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Radiation enhances suicide gene therapy in radioresistant laryngeal squamous cell carcinoma via activation of a tumor-specific promoter

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ABSTRACT

Radioresistant cells have been shown to correlate with poor outcome after radiotherapy. Here, we found that human telomerase reverse transcriptase promoter (hTERTp) had lower activity in laryngeal squamous carcinomas cells than in radioresistant variant cells. Combining radiotherapy with plasmid phTERTp-HRP, in which expression of enzyme horseradish peroxidase (HRP) controlled by hTERTp, resulted in increased apoptosis and necrosis of tumor cells after prodrug indole-3-acetic acid (IAA; converted by HRP into a cytotoxin) incubation. Volume and wet weight of xenograft tumor were reduced more in the combination groups. These data suggest that hTERTp has potential use in targeted cancer gene therapy, especially for radioresistant tumors. Combining radiotherapy with hTERTp-HRP/IAA may overcome radioresistance in laryngeal squamous carcinomas cells and amplify the killing effect in targeted cancer cells.

1. Introduction

Head and neck cancer is the sixth most prevalent type of cancer worldwide, with a global incidence of 700,000 cases per year. The larynx is the commonest head and neck region affected by cancer in the United Kingdom, with the vast majority of tumors being of squamous cell origin [1]. In the United States, laryngeal cancer is estimated to account for almost 0.85% of all new cases of malignancies, and to have caused 0.65% of all cancer deaths in 2008 [2]. Locoregional recurrences, distant metastases, second primaries, and comorbidities remain the main causes of death, with 5-year overall survival in the range of 33–57% [3]. The survival rate of patients with laryngeal cancer has not improved substantially over the past 25 years [2]. Local tumor persistence/recurrence is a major problem affecting long term survival [4]. Radiotherapy is prescribed in more than 70% of these patients, whilst surgery is applied in about 55% and chemotherapy in about 10% [4]. Surgery can cause damage to the larynx and affect the quality of life of patients with laryngeal squamous cell carcinomas (LSCC). Differing radiosensitivities of cancer cells may also be problematic, as some radioresistant cells can survive even after high-dose exposure. The optimal treatment of laryngeal cancer demands both tumor eradication and preservation of laryngeal function.

Gene-directed enzyme prodrug therapy (GDEPT) is a form of tumor-targeted chemotherapy that has entered several clinic trials. The horseradish peroxidase (HRP)/indole-3-acetic acid (IAA) system, a novel GDEPT system, has shown great efficacy in killing tumor cells. It demonstrated a strong bystander effect and significant synergism with radiation, and was more cytotoxic to tumors than the well-known HSVtk/GCV system [5,6]. It involves the delivery of a specific enzyme (HRP) that converts non-cytotoxic

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Abbreviations: GDEPT, gene-directed enzyme prodrug therapy; hTERT, human telomerase reverse transcriptase; hTERTp, human telomerase reverse transcriptase promoter; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; LSCC, laryngeal squamous cell carcinomas.

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IAA into cytotoxic metabolites. When the HRP gene is targeted to tumor cells, only the tumor cells and the surrounding cells are affected by the cytotoxic metabolites, generating a bystander effect [7]. Importantly, HRP is normally absent in mammalian cells and IAA is a poor substrate for mammalian peroxidases, so avoiding the possibility of systemic toxicity [8,9]. Tumor specific expression of the HRP gene is crucial in order to minimize the side effects of treatment. Tumor-targeted suicide gene therapy is an attractive approach for human LSCC therapy, since local gene delivery is feasible.

The human telomerase reverse transcriptase promoter (hTERTp), which is highly activated only in cells with active telomerase, has been widely used in gene therapy for targeting cancer cells [10,11]. It is generally accepted that telomerase activation is important in laryngeal carcinogenesis, as increased levels of hTERT mRNA have been observed in LSCCs [12–14]. These findings suggest that up-regulated hTERT mRNA expression and high telomerase activity represent a major difference between LSCC and normal laryngeal cells. LSCC targeted gene therapy using the HRP/IAA system can make use of this distinction. However, the hTERTp may not be strong enough to achieve therapeutic levels of transgene expression [15,16].

Our previous work demonstrated that the activity of hTERTp could be improved by its combination with radiation [17], suggesting that the combination of radiation and hTERTp-HRP/IAA gene therapy could amplify the killing effect in irradiated telomerase positive cells. In the present study, we investigated the ability of the combination of radiotherapy and hTERTp-HRP/IAA gene therapy to inhibit tumor development in LSCC with different radiosensitivities, both in vitro and in vivo.

2. Materials and methods

2.1. Cell culture

The human HEp-2 LSCC cell line (ATCC CCL-23) was obtained from China Center for Type Culture Collection. The radioresistant human LSCC cell line, HEp-2R, was isolated after repeated radiation exposure [18] and we did find several differences between HEp-2R and HEp-2 cells, including radiosensitivity, morphology, chromosomes, cell growth kinetics, cell distribution, gene expression and significance analysis of microarrays [19].

2.2. Plasmid DNA and cell transfection

The core hTERTp (−385/+40 bp) was kindly provided by Dr. I. Horikawa (NIH/NCI, USA) [20] and cloned into the pGL3-Basic vector (Promega, USA) to generate phTERTp-Luc. For phTERTp, in which the hTERTp does not control the expression of any gene, the hTERTp replaced the CMV promoter in pcDNA3.1(−) (Invitrogen, USA). The plasmid pRK5-HRP was a kind gift of Dr. D.F. Cutler (UCL, UK) [21]. For phTERTp-HRP, the HRP cDNA was cloned into the phTERTp plasmid, driven by the hTERTp. Cells were transfected using the Metafectene method (Biontex, Germany), according to the manufacturer's instructions.

2.3. Reporter gene assay

For transfection, cells were seeded in a 6-well plate at a concentration of 4×10^5 cells/well and allowed to grow overnight to reach 80–90% confluency. phTERTp-Luc or pGL3-Basic plasmid (a negative control) (0.5 μg) were cotransfected into cells with 0.5 μg of pRL-TK (cotransfected standard plasmid, Promega, USA). The mixture was removed after transfection for 5 h and cells were incubated under standard conditions. After transfection for 24 h, cells were lysed at different times post-radiation, and luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, USA), as indicated by the manufacturer. Renilla luciferase (RL) activity was used to control for transfection efficiency, and the relative luciferase activity was then calculated as the difference between the experimental luciferase value and the control level, using pGL3-Basic plasmid.

2.4. RNA isolation and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using TRI Reagent (MRC, USA) and first-strand cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (MBI, Lithuania), according to the manufacturer's instructions. The hTERT cDNA was amplified for 35 cycles (30 s at 94 °C, 40 s at 64 °C, 1 min at 72 °C) using the primers 5′ CTGC CGTCTTCACTTCCCCAC 3′ (forward) and 5′ TTACTCCCACAG CACCTCCCC 3′ (reverse), designed according to complete hTERT mRNA (NM_198253). The HRP cDNA was amplified for 30 cycles (1 min at 94 °C, 1 min at 65 °C, 1 min at 72 °C) using the primers 5′ CCTTGTGGCTCTGTCCGGAGGA 3′ (forward) and 5′ AGTGGGATGGTGTCAGTGGCGT 3′ (reverse), The amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA was used as an internal control. The PCR products were resolved on a 2.5% agarose gel, stained with ethidium bromide (Sigma, USA). Gel images were obtained using the Genesnap gel imaging system and the densities of PCR products were quantified using Genetools gel analysis software (Syngene Bio Imaging, UK). All experiments were repeated at least three times.

2.5. Analysis of telomerase activity

Telomerase activity was determined using the TeloTAGGG Telomerase PCR ELISA Kit (Roche, Switzerland) as recommended by the manufacturer. All solutions were included in the kit. Sample absorbance was measured with Model 550 Microplate Reader (Bio-Rad, USA) at the wavelength of 450/690 nm within 30 min after addition of the stop reagent.

2.6. HRP activity assay

HRP activity was analyzed using a modified 3,3,5,5-tetramethylbenzidine dihydrochloride (Sigma, USA) assay, as described previously [6]. The total protein content in the samples was determined using a commercial BCA protein assay kit (Beyotime, China). HRP activity was expressed as units of enzyme per microgram of total cellular protein.

A unit is defined as the amount of enzyme that produces an increase of 1 unit of absorbance at 652 nm (A652 nm) per minute, using an enzyme kinetics application (Beckman, DU530, USA).

2.7. Clonogenic assays

In order to assess GDEPT, after transfection for 24 h with phTERTp-HRP, cells were exposed to 6 Gy radiation and plated at low density in 25 cm^2 flasks and left to settle overnight. Control cells were not exposed to radiation. Cells were then exposed to culture medium with or without 0.5 mM IAA (Sigma, USA) resolved in phenol red-free Hanks balanced salt solution for 24 h, under standard conditions. The cells were then rinsed in phosphate-buffered saline and irradiated in flasks with various doses of ^{60}Co γ-rays at room temperature (61.2 cGy/min, SSD = 80 cm, 20×20 cm^2), before being cultured under standard conditions for 14 days. After fixation and staining with 1% crystal violet (Sigma, USA) w/v in dehydrated alcohol, colonies of >50 cells were scored and surviving fractions were normalized for plating efficiency by comparison with cells exposed to mock radiation and RPMI 1640 (Gibco, USA) culture medium supplemented with 10% fetal calf serum and HBSS, without IAA.

2.8. Tumor xenograft experiments

Female BALB/C nude mice aged 4–6 weeks were purchased from Hubei Research Center of Laboratory Animals (Wuhan, China) and maintained in specific pathogen-free facilities at the Animal Experiment Center of Wuhan University. The facilities and the protocol for this experiment were consistent with the regulations on animal use for biomedical experiments issued by the Ministry of Science and Technology of China, and approved by the Animal Care Committee of Wuhan University. The animals were injected subcutaneously on their right backs with 0.2 ml of HEp-2 or HEp-2R cell suspension (1×10^7 cells) in complete medium. Tumor growth was monitored every other day by measuring the tumor diameter in two dimensions with calipers. Tumor volumes were calculated as [L (long diameter) $\times$ S^2 (short diameter)]/2. After about 2 weeks, when the volume of the tumor had reached about 30–50 mm^3, the mice were randomly divided into four treatment groups, with seven mice in each group. Mice in the radiation groups were treated with 2 Gy of local radiation from Monday to Friday, for 3 weeks, based on clinical data and our pilot study (total dose = 30 Gy). Mice in the phTERTp-HRP/IAA groups were injected every Monday with phTERTp-HRP (10 μg of plasmid plus 20 μl of Metafectene) and IAA (2 mmol/l IAA 100 μl intraperitoneal injection). In the combination groups, phTERTp-HRP and IAA were administrated every Monday 1 h pre-irradiation. At the same time, mice in the control group were injected with PBS (mock) and exposed to 0 Gy γ-radiation (mock). Tumor volumes were then measured every other day. On the 21st day of treatment, mice in all groups were sacrificed and the tumors were dissected, measured, and weighed. The relative tumor volume ratio was calculated as (mean tumor volume on day 21/mean tumor volume on day 1).

2.9. Apoptosis assays

Apoptosis in LSCC xenografts was identified using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay kit (ZSBiO, Beijing, China), according to the manufacturer's instructions. Brown color indicated apoptotic nuclei, as visualized using diaminobenzidine substrate. The apoptosis index (AI) was calculated as (number of TUNEL positive cells/total number of cells) $\times$ 100. The apoptotic cells were scored in tumor sections for each LSCC xenograft, under a light microscope ($\times$400 magnification) in randomly chosen fields, and the apoptosis index was calculated as a percentage of at least 500 scored cells.

2.10. Hematoxylin–eosin staining

Samples of subcutaneous tumors were carefully dissected to evaluate the mechanism causing the synergism between HRP/IAA gene therapy and irradiation. The xenografted tumor tissues were fixed with 10% neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathologic examination under a light microscope at $\times$50 magnification.

2.11. Statistical analysis

Significance tests were carried out on the data using independent-samples *t* tests. Differences among the treatment groups were analyzed by one-way ANOVA. Differences between the survival fractions in treatment groups were analyzed by chi-squared tests. All data were analyzed using SPSS11.0 software (SPSS, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. hTERT promoter activity, hTERT mRNA expression, and telomerase activity in HEp-2 and HEp-2R cells without irradiation

A dual-luciferase reporter assay was performed to show the different transcriptional activities of the exogenous hTERTp. After cotransfection for 24 h, the exogenous hTERTp in HEp-2R cells showed a 1.8-fold higher transcriptional activity than that in HEp-2 cells (Fig. 1A, relative luciferase activities, 86.2 ± 12.6 from HEp-2R cells versus 47.7 ± 10.6 from HEp-2 cells; $P < 0.01$). An approximately 1.37-fold increase in telomerase activ-

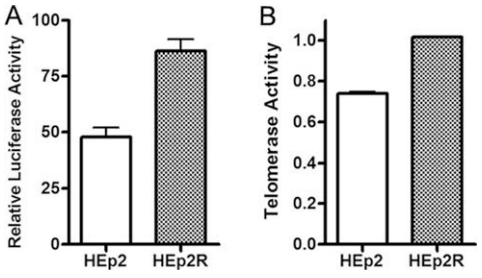

Fig. 1. (A) After contransfection for 24 h, the exogenous hTERT promoter in HEp-2R cells, evaluated by dual-luciferase reporter assay, showed a higher activity than in HEp-2 cells. (B) Endogenous telomerase activity was significantly increased in HEp-2R cells compared with parent HEp-2 cells.

ity was observed in radioresistant HEp-2R cells compared with parent HEp-2 cells (Fig. 1B, telomerase activity, 1.017 ± 0.008 from HEp-2R cells versus 0.740 ± 0.018 from HEp-2 cells; $P < 0.01$), which had a 1.76-fold up-regulation of endogenous hTERT mRNA (lanes 2 and 5, Fig. 2A and B). These results indicated a difference in hTERT promoter activities between radiosensitive and radioresistant LSCC cells.

3.2. Cytotoxicity of hTERT promoter-regulated HRP/IAA GDEPT in vitro

Clonogenic assay is an in vitro cell survival assay to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents [22]. To quantitate any change in cytotoxicity of phTERTp-HRP/IAA, clonogenic assays were done. The surviving fraction was calculated as follows: colony number/(number of cells seeded × plating efficiency). Plating efficiency is equivalent to the colony number divided by the number of mock-transfected cells seeded in the drug-free medium with mock radiation trigger. As shown in Fig. 3, the surviving cell fractions were normalized for plating efficiencies. To evaluate the radiation induction, phTERTp-HRP was transiently transfected into HEp-2 and HEp-2R cells. HRP groups were compared with HRP + IAA groups (in order to eliminate the effect of transfection). Transfected HEp-2 cells were not killed by incubation with 0.5 mM IAA for 24 h ($P > 0.05$), while 21.8% of transfected HEp-2R cells were killed (Fig. 3), due to the different activities of the hTERT promoter and expression of HRP. An IAA concentration of 0.5 mM was chosen because no toxicity was detected in mock-transfected HEp-2 cells (i.e. transfection reagent alone) exposed for 48 h at 0.5 mM IAA.

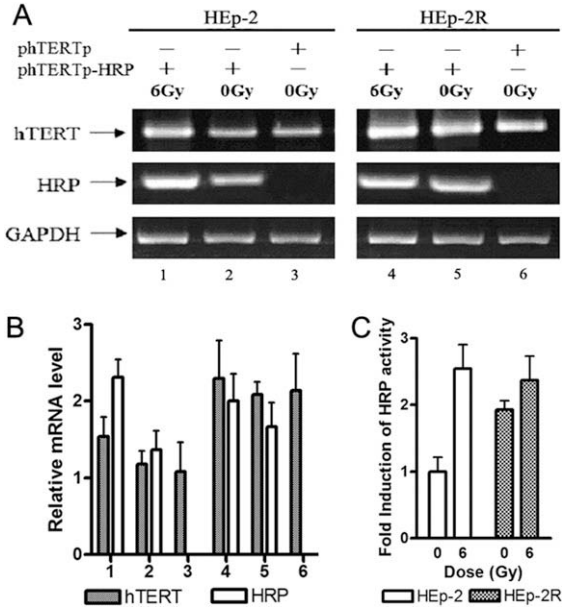

Fig. 2. Increased activity of hTERT promoter induced by radiation in HEp-2 and HEp-2R cells. Cells were transfected with plasmids phTERTp-HRP or phTERTp (negative control). (A) Representative hTERT and HRP mRNA expression were analyzed by semiquantitative RT-PCR. Results for GAPDH mRNA are also shown. HEp-2 cell groups: lane 1, phTERTp-HRP and 6-Gy trigger; lane 2, phTERTp-HRP (no radiation); lane 3, phTERTp (no radiation). HEp-2R cell groups (lanes 4–6) follow the same sequence as HEp-2 cell groups. The control cells (phTERTp) have no banding characteristic of HRP expression. (B) The density of each band was measured. The densities of hTERT and HRP bands were compared with those of GAPDH controls and the results were expressed as density ratios. (C) Peroxidase activity assay. After radiation treatment for 24 h, HRP activities per cellular protein content (A652/μg total cellular protein) were determined. The levels of endogenous peroxidase activity in the untransfected cells (control) were subtracted from the HRP activity values in transfected cells, and the HRP activity was expressed as fold induction relative to that in HEp-2 cells with 0 Gy radiation.

3.3. Radiation-enhanced hTERTp activity and HRP expression

Promoter activation was performed after radiation treatment, and the effectiveness of HRP mRNA expression induction was compared in HEp-2 and HEp-2R cells, using RT-PCR. As demonstrated in Fig. 2A and B, the expression of hTERT mRNA, controlled by the endogenous hTERT promoter was increased (1.3-fold in HEp-2 and 1.1-fold in HEp-2R cells compared with un-irradiated controls) when cells were exposed to a radiation dose of 6 Gy. Accordingly, a 1.69-fold enhancement of HRP mRNA expression controlled by the exogenous hTERT promoter occurred in irradiated HEp-2 cells, and a 1.20-fold enhancement occurred in irradiated HEp-2R cells, compared to un-irradiated cells (Fig. 2A and B). As confirmed by a competent peroxidase activity assay, a 6-Gy trigger induced an increase of 2.54-fold compared with 0 Gy in HEp-2 cells, and an increase of 1.23-fold in HEp-2R cells (Fig. 2C). No HRP mRNA was expressed in cells transfected with phTERTp (lanes 3 and 6, Fig. 2A and B). Very little peroxidase activity was detected in untransfected HEp-2 and HEp-2R cells (negative controls) due to the lack of endogenous HRP in human cells [23]. It was unclear why the hTERT promoter activity and HRP mRNA expression and peroxidase activity after irradiation in HEp-2R cells was not as strong as in HEp-2 cells.

3.4. Enhancement of cytotoxicity of phTERTp-HRP/IAA GDEPT by radiation in vitro

To determine if the combination of radiation with hTERTp-HRP/IAA gene transfer was suitable for cancer gene therapy, HEp-2 and HEp-2R cells were transiently transfected with phTERTp-HRP/IAA plasmid and exposed to 6 Gy–radiation, before incubation with IAA. As shown in Fig. 3, the surviving fraction at 6 Gy for the HEp-2R cells was significantly higher than that of the HEp-2 cells (0.319 ± 0.048 vs. 0.201 ± 0.061, $P < 0.01$). In the HRP-radiation-IAA groups, surviving cell fractions were compared with those of groups exposed to radiation or IAA alone. A significant increase in tumor cell killing was observed when radiation was applied before IAA exposure (survival rate of 1.5% for HEp-2 and 3.4% for HEp-2R cells, all $P < 0.01$, Fig. 3), which indicated that stimulation by radiation could dramatically induce hTERTp activity.

3.5. In vivo inhibition of tumor growth

In order to demonstrate an enhanced effect of radiation together with phTERTp-HRP/IAA, which we had successfully proved in vitro, we developed human xenograft models with subcutaneous HEp-2 or HEp-2R tumors as described in Section 2. When the xenografts were grown to about 30–50 mm^3, the mice were randomly divided into four treatment groups: with phTERTp-HRP/IAA treatment alone, with radiation treatment alone, with combination treatment or control group. Mice were treated with phT-

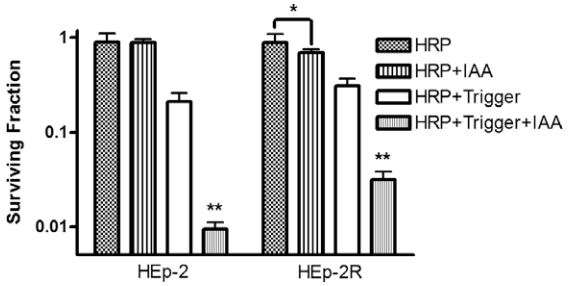

Fig. 3. Clonogenic assays of cancer cells. Cells transfected with phTERTp-HRP plasmid were divided into four groups: (HRP) with mock irradiation and culture medium incubation for 24 h; (HRP + IAA) with mock irradiation then incubation in culture medium with 0.5 mM IAA for 24 h; (HRP + Trigger) with 6 Gy irradiation and culture medium incubation for 24 h; (HRP + Trigger + IAA) with 6 Gy irradiation before 0.5 mM IAA incubation for 24 h. Their surviving fractions were determined. Cell survival of HEp-2R was found to be more significantly reduced than that of HEp-2 in HRP + IAA groups. Combination of 6 Gy irradiation trigger and IAA incubation led to the lowest surviving cell fraction. The means of at least three experiments (triplicate samples) ± SD are shown. Statistically significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$).

ERTp-HRP (10 μg of plasmid plus 20 μl of Metafectene intratumorally injection) and IAA (2 mmol/l IAA 100 μl intraperitoneal injection) on every Monday for 3 weeks, with 2 Gy irradiation from Monday to Friday for 3 weeks, with both or with PBS for the control group. No spontaneous tumor regression was observed in the untreated tumors during the course of the experiment. Tumor growth in the combination treatment groups was inhibited more dramatically in the HEp-2 groups than in any other group. A modest but significant inhibition of tumor growth was demonstrated in the irradiation group and in the phTERTp-HRP/IAA group, compared with the control group, and tumor volumes were reduced by 54.6%, 33.6%, and 14.0%, respectively. In the combination group, irradiation group, and phTERTp-HRP/IAA group, the wet weights of tumors were decreased by 54.2%, 24.9%, and 7.0%, respectively. Similar experiments were performed in HEp-2R tumors. Tumor volumes in animals receiving the combination therapy of phTERTp-HRP/IAA and radiation were reduced more than in controls or in animals treated with single treatment modalities. Compared with the control group, tumor volumes were reduced by 49.2%, 19.2%, and 14.9% in the combination group, phTERTp-HRP/IAA group, and irradiation group, respectively. Wet weights of tumors were decreased by 49.5%, 14.7%, and 3.8%, respectively. The differences in tumor inhibition were significant between the combination group and the single phTERTp-HRP/IAA or irradiation groups (all $P < 0.01$; Fig. 4). These results indicate that the phTERTp-HRP/IAA approach, combined with irradiation, induced synergistic cytotoxicity in solid tumors.

3.6. Histopathologic examination

Hematoxylin–eosin staining of tumors collected on the 21st day of treatment demonstrated the synergistic effect of the combination of phTERTp-HRP/IAA and radiation. In the combination treatment group, large areas of tumor tissue showed massive necrosis (Fig. 5). In contrast, only minor tumor cell damage was observed in the groups subjected to simple radiation or phTERTp-HRP/IAA therapy (Fig. 5). Tumors from mice subjected to mock treatments had few necrotic cell (Fig. 5).

3.7. Combination of phTERTp-HRP/IAA and radiation enhanced apoptosis induction in vivo

Although necrosis, DNA fragmentation, chromatin condensation and apoptosis are all involved in the cytotoxic effect of HRP/IAA GDEPT [5,24], apoptosis is the most important mechanism of cytotoxicity [25]. In order to assess apoptosis induction in vivo in all eight groups, we used the TUNEL assay on tumor sections taken on the 21st day (Fig. 6A). Apoptotic cells with brown nuclei were counted under a light microscope in randomly chosen fields, and the apoptosis index was calculated as a percentage of at least 500 scored cells. As shown in Fig. 6B, a combination of phTERTp-HRP/IAA and radiation resulted in a significantly higher apoptotic index in HEp-2 tumors (18.5%) compared with phTERTp-HRP/IAA alone (6.8%), radiation alone (8.1%), and controls (1.0%) (all $P < 0.01$). The combination of phTERTp-HRP/IAA and radiation also resulted in a significantly higher apoptotic index (17.2%) in HEp-2R tumors, compared with phTERTp-HRP/IAA alone (9.3%), radiation alone (5.2%), and controls (0.8%) (all $P < 0.01$).

4. Discussion

The radiosensitivity of a tumor determinates its responsiveness to radiotherapy. In this report, we observed that hTERTp could be used in LSCC gene therapy, because telomerase activity varies not only between normal laryngeal cells and squamous carcinoma cells [13], but also between radiosensitive and radioresistant LSCC. Our results suggest that efficient tumor-specific cytotoxicity can be achieved in both radiosensitive and radioresistant human LSCC by combining phTERTp-HRP/IAA with radiation.

Radiotherapy is one of the major clinical options for malignant tumors. However, tumor cells are heterogeneous in terms of their radiation responsiveness, which limits the application of radiotherapy in many insensitive tumors. "Radiosensitivity" therefore plays a critical role in radiobiology and is regarded as the fifth "R" in fractionation radiotherapy [26]. Induced radioresistance can be found in tumor cells surviving after radiotherapy and the therapeutic efficacy of these methods is limited when cancer cells develop resistance to radiation [27]. There are several hypotheses that attempt to explain the phenotypic heterogeneity of tumor cells with regard to radiosensitivity

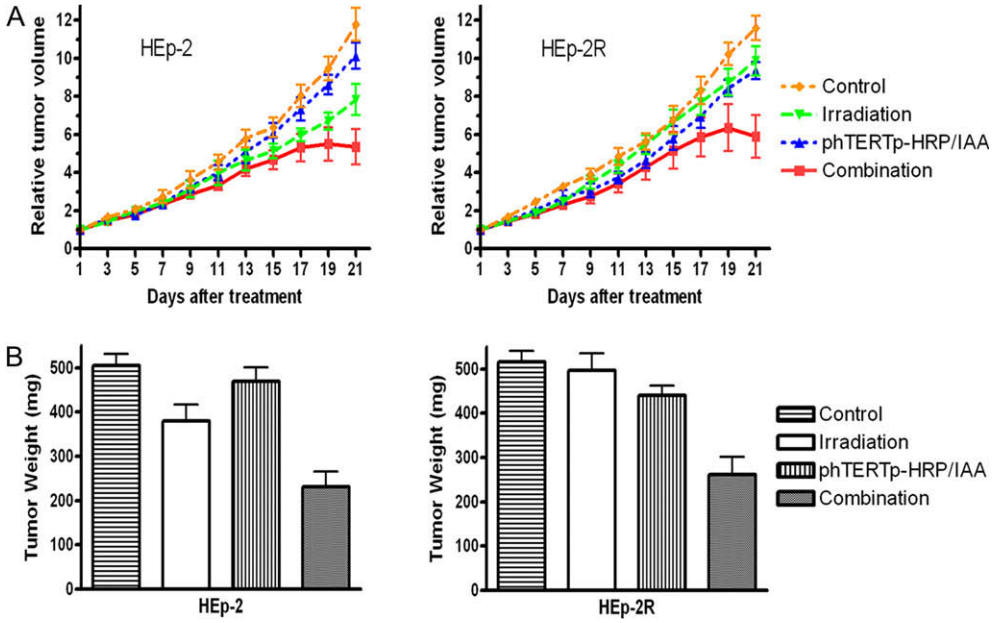

Fig. 4. Combination of phTERTp-HRP/IAA and radiotherapy enhanced the inhibition of both HEp-2 and HEp-2R tumor xenografts. Tumor volumes (A) and weights (B) in mice randomly divided into control, phTERTp-HRP/IAA, irradiation, and combination groups, respectively, as described in Section 2, are shown.

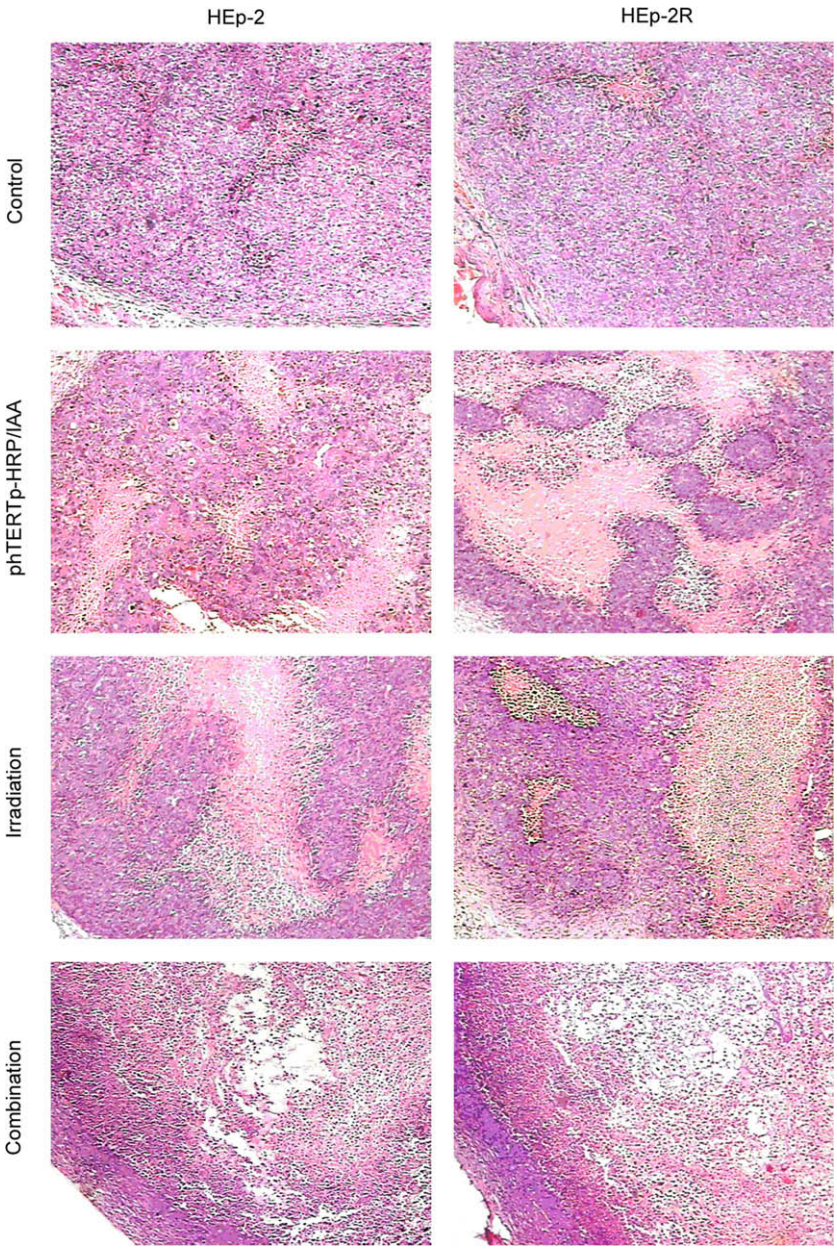

Fig. 5. Histopathologic examination of HEp-2 and HEp-2R xenografts stained with hematoxylin–eosin. Combination of phTERTp-HRP/IAA and radiotherapy enhanced necrosis. Photomicrographs were taken at a magnification of 50×.

[27,28]. However, efficient approaches aimed at overcoming radioresistance are still being explored. In terms of toxicity, radiotherapy commonly causes acute adverse effects and chronic conditions and these complications are the major dose-limiting toxicities associated with radiotherapy. Unfortunately, a relatively low therapeutic index, a lack of specificity, and the emergence of radiation resistant cell subpopulations often hamper the efficacy of LSCC therapy, and there is therefore a need for the development of new alternative therapeutic strategies. The combination of GDEPT (phTERTp-HRP/IAA) and radiotherapy may serve as a novel approach for increasing the therapeutic efficacy of radiotherapy in radiation resistant LSCC.

Target specificity, low in vivo transduction efficacy, and limited cytotoxic effects have attracted increased attention as factors limiting GDEPT efficacy [29]. Thus, tumor-targeted expression of a high level of HRP shows promise as a means of achieving tumor cell killing. Several strategies could be used in order to enhance the efficacy of HRP/IAA molecular chemotherapy. Greco et al. [5] developed gene therapy vectors responsive to both hypoxia and ionizing radiation (IR) which regulate HRP expression in gene therapy strategies aimed at addressing the problem of hypoxia in radiotherapy.

The hTERTp has the ability to specifically target cancer cells [15]. Previous studies have shown that cells with high

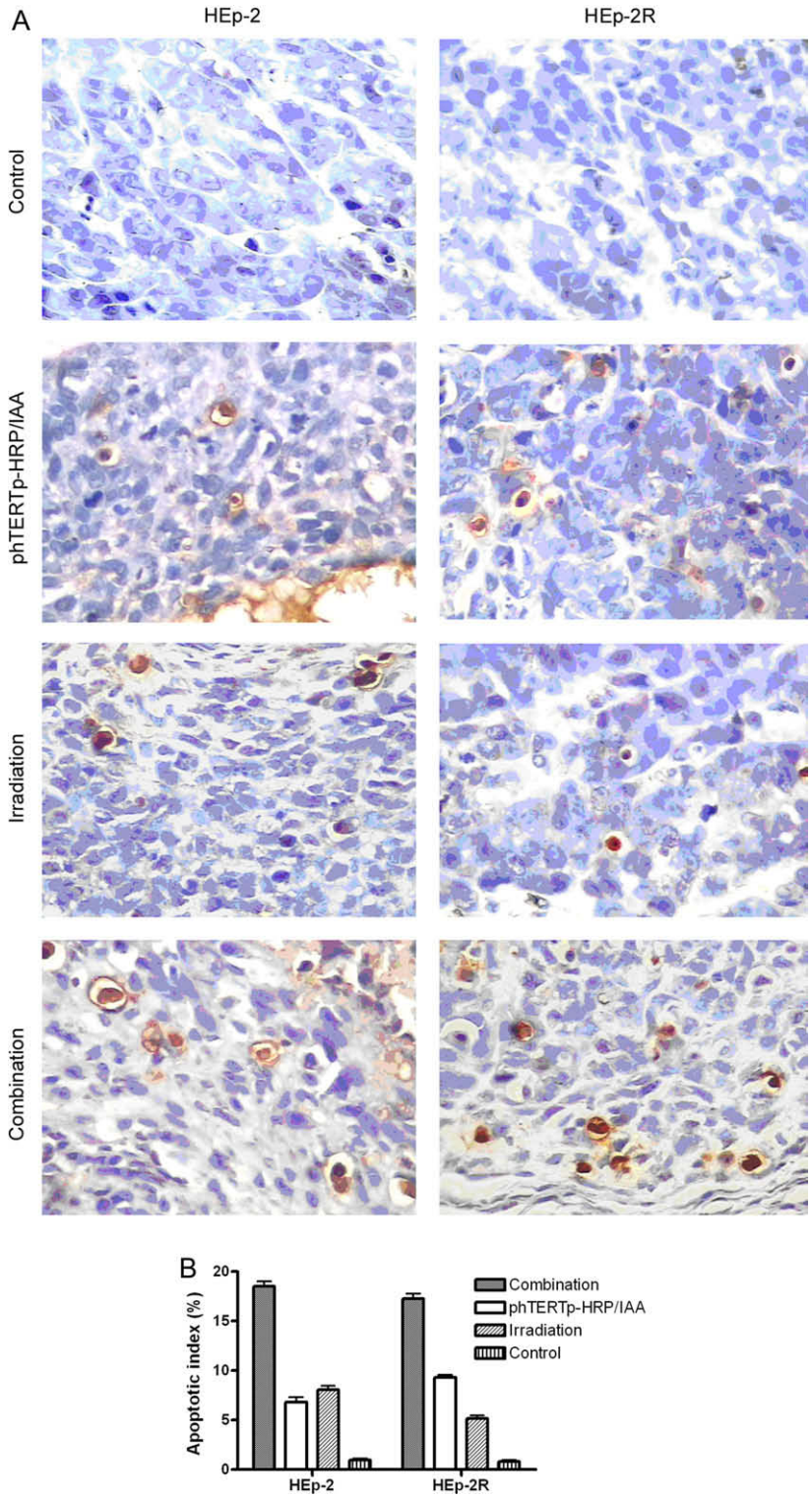

Fig. 6. Combination of phTERTp-HRP/IAA and radiotherapy enhances apoptosis in HEp-2 and HEp-2R xenografts. (A) Representative fields of positive cells by TUNEL assays (as described in Section 2). Photomicrographs were taken at a magnification of 400×. (B) The mean apoptosis index for tumor cells in various treatment groups.

levels of telomerase activity are resistant to radiation and that telomerase activity is up-regulated after failed radiotherapy in LSCC [30–32]. In this study, our results showed that the hTERTp activity and telomerase activity were

higher in HEp-2R than in HEp-2 cells, which supports the correlation between basal levels of telomerase activity and response to radiotherapy in human LSCC cells. This feature suggested that hTERTp could be of use in targeted gene therapy in LSCC, especially in radioresistant cells. Furthermore, although the specific delivery of the DNA vector to cancer cells is one of the technical obstacles to gene therapy, it may not be a problem for LSCC, since local injection is feasible and well tolerated. A higher level of HRP expression regulated by hTERTp was found in HEp-2R cells compared with that in HEp-2 cells, which coincided with the increased cytotoxicity in HEp-2R cells after prodrug IAA incubation. However, the hTERTp may not be strong enough to achieve therapeutic levels of transgene expression [15,16]. Employing an hTERTp with tumor-selective activity should increase the therapeutic efficacy of the HRP/IAA system used in LSCC therapy.

Radiation not only kills tumor cells by damaging DNA strands, but also up-regulates telomerase activity at low doses in many tumor cells from different tissues. Although the underlying mechanism is not clear, many researchers believe that the post-radiation activation of telomerase may be due to the involvement of telomerase in DNA repair and chromosome healing [33,34]. We previously demonstrated that telomerase participated in the process of DNA repair, and the up-regulation of telomerase activity may be a reaction to DNA damage induced by irradiation [35]. Thus, hTERTp may be indirectly enhanced in response to radiation.

Radiation was used in this protocol to increase promoter strength and thus expression of HRP under the control of the radiation-enhanced tumor specific hTERTp was also able to kill radiosensitive tumor cells. Higher levels of HRP expression were observed in HEp-2 and in HEp-2R cells after irradiation: The radiation trigger rendered the HEp-2R and HEp-2 cells more sensitive to IAA-induced killing. In vivo, a radiation dose of 2 Gy was used in our in vivo experimental studies, based on the clinically applicable dose. Tumor growth inhibition was minimal in the radiotherapy only group (30 Gy), suggesting that both the LSCC cell lines HEp-2 and HEp-2R were resistant to this dose of radiotherapy. phTERTp-HRP/IAA alone moderately increased the tumor inhibition effect. However, the combination of radiotherapy and phTERTp-HRP achieved a dramatic improvement, suggesting that hTERT promoter-mediated HRP/IAA GDEPT together with radiotherapy could achieve better therapeutic results in human LSCC cells with different radiosensitivities, since the presence of radioresistant cells in solid tumors has been shown to correlate with poor outcome after radiotherapy.

Clinically, liposomes could provide a safe and effective way to transfect plasmids into tumor cells [36,37]. Moreover, it is not necessary to transfect all the tumor cells with the HRP gene, because even if only a few cells expressed HRP, it would be enough to allow the cytotoxic metabolite to act via the bystander effect [5,6]. However, the low efficiency of transfection should be improved. Several studies have reported that radiation enhances transfection efficiency [38,39], and radiation might enhance liposome-mediated gene transfection in this study too. Thus, radiation not only up-regulated hTERT promoter activity and HRP expression, but also improved liposome-mediated gene transfection.

In this study, both in vitro and in vivo experiments demonstrated that HRP expression increased following radiation exposure of human LSCC cells. We combined the HRP/IAA GDEPT system and radiation therapy. The hTERTp provided tumor specificity and enhanced the expression of HRP in response to radiation. In addition, radiation induced cell killing by causing DNA damage, enhanced hTERTp activity, and improved the transfection efficiency of liposome-mediated plasmid transfer. Furthermore, the HRP/IAA system avoids systemic toxicity and acts synergistically with radiation to kill tumor cells. In vitro and in vivo cytotoxicity assays demonstrated that the combination of phTERTp-HRP/IAA and radiotherapy exerted more than additive effects on LSCC cells with different radiosensitivities. However, tumor volumes still increased, even after combination treatment. Higher gene expression efficiencies might be achieved using more plasmids or virus vectors and cytotoxic agents may be added in the treatment protocol, so enhancing the therapeutic efficacy. Further studies are needed.

In conclusion, precise radiotherapy combined with hTERTp-HRP/IAA transfection may provide a selective and effective therapeutic strategy for both radiosensitive and radioresistant human LSCC. This approach might have great potential for the clinical treatment of many solid tumors.

Conflict of interest

There is no relevant conflict of interest.

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