Combined effects of XAF1 and TRAIL on the apoptosis of lung adenocarcinoma cells

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Abstract. This study aimed to investigate the effects and mechanisms of X-linked inhibitor of apoptosis protein (XIAP)-associated factor 1 (XAF1) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on the apoptosis of A549 lung adenocarcinoma cell lines. Recombinant lentiviral vector of Ad5/F35-XAF1 and controlled lentiviral vector of Ad5/F35-Null were transfected into A549 cells at same multiplicity of infection (MOI), respectively. Based on whether recombinant human TRAIL (rhTRAIL) was added or not, cells were divided into different groups as follows: XAF1 group, XAF1 + TRAIL group, XAF1-Null group, and XAF1-Null + TRAIL group. Following culturing for 48 h, the mRNA and protein expression levels of related genes were determined by reverse transcription-quantitative polymerase chain reaction and western blotting analyses, respectively. Cell proliferationand cell apoptosis were detected by MTT assay and Annexin V-FITC/PI double staining, respectively. Xenograft mice models were established with A549 lung adenocarcinoma cells and treated with recombinant virus Ad5/F35-XAF1 and controlled virus Ad5/F35-Null for immunohistochemical analysis. Expression levels of XAFl at the mRNA and protein levels were significantly higher in the XAF1 group and XAF1 + TRAIL groups when compared with the levels in the other groups (P<0.05). Cleavage of apoptosis-associated proteins, poly ADP-ribose polymerase and caspase-3, was noted in the XAF1 + TRAIL group, whereas they were not detected in other groups. Apoptosis rates of A549 cells in the XAF1, Null + TRAIL and XAFl + TRAIL groups were significantly higher than those in the NOR and Null groups (P<0.05). Apoptotic rates were highest in the XAF1 + TRAIL group. In conclusion, these findings suggest that combined use of XAF1 and TRAIL may synergistically induce the apoptosis of A549 lung adenocarcinoma cells.

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Introduction

Lung cancer is one of the primary causes of cancer associated mortalities worldwide and >80% of lung cancer cases are non-small cell lung cancer (NSCLC). In 2008, >1.6 million people were diagnosed with lung cancer, accounting for 13% of all newly diagnosed cancer cases and 1.4 million succumbed to lung cancer, which accounted for 18% of all cancer associated mortalities (1).

Apoptosis is a process of programmed cell death, which maintains a healthy survival/death balance in metazoan cells. Apoptosis is a key regulator of tissue homeostasis and is tightly regulated by the interactions of activating and inhibitory pathways (2). Aberrant inhibition of cellular apoptosis may result in various diseases, including lung cancer (3). The mechanism of apoptosis is complicated and is regulated at many levels. The signals of carcinogenesis regulate the central control points of the apoptotic pathways, including inhibitor of apoptosis (IAP) proteins. Inhibition of apoptosis has an important role in the development of lung cancer (4). It has been reported that X-linked IAP (XIAP)-associated factor 1 (XAF1) is able to inhibit proliferation and induce apoptosis in tumor cells when combined with XIAP directly (5). Furthermore, our previous study indicated that XAF1 induced apoptosis in lung cancer cells (6). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) specifically induces apoptosis of tumor cells, while no toxicity effect on normal cells has been demonstrated (7-9). In this study, recombinant adenoviral vectors were transiently transfected into lung adenocarcinoma cells to recover the expression of XAF1. Apoptotic effects of XAF1 and TRAIL on A549 lung adenocarcinoma cell lines were investigated, which may provide an experimental basis for the application of this treatment in patients with lung cancer.

Materials and methods

Reagents. Recombinant virus Ad5/F35-XAF1 and controlled virus Ad5/F35-Null were constructed by Shanghai R&S

Biotechnology Co., Ltd. (Shanghai, China) and stored in a laboratory (Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China). rhTRAIL was bought from Peprotech, Inc. (Rocky Hill, NJ, USA). Reverse transcription system was provided by Promega Corp. (Madison, WI, USA). Primers of XAF1 and β-actin and 2X Tap PCR MasterMix were from Sangon Biotech Company (Shanghai, China). BCA protein assay kit was bought from Pierce (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Rat anti-human XAF1 primary antibody was supplied by Abcam (Cambridge, UK). Poly ADP-ribose polymerase (PARP) and capase-3 antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Mouse anti-rat secondary antibody was bought from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). MTT and DMSO were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit and flow cytometry kits were supplied by BD Company (Franklin Lakes, NJ, USA).

Experimental animals. A total of 20 four-week old female BALB/c nude mice were provided by Animal Experimental Centre of Shanghai Institutes for Sciences (Shanghai, China). All animal experiments were approved by and conducted according to the ethical guidelines of Medicine Laboratory Animal Ethics Committee of Shanghai Jiaotong School of Medicine (Shanghai, China).

Cell transfection. A549 cells were cultured in RPMI 1640 complete medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS for 24 h. Adenovirus vectors carrying XAF1 and null genes, respectively, were transfected into lung adenocarcinoma A549 cells (NOR cells) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were divided into different groups based on transfection with or without TRAIL: XAF1 group, XAF1 + TRAIL group, Null group, and Null + TRAIL group, NOR group, and Null + TRAIL group. All transfected cells were cultured in RPMI 1640 serum-free medium with 100 PFU/ml multiplicity of infection (MOI). The blank normal control group was cultured without any lentiviral vector transfection. Differing concentrations of TRAIL (25, 50 and 100 ng/ml, respectively) were added to the associated groups. Following incubation for 4 h, the medium as replenished with RPMI 1640 complete medium supplemented with 10% FBS. Cells were collected after culturing for 48 h at 37˚C (5% CO_2) until collection.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. To detect the expression levels of XAF1, total RNA (10 μg) was extracted from cells using TRIzol, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA was synthesized with a SensiMix™ SYBR-Green One-Step kit (Quantace; Bioline Reagents, London, UK), according to the manufacturer's protocols. The cDNA was treated with polymerase inhibitor and stored in fluid nitrogen cryopreservation to prevent degradation. The RT reaction was performed at 42˚C for 30 min. 2xTaq PCR MasterMix was used for PCR and the total reaction volume was 20 μl. Primers of XAF1 were: 5'-TCCGCAATTCATGCT CCACGAGTCCTACTG-3' (forward) and 5'-ACGCGTCGA CAAACTCTGAGTCTGGACAAC-3' (reverse). Primers of β-actin were: 5'-ATCTGGCACCACACCTTCTACAATGAG CTGC-3' (forward) and 5'-CGTCATACTCCTGCTTGCTGA TCCACATCTGC-3' (reverse) (Shanghai R&S Biotechnology Co., Ltd.). Cycling conditions of PCR were: 95˚C for 3 min, followed by 32 cycles at 95˚C for 45 sec, 57˚C for 45 sec, and 72˚C for 45 sec, and final extension at 72˚C for 8 min. PCR products were detected by 2% agarose gel electrophoresis and analyzed via the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Western blot analysis. Total proteins were extracted using radioimmunoprecipitation assay lysis buffer and phenylmethylsulfonyl fluoride at a ratio of 200:2, and the concentration was quantified by BCA kit. Proteins were separated by 10% SDS-PAGE, transferred to membrane, and subsequently blocked with 5% skim milk. Blots were incubated with specific primary antibodies against β-actin (cat no. ab8227), XAF1 (cat no. ab217178), caspase-3 (cat no. 9662) and PARP (cat no. 9542; all 1:1,000), at 4˚C overnight. Following washing three times by PBS, the secondary antibodies were added and shaken for 2 h at room temperature. The membrane was developed and exposed by adding electrochemiluminescence reagent, and the images were analyzed using the gel imaging system. Relative expression was analyzed using Image J 2.0 software (National Institutes of Health, Bethesda, MD, USA).

MTT assay. Cells were seeded at the density of $1x10^4$ cells/well. After 48 h incubation at 37˚C, MTT (5 mg/ml in DMSO) was added to the wells (20 μl/well) after discarding the supernatant liquid and incubated for an additional 4 h. DMSO-treated cellswere used as control. Following this, MTT was replaced by DMSO (100 μl/well). Absorbance (A value) was measured at the wavelength of 570 nm after shaking for 15 min in the dark. Cell proliferation was calculated as follows: Cell proliferation ratio=(experiment A value-control A value)/control A value x100%. Each group was exposed to different concentrations and replicated three times to calculate an average.

Flow cytometry. Transfected A549 lung adenocarcinoma cells ($1x10^5$ cells/well) were cultured and divided into different groups, as outlined. Annexin V-FITC and PI staining fluid were added after incubation at 37˚C for 48 h. Flow cytometry was used to detect apoptosis of cells in each group. Annexin V-positivity indicates the apoptotic cells are in early stage, while PI positive means the cells are necrotic. Annexin V and PI-double positive cells are late apoptotic cells.

Xenograft mice model. A total of 20 four-week old female BALB/c nude mice were divided into four groups (XAF1, XAF1 + TRAIL, Null and Null + TRAIL), as outlined. A549 cell lines (with $1x10^6$/0.1 ml PBS) were injected into the right side of the back ribs of each mouse. When the tumor could be observed by naked eyes, tumor size was measured using a caliper every 7 days. Tumor volume (V) was calculated according to the following formula: V=V=4/3πxL/2x(w/2) 2; where L is a relatively shorter diameter and w is a relatively longer diameter. Animals were sacrificed on day 30 after injection, and their tumors were weighed and harvested. Images were captured of the tumor specimens, which were subsequently

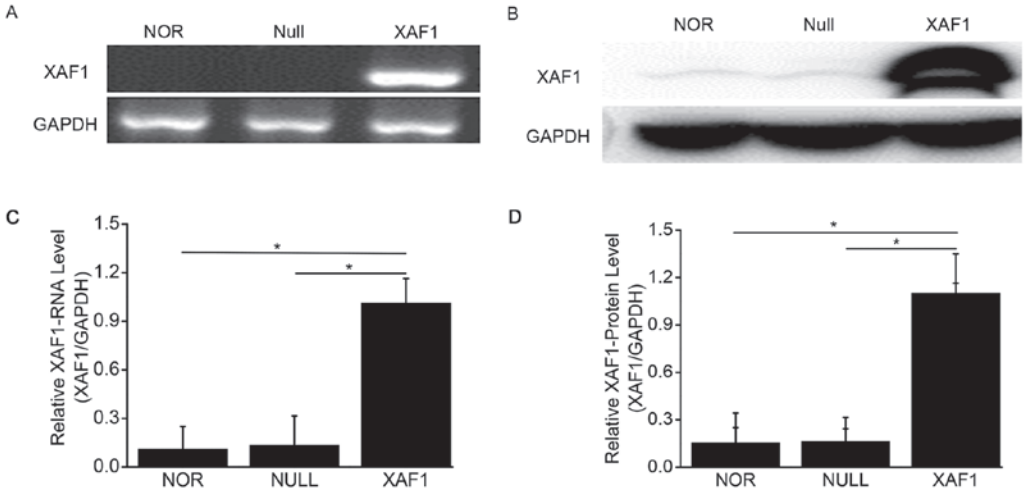

Figure 1. Expression levels of XAF1 mRNA and protein after transient transfection in A549 cells. (A) Reverse transcription-polymerase chain reaction was used to detect the expression of XAF1. (B) Western blot was used to detect the protein expression of XAF1. XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; NOR, normal. (C and D) Quantitative analysis of the expression levels. *P<0.05.

fixed using neutral formaldehyde and paraffin-embedded for further immunohistochemical analysis.

Immunohistochemical (IHC) analysis. Tissue sections were kept at 80˚C for 30 min and subsequently de-paraffinized and rehydrated to retrieve antigen and block peroxidase. Following blocking with non-immune sheep serum, rat anti-human XAF1 primary antibody (1:800; cat no. ab217178; Abcam) was added and incubated at 4˚C overnight. Following this, tumor sections were incubated with horseradish peroxide-conjugated goat anti-rabbit immunoglobulin G (1:1,000; cat no. cw0105; Beijing Cowin Biotech Co., Ltd., Beijing, China) and enough peroxidase substrate was added. A light microscope was used to control staining and PBS was used to terminate the reaction. Nuclei were stained with hematoxylin. Following dehydration and coating with resinene resin, XAF1 IHC slices were observed under a light microscope. Cells stained brown were deemed as positive.

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Analysis of variance (ANOVA) with Tukey's post hoc test was used for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA and protein expression of XAF1 in A549 cells. To explore whether the expression of XAF1 changed in transfected cells, RT-PCR and western blot analysis were employed to detect XAF1 in the different groups of A549 cells. As shown in Fig. 1A, XAF1 mRNA was markedly increased in A549 cells after 48 h of XAF1 group, and XAF1 protein was also upregulated in the XAF1 group when compared with the NOR and Null groups (Fig. 1B). Quantitative analysis of gene and the western blot data were presented respectively (Fig. 1C and D). These results showed that XAF1 was significantly increased after transfection with Ad5/F35-XAF1 vector in A549 cells (P<0.05).

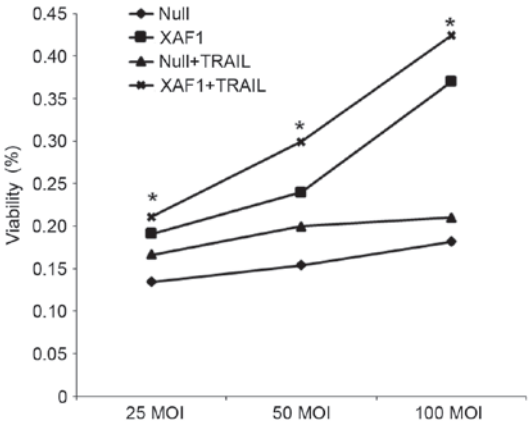

Figure 2. Proliferation of A549 cells after transfection in the four different groups. Proliferation rates of A549 cells in XAFl + TRAIL group were significantly higher than in the XAF1 group (*P<0.05). XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MOI, multiplicity of infection.

Cell proliferation. To investigate the effect of XAF1 and TRAIL on cell proliferation, MTT assay was applied to detect the rate of proliferation in cells of each group. As shown in Fig. 2, the inhibiting effect on the proliferation of A549 cells demonstrated a positive correlation with the concentration of XAF1 in the XAF1 + TRAIL group. Among the Null, XAF1 and Null groups, the inhibition ratio of proliferation was significantly higher in XAF1 group than in the other groups at the same concentration of TRAIL (P<0.05). These findings indicated that TRAIL and different concentrations of XAF1 were able to inhibit the proliferation of A549 cells in a dose-dependent manner.

Cell apoptosis by XAF1. To detect how XAF1 regulated apoptosis in A549 cells, the Annexin V-FITC/PI double staining method was used to assess the different groups. Among the six experimental groups, compared with the XAFl and Null groups, the apoptosis rate in the XAF1 + TRAIL group was significantly higher (P<0.05), whereas no statistically difference was

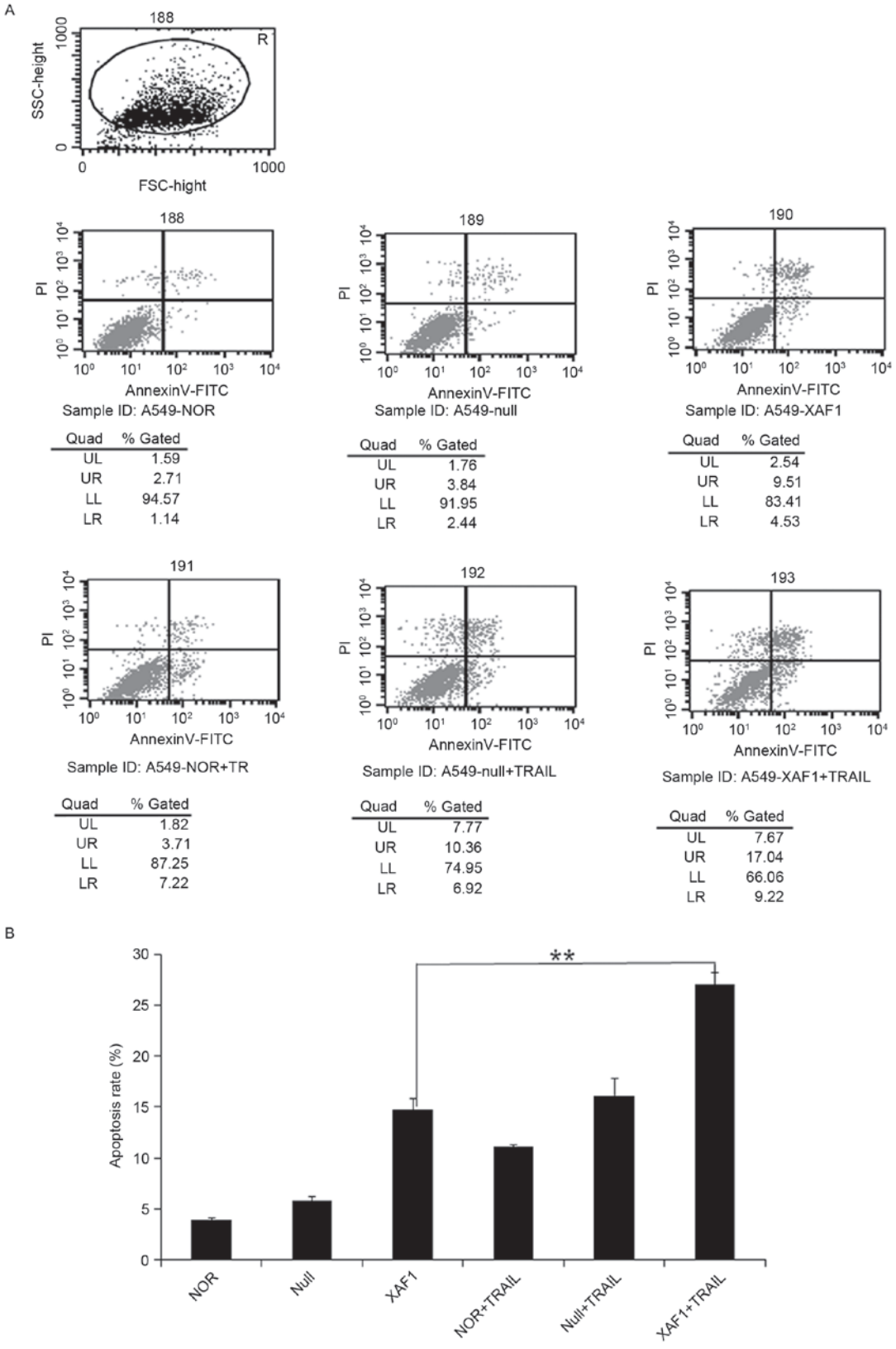

Figure 3. Apoptosis rate of A549 cells after transfection in the different groups. (A) Flow cytometry was used to detect the apoptosis of different groups of cells. Representative flow cytometry results are shown. (B) Apoptosis rate of different groups of cells compared with the NOR group (**P<0.01). XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; NOR, normal; FITC, fluorescein isothiocyanate; PI, propidium iodide; UL, upper left quadrant; UR, upper right quadrant; LL, lower left quadrant; LR, lower right quadrant.

observed between the Null + TRAIL and NOR groups (Fig. 3). These results demonstrated that TRAIL was able to promote the apoptotic effect of Ad5/F35-XAF1 on A549 cells. These findings suggested that a combination of TRAIL and XAF1 may induce cell apoptosis coordinately, with TRAIL enhancing the sensitivity of XAF1 to induce A549 cell apoptosis.

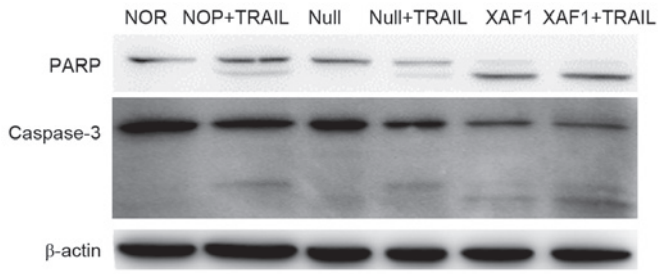

Figure 4. Western blot analysis to detect the expression of PARP and caspase-3 protein in cells of the different groups. XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; NOR, normal; PARP, poly ADP-ribose polymerase.

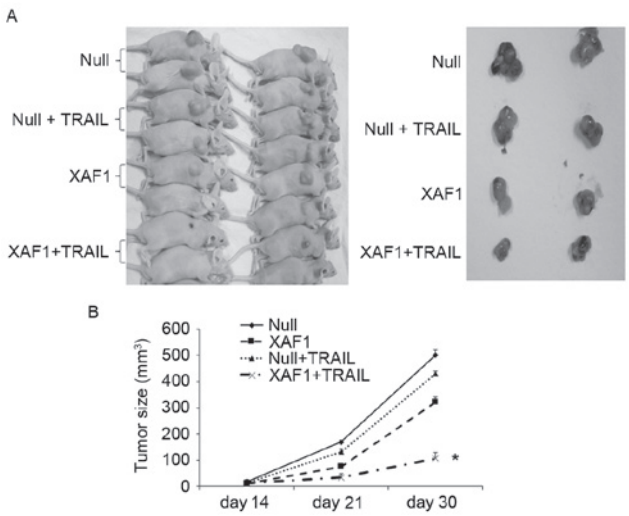

Figure 5. Tumor samples after subcutaneous transplantation of different groups of cells in nude mice. (A) Images of the different tumor samples following transplantation of different groups of cells. (B) Tumor size after subcutaneous transplantation in nude mice. The growth rate of subcutaneously transplanted tumors in the XAF1 + TRAIL group was significantly lower than in the XAF1 group. *P<0.05 compared to XAF1 and NULL+TRAIL group; #P<0.05 compared to NULL and NULL+TRAIL group. XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Expression of apoptosis-related proteins. To detect changes in the expression of apoptosis-related proteins, western blot analysis was used to detect the expression levels of PARP, caspase-3 and XAF1 in A549 cells after transfection with Ad5/F35-XAF1 (MOI 150) for 4 h. As shown in Fig. 4, the results showed that the XAF1 + TRAIL induced marked PARP and caspase-3 cleavage compared with other groups (Fig. 4). No notable difference was observed between the NOR and Null groups.

Transplanted tumor model of nude mice. To determine the impact of XAF1 following combined treatment with TRAIL *in vivo*, a transplanted tumor model of nude mice was generated. Visible tumor tissue was formed 14 days after the injections in the four groups of nude mice (XAF1, XAF1 + TRAIL, Null and Null + TRAIL). Subcutaneously transplanted tumors were stripped from nude mice 30 days after injection (Fig. 5A). Growth rates of subcutaneously transplanted tumors in the XAF1 and XAF1 + TRAIL groups were significantly lower than those of the Null and Null + TRAIL groups ($P<0.05$). Growth rates of subcutaneously transplanted tumors in the XAF1 + TRAIL group were significantly lower than those of the XAF1 group ($P<0.05$) and no significant difference was observed between the Null and Null + TRAIL groups (Fig. 5B). These results indicate that XAF1 + TRAIL inhibited the growth of tumor cells in the murine xenograft model.

Expression of XAF1 protein on transplanted tumor tissue biopsies. To determine the expression of XAF1 protein *in vivo*, tumor tissue biopsies were performed. According to IHC analysis, the expression of XAF1 protein was significantly higher in the XAF1 and XAF1 + TRAIL groups when compared with the Null and Null + TRAIL groups ($P<0.05$; Fig. 6). The results indicate that XAF1 may have important roles in inhibiting the growth of xenograft.

Discussion

Lung cancer, which is one of the most common malignant tumors in China, has become the major cause of cancer-associated mortality due to its gradually increasing morbidity and mortality rates (10). Multiple studies have confirmed that inhibition of cell apoptosis is common and dysregulation of cell apoptosis has an important role in tumorigenesis and tumor progression (3,11). XAF1 is a newly discovered type of XIAP antagonistic protein that was via a yeast two-hybrid system (12). XAF1 is able to directly degrade XAP1 via mitochondrial pathways (13). XAF1 is a tumor suppressor gene that has been demonstrated to decrease multiple tumor cells and tissues in humans (14). Numerous patients with terminal lung cancer who are unsuitable for surgery are administered with conventional chemotherapeutics, which kill tumor cells and normal tissue cells indistinctively, with serious side effects. Therefore, the present study aimed to investigate the combined effects of XAF1 and TRAIL, with the hope that this therapy would kill tumor cells but not normal cells in the A549 lung adenocarcinoma cell line. Whether XAF1 and TRAIL was able to kill lung tumor cells and induce cell apoptosis collaboratively or not was investigated, and the mechanism may provide a novel type of gene therapy in lung cancer.

TRAIL is a tumor-targeted therapy and its specific effect on killing tumor cells has been well-documented (15-17). At present, rhTRAIL alone or combined with chemotherapeutics for the treatment of various human tumors has progressed to phase III clinical trials and no obvious side effects has been observed. This indicates that TRAIL, as a tumor targeted therapy, possesses wide prospects for clinical application (18-20). XAF1 is able to significantly inhibit the growth of gastric cancer and enhance the apoptosis of gastric cancer cells, which prolonged the survival time of the mice under investigation when combined with TRAIL (21,22). In this study, cell proliferation in the XAF1 + TRAIL group was significantly lower than in XAF1 group at the same concentration, while no difference was observed when compared with Null group. This indicates that TRAIL enhanced the killing effect of XAF1 on A549 lung adenocarcinoma cells. Furthermore, XAF1 alone was able to induce lung cancer cell apoptosis and

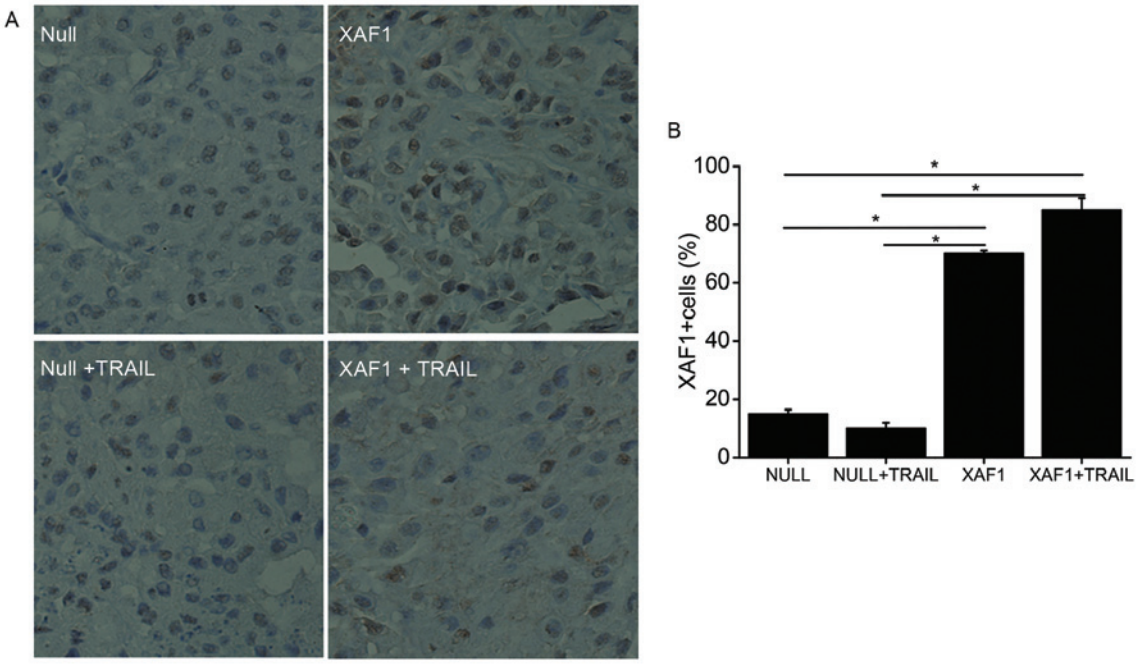

Figure 6. Expression of XAF1 in subcutaneous transplantation of nude mice. (A) Expression of XAF1 was assessed by immunohistochemical analysis (magnification, ´40). (B) Quantitative analysis. *P<0.05.XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

promote apoptosis rates significantly when combined with TRAIL. There was no significant difference between the Null group and Null + TRAIL group, which indicates that XAF1 and TRAIL induce lung cancer cell apoptosis synergistically.

Apoptosis-related proteins have important roles in the progression of programmed cell death. Caspase-3, which is a member of the caspase family of 13 aspartate-specific cysteine proteases that have a central role in the execution of apoptotic mechanisms (23-25) is primarily responsible for the cleavage of PARP during cell death (26). PARP was suggested to contribute to cell death by depleting cells of NAD and ATP (27), as it is activated by binding to DNA ends or strand breaks. The present study investigated the effect of XAF1 and TRAIL on the growth of lung tumors *in vivo* and *in vitro*, and western blotting results confirmed that XAF1 and TRAIL led to activation of caspase-3 and PARP. This result indicates that the mechanism of XAF1 and TRAIL in inhibiting proliferation and inducing apoptosis in A549 lung adenocarcinoma cells is related to caspase-associated apoptosis signaling pathways.

In conclusion, XAF1 combined with TRAIL was able to significantly inhibit proliferation and induce apoptosis synergistically in lung cancer cells *in vivo* and *in vitro*. As TRAIL has been progressed to phase III clinical trials with preferably security, XAF1 + TRAIL may be a potential clinical therapy strategy for the treatment of lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to designing the study, performing experiments, collecting and analyzing data and preparing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by and conducted according to the ethical guidelines of Medicine Laboratory Animal Ethics Committee of Shanghai Jiaotong School of Medicine (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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