Adenovirus vector-mediated FAM176A overexpression induces cell death in human H1299 non-small cell lung cancer cells

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INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality worldwide, and the number of cases and deaths related to lung cancer is on the rise in many parts of the world (1-3). Non-small cell lung carcinoma (NSCLC), which includes adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and bronchioalveolar carcinoma, accounts for nearly 85% of all cases of lung cancer. Cigarette smoking is the most common etiological factor, accounting for nearly 85% of patients with lung cancer. Nonetheless, the number of cases of lung cancer diagnosed in never-smokers has also increased in recent times (4). Despite aggressive efforts, treatments are unsatisfactory and survival rates remain dismal (<20%) (5). Therefore, further understanding of the biology of NSCLC and the development of novel therapeutic approaches for lung cancer treatment are needed.

FAM176A (family with sequence similarity 176 member A) is a novel molecule related to programmed cell death. A decreased expression of FAM176A has been found in several types of human tumors in including lung cancers. In the present study, we investigated the biological activities of FAM176A on the human non-small cell lung cancer cell line H1299 cells. We constructed a recombinant adenovirus 5-FAM176A vector (Ad5-FAM176A) and evaluated the expression and anti-tumor activities in vitro. Cell viability analysis revealed that the adenovirus-mediated increase of FAM176A inhibited the growth of the tumor cells in a dose- and time-dependent manner. This inhibitory effect was mediated by both autophagy and apoptosis that involved caspase activation. In addition, cell cycle analysis suggested that Ad5-FAM176A could induce cell cycle arrest at the G2/M phase, all of which suggested that adenovirus-mediated FAM176A gene transfer might present a new therapeutic approach for lung cancer treatment. [BMB Reports 2014; 47(2): 104-109]

RESULTS

Ad5-FAM176A induces growth arrest of H1299 cells

To explore the potential roles of FAM176A in lung cancer cells, the expression of FAM176A mRNA in three lung cancer cell lines, H1299, A549 and H520, was examined by RT-PCR. As shown in Fig. 1A, the A549 cells expressed high levels of FAM176A mRNA, whereas FAM176A expression was absent in the H1299 and H520 cells. Because H1299 cell fails to express P53 mRNA (Fig. 1A), so we selected the H1299 cells to carry out the subsequent experiments.

We first determined the infection efficiency of type 5 adenovirus in H1299 cells using Ad5-GFP. The cells were infected with Ad5-GFP and flow cytometry analysis suggested that the proportion of Ad5-GFP-positive cells in the H1299
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Fig. 1. Ad5-FAM176A induces growth arrest of H1299 cells in vitro. (A) FAM176A and P53 mRNA expression was analyzed by RT-PCR in H1299, H520 and A549 cells. (B) H1299 cells were infected with Ad5-FAM176A at 100, 200, and 400 MOI or Ad5-Null at 400 MOI for 24 h. The dose-dependent expression of FAM176A was analyzed by western blot. (C) H1299 cells were infected with Ad5-FAM176A or Ad5-Null at 100, 200, and 400 MOI for 48 h. Cell morphological alterations were observed under light microscopy. (D) H1299 cells were infected with either Ad5-FAM176A or Ad5-Null at 100, 200, and 400 MOI for 24 h, 48 h and 72 h. Cell viability was detected by MTT essay. *P < 0.05, **P < 0.001.

Fig. 2. Ad5-FAM176A induces autophagy in H1299 cells. Knockdown of FAM176A inhibits EBSS-induced autophagy in A549 cells. (A) H1299 cells were infected with either Ad5-FAM176A or Ad5-Null combined with Ad5-GFP-LC3 for 22 h. Fluorescence microscopy was used to observe the punctated distribution of GFP-LC3. Representative fluorescence microphotographs are shown. (B) Statistical analysis was used to test the number of cells with GFP-LC3 punctated distribution. ***P < 0.0001. (C) H1299 cells were treated as in (A). The accumulation of GFP-LC3-II were analyzed by western blot. (D) H1299 cells were infected with Ad5-FAM176A or Ad5-Null at 200 MOI for 20 h, and then treated with 10 nM of bafilomycin A1 for 4 h. The levels of endogenous LC3 and FAM176A expression were analyzed by immunoblotting. (E) A549 cells were transfected with either control siRNA or FAM176A siRNA for 20 h, and then treated with EBSS for 2 h. Fluorescence microscopy was used to observe the punctated distribution of LC3 during autophagy. Representative fluorescence microphotographs are shown. (F) A549 cells were transfected with siRNA as (E), treated with 10 nM of bafilomycin A1 with or without EBSS for 4 h. The levels of endogenous LC3 were analyzed by western blot.

Ad5-FAM176A induces autophagy of H1299 cells
We next investigated autophagic effects of Ad5-FAM176A on H1299 cells. The cells were infected with either Ad5-FAM176A or Ad5-Null combined with Ad5-GFP-LC3. After 22 h, we found that the H1299 cells overexpressing FAM176A exhibited greatly punctated GFP-LC3 distribution in contrast to the Ad5-Null-infected cells (Fig. 2A). Quantification of the punctate GFP-LC3 cells from three independent experiments showed that the difference of punctate GFP cells/total GFP cells between the groups was statistically significant (Fig. 2B). We further analyzed
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Ad5-FAM176A induces apoptosis in H1299 cells involving caspase activation

As apoptosis is a factor that affects cell viability, we detected the exposure of phosphatidylserine (PS), which is a key biochemical hallmark of cell apoptosis. The exposure of PS on the surface of apoptotic cells can be easily identified by flow cytometry using FITC-labeled Annexin V, which specifically binds PS. Using this assay, we detected the percentage of cell apoptosis at 24 h, 48 h, and 72 h after transfection with Ad5-FAM176A or Ad5-Null at different MOI. Plasma membrane integrity was assessed by PI staining. Data from flow cytometry analysis revealed that Ad5-FAM176A resulted in a significant dose- and time-dependent increase of apoptotic cells as compared with Ad5-Null. Quantification of the apoptotic cells from three independent experiments showed that the difference between two groups was statistically significant (Fig. 3A). In addition, Ad5-FAM176A also induced cell apoptosis in A549 cells compared with Ad5-Null transfected cells (data not shown).

Caspase-mediated apoptosis is the best-defined cell death program counteracting tumor growth. To confirm the correlation between the activation of caspase cascades and FAM176A, H1299 cells infected with either Ad5-FAM176A or Ad5-Null were assayed for caspase-3, caspase-9 and caspase-8 activities. As shown in Fig. 3C, the Ad5-FAM176A-infected cells exhibited elevated caspase-9 and caspase-3 activities compared with Ad5-Null, but there was no obvious difference for caspase-8 (Fig. 3B). To further determine whether Ad5-FAM176A-induced cell apoptosis was caspase dependent, H1299 cells were pretreated with 50 μM z-VAD-FMK for 2 h before the addition of Ad5-FAM176A for another 24 h. A subsequent Annexin V-binding assay showed that pre-treatment with z-VAD-FMK decreased cell apoptosis (Fig. 3C), indicating that caspase activation at least partly mediated FAM176A-induced cell apoptosis.

Ad5-FAM176A triggers cell cycle arrest at the G2/M phase

Based on the preliminary assays in which we determined the effects of Ad5-FAM176A on cell growth arrest and apoptosis, we next explored the effect of Ad5-FAM176A on cell cycle

![Fig. 3. Ad5-FAM176A induces apoptosis in H1299. (A) H1299 cells were infected with either Ad5-Null or Ad5-FAM176A at 100, 200, 400, and 800 MOI for 24 h, 48 h and 72 h. Apoptotic cells were measured by flow cytometry. The percentages of apoptotic cells were statistical analyzed from three independent experiments. (B) H1299 cells were treated with either Ad5-Null or Ad5-FAM176A at 200 MOI for 22 h. Caspase-3, Caspase-9 and caspase-8 activities were measured using a FLUOstar fluorometer. *P < 0.05, **P < 0.001, ***P < 0.0001, ns, not significant. (C) H1299 cells were pretreated with 50 μM z-VAD-FMK for 2 h before the addition of Ad5-FAM176A for another 24 h. Then treated cells were analyzed by Annexin V-binding assay and flow cytometry.](http://bmbreports.org)
progression. As shown in Fig. 4, cells in the G2/M phase made up 9.04% of the H1299 cells infected with Ad5-Null. Following the infection of H1299 cells with Ad5-FAM176A at 200 and 400 MOI for 48 h, the cell population in the G2/M phase increased to 16.27% and 19.75%, respectively. The results suggested that FAM176A markedly suppressed cell mitotic progression by arresting cells in the G2/M phase, indicating that the inhibition of cell proliferation and induction of cell death in the Ad5-FAM176A-infected H1299 cells may have been associated with the induction of cell cycle arrest at the G2/M phase.

**DISCUSSION**

*FAM176A*, also designated *TMEM166* or *EVA1A* (eva-1 homolog A) by the International Human Gene Nomination Committee, is a novel human gene involved in programmed cell death (6, 9). *FAM176A* is conserved in humans, chimpanzees, rats, mice, and dogs, indicating that it may have important functions in vertebrate animals. Tissue microarray studies indicate that the expression of *FAM176A* protein in most cancer tissues is negative or lower compared with that of normal tissues (7, 8). The restoration of *FAM176A* in some cancer cell lines could induce cell death through both autophagy and apoptosis (9).

In the present study, we evaluated the adenovirus-mediated expression of *FAM176A* in the NSCLC cell line H1299. Flow cytometry and western blotting demonstrated that Ad5-FAM176A significantly increased the level of FAM176A protein. Cell viability analysis showed that Ad5-FAM176A caused significant growth arrest in a time- and dose-dependent manner. Furthermore, we demonstrated that Ad5-FAM176A induced growth arrest through cell autophagy and apoptosis, which may have occurred independently in parallel pathways, or cooperatively, to lead to cell death. In addition, Ad5-FAM176A induced cell cycle arrest at the G2/M phase, which indicated that there might be a relationship between cell cycle arrest and cell death. These data indicate that the adenovirus-mediated expression of *FAM176A* exerted strong anti-tumor activity, implying that it may be potentially used in gene therapy for lung cancer.

A large number of tumor suppressor genes control cell proliferation by regulating cell cycle progression and the apoptotic pathways. Mutations in tumor suppressor genes resulting in their loss of function lead to uncontrolled cell proliferation and tumor development. Most tumors have been shown to contain one or more inactivated tumor suppressor genes that are attributed to their aggressive behavior. The restoration of tumor suppressor gene function through gene delivery can correct the tumor phenotype. For example, p53, one of the most widely studied tumor suppressor genes, is mutated in more than 50% of all human tumors, and is an ideal target for gene replacement therapy (10). The restoration of wild-type p53 by replication-defective adenovirus-mediated gene transfer exhibited an excellent safety profile and resulted in significant anti-tumor effects (11). Furthermore, p53 replacement increases the sensitivity of tumor cells towards chemotherapy and radiotherapy through restoration of the apoptotic pathways, and when used together, a significant increase in anti-tumor efficacy was achieved (12, 13). An adenovirus vector (trademarked as Gendicine) with a p53 expression cassette in the deleted E1 region, has been approved by the State Food and Drug Administration of China for commercial use, thus becoming the first approved gene therapy product for the treatment of cancer (14). Together with previous data, our present study suggests that FAM176A-expressing adenovirus vectors may be able to meet the high expectations for improved cancer treatment and the prevention of a variety of cancers.

Cell death can occur by several mechanisms, and the phenotypic changes that accompany cell death can vary depending on the stimulus and cell setting. In any cell death scenario, the cell decides which pathway to use based on the nature of the stimulus and the particulars of the cell environment. Furthermore, apoptosis and autophagy are not mutually exclusive pathways. They have been shown to act in synergy and in opposition. They share many of the same molecular regulators (15). In a clinical setting, one cannot predict the outcome of the inhibition or activation of one cell death program without considering the effect on another. We demonstrated that recombinant FAM176A could induce cell death characterized by the phenotypes of autophagy and apoptosis. Repeated experiments revealed that the appearance of autophagy-related biochemical parameters preceded apoptosis in the FAM176A-treated cells. Accordingly, the molecular-level cross-talk between apoptosis...
and autophagy induced by FAM176A is under study.

The cell cycle analysis demonstrated that FAM176A markedly increased the percentage of cells in the G2/M phase. It has been proposed that the G1 checkpoint is disabled in the majority of cancers as a prerequisite for tumorigenesis. The G2 checkpoint, however, appears to remain functional in the majority of cancer cells. Many conventional cancer therapeutic agents exert their effect by causing DNA damage; thus, retaining a functional G2 is believed to confer resistance to many such therapeutic agents. To circumvent this, attempts have been made to develop abrogators of this arrest in the G2 phase. Most DNA-damaging agents, including doxorubicin, cause cell death by triggering G2/M-phase cell cycle arrest and inducing apoptosis (16). Here, we propose for the first time that FAM176A also triggers G2/M-phase cell cycle arrest in the H1299 cell line, which interferes with the DNA damage repair processes, followed by the induction of cell death.

In summary, Ad5-FAM176A demonstrated strong anti-tumor activities in the H1299 cell line in vitro through multiple pathways, inducing cell autophagy, apoptosis (via caspase-9 and caspase-3 activation), and cell cycle arrest, all of which contributed to cell death and suppression of tumorigenicity. The current data provide new evidence for the potential application of FAM176A as a valuable anti-cancer strategy for NSCLC treatment.

MATERIALS AND METHODS

Cell culture, treatments and reagents
H1299, H520 and A549 cells were obtained from ATCC (USA) and cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (HyClone, USA). Cell autophagy was induced by nutrient deprivation, through incubation in Earle’s balanced salt solution (EBSS). Autophagy induction was achieved by treating cells with 10 μM bafilomycin A1, a vacuolar-type H+-ATPase (V-ATPase) inhibitor that can block the fusion of autophagosomes with lysosomes. siRNA transfection was performed by MegaTran 1.0 Transfection Reagent (ORIGEN, USA) according to the manufacturer’s instruction. Apoptosis induction was achieved by treating cells with 50 μM z-VAD-FMK. All reagents were purchased from Sigma Aldrich (USA) unless stated otherwise.

Adenoviral vectors
All of the recombinant adenovirus was based on type 5 (E1/E3-deficient) adenovirus. Ad5-Null, Ad5-GFP, and Ad5-GFP-LC3 were purchased from SinoGenoMax (China). The complete coding sequence of FAM176A was subcloned into the BamHI and EcoRI sites of the pShuttle-CMV vector (SinoGenoMax). The expression cassette of FAM176A was then transferred into the adenoviral backbone vector pAd5si, and the recombinant clones of the shuttle plasmid and viral-backbone plasmid of FAM176A were confirmed by DNA sequencing. The recombinant viral plasmid of FAM176A (Ad5-FAM176A) was then linearized by PacI digestion and packaged in HEK293 cells. All of the viral particles were purified by cesium chloride density gradient centrifugation and titrated by TCID50 method.

RT-PCR assay
The detection of FAM176A mRNA expression was carried out by reverse transcription-PCR. The H1299 and A549 cells were collected and the RNA was prepared using TRIzol (Invitrogen Life Technologies, USA). The reverse transcription was carried out with the ThermoScript RT-PCR system (Invitrogen Life Technologies) according to the manufacturer’s protocol. Total RNA (4 μg) was used for the first-strand cDNA synthesis using oligo(dT) as the primer. The complete coding region of FAM176A was amplified by PCR using the forward primer and reverse primer which are available on request. The PCR products were separated via 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

Cell viability assay
Cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 3000-4000 H1299 cells/well were plated in 96-well culture plates and infected by Ad5-Null or Ad5-FAM176A at the indicated multiplicity of infection (MOI) for different durations. The cells were then added to 10 μL MTT and solubilized after incubation for 4-6 h at 37°C. The amount of converted MTT was determined by measuring the absorbance at 570 nm. Cell viability was calculated as follows: cell viability = absorbance of test group/absorbance of uninfected cell group × 100%.

Light and fluorescence microscopy
The H1299 cells were plated on glass coverslips and then infected with various concentrations of Ad5-FAM176A or Ad5-Null for 48 h, and then the cell morphology was observed and photographed under light microscopy. For fluorescence microscopy, cells were plated on glass coverslips and then treated with indicated combinations, the cells were observed using fluorescence microscopy and imaged by a Leica SP2 confocal system (Germany). The accumulation of GFP-LC3 punctate distribution in the cells was considered positive when there were more than 10 dots per cell. The percentages of cells with GFP-LC3 punctate distribution were determined in 10 non-overlapping fields, and statistical analysis was performed on data obtained from three repeated experiments.

Flow cytometry
To detect the efficiency of adenovirus infection, the cells were treated with Ad5-GFP at the indicated MOI for 24 h before harvesting and analysis on a FACSCalibur flow cytometer (Becton Dickinson, USA). An Annexin V-FITC/PI staining detection kit (Beijing Biosea Biotechnology Co., Ltd., China) was used to analyze cell apoptosis according to the manufacturer’s instruction (6).
Caspase activity assay

The H1299 cells were infected with various concentrations of Ad5-FAM176A or Ad5-Null for 22 h, and the activities of caspase-3, caspase-8, and caspase-9 were measured using the CaspaseFluorometric Assay Kit (BioVision, USA). All procedures were carried out according to the manufacturer's instructions. To measure the fluorescence signal, we used a FLUOsstar fluorometer (BMG Labtechnologies, Germany) with a 400-nm excitation filter and a 505-nm emission filter.

Western blot analysis

The treated cells were collected and extracted using RIPA lysis buffer (50 mMTris, pH 7.4, 150 mMNaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and freshly added proteinase inhibitor cocktail). Cell lysates were centrifuged at 16,000 rpm for 15 min at 4°C, and the supernatant was measured using the BCA protein assay reagent (Pierce, USA). Equal amounts of protein were separated by 12.5% or 15% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked and incubated with the indicated antibodies. The protein bands were visualized using an IRDye 800CW-conjugated secondary antibody, and the fluorescence image was obtained using an Odyssey infrared imaging system (LI-COR Biosciences, USA).

Cell cycle analysis

1 × 10^7 H1299 cells were infected with either Ad5-FAM176A or Ad5-Null for 48 h. The cells were then washed and fixed in 70% ethanol overnight. Next, the cells were collected and resuspended in PBS containing 50 μg/ml PI, 0.1 mg/ml RNase, and 5% Triton X-100, and incubated at 37°C for 30 min. Subsequently, the cells were analyzed on a flow cytometer and the percentage of cells in the different phases of the cell cycle was analyzed using ModFit software (BD Biosciences, USA).

Statistical analysis

The data are presented as the mean ± SD. The differences between groups were analyzed using Student's t-test for continuous variables. The statistical significance in this study was set at P < 0.05. All reported P-values are two-sided.

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