Inhibition of human lung adenocarcinoma growth using survivint34a by low-dose systematic administration

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Anti-apoptosis plays an important role in tumour formation and development. Survivin is a member of the inhibitor of apoptosis (IAP) family, which is a target for anti-cancer drug exploitation was replaced as development. We investigated the role of the homo dominant-negative mutant Survivin-T34A in suppressing human lung adenocarcinomas (A549). The anti-tumour activity of HSurvivinT34A plasmid was evaluated in the A549 cell line and nude mice bearing A549 subcutaneous tumours. Low-dose systemic administration was continuously used. The HSurvivinT34A plasmid (5 μg/one) complexed with a cationic liposome (DOTAP/Chol) significantly inhibited tumour growth in our model. We observed microvessel density degradation by CD31 immunohistochemistry and apoptotic cell increase by TUNEL assay, PI staining and flow cytometric analysis in the treated group. The present findings suggest that the HSurvivinT34A plasmid complexed with a cationic liposome may provide an effective approach to inhibit the growth of human lung adenocarcinomas in vitro and in vivo.


Keywords. Apoptosis; cationic liposome; human lung adenocarcinomas; survivin

Abbreviations used: ANOVA, analysis of variance; FBS, foetal bovine serum; H&E, haematoxylin and eosin; IAP, inhibitor of apoptosis; MVD, microvessel density; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction; PI, propidium iodine; P-S, penicilium/streptomycin; TAL, Tachypleus amebocyte lysate; TUNEL, terminal eoxynucleotidyltransferase-mediated dUTP nick-end labelling; VEGF, vascular endothelial growth factor

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basic studies and phase I trials based on the administration of survivin-directed cytotoxic T lymphocytes have been completed and proved to be effective and safe to move on to clinical phase 2 studies (Pisarev et al. 2003; Tsuruma et al. 2004). In this study, we focus on the underexplored question of whether low-dose HSurvivinT34A plasmids (5 μg/one) complexed with cationic liposomes (DOTAP/Chol) would provide a positive anti-tumour effect on human lung adenocarcinomas by systemic administration.

2. Materials and methods

2.1 Recombinant plasmids vector construction

The recombinant plasmids carrying pORF9-HSurvivinT34A or pVITRO2-neo-mcs null were purchased from Invivogen Corporation., San Diego, California (CA). HSurvivinT34A was amplified by polymerase chain reaction (PCR), using the forward primer 5′-CATACGGCGTCACCATGGGTGC-3′ and reverse primer 5′-CGGGCGTCGACTCATACTGCAG-3′ from pORF-9-HSurvivinT34A. After sequence confirmation, the recombinant plasmid was prepared using an Endofree Plasmid Giga kit (Qiagen, Chatsworth, CA). Endotoxin levels of the plasmid DNA prepared were determined by using Tachypleus amebocyte lysate (TAL). The plasmid PVITRO2-null was used as control.

2.2 Cell lines and culture conditions

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 Medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1% penicillin/streptomycin (P-S). The cultures were maintained in a humidified chamber at 37°C in a 5% CO2 atmosphere.

2.3 Assessment of apoptosis in vitro

The DOTAP: chol liposomes were prepared by our laboratory. The ideal plasmid:liposome ratio for the most efficient gene delivery is 1 μg:3 μg. For transfection, 3 × 10^6 A549 cells were plated into 6-well plates (10 cm² growth area) and incubated overnight. Then 2 μg of DNA (HSurvivinT34A plasmid or PVITRO2 null plasmid) with 6 μl of liposomes were dissolved in 500 μl serum-free 1640 media without antibiotics for 30 min and added directly to the cell cultures. After 6 h, the media were replaced with fresh serum-supplemented media with antibiotics. Cells were harvested for apoptotic analysis 48 h later, as described below.

Samples were resuspended in propidium iodine (PI) and incubated in darkness for 5 min. Apoptosis was observed with fluorescence microscopy and by flow cytometry.

2.4 Animals

Female BALB/C nude (nu/nu) mice (6–8 weeks old) were purchased from the Laboratory Animal Center of Shanghai in China. They were maintained under pathogen-free conditions in our laboratory for animal experiments. All studies involving mice were approved by the Institutional Animal Care and Use Committee.

2.5 Lung tumour xenograft models

In our preliminary experiment, the animals were divided into four dose groups (1 μg, 2.5 μg, 5 μg, 10 μg) of HSurvivin T34A, with five mice per group. It was found that tumour growth was inhibited as much in the 5 μg group as in the 10 μg group. Tumour growth was significantly more inhibited in these two groups than in the 1 μg and 2.5 μg groups. Hence, we chose a 5 μg dose for our formal experiment. Female BALB/C nude (nu/nu) mice were injected subcutaneously with 5 × 10^6 of A549 cells in the right flank on day 0. Primary tumours had a mean diameter of about 3 mm on day 15. The tumour-bearing mice were randomly assigned to the following three groups and received the corresponding dose by injection: (a) NS group mice received 100 μl 0.9% NaCl solution (NS); (b) PVITRO2-null group mice received 5 μg PVITRO2-null plasmid/15 μg liposome complexes (volume =100 μl); and (c) HSurvivinT34A group mice received 5 μg HSurvivinT34A plasmid/15 μg liposome complexes (volume = 100 μl). Each group had 7 mice. We began systemic therapy on day 15 and repeated the injections daily for a total of 10 days. On day 38, all mice were euthanized. Tumour net weight of each mouse was measured. Tumour growth was evaluated by the measurement of tumour diameter every 2 or 3 days and the tumour volume was calculated as length × width² × 0.52. All the data are presented as mean ± SE. Normal organs and tumours from each mouse were removed and fixed in a 4% formaldehyde solution for histological analysis.

2.6 Histology and immunohistochemistry

Primary tumours were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 3–5 μm sections. The sections were then stained with haematoxylin and eosin (H&E) for analysis.

CD31 immunohistochemistry was done for tumour microvessel density (MVD) according to the manual. Rabbit anti-human polyclonal CD31 (ab59251, Abcam company) was diluted 1/200 overnight at 4°C, followed.

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by incubation with biotinylated goat anti-rabbit IgG and streptavidin–biotin–horseradish peroxidase complex at 37°C for 1 h. Positive reactions were observed with 3,3′-diaminobenzidine (DAB substrate kit; Vector Laboratories) as a chromagen. Cellular nuclei were counterstained with ameliorative Gill haematoxylin. Quantification of MVD was assessed according to the method of Peng et al. (2008). At least three fields were counted, and the average was taken as the MVD of each section.

### 2.7 Testing of the anti-apoptotic effect in vivo

The presence of apoptotic cells within the tumour sections was evaluated by the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) technique using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) according to the protocol. Per cent apoptosis was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (5 high power fields/slide).

### 2.8 Statistical analysis

The results of statistical analysis are presented as mean±SD. For comparison of condition at individual time points, differences between the groups were tested by performing analysis of variance (ANOVA) and unpaired Student t tests. All P values were two-sided and statistical significance was defined as P<0.05.

### 3. Results

#### 3.1 Anti-apoptotic effect of HSurvivinT34A plasmid in vitro

Treatment with HSurvivinT34A plasmid contributed to morphological changes characteristic of apoptosis of A549 tumour cells: bright red-fluorescent nuclei (fragmented or intact) displayed by fluorescence microscopy of PI-stained tumour cells: bright red-fluorescent nuclei (fragmented or intact) displayed by fluorescence microscopy of PI-stained.

#### 3.2 Anti-tumour effect of HSurvivin T34A plasmid in vivo

The established human lung adenocarcinoma (A549) xenograft model was used to observe the effect of HSurvivinT34A with cationic liposomes (DOTAP/chol) on the tumour-burdened mice. The treatment regimens were as described in Materials and methods. Tumours were monitored every 2–3 days (figure 2A). After day 23, there were statistical differences in the tumour volumes (*P<0.05) between the treatment group and the controls (including the NS group and PVITRO-2 null group). The inhibitory rate of the treatment group was up to 74.21% and the tumour volume of the vector group was up to about four times larger than that of the treated group (figure 2B). At the termination of the experiment, all the animals were euthanized and the tumour weights were measured (figure 2C). We also observed that the average weight of the tumours in the treated group was reduced by 67.95% when compared with the NS group (*P<0.01). The data showed that the HSurvivinT34A plasmid has a significant influence on the suppression of tumour growth. Meanwhile, no severe adverse consequences were detected by gross measures such as weight loss, ruffling of fur, and changes in behaviour and feeding. Furthermore, no pathological changes in the normal tissues such as heart, liver, spleen, lung or kidney were found by H&E staining (data not shown).

#### 3.3 Inhibition of tumour-induced angiogenesis (CD31) and increase of apoptosis (TUNEL)

Tumour sections from each group were stained with H&E (figure 3A), anti-CD31 antibody and TUNEL reagent in order to evaluate the MVD and apoptosis rate as described above. Tumours in the control groups revealed a high MVD while those in the HSurvivinT34A group had lower values (figure 3B). In quantitative morphometric analysis, the treatment group had a ~3-fold suppression of the MVD: microvessel density in A549 xenografts as compared with the control (figure 3D, *P<0.01). In addition, there were large areas of necrosis and many more apoptotic cells in the treated group (figure 3C). Tumour tissues of the treated group tended to display the highest apoptotic indices at 25.82%±2.16%. The control group’s indices were significantly lower, with the NS group’s apoptotic index at 3.36%±0.62% and PVITRO-2 null group at 6.70%±0.76% (figure 3E, *P<0.01). These data suggest that the HSurvivinT34A plasmid could cause inhibition of angiogenesis and directly increase apoptosis of...
tumour cells, which plays an important role in the treatment of lung cancer.

4. Discussion

Lung cancer is the most common cause of cancer-related mortality globally. Currently, adenocarcinoma is the most common histological type in both sexes, races, and in all age groups.

The IAP survivin deserves attention as a target for cancer therapy due to its differential expression in tumours versus normal tissues and as a bifunctional protein that acts as a suppressor of apoptosis and plays a central role in cell division (Altieri et al. 1999). Survivin may be an important prognostic marker of metastasis and proliferative potential (Mohamed et al. 2009) for patients with non-small cell lung cancer (NSCLC). Previous studies targeting survivin in lung cancer have mainly focused on inducing cell apoptosis (Shen et al. 2009; Mehdi et al. 2001; Blanc-Brude et al. 2003) and the sensitizing effects of radiotherapy (Bo et al. 2004) and chemotherapy (Robert et al. 2000) in vitro. There have been few trials in vivo to date. In our study, we evaluated the therapeutic efficacy of low-dose HSurvivinT34A plasmid on the A549 nude mice model by intravenous injection.

In our experiment, we found that HSurvivinT34A plasmid (5 μg)/DOTAP:chol complexes efficiently inhibited the growth of the human lung adenocarcinoma model by systemic administration. We observed that the average weight of tumours in the treated group was reduced by 67.95% compared with the controls. We believe that therapeutically targeting survivin produced significant results due to the following major mechanisms.

(i) The induction of tumour cell apoptosis: The results of PI staining and flow cytometric analysis in vitro were the same as the results of TUNEL staining.
They suggest that HSurvivinT34A induced significantly more apoptotic cells in the A549 group than the control. These data are consistent with those from previous studies. Phosphorylation at Thr34 of survivin played an essential role in its regulation by cyclin-dependent kinase, p34cdc2-cyclinB1 (Muzio et al. 2001). Elevations in p34cdc2 kinase, induced by spindle checkpoint activation, resulted in increased survivin expression (Connor et al. 2002).

The mutant survivin of Thr34-Ala (T34A) cannot be phosphorylated by p34cdc2-cyclinB1. It has been considered that the Thr34 phosphorylation site appears to be in a position to stabilize anti-apoptotic protein–protein interactions through the BIR domain of survivin (0: Verdecia et al. 2000). Failure to phosphorylate Thr34 leads to the dissociation of a survivin–caspase-9 complex and subsequent caspase-9-dependent apoptosis during cell division (Connor et al. 2000). Other researchers have detected that survivinT34A could be worked as responsible for a mitochondrial-dependent apoptosis with release of cytochrome c and loss of mitochondrial transmembrane potential by targeting caspase 9 and other molecules. Survivin could influence the upstream initiation of mitochondrial-dependent apoptosis. This should be considered a viable hypothesis, especially when one considers the association reported with Smac/DIABLO (Du et al. 2000), a mitochondrially released protein, which relieved the inhibitory function of IAP on caspase-9 activation (Shi 2002).

(ii) The inhibition of angiogenesis: The results of CD31 staining in each group detected that the MVD of the HSurvivinT34A plasmid group obviously decreased compared with the control. Research groups using a breast cancer model have also observed this phenomenon. Previous studies have demonstrated that this may involve upregulated survivin expression during the proliferative phase as well as the non-proliferative phase of angiogenesis as a transcriptional target of vascular endothelial growth factors (VEGF) (Connor et al. 2000). The remodelling of blood vessels is attributed to, among others, angiopoietin-1 (Harfouche et al. 2002; Papapetropoulos et al. 2000). Targeting of survivin resulted in the loss of cytoprotection afforded by VEGF against ceramide or tumour necrosis factor-α-induced apoptosis. This suggests that VEGF protects endothelial cells against apoptosis during angiogenesis by upregulating survivin (Mesri et al. 2001). Angiopoietin-1 has been shown to upregulate survivin in endothelial cells, via a PI3-K/AKT pathway, subsequently protecting them from apoptosis-inducing stimuli. However, interference by a dominant negative survivin resulted in the loss of angiopoietin-1. Thus, targeting survivin, in addition to inhibiting tumour cell growth, may also prove to be beneficial in inducing apoptosis in proliferating endothelial cells of the tumour vasculature.

The gene delivery system we utilized was cationic liposomes. This proved to efficiently increase the therapeutic
efficacy of the HSurvivinT34A plasmid. We continuously employed a low-dose systemic therapy. Other research on DOTAP:chol-DNA complexes following intravenous administration indicate that DOTAP:chol liposomes can efficiently deliver genes to various organs and tissues with maximum delivery to the lung (Ito et al. 2003). Chol liposomes as a delivery system have increased uptake by tumour cells and angiogenic endothelial cells in tumours (Thurston et al. 1998). Repeated multiple treatments can increase gene expression when compared to single-dose treatment (Ramesh et al. 2001). Compared with intratumour injection, intravenous administration may additionally control micrometastases throughout the body and can be extended to clinical application with ease. The findings suggest that there is no other systemic injury through this route of administration and we feel this administration technique should be used for subsequent research.

In our laboratory, Peng et al. (2008) used SurvivinT34A plasmid (25 μg/one) complexed with cationic liposome (DOTAP:chol) by intravenous administration to cause anti-tumour effects in female BALB/c mice bearing 4T1 subcutaneous tumours, but in our preliminary tests, we found that HSurvivinT34A plasmid (5 μg/one) given in the human lung adenocarcinoma model had a similar therapeutic effect.
In this study, we have been able to demonstrate that the HSurvivin T34A plasmid complexed with cationic liposome delivered through low-dose systemic administration can efficiently inhibit the growth of human lung adenocarcinoma in vitro and in vivo. The potential positive therapeutic mechanisms derived from current hypotheses, which are confirmed by our data, are the triggering of apoptosis and inhibition of angiogenesis. Moreover, the positive therapeutic effect may be attributed to the autoimmune response stimulated by intravenous injection. In a clinical setting, gene therapy in the form of low-dose systemic administration can be effective, less toxic, affordable and convenient. Our findings may provide some reference for targeting survivin in lung cancer and suggest that the HSurvivinT34A plasmid can be considered as a feasible and effective treatment approach for lung cancer. We feel this trial provides a strong basis for further research into the effects of HSurvivinT34A plasmid by low-dose systemic administration in a clinical setting.

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