

Enhanced tumor radiosensitivity by a survivin dominant-negative mutant

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Abstract. Radiosensitivity of tumors is due to a complex interaction of various factors, it has been reported that survivin also acts as a constitutive and inducible radioresistance factor in a panel of tumor cells and approaches designed to inhibit survivin expression or function may lead to tumor sensitisation to chemical and physical agents. Previously, we found that the plasmid encoding the phosphorylation-defective mouse survivin threonine 34→alanine mutant complexed to DOTAP-chol liposome (Lip-mS) can suppress murine primary breast carcinoma. However, little is known regarding the biological effect of Lip-mS combined with radiation. The present study was designed to determine whether Lip-mS could enhance the anti-tumor activity of radiation. The Lewis Lung Carcinoma (LLC) cells treated with a combination of Lip-mS and radiation displayed apparently increased apoptosis compared with those treated with Lip-mS or radiation alone. Mice bearing LLC tumors were treated with intravenous injections of Lip-mS and radiation, the combined treatment significantly reduced mean tumor volume compared with either treatment alone. Moreover, the anti-tumor effect of Lip-mS combined with radiation was greater than their additive effect when compared with the expected effect of the combined treatment. These data suggest that inhibition of survivin using a dominant-negative mutant, survivin T34A, could sensitize LLC cells to radiation efficiently and the synergistic anti-tumor activity may in part result from increasing the apoptosis of tumor cells,

inhibiting tumor angiogenesis and inducing a tumor-protective immune response in the combined treatment.

Introduction

As the smallest member of the inhibitors of apoptosis protein (IAP) family, survivin is highly expressed in most common neoplasms, but is generally absent or low in most differentiated normal tissues (1-3). Moreover, the overexpression of survivin has been correlated with an enhanced proliferative index, reduced levels of apoptosis, increased rate of tumor recurrence, decreased overall survival and appears to be involved in tumor cell resistance to ionizing radiation (4) and some anti-cancer agents (5,6).

Due to its dual role in suppressing apoptosis and regulating cell division, survivin is vital for cancer cell survival, which makes it an attractive target for new anti-cancer interventions (7,8). In recent years, considerable efforts including antisense oligonucleotides (9), hammerhead ribozymes (10), RNA interference (11,12), cancer vaccines (13) and dominant-negative mutants (14) have been made to counteract survivin and several preclinical studies have already demonstrated that down-regulation of survivin expression or function could inhibit tumor growth potential, increase spontaneous and induced apoptosis and sensitize tumor cells to anti-cancer agents and irradiation.

It was reported that one of the dominant-negative mutants, survivin Thr34→Ala, which abolished a phosphorylation site for cdc2-cyclin B1 and prevented survivin binding to activated caspase-9 (15), could reduce tumor cell proliferative potential and lead to caspase-dependent apoptosis in melanoma cell lines (14). Previously, we also found that the plasmid encoding the phosphorylation-defective mouse survivin threonine 34→alanine mutant complexed to DOTAP-chol liposome (Lip-mS) can suppress murine highly metastatic breast carcinoma (16), however, little is known regarding the biological effect of Lip-mS combined with radiation, so the present study was designed to determine whether Lip-mS could enhance the anti-tumor activity of radiation and to explore the possible mechanisms of interaction between survivin targeting agents and radiation.

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Materials and methods

Cell line and culture conditions. The Lewis Lung Carcinoma (LLC) cell line of C57BL/6 mouse origin was purchased from the American Type Culture Collection (ATCC, Rockville, MD), cultured in DMEM (Gibco BRL, Grand Island, NY) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained in a humidified incubator at 37°C in 5% CO₂ atmosphere.

Plasmid DNA preparation. The recombinant plasmid encoding the phosphorylation-defective mouse survivin threonine 34→alanine mutant (mS-T34A) was purchased from InvivoGen Corporation and confirmed by restriction endonuclease analysis, PCR and DNA sequence analysis. The plasmid was prepared using Endofree Plasmid Giga kit (Qiagen, Chatsworth, CA). Endotoxin levels of the plasmid DNA prepared were determined by Tachypleus Amebocyte Lysate (TAL). No genomic DNA, small DNA fragments, or RNA were detected in the DNA prepared and the OD_{260/280} ratios of the plasmid DNA prepared were between 1.8–2.0. The DNA was eventually dissolved in sterile endotoxin-free water and stored at -20°C before use.

Liposome preparation. DOTAP-chol liposome was prepared using the procedure described previously (17). Briefly, the cationic lipid DOTAP was mixed with the neutral lipid chol at equimolar concentrations. The mixed lipids were dissolved in chloroform in a 100 ml round bottomed flask. Then, the clear solution was rotated on a Buchi rotary evaporator at 30°C for 30 min to make a thin film, the flask containing the thin lipid film was dried under vacuum for 15 min. The film was hydrated in 5% dextrose in water (D5W) to give a final concentration of 7 mM DOTAP and 7 mM chol, referred to as 7 mM DOTAP:chol. The hydrated lipid film was rotated in a water bath at 50°C for 45 min and then 35°C for 10 min. The mixture was allowed to stand in the parafilm-covered flask at room temperature overnight, after which the mixture was sonicated at low frequency for 5 min at 50°C, transferred to a tube and heated for 10 min at 50°C. The mixture was sequentially extruded through Millipore (Billerica, MA) polycarbonate membrane of decreasing size: 0.2 μm for 5 times and 0.1 μm for 3 times using syringes. Liposome were stored under argon gas at 4°C. DOTAP was purchased from Avanti Polar Lipids (Alabaster, AL) and highly purified chol was purchased from Sigma (St. Louis, MO).

Preparation of DOTAP-chol liposome/plasmid DNA. DNA:liposome mixtures were prepared in accordance with a previously described method (18). Briefly, DOTAP-chol (20 mM) and plasmid DNA stock solution diluted in 5% dextrose in water (D5W) were mixed in equal volumes to give a final concentration of 4 mM DOTAP-chol, i.e., 150 μg DNA in 300 μl final volume (ratio, 1:2.6). These reagents were diluted and mixed at room temperature. The DNA solution was added to DOTAP-chol liposome and rapidly mixed by pipetting up and down two times with the pipette tip. The DNA:liposome mixture thus prepared was precipitate-free and used for all the *in vivo* experiments. The size of the DNA

fragments in the DNA:liposome mixture was determined to be in the range of 300–325 nm.

Radiation. Cells and mice were irradiated with Co⁶⁰ γ-rays at a dose rate of 92 cGy/min (Nuclear Power Institute of China, Sichuan, China). During irradiation, unanesthetized mice were immobilized in a special jig and the right hind limb with tumor was put into the radiation field. A 5-mm-thick tissue equivalent bolus was used to direct the maximal radiation dose on the surface of the target tissue.

MTT assay. LLC cells were seeded in a 96-well plate and incubated for 24 h, then treated with NS or Lip-mS (DNA at 1 μg/ml). Forty-eight hours later, the cells were irradiated (2 Gy). After another 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (20 μl, 5 mg/ml; Sigma) was added and it was further incubated for 4 h at 37°C. Then the medium was removed from each well and DMSO (150 μl) was added and incubated for 10 min. Spectrometric absorbance at 540 nm was measured using a plate reader. The cell survival rate was calculated using the following equation: survival rate = experimental group absorbance/control group absorbance × 100%.

Flow cytometric analysis. LLC cells were seeded in a 6-well plate and incubated for 24 h, then treated with NS or Lip-mS (DNA at 1 μg/ml). Forty-eight hours later, the cells were irradiated (2 Gy). After another 48 h, the cells were washed with PBS and resuspended in propidium iodide/RNase A solution (0.5 ml), incubated at 37°C for 30 min and analyzed by flow cytometry.

Animal studies. All studies involving mice were approved by the Institute's Animal Care and Use Committee. Female C57BL/6 mice of 6 to 8 weeks old were purchased from experimental animal center of Sichuan University (Chengdu, Sichuan Province, China) and challenged s.c. with LLC cells (5×10⁵ cells in 50 μl PBS) into the right hind limb. When the size of the tumors reached about 50 mm³, the mice were randomly divided into 4 groups (8 mice per group) and treated with NS, Lip-mS, radiation or Lip-mS + radiation, respectively. Lip-mS was injected into mice via the tail vein at 5 μg per day once daily for 10 days (days 0 to 9) and the tumors were irradiated at 5 Gy per day for 4 days (days 1, 3, 6 and 8). Tumor size was determined by caliper measurement of the largest and perpendicular diameters every two days. Tumor volume was calculated according to the formula $V = 0.52ab^2$, where a is the largest superficial diameter and b is the smallest superficial diameter.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. The mice were sacrificed by cervical dislocation on day 14 after the initiation of Lip-mS administration. Primary tumors were excised, fixed in 10% neutral-buffered formalin solution and embedded in paraffin. Contiguous 3–5 μm sections were mounted. In order to highlight the cells that were undergoing apoptosis, unstained sections mounted in silanized slides were subjected to fluorescent *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay by using an

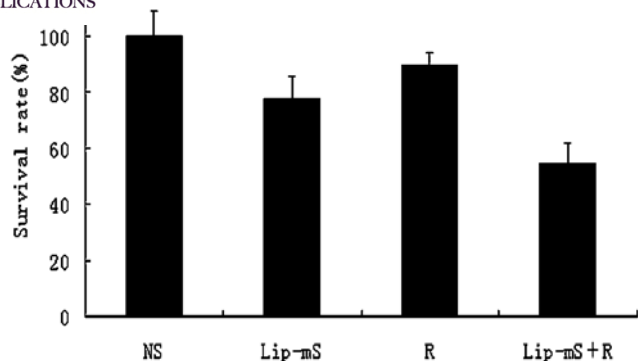


Figure 1. Growth inhibition in LLC cells by treatment with Lip-mS and radiation. LLC cells were treated with NS, Lip-mS (DNA at 1 μ g/ml), radiation (2 Gy), or Lip-mS + radiation. Growth inhibition was measured by the MTT assay. The proportion of surviving cells was calculated as a percentage of the control and data were represented as the mean \pm SE. According to the results of the MTT assays, the combined treatment (54.6%) significantly reduces LLC cell viability compared with the Lip-mS (77.5%) or radiation (89.6%) treatment alone ($P < 0.05$).

in situ apoptotic cell detection kit (Promega, Madison WI, USA), according to the manufacturer's protocol. Representative images were taken under a light microscope (x200) in randomly selected fields.

CD31 immunohistochemical evaluation. Immunohistochemistry analyses of microvessel expression were done with goat anti-mouse CD31 antibody (Santa Cruz Biotechnology) using the labeled streptavidin-biotin method. That is to say, sections were deparaffinized in xylol and rehydrated in graded alcohol series. Antigen retrieval was carried out by autoclaving sections in retrieval buffer (10 mM pH 6.0 EDTA citrate buffer) for 3 min in saturated steam after up-pressure gaining (126°C, 1.6 bars, 23psi). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide at room

temperature free of light for 20 min. Non-specific binding of reagents was quenched by incubation of sections for 20 min in 5% normal rabbit serum. Sections were then incubated with goat anti-mouse CD31 (dilution 1/200) antibodies overnight at 4°C, followed by incubating with biotinylated rabbit anti-goat IgG and then streptavidin-biotin-horseradish peroxidase complex at 37°C for 1 h, respectively. Negative control was included with each run by substituting the primary antibody with non-immune rabbit serum. Cellular nuclei were counter-stained with ameliorative Gill's hematoxylin. Representative images were taken under a light microscope (x400) in randomly selected fields.

Statistical analysis. In order to determine statistical significance, Student's t-test for non-paired data was performed. A value of $P < 0.05$ was considered to be statistically significant.

Results

Enhancement of the anti-tumor effect of radiation *in vitro*. In order to test the combined effect of Lip-mS with radiation *in vitro*, we treated LLC cells with NS, Lip-mS (DNA at 1 μ g/ml), radiation (2 Gy) or Lip-mS + radiation. Growth inhibition was analyzed by measuring cell viability with MTT assay. According to the results of the MTT assays, the combined treatment (54.6%) significantly reduces LLC cell viability compared with the Lip-mS (77.5%) or radiation (89.6%) treatment alone ($P < 0.05$) (Fig. 1).

We also used flow cytometric analysis to evaluate the effect of Lip-mS and radiation on the induction of apoptosis in LLC cells. Lip-mS + radiation treatment (36.2%) significantly increased the proportion of sub-G1 cells (apoptotic cells) compared with the other treatments (NS, 3.0%; Lip-mS, 21.9%; radiation, 9.6%) (Fig. 2).

Moreover, the interactive anti-tumor effect of the combined treatment was greater than additive when compared with the expected effect of the combined treatment *in vitro*. The

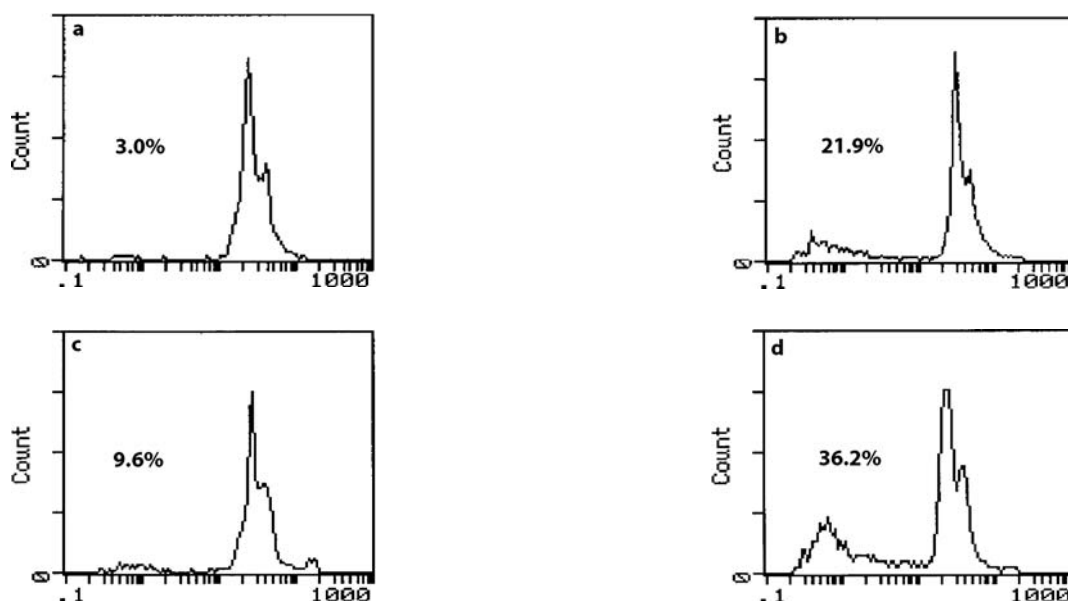


Figure 2. Induction of apoptosis in LLC cells by treatment with Lip-mS and radiation. LLC cells were treated with NS (a), Lip-mS (b), radiation (c), or Lip-mS + radiation (d). Flow cytometric analysis revealed the proportion of sub-G1 cells (apoptotic cells) to be 3.0% (a), 21.9% (b), 9.6% (c) and 36.2% (d), respectively.

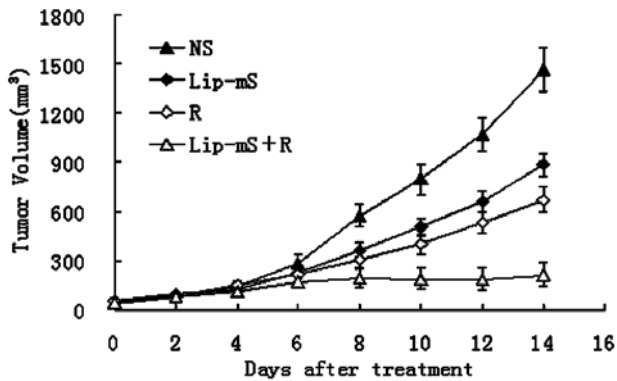


Figure 3. Lip-mS enhanced the anti-tumor effect of radiation *in vivo*. Mice bearing LLC tumors were treated with NS (▲), Lip-mS (◆), radiation (◇) or Lip-mS + radiation (△). The combined treatment significantly reduced the mean tumor volume on day 14 when compared with the Lip-mS or radiation treatment group ($P < 0.05$).

expected combined apoptotic effect was defined as follows: expected combined effect = Lip-mS effect + radiation effect - Lip-mS effect \times radiation effect. The Lip-mS and radiation treatment can induce apoptosis 21.9 and 9.6%, respectively; thus, the expected induction of apoptosis in the combined treatment should be 29.4%. However, the actual induction of apoptosis in the combined treatment is 36.2%, suggesting greater than additive treatment effect.

Enhancement of the anti-tumor effect of radiation *in vivo*. The anti-tumor effect of Lip-mS in combination with radiation was assessed in mice bearing LLC tumors. The tumor growth curves demonstrated that both Lip-mS and radiation individually resulted in effective suppression of tumor growth,

while the combined treatment had a superior anti-tumor effect when compared with the Lip-mS or radiation treatment alone ($P < 0.05$) (Fig. 3). Moreover, the interactive anti-tumor effect of the combined treatment was also greater than their additive effect when compared with the expected effect of the combined treatment. On day 14 after the initiation of Lip-mS administration, the mean tumor volume of radiation group was 670.2 mm³, this represents a 54.3% volumetric reduction. Compared with the 39.6% reduction in tumor size observed in the Lip-mS treatment group, the expected volumetric reduction in tumor size of the combined treatment group should be 72.4%. However, the percent reduction in the combined treatment group is 85.5%, suggesting the greater than additive effect of the combined treatment.

In order to test by which possible mechanisms Lip-mS enhanced the anti-tumor effect of radiation *in vivo*, tumor sections of each group were stained with TUNEL reagent and anti-CD31 antibody to evaluate apoptosis rate and microvessel density as described above. An apparent increase in the number of apoptotic cells was observed within the tumors treated with the combination of Lip-mS and radiation compared with the other treatments (Fig. 4). Tumors of the NS and radiation treatment groups demonstrated high microvessel density, while those of the Lip-mS alone and combined treatment groups had decreased values (Fig. 5). These data suggest Lip-mS can cause the increased apoptosis of tumor cells and inhibition of tumor angiogenesis, which may play important roles in the enhancement of the anti-tumor effect of radiation *in vivo*.

Discussion

Radiotherapy is one of the most widely used treatments for cancer, with over half of all cancer patients receiving radiation

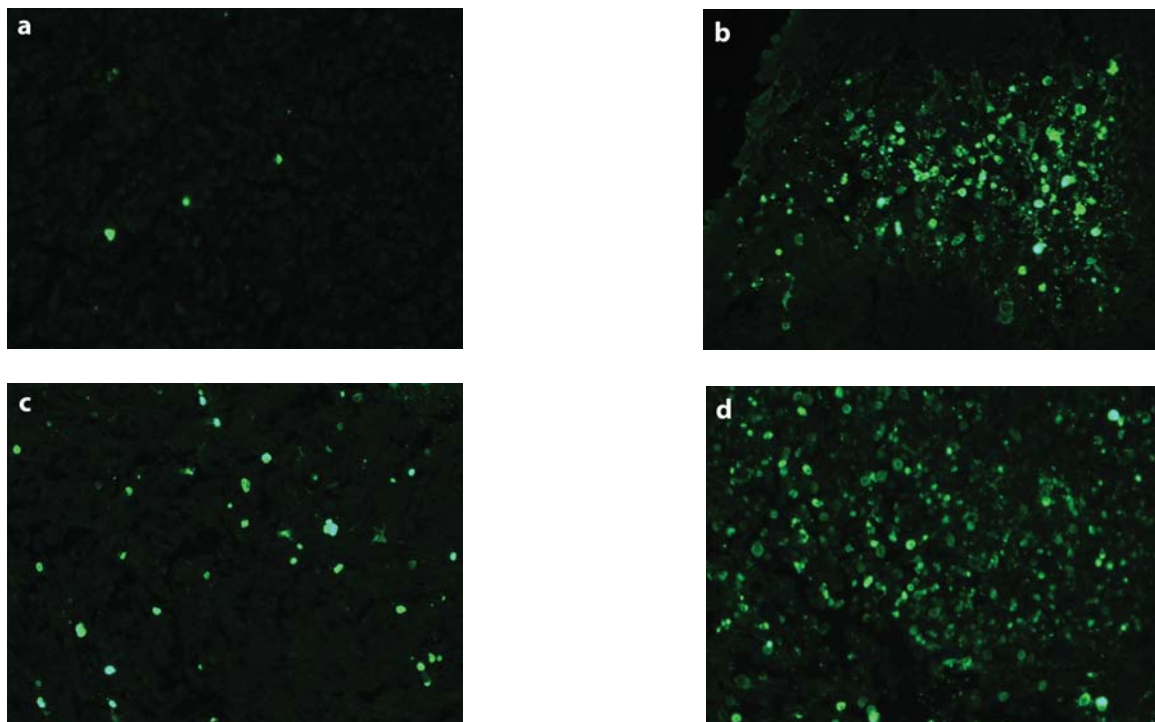


Figure 4. Combination of Lip-mS and radiation enhanced the induction of apoptosis *in vivo*. Tissue sections from tumor-bearing mice treated with NS (a), Lip-mS (b), radiation (c), or Lip-mS + radiation (d) were stained with FITC-DUTP. An apparent increase in the number of apoptotic cells was observed within tumors treated with a combination of Lip-mS and radiation compared with the other treatments.

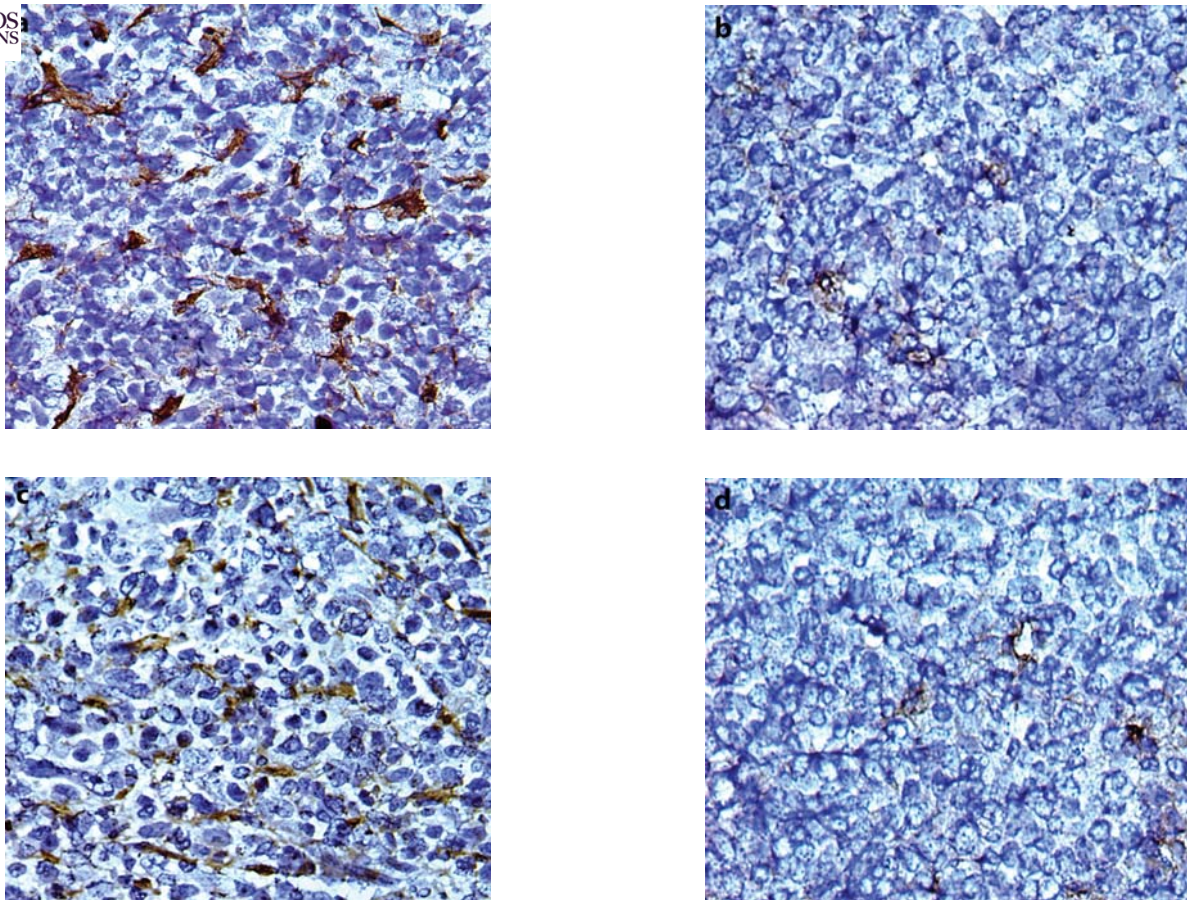


Figure 5. Inhibition of intratumoral angiogenesis assayed by CD31 staining of microvessel. Vascularization within tumors was detected by an antibody to CD31, representative images were taken under a light microscope (x400) in randomly selected fields. Tumors of the NS (a) and radiation (c) treatment groups demonstrated high microvessel density, while those of the Lip-mS (b) and Lip-mS + radiation (d) treatment groups showed apparent inhibition of angiogenesis.

at some point during their course of treatment (19), but to varying degrees of success, some tumors are highly responsive to radiation while others are typically very radioresistant and tend to progress even after high radiation doses (20), thereby increasing the radiosensitivity of tumor cells has the potential to significantly improve the rate of recovery from many malignancies. Radiosensitivity is due to a complex interaction of various factors (21), previously, it was reported that survivin also acts as a constitutive and inducible radio-resistance factor in a panel of tumor cells (4) and approaches designed to inhibit survivin expression or function may lead to tumor sensitisation to chemical and physical agents (22).

In the present study, the combination of Lip-mS gene therapy and radiation significantly enhanced the anti-tumor effect of radiation. Moreover, the interactive anti-tumor effect of the combined treatment was greater than their additive effect when compared with the expected effect of the combined treatment. These data suggest that inhibition of survivin using a dominant-negative mutant, survivin T34A, can sensitize LLC cells to radiation efficiently. The exact mechanism of interaction between survivin targeting agents and radiation, although remained to be determined, may be in part involved in three aspects as follows: increasing the apoptosis of tumor cells, inhibiting tumor angiogenesis and inducing a tumor-protective immune response.

Apoptosis, an evolutionarily conserved and genetically regulated process, preserves tissue and organ homeostasis by

eliminating senescent or damaged cells (23). Aberrations of apoptosis are known to contribute to a variety of diseases including cancer and play an important role in tumor initiation, progression, and radioresistance (24). One cellular mechanism common to radiation is to kill tumor cells via apoptosis (25,26). Radiation can directly damage DNA, resulting in mitochondrial damage due to the breakdown of the mitochondrial membrane potential, which will stimulate the initiation of apoptosis by releasing cytochrome c (27-29). Alternatively, radiation may directly damage the cellular membrane, resulting in the release of ceramide. Once released into the cytoplasm, ceramide can directly damage the mitochondrial membrane and then stimulate the release of cytochrome c (30). Released from mitochondria, cytochrome c can bind and activate the apoptotic protease-activating factor-1 (Apaf-1), causing activation of caspase-9 within the apoptosome complex and leading to a protease cascade ultimately to apoptosis (31,32). In this process of apoptosis, activation of caspases, a common step downstream of mitochondrial cytochrome c release, may be controlled by the intracellular IAP family (33).

As a structurally unique member of the IAP family, survivin plays an important role in the suppression of apoptosis by either directly or indirectly interfering with the function of caspases, the downstream effectors of apoptosis. XIAP, another IAP family member which contains three Baculovirus IAP Repeat (BIR) domains, can inhibit caspase-3 and -7 by a linker region between the first two BIR domains and can also bind

and inhibit caspase-9 via its third BIR (BIR3) domain. While survivin contains only one BIR domain, which appears in three-dimensional structure closely related to the BIR3 domain of XIAP, suggesting the possibility of binding and inhibiting caspase-9 directly (34,35). Smac/Diablo, released from mitochondria into the cytosol along with cytochrome c, can increase Apo-2L/TRAIL-induced caspase-3 activity and downregulate the activity of IAPs during execution of the mitochondrial apoptosis pathway. Survivin may bind and sequester Smac/DIABLO, thus preventing Smac/DIABLO binding to other IAPs (36). Survivin has also been shown to interact with Cdk4, which releases p21 to complex with and inactivate caspase-3 (37). Therefore it is not difficult to understand that inactivation of IAPs such as survivin, as a barrier to apoptosis, should be possible to reverse malignant cell radioresistance, effectively promote the apoptotic signals conveyed by radiation and ultimately drive tumor cells into the apoptotic suicide process.

Enhancement of tumor response to radiation by survivin targeting agents has also been explained by a decrease in tumor angiogenesis after treatment. To supply oxygen and other nutrients, angiogenesis plays an important role in the growth and progression of solid tumors (38). Survivin is also overexpressed in endothelial cells of the tumor vasculature during the proliferative and remodeling phases of angiogenesis and generates a cytoprotective mechanism for these cells (39). Moreover, radiation may induce tumor cells to secrete protective cytokines, such as VEGF, which could further up-regulate survivin expression, inhibit apoptosis and enhance radioresistance in vascular endothelial cells (40). These findings identify survivin as a novel protective gene expressed by endothelial cells during angiogenesis, suggesting that survivin targeting may be able to induce apoptosis in endothelial cells and sensitise the tumor vasculature to different anti-cancer modalities including radiation. In the previous and present studies (16), we have demonstrated that inhibition of survivin by a T34A dominant-negative mutant significantly increased apoptosis of endothelial cells and reduced tumor-associated angiogenesis, which may retard or prevent adequate nourishment of tumors, result in tumor growth stasis and therefore is complementary to the anti-tumor effect of radiation.

As a strategy for cancer therapy, anti-angiogenic therapy attempts to stop new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feeds the cancerous mass (41), but it is well known that oxygen is a potent radiosensitizer, the combination of anti-angiogenic targeting agents with radiation appears to be a counterintuitive strategy because a reduction in tumor vasculature would be expected to reduce blood perfusion and oxygen concentration, so to result in hypoxia in tumor cells which is resistant to radiotherapy (42). However, accumulating experimental evidence revealed that anti-angiogenic agents may actually increase oxygen levels, reduce tumor hypoxia and provide a rationale for combining these agents with radiation (43,44). Tumor oxygenation is a function of perfusion and oxygen consumption, anti-angiogenic agents may increase perfusion by reducing the number of immature and inefficient vessels, and reduce the overall consumption by reducing the number of oxygen-consuming tumor cells and endothelial cells, all of

which give rise to more oxygenated tumor cells that are sensitive to radiation (45).

Survivin is also an attractive target for novel immunotherapies against cancer (46). Previously, the immunological properties of survivin were demonstrated by the detection of survivin-reactive antibodies (47) and cytolytic T-cell clones (48,49). In particular, the survivin-based DNA vaccine can inhibit tumor growth and metastases by evoking an effective T cell-mediated immune response, which simultaneously attacks both the tumor and endothelial cells of tumor vessels, thereby triggering tumor cell apoptosis and suppression of angiogenesis (50) and eventually rendering tumor cells more sensitive to the anti-tumor effect of radiation.

Many attempts have been undertaken in order to understand the mechanisms of radiosensitivity of tumors, our data suggest that a dominant-negative mutant, survivin T34A, could sensitize LLC cells to radiation efficiently and the synergistic anti-tumor activity may in part result from increasing the apoptosis of tumor cells, inhibiting tumor angiogenesis and inducing a tumor-protective immune response in the combined treatment. These findings may serve as a basis for the rational design of future strategies and it appears to be only a matter of time before our knowledge of the mechanisms of radiosensitivity of tumors can be merged and applied to improve the clinical results of tumor radiotherapy.

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