assay. GAPDH was taken as the internal reference. The images were scanned by computers, and the gray value of each bands was detected by gel imaging system (Bio-Rad iQ5, Bio-Rad, Inc., CA, USA). The relative content of proteins in each group = gray value of target proteins/gray value of GAPDH.

Subcutaneous tumorigenesis in nude mice

Cells in the logarithmic growth phase in Hep3B human HCC cell line were made into single-cell suspension. PBS together with Matrigel were mixed up at 1: 1, and the mixed liquid was applied to resuspend the cells, the final cell concentration was diluted to $1 \times 10^6$ cells/200 μL. Male BALB/c nude mice (aged 4–6 w, weighed 18–22 g) were fed at specific pathogen-free environment, with the temperature at 22°C and the humidity at 50%-70%. Standard circadian system (12 h illumination + 12 h night) was used for the adaptive feeding, and the mice had free access to food and water. A total of 18 mice were separated
into three groups, 6 mice in each group: the blank group, the NC group and the TP73-AS1-siRNA group. Mice in each group were, respectively, subcutaneously injected with Hep3B cells at the back of right hind leg that transfected with empty vector, NC-siRNA or TP73-AS1-siRNA at $1 \times 10^6$ cells (200 μL), then fed at the same environment. The size and volume of tumors were determined every 3 d, three mice in each group were X-rayed at 4 Gy when the tumor volume reached 50 mm$^3$, the remained three mice in each group were taken as the control group of 0 Gy. Then, the mice were observed every seven d, the length and width of the tumors were recorded, tumor volume = length × width$^2$/2. The nude mice were euthanized at the 35th d and three tumors in each group were taken out.

**Statistical analysis**

All data analyses were conducted using SPSS 21.0 software (IBM-SPSS, Inc, Chicago, IL, USA). The measurement data conforming to the normal distribution were expressed as mean ± standard deviation. The unpaired t-test was performed for comparisons between two groups and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups, Fisher’s least significant difference t-test (LSD-t) was used for pairwise comparisons after the ANOVA. $p$-Value < 0.05 was indicative of statistically significant difference.

**Results**

**LncRNA TP73-AS1 is highly expressed in HCC tissues and cells**

TP73-AS1 expression of HCC tissues and the adjacent normal tissues was detected by RT-qPCR, and the outcomes unraveled that (Figure 1a) the expression of LncRNA TP73-AS1 in HCC tissues was apparently higher than that in the adjacent normal tissues ($P < 0.05$). Expression of LncRNA TP73-AS1 of human normal liver cells and human HCC cell lines was also evaluated using RT-qPCR, and the outcomes implied that (Figure 1b) LncRNA TP73-AS1 expressed in the four cell lines, while when compared with HL-7702 human normal liver cell line, LncRNA TP73-AS1 expression was observable increased in HepG2 cell line, Hep3B cell line and SMCC-7721 cell line (all $P < 0.05$). Meanwhile, in contrast to Hep3B cell line, LncRNA TP73-AS1 expression was reduced in HepG2 cell line and SMCC-7721 cell line (both $P < 0.05$).

**Radioresistance of different HCC cell lines**

After the HCC cell lines (Hep3B, HepG2 and SMCC-7721) were X-rayed at different dose (0, 2, 4, 6, 8 Gy), the cell survival was determined by MTT assay. Relative proliferation of each HCC cell lines was restrained with the increasing of radiation dose, while the relative proliferation of Hep3B cells was the

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**Figure 1.** LncRNA TP73-AS1 is up-regulated in HCC tissues and cells. a, comparison of LncRNA TP73-AS1 expression between HCC tissues and adjacent normal tissues; b, comparison of LncRNA TP73-AS1 expression among different cell lines, * $P < 0.05$ vs HL-7702 cells; # $P < 0.05$ vs Hep3B cells.
highest and that of SMCC-7721 cells was the lowest at the same dose (Figure 2a). The outcomes proved that at the same radiation dose, the radioresistance of Hep3B cells was the strongest, which was followed by HepG2 cells, and the radioresistance of SMCC-7721 cells was the weakest.

After the HCC cell lines (Hep3B, HepG2 and SMCC-7721) were X-rayed at different dose (0, 2, 4, 6, 8 Gy), as shown in colony formation assay (Figure 2b, c) and flow cytometry (Figure 2d, e) revealed that SF of each HCC cell lines was abated with the augment of radiation dose, while SF of Hep3B cells was the highest and the total apoptotic rate was the lowest, while SF of SMCC-7721 cells was the lowest and the total apoptotic rate was the highest at the same dose. The results clarified that at the same radiation dose, the radioresistance of Hep3B cells was the strongest, which was followed by HepG2 cells, and the radioresistance of SMCC-7721 cells was the weakest.

Reduced LncRNA TP73-AS1 up-regulates PTEN and declines Akt phosphorylation in HCC cells after radiotherapy

After different doses (0, 2, 4, 6, 8 Gy) of irradiation of non-transfected Hep3B cells, TP73-AS1 expression was gradually heightened. Particularly, compared with 0 Gy, lncRNA TP73-AS1 expression in Hep3B cells was evidently elevated at doses of 2, 4, 6, and 8 Gy (all \( P < 0.05 \), Figure 3a). After treated with different doses (0, 2, 4, 6, 8 Gy), the expression of PTEN gradually lowered; relative to 0 Gy, PTEN expression in Hep3B cells was markedly restrained in 2, 4, 6, and 8 Gy (all \( P < 0.05 \), Figure 3b).
Figure 3. Reduced IncRNA TP73-AS1 up-regulates PTEN and declines pAkt/Akt in HCC cells after radiotherapy. a, expression of IncRNA TP73-AS1 after Hep3B cells were conducted with different doses of irradiation (0, 2, 4, 6, 8 Gy); * P < 0.05 vs 0 Gy; b, expression of PTEN after Hep3B cells were conducted with different doses of irradiation (0, 2, 4, 6, 8 Gy); * P < 0.05 vs 0 Gy; c, expression of IncRNA TP73-AS1 before and after Hep3B cells that have been transfected with silenced TP73-AS1 vectors were treated with radiotherapy, * P < 0.05 vs the blank group at the same dose; # P < 0.05 vs at dose of 0 Gy in the same transfected cells; d, mRNA expression of PTEN before and after Hep3B cells that have been transfected with silenced TP73-AS1 vectors were treated with radiotherapy, * P < 0.05 vs the blank group; # P < 0.05 vs at dose of 0 Gy in the same transfected cells; e, protein expression of PTEN and pAkt/Akt before and after Hep3B cells that have been transfected with silenced TP73-AS1 vectors were treated with radiotherapy; f, TP73-AS1 expression before and after Hep3B cells that have been transfected with overexpressed TP73-AS1 vectors were treated with radiotherapy; g, PTEN mRNA expression before and after Hep3B cells that have been transfected with overexpressed TP73-AS1 vectors were treated with radiotherapy, & P < 0.05 vs the blank group at the same dose of irradiation; # P < 0.05 vs at dose of 0 Gy in the same transfected cells; h, comparisons of protein expression of PTEN and pAkt/Akt before and after Hep3B cells that have been transfected with overexpressed TP73-AS1 vectors were treated with radiotherapy among the groups, & P < 0.05 vs the blank group at the same dose of irradiation; # P < 0.05 vs at 0 Gy in the same transfected cells.

The transfected Hep3B cells were X-rayed at 0 Gy or 4 Gy and cultured for 48 h, then TP73-AS1 expression was measured by RT-qPCR, the outcomes of which suggested that (Figure 3c) when the dose was 0 Gy, there was no apparent difference in expression of IncRNA TP73-AS1 between the blank group and the si-NC group (P > 0.05), while that of the TP73-AS1-siRNA group was remarkably lowered (P < 0.05), implying that Hep3B cells were successfully transfected, while at the dose of 4 Gy, IncRNA TP73-AS1 expression was considerably increased in the blank group, the si-NC group and the TP73-AS1-siRNA group, which was contrasted to the dose of 0 Gy (all P < 0.05).

After 4 Gy dose of irradiation, the transfected Hep3B cells were cultured for 48 h, the outcomes of RT-qPCR revealed that (Figure 3d) when the dose was 0 Gy, there was no evident difference in mRNA expression of PTEN between the blank group and the si-NC group (P > 0.05), while mRNA expression of PTEN in the TP73-AS1-siRNA group was observably heightened (P < 0.05); when the dose was 4 Gy, it came out that the mRNA expression of PTEN in the blank group, the si-NC group and the TP73-AS1-siRNA group were suppressed in contrast to the dose of 0 Gy (all P < 0.05).

The outcomes of Western blot analysis indicated that (Figure 3e) when the dose was 0 Gy, there was no obvious difference in protein expression of PTEN and pAkt/Akt between the blank group and the si-NC group (P > 0.05), while protein expression of PTEN in the TP73-AS1-siRNA group was noticeably increased (P < 0.05), and that of pAkt/Akt was broadly reduced (P < 0.05); when the dose was 4 Gy, the protein expression of PTEN was markedly
restricted in the blank group, the si-NC group and the TP73-AS1-siRNA group, and the levels of pAkt/Akt were all amplified, which was relative to the dose of 0 Gy (all $P < 0.05$).

To further investigate the effect of TP73-AS1 on the protein expression of PTEN/Akt signaling pathway, the expression of PTEN and pAkt/Akt in cells that have been transfected with overexpressed TP73-AS1 plasmids of each group was measured, and the outcomes reflected that at 0 Gy, TP73-AS1 and pAkt/Akt expression was heightened, while both mRNA and protein expression of PTEN was noticeably repressed in the oe-TP73-AS1 group, which was relative to the oe-NC group (all $P < 0.05$); in comparison to 0 Gy, TP73-AS1 and pAkt/Akt expression was advanced, but mRNA and protein expression of PTEN was lowered at 4 Gy in both the oe-TP73-AS1 group and the oe-NC group (all $P < 0.05$, Figure 3F–h).

**LncRNA TP73-AS1 knockdown promotes radiosensitivity of Hep3B cells**

Hep3B cells were, respectively, conducted with 0 Gy and 4 Gy irradiation, the cell proliferation was detected by MTT assay, the cell colony formation ability was determined by colony formation assay and apoptosis was evaluated using flow cytometry, the impacts of down-regulated lncRNA TP73-AS1 on radiosensitivity of Hep3B cells were assessed.

The cell proliferation was detected by MTT assay, OD value of the blank group at 0 Gy was taken as the control to calculate the relative proliferation ability in each group, the results illustrated that (Figure 4a) contrasted to 0 Gy of irradiation, the relative proliferation ability was declined in the blank group, the si-NC group and the TP73-AS1-siRNA group after 4 Gy of irradiation (all $P < 0.05$), there was no broad difference in relative proliferation ability between the blank group and the si-NC group at the same dose of irradiation ($P > 0.05$, Figure 4b, c) after 4 Gy of irradiation, the SF in the blank group, the si-NC group and the TP73-AS1-siRNA group was broadly lowered, which was relative to the dose of 0 Gy (all $P < 0.05$). No obvious difference could be found in SF between the blank group and the si-NC group at the same dose of irradiation ($P > 0.05$), while that of the TP73-AS1-siRNA group was evidently decreased ($P < 0.05$).

**Figure 4.** LncRNA TP73-AS1 knockdown promotes radiosensitivity of Hep3B cells. a, cell proliferation before and after transfected Hep3B cells were treated with radiotherapy was detected by MTT assay; b, cell colony formation ability before and after transfected Hep3B cells were treated with radiotherapy was detected by colony formation assay; c, representative images of colony formation assay of each group; d, apoptosis rate before and after transfected Hep3B cells were treated with radiotherapy was detected by flow cytometry; e, representative images of apoptotic results were detected by flow cytometry; * $P < 0.05$ vs the blank group at the same dose of irradiation; # $P < 0.05$ vs at dose of 0 Gy in the same transfected cells.
Apoptosis was detected by Annexin V-FITC/PI double staining, the outcomes implied that (Figure 4d,e) the total apoptosis of cells in the blank group, the si-NC group and the TP73-AS1-siRNA group was considerably elevated after 4 Gy of irradiation, which was relative to the dose of 0 Gy (all \( P < 0.05 \)). There was no obvious difference in total apoptosis between the blank group and the si-NC group at the same dose of irradiation (\( P > 0.05 \)), while that of the TP73-AS1-siRNA group was substantially heightened (\( P < 0.05 \)).

To sum up, the radiosensitivity of Hep3B cells was evidently strengthened after transfected with TP73-AS1-siRNA.

\[ \text{Reduced LncRNA TP73-AS1 up-regulates PTEN and declines Akt phosphorylation in HCC tissues in nude mice after radiotherapy} \]

After rats of each group were treated with 0 and 4 Gy of irradiation, the impacts of down-regulated lncRNA TP73-AS1 on the activation of the PTEN/Akt signaling pathway was measured. The results of RT-qPCR revealed that (Figure 6a,b) lncRNA TP73-AS1 expression in the blank group, the si-NC group and the TP73-AS1-siRNA group was noticeably augmented and the mRNA expression of PTEN was restrained after 4 Gy of irradiation, which was contrasted with the dose of 0 Gy (all \( P < 0.05 \)). At the same dose (4 Gy) of irradiation, no evident difference could be found in TP73-AS1 expression and PTEN mRNA expression of the blank group and the si-NC group (all \( P > 0.05 \)), while lncRNA TP73-AS1 expression was declined and mRNA expression of PTEN was increased in the TP73-AS1-siRNA group (both \( P < 0.05 \)).

The outcomes of Western blot analysis suggested that (Figure 6c–e) the protein expression of PTEN was evidently suppressed and expression of pAkt/Akt was apparently heightened in the blank group, the si-NC group and the TP73-AS1-siRNA group at 4 Gy of irradiation, which was in contrast to the dose of 0 Gy (all \( P < 0.05 \)). At the same dose (4 Gy) of irradiation, no broad difference could be found in the protein expression of PTEN and pAkt/Akt in the blank group and the si-NC group (all \( P > 0.05 \)), while protein expression of PTEN was elevated and expression of pAkt/Akt was reduced in the TP73-AS1-siRNA group (both \( P < 0.05 \)).

**Figure 5.** Down-regulated lncRNA TP73-AS1 represses tumorigenesis by promoting radiosensitivity in HCC mice. a, tumor growth curve of nude mice in each group before and after radiotherapy; b, analysis of tumor volumes of mice in each group before and after transfection and radiotherapy; c, representative images of tumors of mice in each group; * \( P < 0.05 \) vs the blank group, # \( P < 0.05 \) vs at dose of 0 Gy in the same transfected cells.
Discussion

HCC is the 3rd factor causing death that is related to the cancers in the world, and also the major reason of mortality in patients with cirrhosis [23]. It has been clarified that lncRNAs play a key part in the progression of many intricate diseases [24]. Moreover, there were several researches implied that the mechanism of lncRNA TP73-AS1 might be associated with human diseases, for instance, lncRNA TP73-AS1 could regulate the development of glioma through the interaction with microRNA-124 [11]. What’s more, TP73-AS1 could also play a part in the prognosis of bladder cancer patients and function as a repressor of bladder cancer via the epithelial–mesenchymal transition signaling pathway [25]. Our research aimed to investigate the impacts of lncRNA TP73-AS1 on radioresistance of HCC via the PTEN/Akt signaling pathway, and we could conclude that lncRNA TP73-AS1 participated in radioresistance of HCC via the PTEN/Akt signaling pathway, and reduced lncRNA TP73-AS1 regulated expression of PTEN, phosphorylation of Akt and radioresistance of HCC cells.

Among the vital results in our research, one of them suggested that lncRNA TP73-AS1 was up-regulated in HCC, implying that there was a dysregulation of lncRNA TP73-AS1 in HCC. This dysregulation of lncRNA TP73-AS1 has also been identified in other human diseases, for example, a research has revealed that TP73-AS1 expression was conspicuously enhanced both in HCC tissues and cell lines, which were associated with advanced tumor stage and poor prognosis. In addition, Li et al. have proved that TP73-AS1 might be an oncogenic lncRNA that promoted proliferation of HCC and could be regarded as a therapeutic target in human HCC [14]. These documents indicated that TP73-AS1 was aberrantly expressed in HCC, and the up-regulation of TP73-AS1 performed as a oncogene in HCC tissues. Moreover, we have found in the results that the restrained TP73-AS1 was able to inhibit
the cell proliferation and promote apoptosis in HCC, and it has been elucidated that restrained TP73-AS1 could suppress cell proliferation and elevate apoptosis in esophageal squamous cell carcinoma [26]. A study has also proved that IncRNA TP73-AS1 has the capacity to accelerate proliferation of breast cancer cells by miR-200a-mediated mitochondrial transcription factor A [13]. Furthermore, we have found that with the augment dose of the radiation, the relative proliferation activity of the HCC cells was gradually declined, while the survival fraction was gradually enhanced, indicating that radiotherapy contributes to the treatment of HCC, which has been proved in other literatures as well [27,28].

Another essential finding was that the restrained IncRNA TP73-AS1 could suppress the expression of PTEN. It was similar to this result that Guo et al. have provided the evidence that IncRNA GAS5 could induce the expression of PTEN in endometrial cancer cells [29], and IncRNA FER1L4 was identified to decelerate cell proliferation and cycle by promoting the expression of PTEN and inhibiting the phosphorylation of Akt in HCC. Similarly, a recent research has illuminated that TP73-AS1 knockdown could regulate the levels of Akt [12]. The most important finding in this study is that the reduced IncRNA TP73-AS1 could improve radiosensitivity of HCC cells, which may be a novel biomarker that function as a candidate for the therapy of HCC. Although it is a novel finding that has not been found before, we can still find some related demonstrations. Hou et al. have mentioned in their study that down-regulated TP73-AS1 was able to promote the chemosensitivity of esophageal cancer cells [31]. More than that, the down-regulation of IncRNA NEAT1 was also verified to improve the radiosensitivity by regulating the miR-204/ZEBl axis in nasopharyngeal carcinoma [32]. All the data contributed to clarifying the role of IncRNA TP73-AS1 in radioresistance of human diseases.

To sum up, this research clarified that IncRNA TP73-AS1 is highly expressed in HCC, and reduced IncRNA TP73-AS1 represses the development of HCC and promotes radioresistance in HCC via the PTEN/Akt signaling pathway. However, further investigation on the mechanisms of TP73-AS1 in HCC cell radioresitivity is needed, and more efforts, such as enlarging the sample size or verify our results with more cell lines, are required to further assess the mechanisms of IncRNAs in HCC, which could augment our comprehension in the progression of HCC and further promote the diagnosis and treatment of HCC.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**ORCID**

Miaomiao Sun http://orcid.org/0000-0002-7338-4522

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