Retinoblastoma 94 Enhances Radiation Treatment of Esophageal Squamous Cell Carcinoma in Vitro and in Vivo

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Esophageal squamous cell carcinoma/Radiotherapy/Retinoblastoma 94/Gene transfection/Xenograft.

We performed the study to investigate whether adenovirus-mediated retinoblastoma 94 (RB94) gene transfer could enhance radiation treatment of esophageal squamous cell carcinoma (ESCC) in vitro and in vivo. ESCC cells (Kyse150 cell line) were cultivated in vitro and tumors originated from the cell line were propagated as xenografts in nude mice. Treatment with Ad-RB94 and/or ionizing radiation (IR) was carried out both in vitro and in vivo with Ad-LacZ control vector and blank control. Cell viability, cell cycle distribution, cell apoptosis, tumor growth and transfected gene expression were evaluated and tumor degeneration was analyzed. The data of quantification real-time PCR assays and immunohistochemistry staining using RB antibody indicated that RB94 was efficiently transfected into Kyse150 cells. In vitro, data of cell growth assay indicated that treatment with Ad-RB94 improved radiation treatment of Kyse150 cells. Tumor xenograft studies, pathological analysis of H.E. staining and Ki67 staining suggested transfecting RB94 enhanced tumor regression induced by radiation treatment in vivo. In addition, data of Annexin V, TUNEL and cell cycle distribution assays proposed combination treatment effectively induced cell apoptosis and cell cycle arresting in G2/M phase. In conclusion, transferring RB94 gene by the adenoviral vector enhances radiation treatment of ESCC.

INTRODUCTION

Esophageal cancer (EC) is a highly malignant neoplasm. Once diagnosed, the 5-year survival rate of patients is only 40%.1 In China, the most frequent subtype of EC is esophageal squamous cell carcinoma (ESCC) which is the fourth most frequent cause of cancer–related deaths.2

Conventional radiotherapy is widely used for ESCC, but high doses of ionizing radiation always results in side effects due to irradiating the adjacent structures.3,4 Novel approaches to enhance the antitumor effect of radiotherapy could decrease doses of radiotherapy with a consequential reduction in side effects and better quality of life. The local-regional biology of ESCC makes this cancer an ideal target for tumor suppressor gene transfer.

The retinoblastoma gene (RB), located on chromosome 13q14, encodes a 928 amino acid, 110 kDa nuclear phosphoprotein which plays a key role in regulating the ability of cells to enter S phase.5 Deregulation of the RB gene is important in a wild array of cancers, such as hepatoma, leukemia, esophagus, bladder, gastro, and breast.5–10 More evidences indicate that gene therapy using wild-type RB can suppress tumor cell growth in vitro and in vivo.11,12 However, the effectiveness of wild-type RB gene therapy is limited by the rapid inactivation caused by phosphorylation of the ectopically expressed RB protein (pRB).13,14 A truncated variant pRB94, lacking the NH2-terminal 112 amino acid residues of the full-length pRB, has been found to have better efficacy than wild-type pRB110 in tumor suppression.11,12 Furthermore, pRB94 showed an increased antitumor efficacy not only in RB-negative but also in RB-positive human pancreatic tumor, head and neck squamous cell carcinoma and bladder cancer.15–17

The findings of pRB94 as a potent tumor suppression agent led us to investigate whether transflecting RB94 gene can enhance radiation treatment of ESCC.

MATERIALS AND METHODS

The cell line
The human esophageal squamous cell carcinoma (ESCC)
cell line Kyse150 (provided by Cancer Institute, Chinese Academy of Medical Sciences, China) was used in all experiments. Cells were propagated in RPMI 1640 with 10% FBS and 1% Penicillin and Streptomycin in humidified atmosphere with 5% CO₂ at 37°C.

**Animals**

Animal experiments were performed on 6 weeks old male nude mice (athymic, BALB/C nu/nu). The care and use of all animals were in accordance with the guidelines of the animal welfare committee of Institute of Radiation Medicine, Chinese Academy of Medical Sciences.

**Construction of recombinant adenoviral vectors**

In previous work, we constructed replication-defective adenoviral vector containing the RB94 gene as described. The replication-defective control adenovirus not carrying the RB94 gene, but Ad-LacZ was obtained from Invitrogen Inc. (USA). Viruses were amplified and plaque-purified. Titers were determined using Trizol reagent (Invitrogen Inc.) according to the manufacturer’s instruction. Total RNA of each group were prepared using Trizol reagent (Invitrogen Inc.) according to the manufacturer’s instruction. Total RNA of each group were used to synthesize the first strand of cDNA using RNA PCR Kit (AMV) Ver.3.0 (Takara Bio Inc., Japan). RB94 transgene expression in vitro was evaluated by qRT-PCR on an ABI Prism 7500 (Applied Biosystems Inc.) with SYBR Green PCR core reagent (ABI Bioscience Inc.) according to the manufacturer’s protocol and triplicate samples were setup. Primers designed for RB110 were 5'-CCT CTC TCT AGG CTT GAG TTT G-3’ as forward and 5'-TTG AGC AAC ATG GGA GGT GAG-3’ as reverse, which cover a fragment of RB110 but not the RB94. Primers of RB were 5’-GAA CAG GAG TGC ACC AGT AG-3’ as forward and 5’-GGC GCC ATT ACA ACC TCA AG-3’ as reverse which span a segment of both RB110 and RB94. Primers of GAPDH, set as the reference, were 5’-CGG GGA AGC TTG TGA TCA ATG G-3’ as forward and 5’-GCC AGT GAT GGC ATG GAC TG-3’ as reverse. Results of the experiment are presented as Ct values, which is defined as the threshold PCR cycle number at which an amplified product is first detected. \( \Delta C_t \) was determined as the mean of triplicate Ct values for RB or RB110 minus the mean of triplicate Ct values for GAPDH. The \( \Delta \Delta C_t \) represents the difference between the total RB and RB110, as calculated by the formula \( \Delta \Delta C_t = (\Delta C_t^{RB} – \Delta C_t^{RB110}) \). The relative expression fold of RB94 with RB110 was calculated by \( 2^{-\Delta \Delta C_t} \).

**Cell cycle analysis**

Cells were seeded at a density of \( 1 \times 10^5 \) cells/well in 6-well tissue culture plates. After cells adhered, quadruplex samples were infected and/or exposed to IR at a dose of 4 Gy and cultured for 24 h aforementioned. Harvested cells samples were infected and/or exposed to IR at a dose of 4 Gy and cultured for 24 h aforementioned. Apoptosis was evaluated by Annexin V-FITC Kit (Beckman Coulter Co.). Cells were resuspended in 1 ml of binding buffer containing 0.5 μg/ml Annexin-V-FITC and 5 μg/ml propidium iodide. Cells were incubated for 30 min at 4°C in the dark, and the fluorescence was read on a LSR II Flow Cytometer (BD Bioscience Inc.). Data from ≥ 10,000 cells were collected and analyzed by FACS Diva software (BD Bioscience Inc.).

**Annexin-V assays**

Cells were seeded at a density of \( 1 \times 10^5 \) cells/well in 6-well tissue culture plates. After cells adhered, quadruplex samples were infected and/or exposed to IR at a dose of 4 Gy and cultured for 24 h aforementioned. Apoptosis was evaluated by Annexin V-FITC Kit (Beckman Coulter Co.). Cells were resuspended in 1 ml of binding buffer containing 0.5 μg/ml Annexin-V-FITC and 5 μg/ml propidium iodide. Cells were incubated for 30 min at 4°C in the dark, and the fluorescence was read on a LSR II Flow Cytometer (BD Bioscience Inc.). Data from ≥ 10,000 cells were collected and analyzed by FACS Diva software (BD Bioscience). Propidium iodide-negative and Annexin-V-FITC-positive cells were considered as the apoptotic population.

**Tumor xenograft studies**

Mice were injected S.C. into posterior flank with \( 1.0 \times 10^7 \) Kyse150 cells suspended in 50 μL serum-free medium. When xenografts reached a mean volume of 35 mm³, mice were divided into five groups (n = 6–8/group) randomly: (a)
Control group; (b) Ad-lacZ infection group; (c) IR group; (d) Ad-RB94 infection group; and (e) Combination treatment with Ad-RB94 infection and IR group. In group (b), (d) and (e), tumors were injected using a 100 μL Hamilton syringe with Ad-LacZ, Ad-RB94, and Ad-RB94 at a MOI of 1:40 in 50 μL volume at days 0, 3, and 7 after the establishment of the groups. Mice of group (c) and (e) were exposed to ionizing radiation at a dose of 4 Gy at days 1, 4, and 8. Tumors were measured over the skin in two dimensions using calipers at days 0, 3, 7, 11, 15. Volumes were calculated according to the formula: \( V (\text{mm}^3) = \text{larger diameter (mm)} \times \text{smaller diameter}^2 \times \pi / 6 \). Eighteen days after establishment of the groups, mice were sacrificed and residual tumor mass was measured in three dimensions and harvested for pathological and immunohistochemical study.

Pathological analysis
Harvested tumors were fixed in 4% neutral buffered formalin and embedded in paraffin using standard procedures. Paraffin embedded tumors were cut in 5 μm sections at the maximum cross-section. Sections were stained with haematoxylin-eosin (H.E.) and analyzed under a microscope (BX51, Olympus, Japan). Apoptosis bodies of each section were counted at least 4 fields observed at 400-fold magnification. The number was semiquantitatively graded as: – (no one), 1+ (1–5 bodies per field), 2+ (6–10 bodies per field), 3+ (> 10 bodies per field). The images were captured and areas of tumors maximum cross-section were calculated by Image-Pro Plus 5.1 software (IPP, Media Cybernetics, Inc. USA).

Immunohistochemistry
The 4 μm thick sections from paraffinembedded tumors were deparaffinized and rehydrated, respectively, using xylene and ethanol and immersed in 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase. Sections were then boiled for 30 min in 10 mmol/L citrate buffer solution (pH6.0) for antigen retrieval. Sections were then incubated with 5% skimmed milk for 20 min and incubated with rabbit anti-human RB protein antibody (Santa Cruz, 1:600), rabbit anti-human Ki67 protein antibody (Abcam, 1:2000) at 37°C for 1 h and visualized using PV-6001 Polymer Detection System (Golden Bridge International Inc.) following the manufacturer’s instructions. Subsequently, slides were incubated with 3, 3’-diaminobenzidine tetrahydrochloride-H₂O₂ solution for visualization and counterstained with haematoxylin. For analysis, four views at 400-fold magnification were chosen randomly on each slide. The images were captured and positive staining was quantified objectively by IPP software. The staining quantitation in control group provided a relative referenced baseline for endogenous pRB110 expression. Exogenous pRB transfected by Ad-RB94 can be calculated by the heightened staining quantitation compared with the baseline.

TUNEL assay
The 4 μm thick sections from paraffinembedded tumors were deparaffinized and rehydrated, respectively, using xylene and ethanol. The slides were rinsed twice with PBS and treated with proteinase K (15 μg/ml in 10 mM Tris/HCl, pH 7.4–8.0) for 15 min at 37°C. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in methanol at room temperature for 10 min. The tissue sections were then analyzed with an in situ Cell Death Detection Kit, POD (Roche, Germany) in accordance with the manufacturer’s instructions. As a negative control, the terminal transferase was omitted. All slides were incubated for 1 min with diaminobenzidine for visualization and counterstained with haematoxylin. Four randomly selected views under a microscope at 400-fold magnification were digitally recorded for each tumor and all cells stained positive for apoptosis within the view were counted with the assistance of IPP software.

Fig. 1. Ad-RB94 transfection and/or IR suppressed tumor cell growth in vitro. Mean viable cell numbers were inspected for 8 days by MTT assays. Cells of control group and Ad-LacZ group grew steadily by linear growth. Combination treatment with Ad-RB94 and IR resulted in more efficient inhibition of cell growth than IR or Ad-RB94 alone.

Fig. 2. Treatment with Ad-RB94 and/or IR suppressed tumor growth in vivo. Tumor growth curve in vivo in five different treatment groups showed that tumors of control group and Ad-LacZ group grew steadily by linear growth. Treatment with Ad-RB94 and/or IR resulted in better inhibition of tumor growth than IR or Ad-RB94 alone at day 18.
Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc.) to determine statistical significance by using the nonparametric tests of Mann-Whitney and Kruskal-Wallis H.

Fig. 3. (A) All pictures were digitally captured and analyzed with assistance of Image-Pro Plus software. (B) Tumor sections were stained with H.E. and tumor cross-section area was calculated. (C, D) Tumor sections were subjected to immunohistochemical analysis using anti-RB and anti-Ki67 antibodies, then RB and Ki67 expression levels were quantified by AOI analyzing. (E) TUNEL assay was performed using In Situ Cell Death Detection Kit POD, tumor cells stained positive for apoptosis were counted.
RESULT

Inhibitory effects on tumor cells in vitro

In our study, Kyse150 cells were treated with Ad-RB94 and/or IR, and cell growth curves were plotted (Fig. 1). It was observed that: (a) Cells of control group and Ad-LacZ group grew steadily by linear growth; (b) Cells of IR group and combination group stepped down their growth since day 4; (c) Combination treatment with Ad-RB94 and IR resulted in better inhibition of cell growth compared with treatment with Ad-RB94 alone or IR alone at day 8 (p < 0.01).

Tumor growth inhibition in vivo

In vivo, the effectiveness of treatment with Ad-RB94 and/or IR to inhibit the growth of planted ESCC tumors was examined (Fig. 2). Till day 7 after groups arrangement, tumor growth in each groups were identical (p > 0.05). Since day 11, tumors of control and Ad-LacZ groups grew abruptly, but tumor growth of Ad-RB94, IR and combination groups was arrested. At day 18, volume of tumors treated with Ad-RB94 and IR were less than that of Ad-RB94 or IR alone, and statistical significant differences (p < 0.01) could be identified.

Mice were sacrificed at day 18. Tumors were harvested and submitted to pathological analysis. The pathological examination of tumor sections stained by H.E. revealed more details of anti-tumor efficiency of Ad-RB94 and/or IR (Fig. 3A). Tumors of control group and Ad-LacZ group grew actively. Meanwhile, treatment with Ad-RB94 and/or IR resulted in cancer cell growth inhibition and apoptosis. Compare with other groups, tumors of combination treatment group had fewer karyokinesis, lower level of nuclei hyperchromasia, and more apoptotic bodies (Table 1), which suggest better anti-tumor potency of combination treatment than that of Ad-RB94 or IR alone. As well, the data suggest combination treatment has better efficacy in inhibiting tumor cell growth than that of Ad-RB94 or IR alone (p < 0.05).

Evaluation of RB94 gene transfer in vitro and in vivo

Quantification Real-Time PCR assays was performed to evaluate the transfection efficiency of Ad-RB94 in vitro (Fig. 4). The RB94 expression can be identified in Ad-RB94 and combination groups, but not in control, Ad-LacZ and IR groups.

In vivo, immunohistochemical assays of RB expression was performed on 4 μm tumor sections (Fig. 3A). RB expression level was quantified by IPP software (Fig. 3C). The calculated RB staining of control group was considered as a baseline and exogenous RB94 could be evaluated. The expression levels in tumors of control and Ad-LacZ group were identical (p > 0.05). Compared with control group, heightened RB staining level, considered as transfected RB94 staining, was seen in Ad-RB94 group and combination group (131.8% and 95.3%, p < 0.01).

Cell cycle and apoptosis

To investigate the mechanism behind the tumor growth inhibiting activity, cell cycle distribution assays were performed in vitro (Fig. 5A). In repeated experiment, flow cytometry showed that cells of IR and combination groups arrested in the G2/M phase, while cells of Ad-RB94 group maintained a cell cycle distribution as control group without cell cycle arrest.

### Table 1. Pathological analysis of harvested tumors.

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<th>Control</th>
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<th>Ad-RB94</th>
<th>Combination</th>
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Fig. 4. Quantification Real-time PCR analysis of comparing relative Rb94 expression in cells of difference groups. The folds of transfected Rb94 expression compared with endogenic Rb110 were calculated. The Rb94 expression could be identified in Ad-Rb94 and combination groups, but not in other 3 groups.
Annexin-V assays analysis revealed early apoptosis of cells treated with Ad-RB94 and/or IR in vitro (Fig. 5B). The apoptosis percentage of cells treated with Ad-RB94 and/or IR raised by 1.07–4.81 folds compared with cells treated with Ad-lacZ or no treatment (p < 0.01). For cells treated with Ad-RB94 and IR, the percentage reached 15.7% which was higher than cells treated with Ad-RB94 or IR alone.

TUNEL assays revealed advanced stage apoptosis of tumor cells (Fig. 3A). DAB coupling showed that the level of TUNEL-positive implanted tumor cells. Comparison with mock treatment (2.6 versus 24.8 cells, P < 0.01), Ad-LacZ treatment (3.1 versus 24.8 cells, P < 0.01), Ad-RB94 alone (14.6 versus 24.8 cells, P < 0.01), and IR alone (17.8 versus 24.8 cells, P < 0.05) revealed a statistically greater apoptotic index for the combination treatment group (Fig. 3E).

DISCUSSION

The study demonstrated that Ad-RB94 enhanced radiation treatment of ESCC in vivo and in vitro. Firstly, it is confirmed that the adenoviral vector can efficiently delivers the RB94 gene into Kyse150 cells in vitro using qRT-PCR assays (Fig. 4) and in vivo using immunohistochemistry analyses (Fig. 3C). Then, the anti-tumor efficiency of Ad-RB94 and IR was confirmed by cell growth assays in vitro (Fig. 1) and tumor xenograft studies in vivo (Fig. 2). In addition, to investigate mechanism of enhanced anti-tumor efficiency, cell cycle distribution (Fig. 5A) and cell apoptosis assays (Fig. 5B, 3E) were performed in vitro and in vivo.

Systematic works were performed to clarify anti-tumor effect of Ad-RB94 and IR. In tumor xenograft studies, tumor volumes were calculated. The growth curves of tumors indicated that radiation induced tumor growth inhibition was enhanced by treatment with Ad-RB94 (Fig. 2). In addition, harvested tumors were presented to pathological examination. By observing H.E. staining slides (Fig. 3A), tumor features, such as tumor margins, cellular atypia, karyokinesis, apoptotic bodies and nuclei hyperchromasia were analyzed, and tumor maximum cross-section area was calculated. The data (Table 1, Fig. 3B) advocate that combination treatment has better anti-tumor effect than treatment with Ad-RB94 or IR alone. Furthermore, Ki67 expression of harvested tumors were identified by immunohistochemistry staining and the expression was quantificated (Fig. 3D). This nuclear antigen, a marker of cell growth, expressed during all phases of the cell cycle, is related to tumor growth, invasion, metastasis and prognosis.

In our work, a declined level of Ki67 expression was discerned in groups treated with Ad-RB94 and/or IR compared with control group and Ad-LacZ group (Fig. 3D), which suggests treatment with IR and/or Ad-RB94 can restrain tumor growth and lead to better prognosis. Furthermore, the level of Ki67 expression in combination group was significantly lower than that of IR or Ad-RB94 alone group, which proposes Ad-RB94 enhanced radiation treatment of ESCC in vivo.

The wild-type RB protein (RBwt) binds to members of the cell cycle progression essential transcription factor family, E2F, thereby suppressing the transcriptional activity of E2F and inhibiting G1/S cell cycle progression. There is growing evidence that RB also has regulatory effects beyond the G1/S boundary, mediating additional cell division control at the S and G2 phases. RB94, lacking only 1 (Thr 5) of the 16 phosphorylation sites reported for the RBwt protein, might behave in a similar way to the phosphorylation mutants RBwt, allowing cells to enter S phase. In a preclinical study, adenovirus-mediated RBwt and RB94 gene were transferred into human pancreatic carcinoma cell lines NP-9, NP-18, and NP-31. Interestingly, treated with Ad-RBwt, NP-9 cells demonstrated a significant increase in the G2 population, but not NP-18 and NP31; treated with Ad-RB94, NP-9 cells resulted in a sharp increase in an accumulation at the S-G2 phase of the cell cycle, but not of NP-18 and NP31 cells. It has been also reported that Ad-RB94 gene therapy in pancreatic cancer cells results in S-G2 cell cycle arrest in tumor cells. But in our works, any change of cell cycle distribution (Fig. 5A) is not observed by RB94 treatment alone. These studies suggest that exogenous RB94 or
RBwt could affect cell cycle distribution of cancer cells in different way due to their individual genetic background, and more works were needed to discover the exact mechanism.

A recent work showed that Ad-RB94 gene transfer in combination with radiation therapy significantly suppresses human head and neck squamous cell carcinoma (HNSCC) tumor growth both in vitro and in vivo. The work suggests that the transgene expression of RB94 in tumor cells may result in G2/M cell cycle arrest. This is advantageous for treatment with radiation therapy because the cells arrested in G2/M phase have greater sensitivity to radiation than the ones arrested in other phases. Similar with the study, our works advocate treatment with Ad-RB94 enhanced radiation treatment of ESCC tumor, the fourth most frequent cause of cancer–related deaths in China, in vivo and in vitro. It is suggested that treatment with Ad-RB94 could be a potential way to enhance radiotherapy on other local-regional squamous cell carcinomas. Different from the HNSCC work, not any change of cell cycle distribution was observed by treatment with Ad-RB94 alone in our work. For treatment with Ad-RB94 or IR alone could induced cell apoptosis in vivo and in vitro (Fig. 3E, 5B), it is supposed that Ad-RB94 enhanced radiation treatment of ESCC by duplicate effect of cell apoptosis induction or other unclear synergetic mechanisms.

RB influences the function of at least two proteins that are important in control of cell death, ABL kinase and c-Jun N-terminal kinase (JNK). The C pocket region of RB that spans amino acids 772–870 binds to the ABL protein, which can interact with a variety of downstream substrates to stimulate cell death pathways. ABL kinase is activated within minutes of IR, but in the presence of RB expression, ABL kinase activation was enhanced 48 h after IR, coincident with the onset of cell death. The C-terminal end of RB, amino acids 768–928, is the site of interaction with JNK. JNK, a kinase mediator of cell death, is an effector of the MAP kinase phosphorylation cascade that phosphorylates JUN at serines 63 and 73 after exposure of the cell to irradiation. Activation of JNK depended on the presence of RB and occurred within 8 h of irradiation. Our study, cells or tumors were treated with Ad-RB94, and were exposed to IR 24 h later. The data of cell apoptosis assays (Fig. 3E, 5B) indicate that combination treatment of Ad-RB94 and IR induces tumor cell apoptosis more than that of Ad-RB94 or IR alone. The exogenous RB94 might enhance IR induced cell apoptosis related to JNK and ABL kinase.

Above all, the study indicates that treatment with adenovirus-mediated RB94 gene transfer and IR significantly inhibits human Kyse150 cell proliferation in vitro and in vivo. Combination treatment of Ad-RB94 and IR can restrain the tumors more effectively than IR alone which proposes that transferring RB94 gene could enhance the antitumor effects of radiotherapy.

ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China (No. 30770638 & 30870583) and Institute of Radiation Medicine Foundations (SF0825 & SF0822).

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Received on April 11, 2011
Revision received on August 19, 2011
Accepted on August 22, 2011