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Adenovirus KH901 promotes 5-FU antitumor efficacy and S phase in LoVo cells

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1. ABSTRACT

A combination of oncolytic and chemotherapeutic agents has been used to kill cancer cells. However, the effect of oncolytic adenoviruses on the cell cycle remains to be determined. Cytotoxicity assays were performed to determine cell death in cells treated with 5-fluorouracil (5-FU) alone or in combination with the oncolytic adenovirus KH901. Dynamic changes in the cell cycle, cell proliferation, and apoptosis-related proteins including p-AKT, Bcl-2, Bax, and caspase 3 were investigated after treatment with 5-FU with or without KH901. A higher proportion of S-phase cells were observed after treatment with KH901 and 5-FU than with 5-FU alone. p-AKT, Bcl-2, and Bax expression was increased upon treatment with KH901, whereas the expression of caspase-3 was not induced upon treatment with KH901 with or without 5-FU. KH901 exhibited significant potential as an oncolytic adenovirus and increased cell death in combination with 5-FU in LoVo cells, as compared to 5-FU alone. In conclusion, KH901 stimulates LoVo cells to enter the S-phase by activation of p-AKT, which could partly explain its synergistic effect with 5-FU on LoVo cell cytotoxicity.

2. INTRODUCTION

The use of oncolytic adenoviruses for the treatment of tumors has gained popularity in recent years, and several phase I and II clinical trials to study their efficacy have been performed. Several recombinant adenoviruses have been designed with innovative modifications, in order to improve their specificity and efficacy of replicating in tumor cells and increasing cell killing. The E1A gene promoter of the virus has been replaced with either the TERT (telomerase reverse-transcriptase) gene (1), E2F (2, 3), or survivin (4), in order to induce conditional virus replication in targeted tumors. One of the most universal tumor-specific genes identified thus far is the TERT gene. TERT is a telomerase catalytic subunit that is responsible for the maintenance of the normal length of telomeres. It is believed that TERT is active and highly expressed in >90% of malignant tumors (5) but inactive in the overwhelming majority of somatic human cells (6). KH901 is a conditionally replicating oncolytic serotype 5 adenovirus (Ad5) in which the E1A gene is driven by a modified human TERT promoter. Shen reported that the replication ability of KH901 was more rapid in LNcap, Hep3B, A549, SW620, and HeLa cancer cells and lower in normal

somatic cell lines such as MRC25 and WI38 than that of wild-type Ad5 (7).

In addition to the specific tumor-lysis function, KH901 also induces local and systemic antitumor activity due to the cDNA of human granulocyte macrophage colony-stimulating factor (GM-CSF) that is inserted into the E3 region under the control of the endogenous viral E3 promoter, and which is transactivated by E1A. Thus, the modified telomerase promoter possibly controls GM-CSF expression. Many studies have validated the therapeutic potential of manipulating the cytokine balance in the tumor microenvironment to promote immune-mediated tumor destruction. A comparative analysis of the relative abilities of multiple immunostimulatory molecules to enhance host responses by gene transfer into tumor cells has shown that GM-CSF is the most potent of 30 gene products tested. GM-CSF engenders protective immunity primarily by stimulating recruitment, maturation, and function of dendritic cells. The elevated level of systemic GM-CSF and its benefit in the treatment of tumors have been demonstrated in preclinical and clinical studies of KH901 (9).

5-Fluorouracil (5-FU) is a pyrimidine analog that requires cellular uptake and metabolic activation to exert its cytotoxic effects (10). It is widely used in the treatment of a range of cancers, particularly colorectal cancer, but also those of the breast, head and neck, and digestive tract (11).

Oncolytic adenoviruses have been shown to exert a synergistic effect on chemotherapeutic drugs (12–15), but the exact mechanism is not clear. In the present study, we aimed to evaluate the mechanism of action of the combination treatment of KH901 and 5-FU in colon cancer. We examined the effects of KH901 alone and in combination with 5-FU on the dynamic changes in the cell cycle as well as the expression of apoptosis-related proteins.

3. MATERIALS AND METHODS

3.1. Cell lines, cell culture, and drugs

Human colorectal carcinoma cell lines were purchased from the Shanghai Cell Collection (Shanghai, China). HEK293 and LoVo cells were maintained in RMPI-1640 supplemented with 2% or 10% heat-inactivated fetal bovine serum (Life Technologies Incorporation, Carlsbad, CA, USA), respectively, at 37°C in a 95% air/5% CO_2 humidified incubator (Thermo Fisher Scientific, Massachusetts, USA). 5-FU was commercially purchased (Sigma High-Tech Co. Ltd., Shanghai, China).

3.2. CPE assay of KH901

The conditionally replicating oncolytic adenovirus KH901 and the wild-type adenovirus were obtained from Kanghong Pharmaceuticals (Chengdu, China). To determine the infectivity of KH901, MOI (multiplicity of infection) was used as a parameter in the cytopathic-effect assay (CPE). HEK293 cells were cultured in RPMI-1640 containing 2% fetal bovine serum, and 10^5 cells per well were plated in 10-well plates, incubated for 24 hours, and then infected with either KH901 or wild-type adenovirus at an increasing MOI of 0, 0.001, 0.01, 0.1, and 1 pfu/cell for an additional 48 hours. The culture medium was then removed, and the cells were stained with crystal violet (0.5% in 20% methanol; Sigma).

3.3. Cell-viability assay

For crystal-violet staining, 2×10^5 LoVo cells were incubated for 24 hours in 6-well plates, treated with 0.5 or 1 mg/mL 5-FU, alone or in combination with KH901 at an MOI of 10 pfu, and incubated for an additional 48 hours. The culture medium was then removed, and the cells were stained with crystal violet (0.5% in 20% methanol; Sigma).

For the quantitative cell-viability assay, 4000 LoVo cells were incubated in 96-well tissue culture plates for 24 hours and treated with various concentrations of 5-FU, alone or in combination with KH901, in PBS for an additional 72 hours. The medium was removed and 100 μL fresh medium containing cholecystokinin-8 (Dojindo, Kyushu Island, Japan) was added to each well. Cells were then incubated at 37°C for 3 hours, and the absorbance of each well was read on a Bio-Rad microplate reader (Hercules, CA) at 450 nm. The cell-inhibition rate is assessed by the equation: inhibition rate = 100% - absorption of the treated group/control.

3.4. Flow cytometry assay

LoVo cells were seeded in 6-well plates at 2×10^5 cells per well and cultured for 24 hours before treatment with 0.5 mg/mL 5-FU alone or in combination with KH901 at an MOI of 4 pfu for 24, 48, and 72 hours. Control cells received only PBS for the times indicated above. Cells were collected, trypsinized, pelleted, and washed in pre-cooled PBS, followed by addition of 100% ethanol to a final concentration of 75% ethanol. Next, cells were washed twice with pre-cooled PBS, re-suspended in 300–500 μL PBS (including 20 μg/mL RNase A, 0.2% Triton X-100, and 0.2 mM EDTA), and placed in a water bath at 37°C for 15–30 minutes. A FACSCalibur flow cytometer (Becton, Dickinson and Company) was used to examine the DNA content of the cells, and cell cycle analysis was performed using the ModifitLT cell cycle analysis software.

3.5. Western blotting

The protein expression of cell cycle-regulated genes was examined by western blotting. Cells were treated with 5-FU at 0.5 mg/mL, alone or in combination with KH901 at an MOI of 4 pfu, for 24 and 48 hours. Cells treated with PBS were used as a control. The cells were lysed in cell-lysis buffer for western blotting. Samples containing equal amounts of protein were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes, followed by blocking with 5% (w/v) non-fat dry milk. The membranes were then incubated with the primary antibody, followed by the appropriate secondary antibody. Signals and images were visualized by enhanced chemiluminescence, according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The primary antibodies used were mouse anti-human p-AKT,

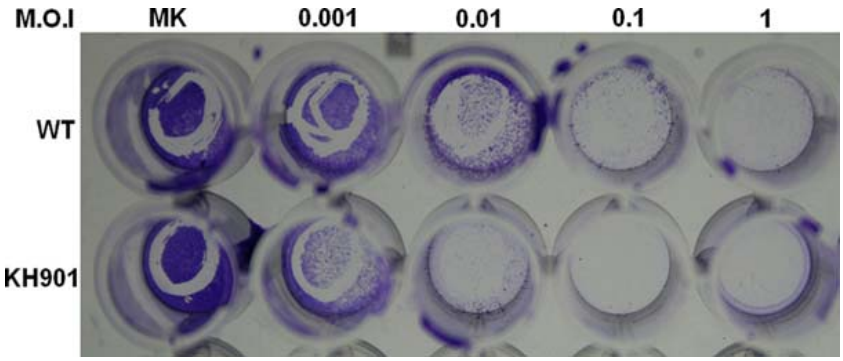

Figure 1. The CPE assay of KH901 and wild-type adenovirus in HEK293 cells, it showed that KH901 could effectively replicate in HEK293 cells, and the CPE of KH901 was much stronger than that of wild-type adenovirus.

Bcl-2, Bax-1, and caspase-3 (Merck & Co, Darmstadt, German).

3.6. Statistical analysis

Data are displayed as means. The statistical significance of differences between various groups was analyzed by analysis of variance (ANOVA) or Student's *t* test, as appropriate. The statistical analysis was performed using SPSS version 13.0 software. Differences among groups were regarded as significant if $p < 0.05$.

4. RESULTS

4.1. Synergistic effect of the adenovirus KH901 and 5-FU on cell death

The CPE assay of KH901 and the wild-type adenovirus in HEK293 cells demonstrated that KH901 could effectively replicate in HEK293 cells and that it was more potent than the wild-type adenovirus (Figure 1). A greater level of cell death was noted by crystal-violet staining of cells treated with both KH901 and 5-FU together (Figure 2A). Cell killing was assessed using various concentrations of 5-FU, alone or in combination with various MOIs of KH901, in LoVo cells. An increase in the MOI of KH901 from 1 to 100 was accompanied by a sequential enhancement in cell killing ($p < 0.01$). Inhibition rates of 50%, 40%, 30%, and 20% were noted at 6, 3, 1.5, and 0.7 pfu, respectively (Figure 2B). A progressive killing ability was also noted with increasing concentrations of 5-FU alone ($p < 0.01$; Figure 2C). Inhibition rates of 50%, 40%, 30%, and 20% were observed at concentrations of 9.84×10^{-6} mol/L , 3.76×10^{-6} mol/L, 1.44×10^{-6} mol/L, and 5.50×10^{-6} mol/L, respectively.

A 2-factor, 4-level orthogonal experimental design was used for the assessment of the killing of LoVo cells by a combination of KH901 and 5-FU (Table 1). MOIs of KH901 were fixed at 0 (as control) and 0.7, while 5-FU at concentrations of 0, 5.50×10^{-7} mol/L, 1.44×10^{-6} mol/L, 3.76×10^{-6} mol/L, and 9.84×10^{-6} mol/L were added to each serial dose of KH901. KH901 and 5-FU had a synergistic effect in killing LoVo cells ($F = 497.349$, $p < 0.01$; Figure 2D).

4.2. KH901 induced LoVo cells to enter the S-phase

The proportions of G0/G1 and S-phase cells in the control, 5-FU-treated, KH901-treated, and 5-FU/KH901-treated groups are shown in Figure 3. Compared to the 5-FU group, the proportion of cells in the S-phase in the KH901 and combination groups was significantly higher at 24 and 48 hours ($p < 0.01$; Table 1)

4.3. The expression of p-AKT, Bax, and Bcl-2 was significantly elevated in cells treated with KH901

The expression of p-AKT was significantly elevated and that of bcl-2 was markedly increased in the KH901-treated and 5-FU/KH901-treated groups, as compared to the control and 5-FU groups. Expression of Bax was the highest in the KH901group, followed by the 5-FU/KH901 group, and least in the 5-FU group. There were no differences in the expression of caspase 3 among the 4 groups. Western blotting results are presented in Figure 4.

5. DISCUSSION

Oncolytic adenoviruses have been shown to exert a synergistic effect with chemotherapeutic agents, resulting in a better response and reduced toxicity of the chemotherapeutic agents in several types of cancers (16, 17).

In concurrence with previous results (16–18), we observed that KH901 markedly increased the killing ability of 5-FU in a synergistic manner. Additionally, as previously demonstrated (19), we found a significant increase in cells in the S-phase in the KH901 and the 5-FU/KH901 groups, as compared to the 5-FU group. It has been shown that the S-phase of cells facilitates the replication and proliferation of adenovirus, and that the adenovirus infection in turn induces infected cells to enter the proliferative stage (20). Retinoblastoma (RB) and RB-related proteins can bind to E2F and regulate the cell cycle, whereas the conservative region of E1A (CR1 and CR2) can bind to and displace RB and RB-related proteins from E2F. This activates the E2F gene and stimulates the cells to move from the G0 to the S-phase (21, 22). Kolli demonstrated that the conservative region of E1A can bind to and inactivate p300, which can lead to an enhanced activity of the c-myc gene, which also stimulates the cells to move from the G0 to the S-phase (23). Cherubini also showed that the ONYX-015 oncolytic virus induces cells with a normal p53 expression to bypass the G1/S and G2/M cell-cycle checkpoints, thus

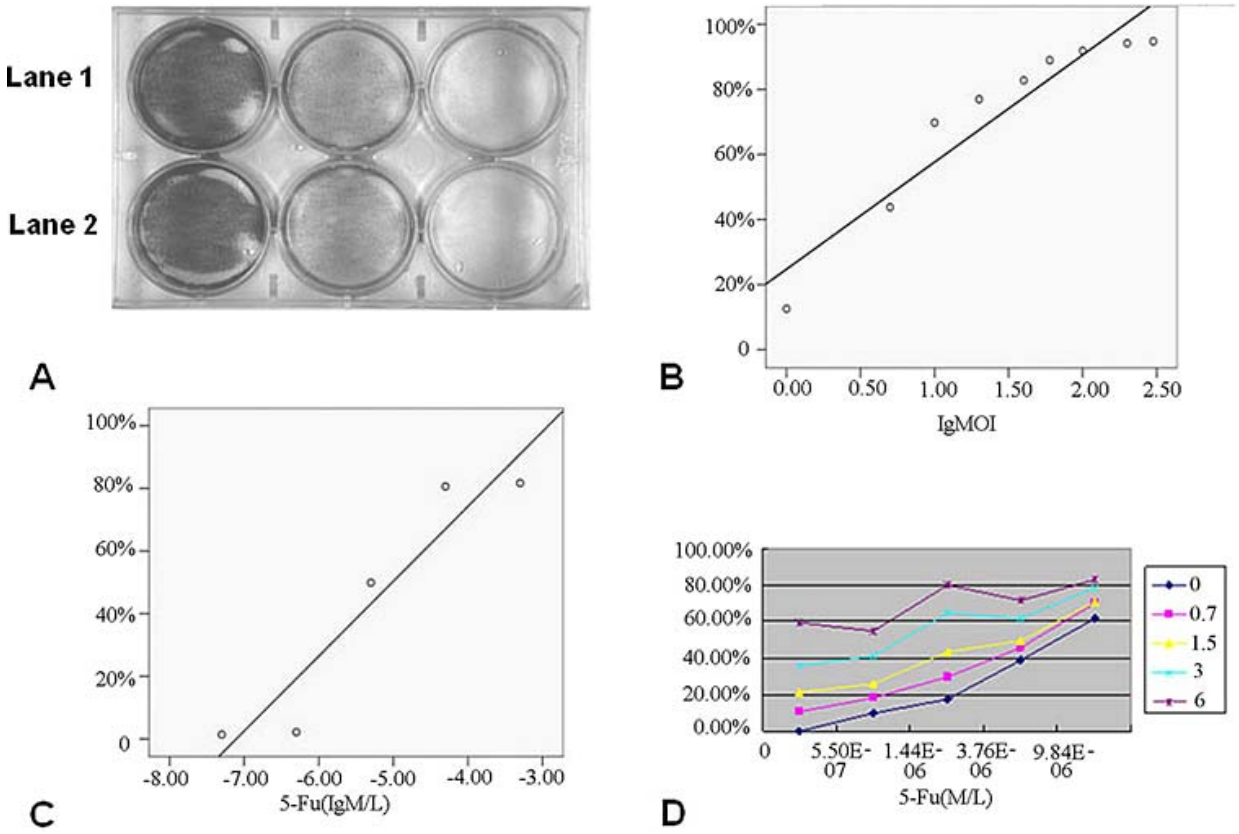

Figure 2. Cytotoxicity assay. A: Lane 1, from left to the right, cells was treated with 1.0 mg/ml 5-FU, 1.0 mg/ml 5-FU plus KH901 at an MOI of 1, and 1.0 mg/ml 5-FU plus KH901 at an MOI of 10. Lane 2, from left to the right, cells were treated with 0.5 mg/ml 5-FU, 0.5 mg/ml 5-FU plus KH901 at an MOI of 1, and 0.5 mg/ml 5-FU plus KH901 at an MOI of 10, respectively. B: Inhibition rate was assessed after 72 hours infection with KH901. C: Inhibition rate of LoVo cells was assessed after 72 hours treatment with 5-FU at various concentrations. D: Inhibition rate was assessed after treatment with 5-FU and/or KH901 for 72 hours.

accelerating cell proliferation (19).

Our study showed that the expression of p-AKT was significantly upregulated in the 5-FU and 5-FU/KH901 groups as compared to the control and the 5-FU groups. Activation of AKT is known to stimulate cell proliferation through multiple downstream targets that influence cell-cycle regulation. AKT phosphorylates the cyclin-dependent kinase inhibitors p21Cip1/WAF1 and p27Kip1, promoting their cytosolic localization and preventing their cell-cycle inhibitory effects. AKT phosphorylation of other targets such as GSK3, TSC2, and PRAS40 also possibly induces cell proliferation through the regulation of the stability and synthesis of proteins involved in cell-cycle entry (24, 25). Icyuz has demonstrated that wild-type Ad5 infection promotes the expression and activation of AKT1 in normal pancreatic islets (26). They have suggested that adenovirus infection can activate cell survival and proliferation, in particular, by operating through the PI3K/AKT signaling pathway (26).

We found that the expression of Bax was markedly elevated by KH901. We suggest that the Bax gene accelerated the progression of the cells into the S-phase, and together with the activation of p-AKT, contributed to the S-phase stimulation induced by KH901.

In our study, expression of Bax was elevated in the KH901 group, but the expression of the apoptotic gene, caspase 3, was not. This could be explained by the structure of KH901. The E1B-55k gene in KH901 inhibits p53-dependent apoptosis (27), whereas E1B-19k binds to Bax, thus blocking activation of caspase-3 and -9 and the ensuing apoptosis (28). Additionally, the expression of Bcl-2, an inhibitor of apoptotic proteins, was also elevated, which may act together with E1B-19k to block p53-independent apoptosis. Furthermore, we found that 5-FU inhibited the expression of Bax. Further investigation needs to be conducted to elucidate this process.

Bcl-2 is an anti-apoptotic gene that enhances the G0 phase and delays the G0 to S transition, whereas Bax is a pro-apoptotic gene. As observed in our study, there was simultaneous elevated expression of Bcl-2 and Bax in the KH901 group. This seemingly paradoxical expression of the 2 genes could be attributed to the complexity of the Bcl family and its interplay (29–31) and needs to be further investigated. In conclusion, our study revealed that the

Table 1. Cell distribution assay results

Groups		24 h	48 h
Control	G0/G1	34.32%	37.47%
	S	60.40%	42.89%
5-FU group	G0/G1	44.66%	49.26%
	S	28.28%	23.27%
KH901 group	G0/G1	37.26%	47.05%
	S	42.90%	41.38%
5-FU plus KH901 group	G0/G1	43.14%	37.29%
	S	41.44%	60.75%

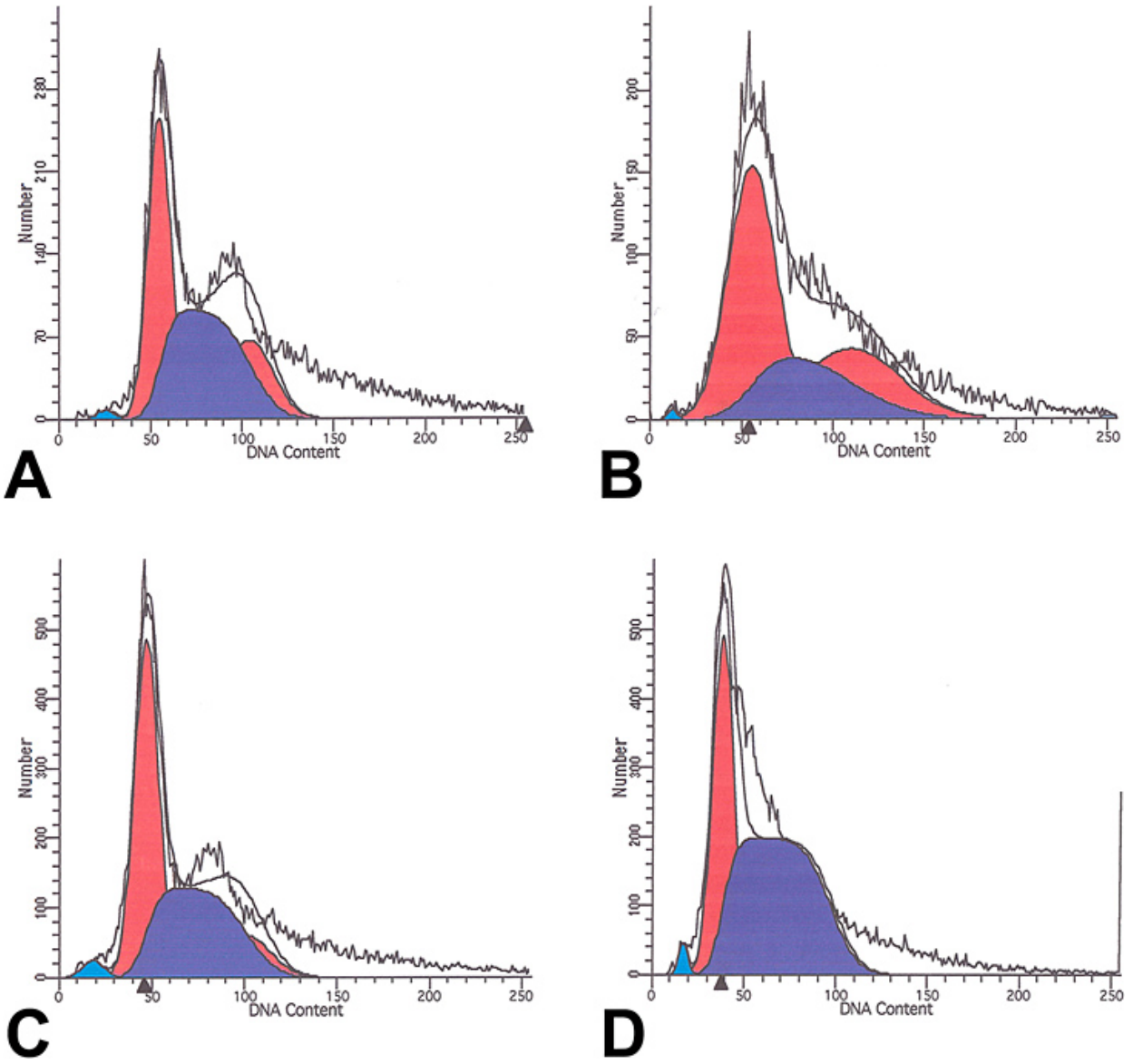

Figure 3. Cell cycle analysis by flow cytometry. A: Cell distribution of the control group after 48 hours. B: Cell distribution after treatment with 5-FU for 48 hours. C: Cell distribution after treatment with KH901 for 48 hours. D: Cell distribution after treatment with KH901 plus 5-FU for 48 hours (blue part represents S-phase cells).

oncolytic adenovirus KH901 had a synergistic effect on 5-FU-mediated antitumor activity, possibly due in part to an increased number of cells in the S-phase. We also demonstrated that p-AKT activation by KH901 stimulated the entry of cells into the S-phase. Our results showed in vitro evidence of the effectiveness of the combined application of KH901 and 5-FU. Because 5-FU is currently being used in chemotherapy of colorectal cancer, our study indicates that a combination of KH901 and 5-FU could have a potential application in the treatment of colorectal cancer. However, further investigation including animal studies and

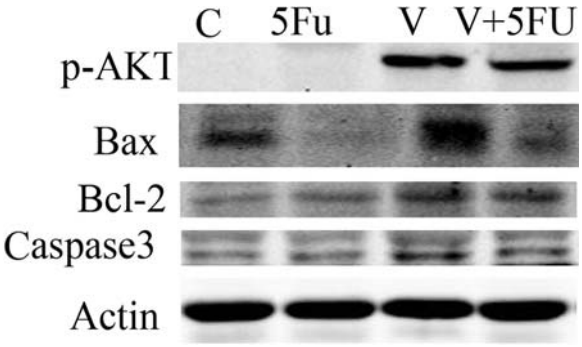

Figure 4. Results of western blotting: showing of p-AKT, Bcl-2, Bax and caspase 3 in the four groups of cells. Lane C: control cells harvested at 24 hours;5-FU, 5-FU-treated cells harvested at 48 hours; V, cells infected with KH901 virus, harvested at 48 hours; V+5-FU,KH901 infected cells treated with 5-FU,harvested at 48 hours.

clinical trials is required.

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Abbreviations: 5-FU: 5-fluorouracil, TERT: telomerase reverse-transcriptase, GM-CSF: granulocyte macrophage colony-stimulating factor, RB: Retinoblastoma

Key Words: KH901, 5-fluorouracil, p-AKT, LoVo cells, Antitumor efficacy, S phase

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