Original Article
AZT-mediated inhibition of telomerase activity suppresses pancreatic cancer cell proliferation and enhances their radiosensitivity

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Abstract: Objective: To investigate the effects of AZT-mediated inhibition of telomerase activity on the proliferation, apoptosis and radiosensity of panc1 cells, and the growth of pancreatic tumor in vivo. Methods: panc1 cells were divided into four groups: A, control group; B, 0.4 mmol/L AZT; C, 0.6 mmol/L AZT; and D, 0.8 mmol/L AZT group. Cell proliferation was assessed by MTT assay. Cell apoptosis was detected by flow cytometry. Telomerase activity was measured by the quantitative TRAP-ELISA and the level of hTERT mRNA expression was determined by RT-PCR analysis. The changes in the radiosensitivity of cells were analyzed by colony formation assay and cell survival curve. Further, a xenograft mouse model of human pancreatic cancer was constructed and the effect of AZT-mediated telomerase activity on the tumor growth in vivo was evaluated. Results: Telomerase activity in group B, C and D was decreased by 8.4%, 14.6% and 24%, respectively compared with the control group. The expression of hTERT mRNA in group B, C and D was significantly decreased (P < 0.05). ATZ significantly reduced the cell viability in group B, C and D (P < 0.05), but significantly increased the percentage of early apoptotic cells in the three groups in a dose-dependent manner (P < 0.05). Moreover, ATZ dose-dependently increased the radiosensitivity of cells in group B, C and D compared with the control group. In animal studies, the tumor volume of nude mice in AZT, radiation and the combination group of AZT and radiation was decreased compared with the blank control, whereas the tumor volume in the combination group was significantly smaller than that in the other two treatment groups (P < 0.05). Conclusion: AZT-mediated inhibition of telomerase activity suppresses the proliferation of pan1 cells, induces their apoptosis, increases their radiosensitivity, and inhibits the growth of pancreatic tumor in vivo.

Keywords: Panc-1 cells, AZT, hTERT, telomerase, radiosensitivity

Introduction

Pancreatic cancer is one of the most lethal malignancies with a 5-year survival rate of only 5.5% [1]. In recent decades, the incidence and mortality rates of pancreatic cancer have been gradually increasing [2, 3]. Radiotherapy has become an important method for the treatment and prognosis of pancreatic cancer. However, the clinical effect of radiotherapy has been far from satisfactory largely due to the low radiosensitivity of pancreatic cancer cells. Therefore, there is currently an urgent need for a clinically effective and practical method to improve the radiosensitivity of pancreatic cancer.

Telomeres are the special structures at the ends of linear chromosomes that are essential for both the integrity of genes and the stability of cells [4]. Telomerase, the reverse transcriptase enzyme responsible for the synthesis of telomeres, maintains the length of telomeres, and compensates for the DNA loss during cell division. It has been shown that the activity of telomerase is primarily determined by hTERT, one of the essential components of telomerase [5]. Under the catalysis of hTERT, telomerase compensates for the DNA loss, which thereby makes tumor cell immortal. It has been well known that telomerase activation is the distinct difference between normal and tumor cells [6, 7], which may be involved in the malignant transformation of cells [8, 9]. Moreover, telomerase can repair radiation-induced DNA damages and double-strand breaks. Therefore, telomerase may be a good molecular target for radiotherapy, and the radiosensitivity of cells may be increased by inhibition of telomerase

activity. Zidovudine, also called AZT, is one of the most common reverse transcriptase inhibitors to date. Numerous studies have shown that AZT can reduce telomerase activity by decreasing the expression of hTERT [10, 11].

In this study, we used AZT to inhibit the expression of hTERT in panc1 cells in order to investigate the effects of AZT-mediated inhibition of telomerase activity on the proliferation, apoptosis and radiosensitivity of panc1 cells, and the growth of pancreatic tumor in vivo. This study will provide a theoretical basis for the development of a clinically effective and practical method to improve the radiosensitivity of pancreatic cancer.

Materials and methods

Cells and reagents

Panc1 cells were purchased from Fumeng Gene Biotech (Shanghai, China). DMEM culture media, fetal bovine serum (FBS) and penicillin-streptomycin double-resistance were purchased from Sigma (Gbico, St. Louis, MO, USA). Tetrazolium reagent (MTT) and AZT were purchased from Sigma (St. Louis, MO, USA). AnnexinV-FITC/PI apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA). RNA extraction kit and reverse transcription kit were purchased from Fermentas (Hanover, MD, USA). The primers were synthesized by Invitrogen Corporation (Shanghai, China). TRAP-ELISA kit was purchased from Roche (Indianapolis, IN, USA). All other reagents were purchased from Sigma unless otherwise specified.

Culture of cells and grouping

Panc1 cells were cultured in DMEM medium containing 10% FBS at 37°C in an incubator with 5% CO_2, and divided into four groups: A. control group; B. 0.4 mmol/L AZT group; C. 0.6 mmol/L AZT group; and D. 0.8 mmol/L AZT group. While the control group was cultured in standard condition, cells in the other three groups were treated by AZT for 48 h. Cells in the exponential phase were used for subsequent experiments.

MTT assay

Panc1 cells (100 µl) in all groups were inoculated into 96-well plates and cultured at 37°C in a 5% CO_2 incubator. After 24 h, 20 µl of 5 mg/ml MTT was added to each well and cells were incubated for an additional 4 h. Medium was discarded and 150 µl of DMSO was added to each well. The optical density of dissolved MTT crystals was measured by a plate reader (Bio-rad, Hercules, CA, USA) at 490 nm every 24 h for 5 days.

Detection of cell apoptosis by Annexin V-FITC/PI double staining

Panc1 cells in exponential phase in all groups were digested with trypsin, collected, washed twice with cold PBS, and resuspended in 100 µl of Annexin V binding buffer. A total of 5 µl of Annexin V-FITC solution were added and the mixture was incubated at room temperature in the dark for 15 min. Next, 5 µl of PI staining solution were added and the mixture was incubated for another 5 min. A total of 400 µl of Annexin V binding buffer was added and cell apoptosis was detected by flow cytometry within 1 h. The experiment was repeated three times and results were analyzed using Cellquest Pro software. The left lower quadrant (Q3) represents normal viable cells with low staining intensity of both Annexinv and PI, whereas the lower right quadrant (Q4) represents annexin V-FITC stained (early apoptotic) cells. Q1 and Q2 represent necrotic or late apoptotic cells with strong staining of both Annexinv and PI.

Measurement of telomerase activity

Panc1 cells in exponential phase in all groups were collected and telomerase activity was measured using a quantitative TRAP (telomeric repeat amplification protocol) ELISA kit according to the manufacture's instructions. Positive control was the extract of telomerase-positive immortalized human kidney cells (293 cells), and negative control was denatured cell extracts at 65°C in a water bath. Three parallel samples in each group were measured and the experiment was repeated three times.

Quantification of hTERT-mRNA expression

Panc1 cells in exponential phase in all groups were collected and total RNA was extracted using RNA extraction kit and reverse transcribed into cDNA using reverse transcription kit. RCR amplification was performed using the following program: initial denaturation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and final extension at 72°C for 10 min, followed by cooling to 15°C for 20
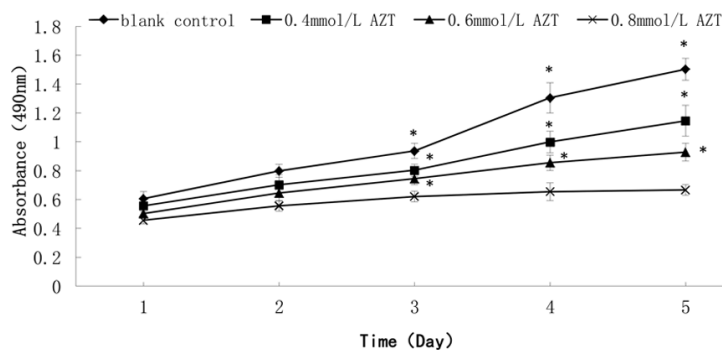
Figure 1. MTT assay of cell viability in each group. Cell viability was measured by the MTT assay every day for 5 days. Data are expressed as mean ± standard deviation from three independent experiments. *, P < 0.05 compared with blank control group.

min. The sequences of primers for hTERT cDNA: forward: 5'-CCTGCCGTCTTCACTTCC-3'; reverse: 5'-TGAACAATGGCGAATCTGG-3'. The sequences of primers for house-keeping gene GAPDH were as follows: forward: 5'-CGTATTGGGCGCCTGG-TCACCAG-3'; reverse: 5'-GTCCTTGCCCACAGC-CTTGGCAG-3'. PCR product was analyzed by electrophoresis, and gel was observed using a gel imager to compare the expression of hTERT mRNA in different groups.

Colony formation assay

Panc1 cells in exponential phase in all groups were trypsinized and plated in 6-well dishes at different densities depending on the potency of the treatments (from 50 to 10^4 cells/well). After 12 h of incubation, cells were exposed to radiation therapy (0, 2, 4, 6, 8 Gy) generated by a 23EX accelerator at a dose rate of 400 cGy/min. The plates were incubated at 37°C until visible colonies were observed. The cells were fixed with methanol and stained with Giemsa. The number of colonies, defined as >50 cells/colony were counted, and the surviving fraction was calculated as the ratio of the number of colonies in the treated sample to the number of colonies in the untreated sample. Triplicate wells were set up for each condition, and the experiment was repeated three times. The survival curves were analyzed by the linear quadratic (LQ) model, and the changes in the radiosensitivity of cells were analyzed based on the values of α, β and α/β ratio.

Construction of xenograft mouse models

A total of 20 BalB/c inbred healthy nude mice weighting 18-20 g were purchased from the

Experimental Animal Center at the Harbin Medical University. 5×10^6 panc1 cells were injected into the left abdomen of each mouse. These tumor-bearing mice were randomly divided into 4 groups: the control group, AZT group, radiation group, and the combination of AZT and radiation group. Mice in AZT group were administered AZT intragastrically twice daily at a dose of 300 mg/kg body weight/day. Mice in the combination group received radiation daily (2 Gy/day) at 2 h after administration of AZT, 5 days a week for 3 weeks. Tumor volume was measured once every three days. The mean tumor volume (mm^3) was calculated according to the formula: $(d^2 \times D)/2$, where d and D are the shortest and longest diameters of the tumor. This animal study was approved by the Animal Care Committee of the Harbin Medical University and performed under strict accordance with the guideline for Laboratory Animal Care of the university.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analyses were performed using SPSS 18.0. Difference between groups was analyzed by t tests. Differences among groups were compared by one-way analysis of variance (ANOVA). P < 0.05 is considered statistically significant.

Results

Effects on cell proliferation

As shown in **Figure 1**, cell proliferation in all AZT groups was significantly lower compared with the blank control group on day 3, 4 and 5 (P < 0.05). Cell proliferation in 0.8 mmol/L AZT group was the lowest, followed by the 0.6 mmol/L AZT group, suggesting that AZT inhibited the proliferation of panc1 cells in a dose-dependent manner.

Effects on cell apoptosis

As shown in **Figure 2**, the percentage of early apoptotic cells in 0.4, 0.6 and 0.8 mmol/L AZT groups was 4.49 ± 0.02%, 7.32 ± 0.05%, and 11.2 ± 0.08%, respectively, which was signifi-
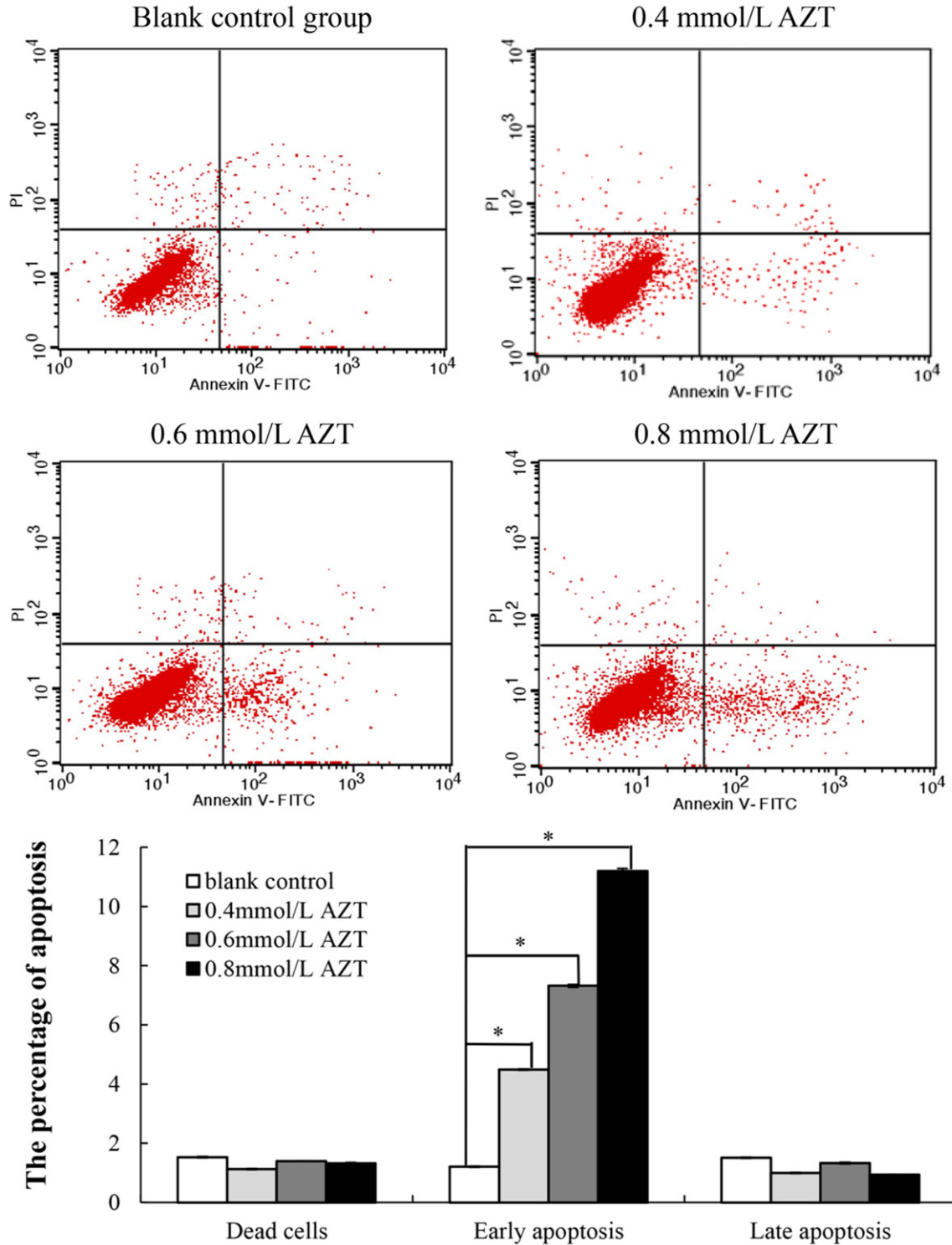
Figure 2. Analysis of apoptotic cells. Q1: dead cells; Q2: late apoptosis; Q3: normal cells; Q4: early apoptosis. Data are expressed as mean ± standard deviation from three independent experiments. *, P < 0.05 compared with blank control group.

cantly higher compared with the control group (1.21 ± 0.01%, P < 0.05), indicating that AZT dose-dependently promoted early apoptosis of panc1 cells. Nevertheless, the percentage of late apoptotic and necrotic cells in all AZT groups was similar to that in the control group (P > 0.05), suggesting that AZT did not induce late apoptosis and necrosis of panc1 cells.
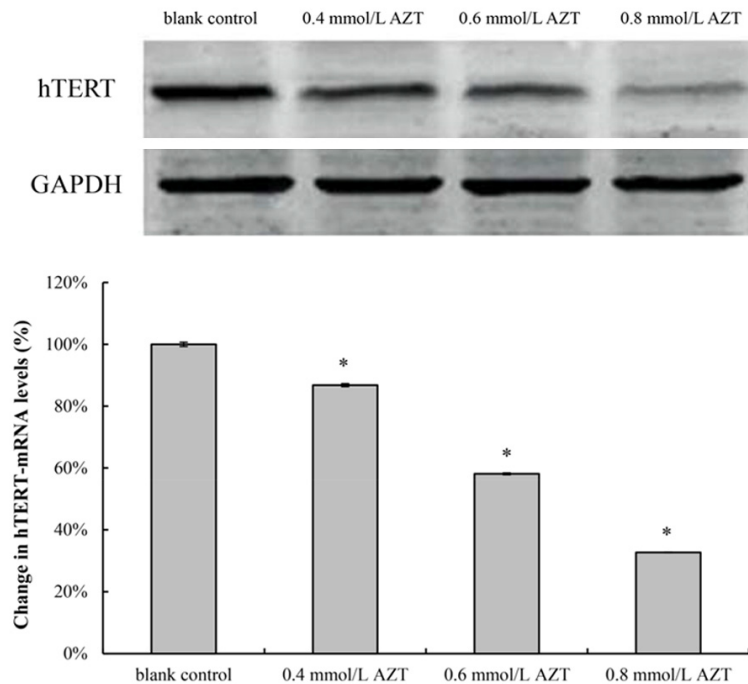
Figure 3. RT-PCR analysis of hTERT mRNA expression in each group. Data are expressed as mean ± standard deviation from three independent experiments. *, P < 0.05 compared with blank control group.
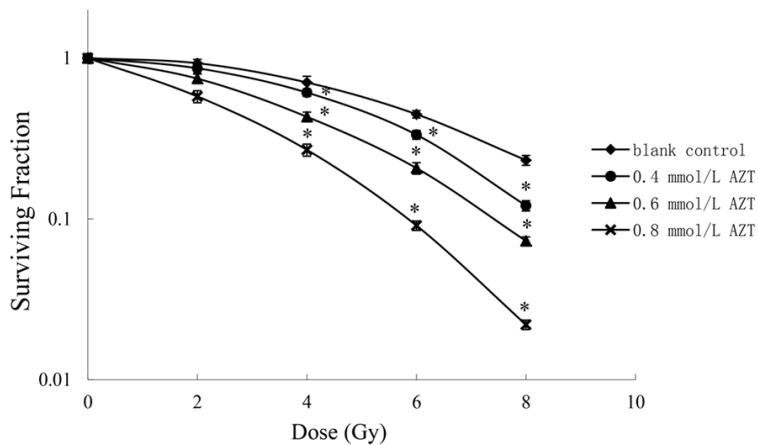

Figure 4. Radiosensitivity of panc1 cells in each group. Cells in each group were treated by radiation at various doses (0, 2, 4, 6, and 8 Gy). Data are expressed as mean ± standard deviation from three independent experiments. *, P < 0.05 compared with blank control group.

Inhibition on telomerase activity and hTERT-mRNA expression

Telomerase activity in 0.4, 0.6 and 0.8 mmol/L AZT groups was 2.365 ± 0.015, 1.847 ± 0.008, and 1.203 ± 0.005, respectively, which was significantly lower than that in the control group (2.631 ± 0.003, P < 0.05), suggesting a dose-dependent inhibitory effect of AZT on telomerase activity. As shown in **Figure 3**, the relative expression level of hTERT-mRNA in 0.4, 0.6 and 0.8 mmol/L AZT groups was 86.8%, 58.1%, and 32.7%, respectively, and was significantly reduced compared with the control group (P < 0.05). These results demonstrated that AZT dose-dependently inhibited both telomerase activity and hTERT-mRNA expression.

Effects on radiosensitivity of cells

The colony formation rate in all AZT groups was significantly lower compared with the blank control group (P < 0.05). The survival curves of cells in each group are shown in **Figure 4**. The radiosensitivity parameters in the blank control group were α = 0.13, β = 0.020, and α/β = 6.5, whereas the radiosensitivity parameters in 0.4, 0.6 and 0.8 mmol/L AZT groups were α = 0.27, 0.40 and 0.53, β = 0.025, 0.028 and 0.030, α/β = 10.80, 14.29 and 17.67, respectively, suggesting that the radiosensitivity of cells was significantly increased in a AZT dose-dependent manner (P < 0.05).

Effects on tumor growth in vivo

The tumor growth curve of each group was shown in **Figure 5**. The tumor in the control group had grown rapidly, whereas the tumor in AZT, radiation, and the combination of AZT and radiation group was reduced by 28%, 35.9% and 54.3% (P < 0.05), respectively at 21 days after the start of the treatment, which suggested the therapeutic effects of AZT, radiation, and the combination of AZT and radiation on tumor growth. The inhibitory effect in the combination group was significant higher than that in AZT
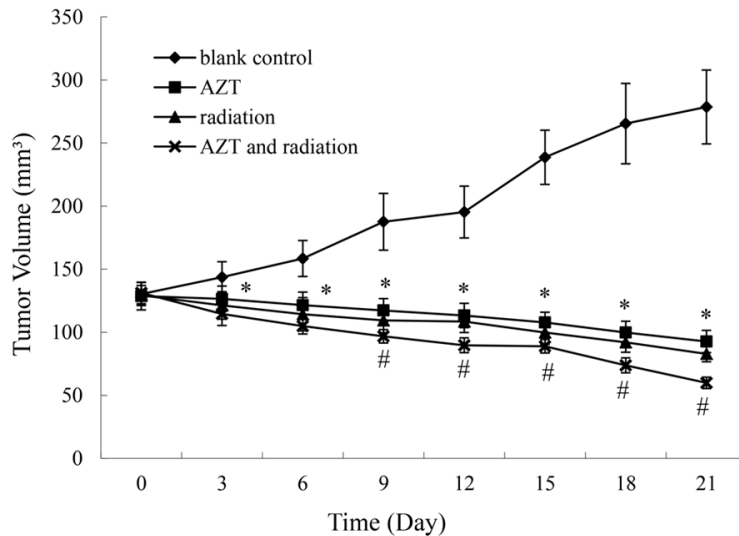
Figure 5. Tumor growth curve analysis. The tumor volume of tumor-bearing mice in each group was measured. The mean tumor volume (mm^3) was calculated using the following formula: $(d^2 \times D)/2$, where d and D are the shortest and longest diameters of the tumor. All measurements were performed in a coded and blinded fashion. Data are expressed as mean ± standard deviation from three independent experiments. *, $P < 0.05$ compared with blank control group. #, $P < 0.05$ compared with single treatment.

and telomerase activity in pancreatic cancer cells. We further demonstrated that AZT-mediated inhibition of telomerase activity suppressed pancreatic cancer cell proliferation, promoted their early apoptosis, and enhanced their radiosensitivity in a dose-dependent manner. Moreover, we revealed the synergistic effect of AZT and radiation by comparing the changes in tumor volume of pancreatic tumor-bearing mice in AZT, radiation and combination treatment groups. Our results suggested that reduced telomerase activity inhibited tumor growth both in vitro and in vivo, and increased the radiosensitivity of pancreatic cancer cells, which was consistent with previous studies on other types of cancers [20, 21].

and radiation groups ($P < 0.05$), which suggested that the telomerase inhibitor AZT significantly increased the radiosensitivity of the tumor.

Discussion

Telomeres are responsible for the maintenance of chromosomal integrity and the repair of radiation damage, and therefore are closely associated with the immortalization and apoptosis of cells [12]. Telomerase maintains the length of telomeres, and is crucial for the functions of Telomeres [13, 14]. It has been widely known that telomerase activation is a common feature of all tumor cells and telomerase dysfunction is closely related to the occurrence of tumors [15]. As the important component of telomerase, hTERT has been considered as the marker for telomerase activity [16, 17]. hTERT expression has been detected in almost all tumor cells with abnormal telomerase activity [18]. Therefore, hTERT may be an effective molecular target for reducing telomerase activity. Previous studies have shown that a commonly used telomerase inhibitor AZT suppresses telomerase activity by down-regulating the expression of hTERT [19]. Consistent with previous findings, our results confirmed the inhibitory effects of AZT on the expression of hTERT

Recently, the association between radiosensitivity, and telomerase has become a research hotspot. It is generally believed that telomerase activity was negatively correlated with radiosensitivity. When suppressed telomerase activity can not maintain the normal function of tumor cells, telomeres gradually start to lose, which promotes apoptosis of cells, and thus increased their radiosensitivity [22]. Some studies have suggested that the radiosensitization effect of reduced telomerase activity might be associated with radiosensitive sites on telomeres [23, 24]. After radiation, the sequences of these sites are broken, and short DNA sequence of telomeres and increased telomerase activity are detected, indicating that telomerase is involved in the repair of DNA damage. Therefore, with decreasing telomerase activity and hTERT expression, the repair capacity of telomerase is reduced, leading to heavier chromosomal damages. The damaged telomeres are no longer extended, and tumor growth is prohibited [25]. Nevertheless, other studies have suggested that telomerase activity is positively correlated or uncorrelated with radiosensitivity [26, 27]. According to the positive correlation theory, the DNA repair of telomerase is erroneous, leading to irreversible chromosomal breakage. The scholars of uncorrelation theory

believe that telomerase activity is irrelevant to radiosensitivity because the repaired DNA by telomerase is only a very small proportion of all chromosomal damages. Moreover, radiosensitivity is related to double-strand breaks, whereas telomerase is only responsible for the repair of single-strand break [28]. Additionally, telomeric proteins that are necessary for the repair of telomeres are not detected in numerous sites of DNA damages, indicating that changes in telomerase activity are uncorrelated with radiosensitivity.

The regulation of telomerase is a complex process. Although the specific mechanism behind the regulation has not yet been clarified, telomerase might become an important molecular target for radiation therapy. The optimal method of increasing radiosensitivity should involve minimum injuries to the body. Since telomerase is not expressed or is expressed at very low levels [4], inhibition of telomerase activity does not cause any damages to normal tissues. Therefore, downregulation of telomerase activity might be an important approach for the treatment of cancer. Furthermore, the therapeutic effect of combination therapy is markedly higher than that of a single treatment due to the high degree of malignancy of pancreatic cancer. The approach of enhancing radiosensitivity of pancreatic cancer by regulating telomerase activity is simple and feasible. We believe that with increasing recognition of pancreatic cancer radiotherapy and more researches on telomerase, more patients will benefit from effective radiotherapy. Our study has provided experimental and theoretical basis for pancreatic cancer radiotherapy.

Disclosure of conflict of interest

None.

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