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Antitumor and radiosensitization effect of $^{12}C^{6+}$ heavy-ion irradiation mediated by radiation-inducible gene therapy

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Abstract Radio genetic therapy which combines gene therapy with radiotherapy has shown promising results in cancer treatment. In this study, an oncolytic adenovirus-based gene therapy system regulated by radiation was constructed to improve the cancer curative effect. This gene therapy system incorporated the radiation-inducible early growth response gene (Egr-1) promoter and the anticancer gene tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). To confirm the antitumor effect of Ad-ET combined with $^{12}C^{6+}$ ion irradiation, the survival and apoptosis fraction of tumor cells HT1080 and normal cells MRC-5 in combination treatment were detected by CCK-8 assay and FACS analysis. Then the expression levels of TRAIL gene and protein were tested by real-time PCR and western blotting. The results show that $^{12}C^{6+}$ ion irradiation could induce cell growth inhibition and apoptosis by activating the TRAIL gene expression in tumor cells, while exhibiting no obvious toxicity to the normal lung cell line MRC-5. The results also demonstrate that use of an oncolytic adenovirus-based radiation-inducible gene therapy system together with $^{12}C^{6+}$ ion irradiation could cause synergistic antitumor effect specifically in tumor cells but not in normal cells. The results indicate that the novel radio genetic therapy could potentiate radiation treatment by improving the safety and efficiency of monotherapy, and provide theoretical support for clinical application of combination treatment.

Keywords Radio genetic therapy · ^{12}C ion irradiation · Apoptosis · Egr-1 prompter · Tumor necrosis factor-related apoptosis-inducing ligand

1 Introduction

Adenovirus-based gene therapy has been proven to be a promising candidate for cancer treatment, with excellent safety and efficacy, as demonstrated by a wealth of clinical data. In clinical research, many strategies were applied in gene therapy [1–3]. Oncolytic adenovirus H101, as a gene therapy vector, is modified on the basis of ONYX-015. H101 can specifically infect and lyse P53-mutated tumor cells with deletion of E1B55K and part of E3 region [4]. It has been demonstrated that many tumor cell lines are deficient or mutant of P53 gene. Thus, H101 can be widely used in targeted gene therapy.

The suicide gene transferred by adenovirus vector is the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) gene, which is a member of the TNF family of type 2 transmembrane proteins. TRAIL can specifically induce apoptosis in a variety of tumor cell types by binding two pro-apoptotic receptors: TRAIL-R1 (DR4) and TRAIL-R2 (DR5) [5], and was reported to

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induce apoptosis resulting in receptor cross-linking and caspase activation [6]. However, TRAIL is almost non-toxic for most normal tissues due to existence of two decoy receptors: TRAIL-R1 (DcR1) and TRAIL-R2 (DcR2) [7]. Studies show that TRAIL is a potential method for cancer treatment when combined with conventional therapy module, such as radiotherapy and chemotherapy, because of synergistic antitumor effects, including apoptosis and tumor growth inhibition [8–10].

Compared with X-rays and γ -rays, heavy ions are of high-LET (linear energy transfer) value. Also, ion beams exhibit Bragg peak effect which means most energy of an ion is deposited on the target region with little normal tissue toxicity. These make heavy-ion beams an advanced radiotherapy modality, and bring outstanding results in clinical researches [11, 12]. Using heavy-ion beams to initiate suicide gene expression in combination gene therapy will enhance the antitumor activity of monotherapy. The activity of antitumor monotherapy will be enhanced when the advantage of heavy-ion treatment is combined with gene therapy.

To further control the antitumor apoptosis effect of TRAIL protein specifically acting in tumor cells within the radiotherapy target region, early growth response-1 (Egr-1) is incorporated into the adenovirus as a promoter that can be initiated in response to various stimuli including radiation. Egr-1 is a zinc-finger transcription factor, which can be rapidly and transiently induced when exposed to radiation. In the presence of zinc ions, the three zinc-finger motifs included in Egr-1 protein enable Egr-1 to recognize and combine with a specific DNA sequence, 5′-CGCCCCCGC-3′, and finally regulate downstream genes' expression by activating the Egr-1 promoter transcription [16, 17]. Under the control of Egr-1 promoter, the therapeutic gene can be effectively regulated by irradiating the specific target region for a chosen period of time. This combination treatment of gene therapy and heavy-ion radiotherapy improves the safety and efficiency of gene therapy [18, 19].

In this study, we used an adenoviral delivery system to construct the Egr-1 promoter/TRAIL therapeutic cassette into the backbone of an E1B55K-/E3-deleted oncolytic adenovirus. Although Wang et al. applied Ad-ET to tumor cells exposed to X-rays and observed enhanced tumor targeting effect [20], no research has been conducted, to the authors' knowledge, to combine Ad-ET with heavy-ion radiotherapy as a synergistic antitumor effect. In this paper, we investigate cell apoptosis in tumor and normal cells following combined treatment of Ad-ET and $^{12}C^{6+}$ ion beams to evaluate whether it can cause the synergistic pro-apoptotic effect. The results strongly support the novel treatment strategy of heavy-ion radiotherapy and radiation-inducible gene therapy, and the preliminary data are useful for future clinical applications of combination treatment.

2 Materials and methods

2.1 Cell lines and cell culture

The human fibrosarcoma cell line HT1080 and normal human lung embryonic cell line MRC-5 were kindly provided by Institute of Modern Physics, Chinese Academy of Sciences, and obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Both of the cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, Thermo Scientific) and cultured at 37 °C in a humidified 5 % CO_2 incubator (Thermo Scientific). The population doubling time of MRC5 was about 24 h, and that of HT1080 was 12 h.

2.2 Heavy-ion irradiation

The cells were exposed to 160 MeV/u $^{12}C^{6+}$ ion beams at a dose rate of 0.2 Gy · min^{-1} derived from the Heavy-ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). The LET value is 80 at peak region and 17 at plateau region. Exponentially growing HT1080 and MRC-5 cells were seeded in 96-well microtiter plate or 24-well microtiter plate, and the culture medium was replaced 24 h later before exposed to $^{12}C^{6+}$ ion beams performed through the plate bottom.

2.3 Adenovirus vector and infection

Three kinds of recombinant adenoviruses were used: PPE3-EGR1-TRAIL (Ad-ET), PXC20-Δ E1b55Kda (Ad) and PPE3-GFP(Ad-GFP). These were commercially packaged and purified by Minghong Biotech (Shanghai, China). All the adenoviruses were constructed on the basis of the oncolytic adenovirus H101 backbone. The new antitumor adenovirus was constructed and packed as described [21].

After being cultured for 24 h, the cell medium was replaced by fresh medium without FBS. The adenovirus was added into the medium for transfection. Four hours later, the same amount of medium with 20 % FBS was added for the subsequent cell culturing.

2.4 Cell growth inhibition

The CCK-8 assay (Cell Counting Kit-8, Beyotime, Jiangsu, China) was used for analyzing the cell growth by quantitatively measuring cell viability. The action principle is that WST-8 can be deoxidized by dehydrogenases of viable cells to an orange formazan. Approximately 5×10^3

exponentially growing cells were seeded into the 96-well microtiter plates. Cells were irradiated by $^{12}C^{6+}$ ion beams to 1 Gy, 2 Gy and 4 Gy, so as to determine the optimal dose that could cause the maximal synergistic tumor cell-killing effect. After $^{12}C^{6+}$ ion irradiation, cells were transfected with the Ad or Ad-ET adenovirus at a MOI (multiplicity of infection) of 10 and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for 48 h.

To test cell growth and viability, 20 μL of a CCK-8 reaction mixture in fresh medium was added to each well after incubation. Cells were incubated at 37 °C for 1 h with gentle shaking, and then, absorbance was measured at 450 nm in a SpectraMax M5 multifunctional microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance values in the treated groups were normalized to the values of untreated cells to calculate the survival fraction: [1-(*OD cells treated*/*OD cells treated with PBS*)] × 100. Each experiment was performed in quadruplicate and repeated at least three times.

2.5 Apoptosis assay

To analyze whether the synergistic apoptosis effect can be induced by Ad-ET treatment combined with $^{12}C^{6+}$ heavy-ion irradiation, first HT1080 and MRC-5 cells were seeded into 96-well microtiter plates. After exposure to $^{12}C^{6+}$ ion irradiation at a dose of 0.5 Gy or 1 Gy, Ad or Ad-ET was immediately added into the medium for transfection.

To detect the cell apoptosis fraction, both floating and attached cells were harvested after 48-h incubation following the treatment. After being washed two times with cold PBS, the cells were resuspended in 100 μL of 1 × binding buffer (Invitrogen, Carlsbad, CA). Following the protocol provided with the Invitrogen Apoptosis Detection Kit, total cells were stained with both annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide and analyzed using a FACScan flow cytometer with the CellQuest program (Becton Dickinson) as soon as possible.

2.6 Real-time PCR

After treated with Ad-ET and $^{12}C^{6+}$ ion irradiation, total RNA was isolated from cells using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized with RNA reverse transcriptase. Quantitative real-time RT-PCR amplification was carried out by using QuantiTect SYBR Green (Qiagen, Valencia, CA) as described in previous article [20]. Primers for amplifying TRAIL and GAPDH mRNA were expressed as follows:

TRAIL sense: GAGCTGAAGCAGATGCAGGAC, and TRAIL antisense: TGACGGAGTTGCCACTTGACT;

GAPDH sense: CATCAAGAAGGTGGTGAAGCAGG, and GAPDH antisense: AGCGTCAAAGGTGGAGGAG TGG.

The CT (threshold cycle) value of TRAIL was quantitated by Q-PCR in triplicate using an ABI Prism 7900 HT sequence detector (AB Applied Biosciences, CA) following the manufacturer's protocol and was normalized over the CT of the GAPDH control.

2.7 Western blot analysis

Further detection of the TRAIL gene expression level and the relative apoptosis pathway, the TRAIL protein was quantified by western blotting. The cells were harvested and lysed in lysis buffer (Beyotime, Jiangsu, China) and analyzed using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Cell lysates containing 40 μg of soluble total cellular protein were separated by 12 % SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche, Basle, Switzerland). These membranes were blocked for 1 h with a buffer containing 1 % fat-free dried milk and TBST (TBS with 0.1 % Tween). The membrane was washed three times with TBST and incubated overnight with primary antibodies at 4 °C. Primary antibodies were used against TRAIL (1 : 500, Cell Signaling Technology, Danvers, MA) and β-actin (1 : 2000 Cell Signaling Technology). After being washed with TBST three times, the membranes were incubated for 1 h with a horseradish peroxidase-conjugated antimouse IgG secondary antibody (Cell Signaling Technology, 1 : 4000) or antirabbit IgG secondary antibody (Cell Signaling Technology, 1 : 4000). Signals were visualized by enhanced chemiluminescence (Applygen, Beijing, China) using FluorChem E (Cell Biosciences, Santa Clara, CA) according to the manufacturer's instructions.

2.8 Statistical analysis

Data were presented as means and standard derivations. Significance levels were assessed using Student's t test. A p value of 0.05 or less between groups was considered significant.

3 Results

3.1 Cell viability of the cells following combined treatment of Ad-ET and $^{12}C^{6+}$ ion irradiation

To test whether the combined treatment with Ad-ET and $^{12}C^{6+}$ ion irradiation could cause a synergistic cell growth inhibitory effect on HT1080 tumor and MRC-5 normal cells, the cell viability was determined by the CCK-8 assay after

combination treatment *in vitro*. To maximize the synergistic effect of combination treatment, three doses of 1 Gy, 2 Gy and 4 Gy were used at the MOI of 10 in both tumor and normal cells.

The viability of HT1080 decreased with increasing doses within 2 Gy, and it increased under the combined treatment. Comparing the treatment group of Ad plus $^{12}C^{6+}$ ion irradiation with the control group of irradiation, the survival fraction of the combined treatment decreased by varying degrees at all the three doses (Fig. 1a). This is probably due to synergistic effect of combination treatment and the efficiency of Ad-ET, which could reach the highest value when receiving the 2 Gy irradiation dose. The results indicate that $^{12}C^{6+}$ ion irradiation can effectively activate the cell-killing capacity of the radiation-inducible adenovirus Ad-ET in tumor cells HT1080. The MRC-5 cell line was also in combination treatment on normal tissues. The results show, however, that the survival fraction did not decrease significantly (Fig. 1b). All these indicate that combination treatment with Ad-ET and irradiation could significantly enhance the cell growth inhibition in tumor cells and is nontoxic to normal cells.

3.2 Cell apoptosis enhanced by the combined treatment

For further investigation of mechanism of the cell death, an annexin-V-FITC/PI assay was used by flow cytometry after the tumor cell line HT1080 and normal cell line MRC-5 was treated with Ad-ET plus ^{12}C ion irradiation. According to the results in Sect. 3.1, the ^{12}C ion irradiation dose of 2 Gy was adopted in the combined treatment for apoptosis detection. As shown in Fig. 2a, the apoptotic fraction in the Ad-ET group and the RAD group was only 5.8 % and 5.46 %, respectively, and it increased to 20.7 % with the Ad-ET+RAD treatment. The survival fraction was much higher than that of the Ad plus RAD group, indicating that increasing apoptosis effect can be induced by $^{12}C^{6+}$ ion irradiation only when combined with Ad-ET. That means $^{12}C^{6+}$ ion irradiation can greatly sensitize the cells to the effect of Ad-ET by activating TRAIL-induced apoptosis in HT1080 cells. However, similar synergistic apoptosis effect was not observed in the MRC-5 cells when treated with Ad-ET+ RAD. As shown in Fig. 2b, the degree of apoptosis just slightly increased in MRC-5 cells with the Ad-ET+RAD group. These demonstrate that the combined treatment of Ad-ET and $^{12}C^{6+}$ ion irradiation could only induce a significant synergistic cell apoptosis effect specifically in tumor cells, but not in normal lung cells. This is probably due to the different sensitivity of TRAIL-induced apoptosis between tumor cells and normal cells.

3.3 TRAIL gene expression in HT1080 cells treated with Ad-ET+RAD

To elucidate mechanism of the synergistic apoptosis induced by Ad-ET combined with $^{12}C^{6+}$ ion irradiation, we

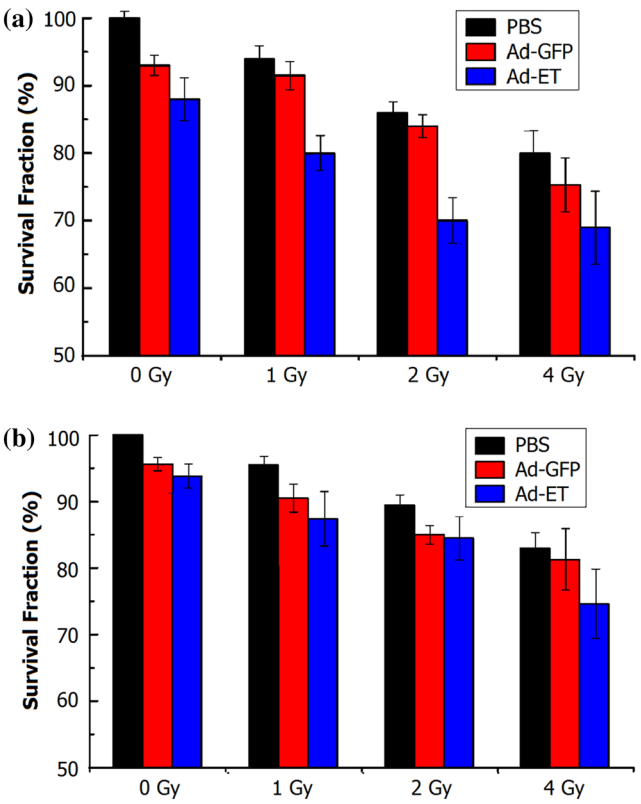

Fig. 1 (Color online) Relative cell viability of HT1080 (**a**) and MRC-5 (**b**) cells infected with Ad-ET and $^{12}C^{6+}$ ion irradiation. The cell survival fraction was determined using the CCK-8 assay and normalized to PBS-treated control cells (survival fraction = 100 %)

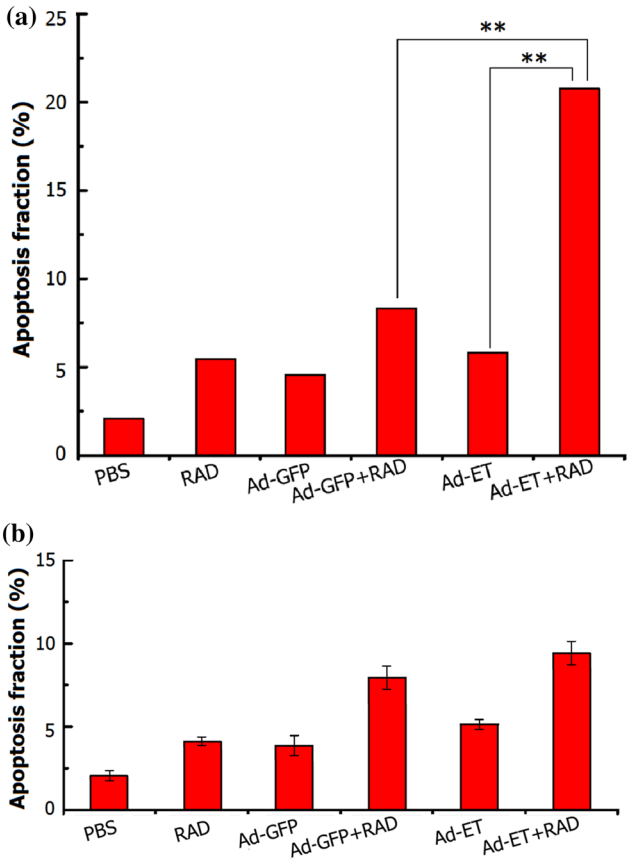

Fig. 2 (Color online) Cell apoptosis of HT1080 (**a**) and MRC5 (**b**) cells infected with Ad-ET and $^{12}C^{6+}$ ion irradiation. The proportion of apoptotic cells was determined by annexin-V-FITC/PI assay. $^{*}p < 0.05$, Student's *t* test

detected the level of TRAIL gene expression by real-time PCR. As shown in Fig. 3, the fold of increase in the RAD, Ad-GFP, Ad-GFP+RAD and Ad-ET groups was less than 20 when the TRAIL gene expression in PBS control group was set as 1, while in the group of Ad-ET plus $^{12}C^{6+}$ ion irradiation, the TRAIL gene increased to 100. The results show that $^{12}C^{6+}$ ion irradiation could enhance the pro-apoptotic TRAIL gene expression in tumor cells treated with Ad-ET. The increased cytotoxicity observed in apoptosis analysis was mostly due to the elevated TRAIL gene expression when activated by $^{12}C^{6+}$ ion irradiation.

3.4 Expression of TRAIL proteins following the combined treatments of HT1080 cells

For further detection of the TRAIL protein expression following TRAIL gene activation in HT1080 cells treated with Ad-ET and $^{12}C^{6+}$ ion irradiation, we detected the level of TRAIL protein by western blotting. As shown in Fig. 4, the band in Ad-ET plus $^{12}C^{6+}$ ion irradiation group was much thicker than other treatment groups, showing

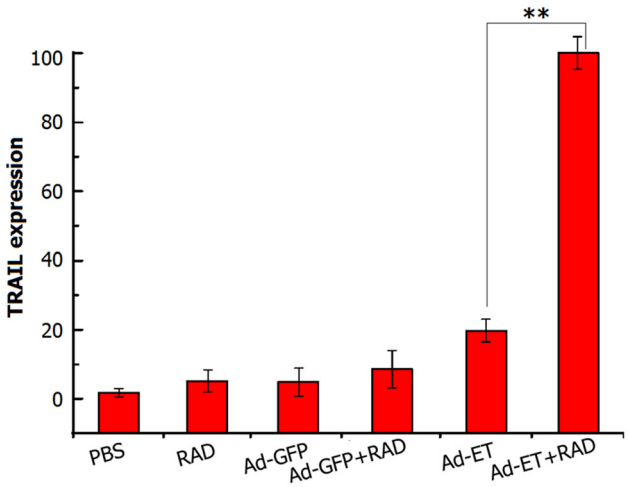

Fig. 3 (Color online) TRAIL gene expression in HT1080 cells infected with Ad-ET and $^{12}C^{6+}$ ion irradiation. The level of TRAIL gene expression was detected by real-time PCR and normalized to PBS-treated control cells with their survival fraction set as 1. $^{*}p < 0.05$, Student's *t* test

that the expression of TRAIL protein in Ad-ET-infected HT1080 cells was dramatically enhanced following exposure to $^{12}C^{6+}$ ion beams. The result indicates that the pro-apoptotic effect in Ad-ET-treated tumor cells could be enhanced by $^{12}C^{6+}$ ion irradiation by activating the TRAIL expression.

4 Discussion

Radiotherapy is a main modality of cancer treatment, though it has limitation in treatment accuracy [22–33]. Each dose of radiotherapy is usually below 2 Gy, so as to save the normal tissue. In clinical, radiotherapy is often used in conjunction with other treatments, such as surgery and chemotherapy. In our study, radiotherapy is applied in combination with gene therapy. While showing its

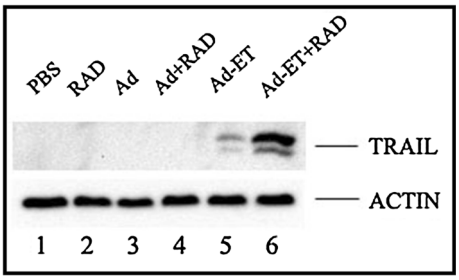

Fig. 4 Western blot analysis of TRAIL protein expression in HT1080 cells. Actin was used as the internal control. The expression level of each band was standardized to actin and expressed as a *fold* increase with the control set as 1

promising prospect in the future for tumor therapy, this approach has limitations, too, such as the low efficiency of tumor target [1]. Combining radiotherapy with gene therapy, radio genetic therapy can overcome weakness of the two treatment methods. This method can constrain the therapeutic gene expression in radiotherapy target region to realize the double action of radiation and therapeutic gene in tumor cells, and improve the therapeutic efficacy. It takes full advantages of radiotherapy and gene therapy, so as to reduce the dosage of radiotherapy and gene therapy, with decreased cytotoxicity in normal tissue.

Some studies reveal that radio gene therapy induces the DNA damage through different pathways, i.e., combining the two methods probably induces the synergistic effect in tumor cells [34, 35]. In our adenovirus gene therapy system, early growth response-1 (Egr-1) is constructed as a promoter to selectively activate the therapeutic gene in the target tumor region and avoid probable cytotoxic effects in normal tissues. The Egr-1 gene acted as a promoter in radio genetic therapy and has demonstrated that it can regulate the downstream therapeutic gene expression in radiation target region effectively to improve the therapeutic gain. Weichselbaum et al. [36] constructed a recombinant expression plasmid pEgr-TNF-α to include Egr-1 promoter and TNF-α gene. After exposure to X-rays, the tumor cells treated with pEgr-TNF-α were significantly inhibited without increase of the whole body toxicity

The tumor necrosis factor (TNF)-related apoptosis-inducing TRAIL ligand, which was inserted into the radiation-inducible gene therapy system, has been confirmed as being able to selectively induce apoptosis in a broad range of cancer cells but spares normal cells. However, some studies revealed that TRAIL has limitation in eradicating tumors when used as monotherapy. This is probably due to deficiency of TRAIL death receptors or competition of antagonistic receptor. Thus, in our research, by combining TRAIL gene with radiotherapy in radio genetic therapy, one could potentiate its antitumor activity and specificity in a variety of radio-resistant tumor cells [37]. In addition, without understanding the mechanisms, it has been revealed that TRAIL activated a pro-apoptotic effect in human liver cells [38]. This radiation-inducible strategy in our research could offer a double guarantee of specific antitumor activity. On the other hand, radiation could also enhance the sensitivity of TRAIL-resistant cells in combination treatment by up-regulating the death receptors of TRAIL or activating the caspase pathway [39].

Also we tested the tumor killing capacity and apoptosis fraction of radiation-inducible TRAIL gene therapy. The results demonstrate that the tumor killing capacity and apoptosis fraction are significantly increased in Ad-ET combined with $^{12}C^{6+}$ ion irradiation than monotherapy of $^{12}C^{6+}$ ion beams or Ad-ET. The results also indicate that the tumor killing and apoptosis induction capacity in combination treatment was more than the sum of monotherapy of $^{12}C^{6+}$ ion beams and Ad-ET. We further detected the TRAIL gene and followed TRAIL protein expression to explore the probable mechanisms of the synergistic pro-apoptotic effect. The result suggests that irradiation could activate the TRAIL gene expression and cause the synergistic antitumor activity in combination treatment of Ad-ET and $^{12}C^{6+}$ ion irradiation.

In conclusion, our study demonstrates that Ad-TRAIL administration can work in concert with irradiation to improve tumor cell eradication. The evidence suggests that combining irradiation and Ad-TRAIL will be a promising strategy to improve tumor treatment efficacy by specifically targeting tumor cells.

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References

1. I.M. Verma, N. Somia, Gene therapy-promises, problems and prospects. Nature **389**, 239–242 (1997). doi:10.1038/38410
2. M.L. Edelstein, M.R. Abedi, J. Wixon et al., Gene therapy clinical trials worldwide 1989–2004-an overview. J. Gene. Med. **6**, 597–602 (2004). doi:10.1002/jgm.619
3. R.D. Alvarez, J. Gomez-Navarro, M. Wang et al., Adenoviral-mediated suicide gene therapy for ovarian cancer. Mol. Ther. **2**, 524–530 (2000). doi:10.1006/mthe.2000.0194
4. K. Garber, China approves world's first oncolytic virus therapy for cancer treatment. J. Natl. Cancer. Inst. **98**, 298–300 (2006). doi:10.1093/jnci/djj111
5. H. LeBlanc, A. Ashkenazi, Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ, **10**, 66–75 (2003). doi:10.1038/sj.cdd.4401187
6. A. Ashkenazi, R. Pal, S. Fong et al., Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Invest. **104**, 155–162 (1999). doi:10.1172/JCI6926
7. G. Pan, G. Ni, Y.F. Wei et al., An antagonist decoy recep-tor and a death domain-containing receptor for TRAIL. Science **277**, 815–818 (1997). doi:10.1126/science.277.5327.815
8. T. Hori, T. Kondo, M. Kanamori et al., Ionizing radiation enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through up-regulations of death receptor 4 (DR4) and death receptor 5 (DR5) in human osteosarcoma cells. J. Orthop. Res. **28**, 739–745 (2009). doi:10.1002/jor.21056
9. A.M. Chinnaiyan, U. Prasad, S. Shankar et al., Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc. Natl. Acad. Sci. **97**, 1754–1759 (2000). doi:10.1073/pnas.030545097
10. W. Liu, E. Bodle, J. Chen et al., Tumor necrosis factor-related apoptosis-inducing ligand and chemotherapy cooperate to induce apoptosis in mesothelioma cell lines. Am. J. Respir. Cell Mol. **25**, 111–118 (2001). doi:10.1165/ajrnmeterb.25.1.4472

11. P. Todd, Heavy-ion irradiation of cultured human cells. Radiat. Res. Suppl. **7**, 196–207 (1967). doi:10.2307/3583713
12. T. Kanai, M. Endo, S. Minohara et al., Biophysical characteristics of HIMAC clinical irradiation system for heavy-ion radiation therapy. Int. J. Radiat. Oncol. **44**, 201–210 (1999). doi:10.1016/S0360-3016(98)00544-6
13. H. Tsujii, J. Mizoe, T. Kamada et al., Clinical results of carbon ion radiotherapy at NIRS. J. Radiat. Res. **48**, A1–13 (2007). doi:10.1269/jrr.48.A1
14. H. Tsujii, J. Mizoe, T. Kamada et al., Overview of clinical experiences on carbon ion radiotherapy at NIRS. Radiother. Oncol. **73**, S41–S49 (2004). doi:10.1016/S0167-8140(04)80012-4
15. J.R. Castro, D.H. Char, P.L. Petti et al., 15 years experience with helium ion radiotherapy for uveal melanoma. Int. J. Radiat. Oncol. **39**, 989–996 (1997). doi:10.1016/S0360-3016(97)00494-X
16. A. Gashler, V.P. Sukhatme, Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. Prog. Nucleic Acid Res. Mol. Biol. **50**, 191–224 (1995). doi:10.1016/S0079-6603(08)60815-6
17. R. Datta, E. Rubin, V. Sukhatme et al., Ionizing radiation activates transcription of the EGR1 gene via CArG elements. Proc. Natl. Acad. Sci. **89**, 10149–10153 (1992)
18. R.G. Meyer, J.H. Küpper, R. Kandolf et al., Early growth response-1 gene (Egr-1) promoter induction by ionizing radiation in U87 malignant glioma cells in vitro. Eur. J. Biochem. **269**, 337–346 (2002). doi:10.1046/j.0014-2956.2001.02658.x
19. T. Joki, M. Nakamura, T. Ohno, Activation of the radiosensitive EGR-1 promoter induces expression of the herpes simplex virus thymidine kinase gene and sensitivity of human glioma cells to ganciclovir. Human Gene Ther. **6**, 1507–1513. doi:10.1089/hum.1995.6.12-1507
20. H.B. Wang, X. Song, H. Zhang, Potentiation of tumor radiotherapy by a radiation-inducible oncolytic and oncoapoptotic adenovirus in cervical cancer xenografts. Int. J. Cancer **130**, 443–453 (2012). doi:10.1002/ijc.26013
21. C.A. Heid, J. Stevens, K.J. Livak et al., Real time quantitative PCR. Genome Res. **6**, 986–994 (1996). doi:10.1101/gr.6.10.986
22. Y.C. Wu, G. Song, R.F. Cao et al., Development of accurate/advanced radiotherapy treatment planning and quality assurance system (ARTS). Chin. Phys. C **32**, 177–182 (2008)
23. S. Tao, Y. Wu, Y. Chen et al., Repeated positioning in accurate radiotherapy based on virtual net technique and contrary reconstruction scheme. Comput. Med. Imaging Graph. **30**, 273–278 (2006). doi:10.1016/j.compmedimag.2006.05.006
24. S. Tao, A. Wu, Y. Wu et al., Patient set-up in radiotherapy with video-based positioning system. Clin. Oncol. **18**, 363–366 (2006). doi:10.1016/j.clon.2005.11.018
25. R.F. Cao, Y.C. Wu, X. Pei et al., Multi-objective optimization of inverse planning for accurate radiotherapy. Chin. Phys. C **35**, 313–317 (2011). doi:10.1088/1674-1137/35/3/019
26. Y.C. Wu, Development of reliability and probabilistic safety assessment program RiskA. Ann. Nucl. Energy **83**, 316–321 (2015). doi:10.1016/j.anucene.2015.03.020
27. J. Song, G. Sun, Z. Chen et al., Benchmarking of CAD-based SuperMC with ITER Benchmark Model. Fusion Eng. Des. **89**, 2499–2503 (2014). doi:10.1016/j.fusengdes.2014.05.003
28. L. Hu, Y. Wu, Probabilistic safety assessment of the dual-cooled waste transmutation blanket for the FDS-I. Fusion Eng. Des. **81**, 1403–1407 (2006). doi:10.1016/j.fusengdes.2005.08.091
29. H. Zheng, G. Sun, G. Li, et al., Photon dose calculation method based on Monte Carlo finite-size pencil beam model in accurate radiotherapy. Commun. Comput. Phys. **14**, 1415–1422 (2013)
30. Y. Wu, G. Li, S. Tao et al., Research and development of an accurate/advanced radiation therapy system (ARTS). Chin. J. Med. Phys. **22**, 683–690 (2006). doi:10.3969/j.issn.1005-202X.2005.06.001
31. W.Y. Li, R.F. Cao, X. Pei et al., Radiotherapy reliability analysis based on PSA method. Nucl. Sci. Tech. **25**, 060501 (2014). doi:10.13538/j.1001-8042/nst.25.060501
32. K. Zhao, M.Y. Cheng, P.C. Long et al., A hybrid voxel sampling method for constructing Rad-HUMAN phantom. Nucl. Sci. Tech. **25**, 020503 (2014). doi:10.13538/j.1001-8042/nst.25.020503
33. J. Wang, X. Pei, R.F. Cao et al., A multiphase direct aperture optimization for inverse planning in radiotherapy. Nucl. Sci. Tech. **26**, 010502 (2015). doi:10.13538/j.1001-8042/nst.26.010502
34. R.R. Weichselbaum, D.E. Hallahan, M.A. Beckett et al., Gene Therapy targeted by radiation preferentially radiosensitized tumor cells. Cancer Res. **54**, 4266–4269 (1994)
35. V.K. Gupta, J.O. Park, N.T. Jackowiak et al., Combined gene therapy and ionizing radiation is a novel approach to treat human esophageal adenocarcinoma. Ann. Surg. Oncol. **9**, 500–504 (2002). doi:10.1007/BF02557275
36. R.R. Weichselbaum, D.E. Hallahan, V.P. Sukhatme et al., Gene therapy targeted by ionizing radiation. Int. J. Radiat. Oncol. **24**, 565–567 (1992). doi:10.1016/0360-3016(92)91075-X
37. E. Wissink, I. Verbrugge, S. Vink et al., TRAIL enhances efficacy of radiotherapy in a p53 mutant, Bcl-2 overexpressing lymphoid malignancy. Radiother. Oncol. **80**, 214–222 (2006). doi:10.1016/j.radonc.2006.07.030
38. D. Lawrence, Z. Shahrokh, S. Marsters et al., Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat. Med. **7**, 383–385 (2001). doi:10.1038/86397
39. C. Belka, B. Schmid, P. Marini et al., Sensitization of resistant lymphoma cells to irradiation-induced apoptosis by the death ligand TRAIL. Oncogene **20**, 2190–2196 (2001)