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Effects of adenovirus vectors mediated human lactoferrin cDNA on mice bearing EMT6 breast carcinoma

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Human lactoferrin (hLTF) is an 80KD iron-binding protein. It has been reported that hLTF exists anti-tumor effects. In this study Adenovirus Vectors Mediated Human Lactoferrin cDNA (ad-rhLTF) was constructed and an antitumor effects of ad-rhLTF were investigated in mice bearing EMT6 breast carcinoma. The results demonstrated that ad-rhLTF (5×10^8 and 25×10^8 pfu/ml local injection) had high expression in tumor tissues and effectively reduced the weight of EMT6 breast tumors. Compared with the control group, cell cycle assay by flow cytometry showed that ad-rhLTF increased the percentage of tumor cells in the Sub-G_1 phase and G_0/G_1 phase and the apoptotic number reached to 23.2% in ad-rhLTF group (25×10^8 pfu/ml). Ad-rhLTF treatment also resulted in a decrease of Bcl-2 and an increase in Bax and caspase 3 expressions, which was demonstrated by immunohistochemical analysis and RT-PCR. These data suggest that the antitumor effects of ad-rhLTF might be associated with arresting tumor cells in the G_0/G_1 phase, inducing cell apoptosis and regulation of the expression of Bcl-2, Bax and activation of caspase 3.

1. Introduction

The human lactoferrin (hLTF) is an 80-kDa iron-binding glycoprotein (González-Chávez et al. 2009). It exhibits many useful biological activities that have been used in antibacterial, antivirus, antioxidant and immunoregulation (Mulder et al. 2008; Connely 2001; Ward et al. 2002). It has been reported that hLTF has an antitumor effect and inhibits the proliferation of different tumor cells which include esophageal carcinoma, oral cancer, lung cancer, liver cancer, colon carcinoma and blander cancer (Maden et al. 2005; Hiroyuki et al. 2002). But there is no report on pharmacologic mechanisms of hLTF against breast cancer.

However, it is quite difficult to get natural hLTF because of its complex extraction, low content and activity (Keiichi 2000). In previous works, we had constructed the adenoviral vectors containing the hLTF cDNA and green fluorescent protein (GFP) gene as reported gene and obtained high-level expression of hLTF in the milk of goats and rabbits (Han et al. 2007, 2008).

In this study, we injected ad-rhLTF that had been purified and titrated into the tumor site in different concentrations. The purpose was to explore the therapeutic potential of ad-rhLTF for breast cancer, to unravel possible mechanisms responsible for the antitumor activity of ad-rhLTF, and to provide experimental evidence for breast cancer therapy of ad-rhLTF.

2. Investigations and results

2.1. Virus Preparation

We have generated an adenovirus vector, ad-rhLTF, which carries the complete coding sequence of the human LTF gene. The intensity of GFP fluorescence indicated the high-expression

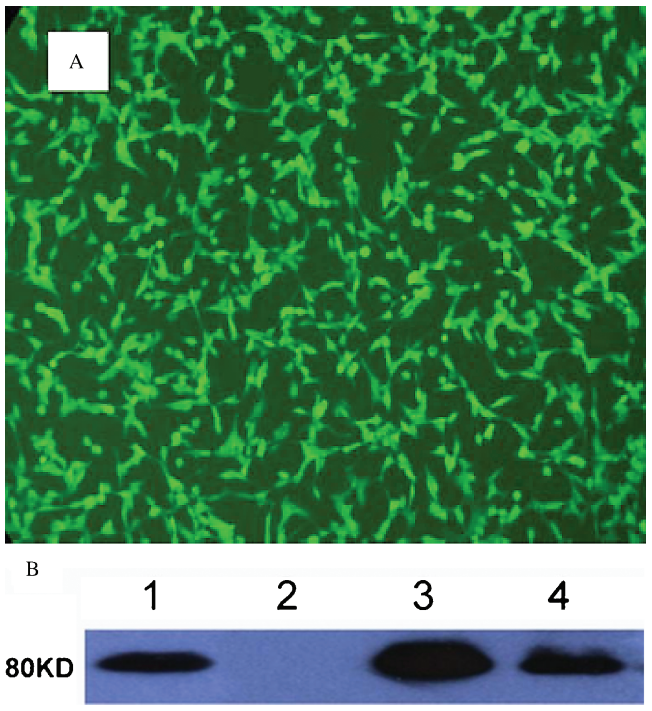


Fig. 1: A the fluorescent picture of 293 cells transfected ad-rhLTF (× 200). B Western blotting analysis of the expression of ad-rhLTF in tumor tissues. 15 μl of protein samples was loaded in each well. Lane 1, the standard of hLTF protein; lane 2, protein sample from the control group; lanes 3–4, protein samples from administrated with ad-rhLTF (25×10^8 pfu/ml and 5×10^8 pfu/ml).

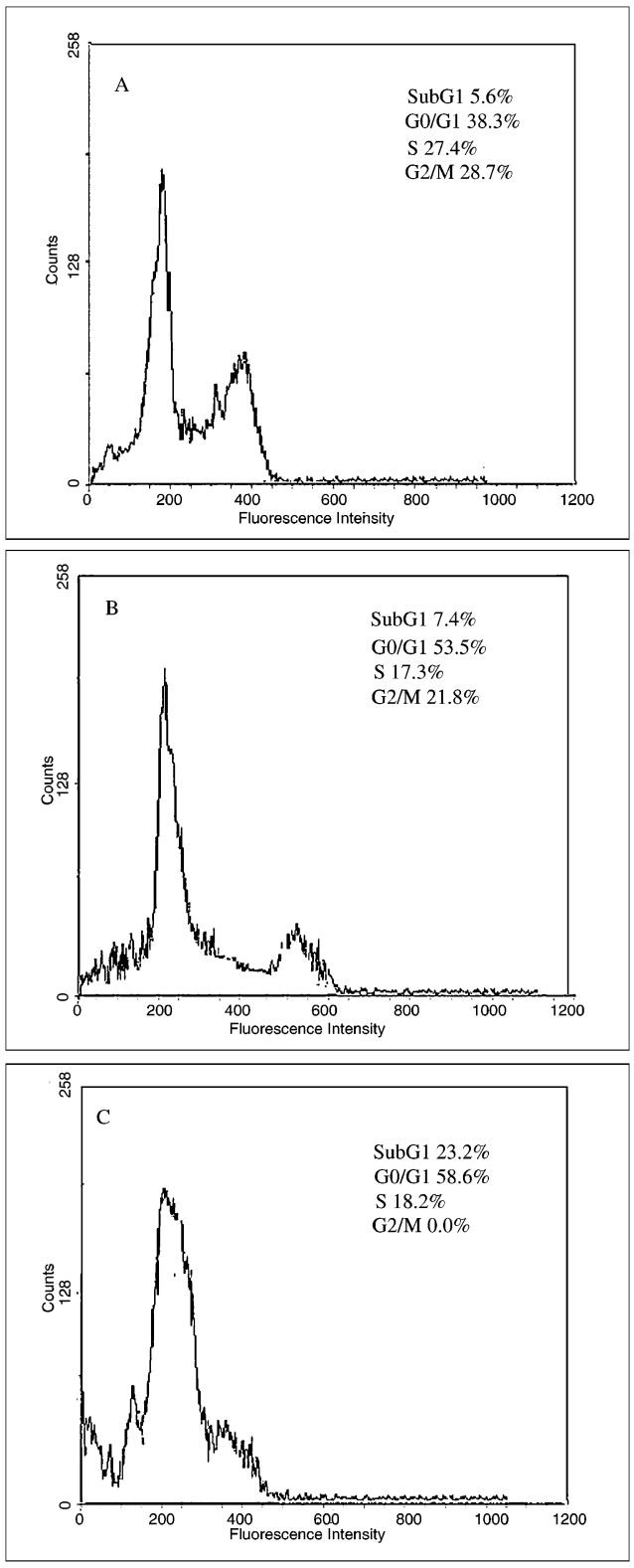


Fig. 2: Effect of ad-rhLTF on tumor cell cycle. A control group B CTX group C ad-rhLTF group.

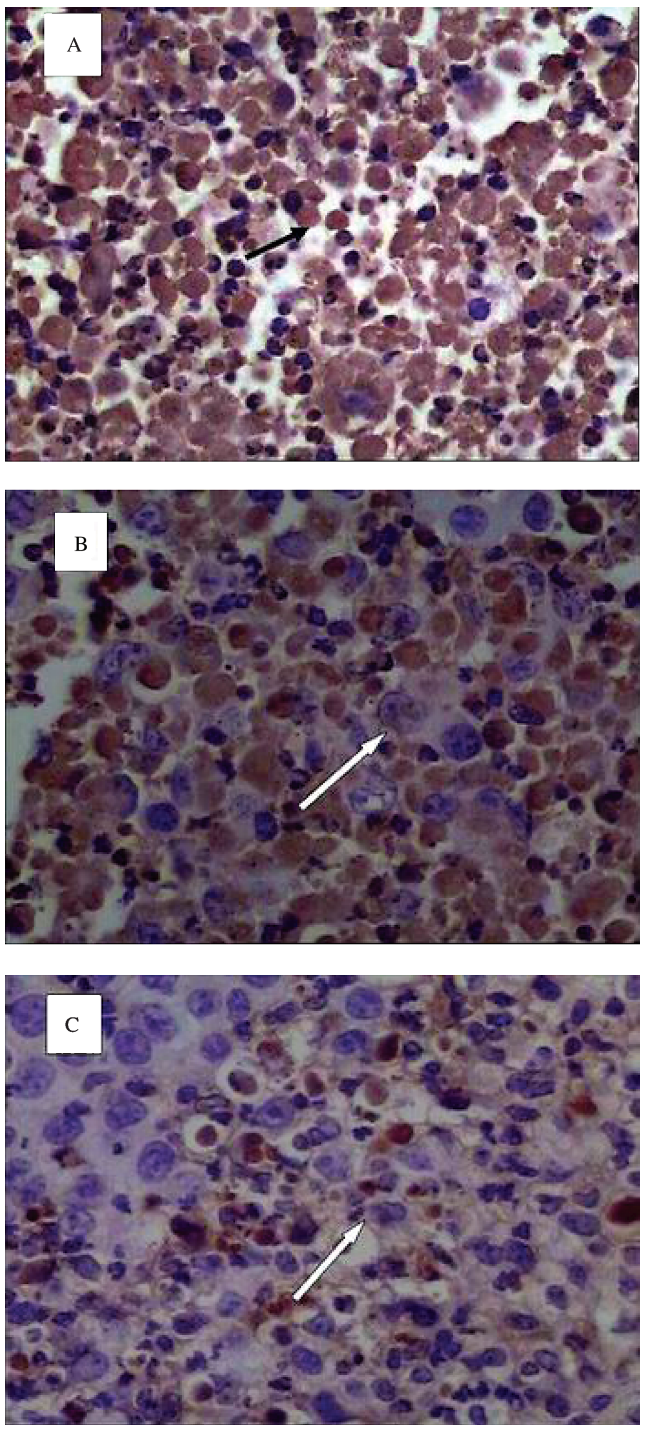


Fig. 3: Effect of ad-rhLTF on the expression of Bcl-2 protein in EMT6 tumor tissues (S-P × 400). Black arrows indicate that nucleus is stained brown, which is a Bcl-2 protein positive nucleus. White arrows indicate that nucleus is stained blue, which is a Bcl-2 protein negative nucleus. A control group B CTX group C ad-rhLTF group.

level of ad-rhLTF in 293 cells under fluorescence microscope (Fig. 1A). This result indicated that the ad-rhLTF could be used effectively in further experiments.

2.2. Expression of ad-rhLTF in tumor tissues

The result of western blotting indicated that the expression of ad-rhLTF in tumor tissues was a very similar migration with the standard of rhLTF and the expression of ad-rhLTF also obviously increased in a dose-independent manner with 25×10^8 pfu/ml being the most effective concentration for exogenous LTF expression. However, there was no expression of ad-rhLTF in the control group (Fig. 1B). The results proved that ad-rhLTF had been successfully transfected into the tumor cells of mice.

2.3. Effect of ad-rhLTF on solid tumor growth

Tumor mice received ad-rhLTF (5×10^8 pfu/ml and 25×10^8 pfu/ml for 7 times) or CTX, the body weight of mice was not significantly affected. However, compared with the control group,

Table 1: Inhibitory effect of ad-rhLTF on EMT6 solid tumor ($x \pm s$)

Group	n	Treatment	Body weight (g)			
			Beginning	End	Mean weight of tumor (g)	Inhibition Rate (%)
Control	10	Vehicle	19.71 ± 1.84	21.18 ± 2.01	2.631 ± 0.46	
CTX	10	25 mg/kg	20.96 ± 1.72	19.24 ± 1.20	$1.166 \pm 0.19^{*}$	55.68
Ad-rhLTF	10	5×10^8 pfu/ml	20.17 ± 1.84	21.65 ± 2.59	$1.446 \pm 0.27^{*}$	45.04
	10	25×10^8 pfu/ml	20.86 ± 1.74	22.57 ± 2.04	1.121 ± 0.17	57.39

$^{*}p < 0.05$ as compared with control group, values are mean $\pm$ SD

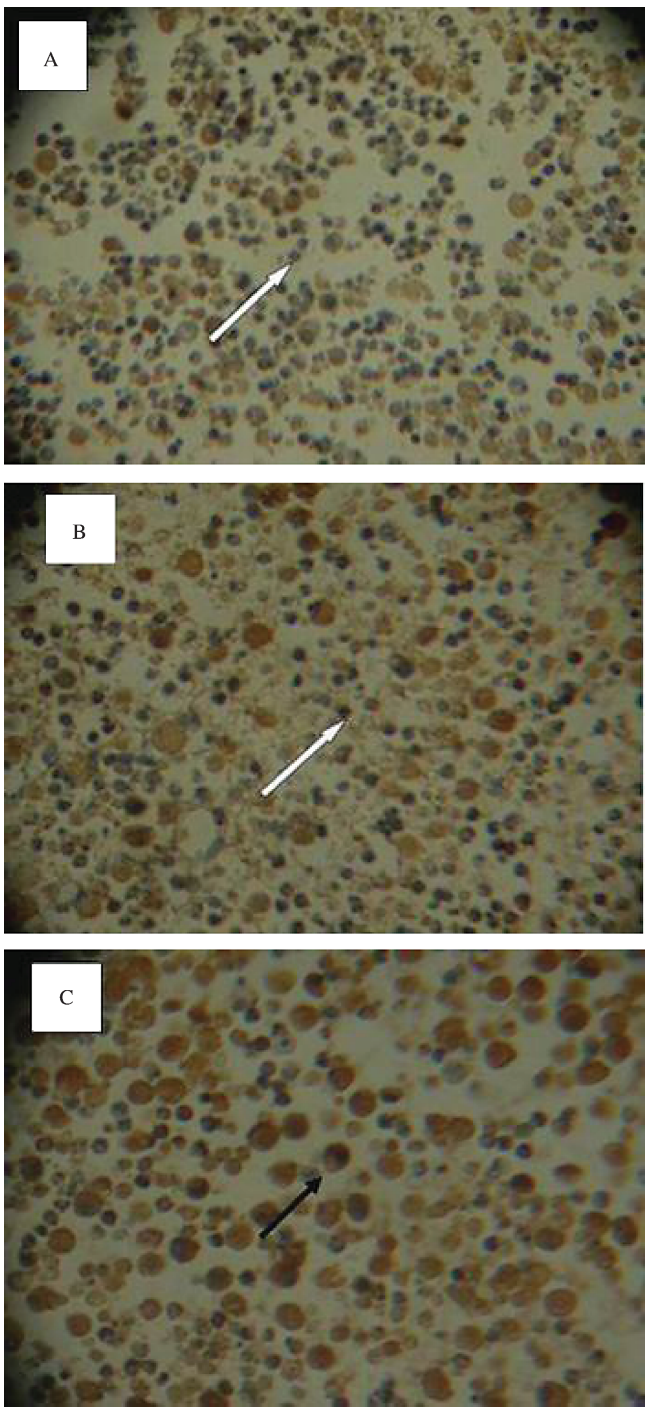


Fig. 4: Effect of ad-rhLTF on the expression of Bax protein in EMT6 tumor tissues (S-P $\times$ 400). Black arrows indicate that nucleus is stained brown, which is a Bax protein positive nucleus. White arrows indicate that nucleus is stained blue, which is a Bax protein negative nucleus. A control group B CTX group C ad-rhLTF group

ad-rhLTF could significantly decrease the tumor weight and the tumor inhibition rates reach 45.04% and 57.39%, respectively. CTX being considered as the receipted chemotherapeutics, the tumor inhibition rate reach 55.68% (Table 1).

2.4. Effect of ad-rhLTF on tumor cell cycle

The percentage of Sub-G_1 cells as apoptotic cells was significantly increased from 5.6% in the control group to 7.4% in the CTX group and 23.2% in the ad-rhLTF group by flow cytometry assay. Similarly, the percentage of tumor cells in the G_0/G_1 phase was increased. However, the proportion of tumor cells in S and G_2/M phase was decreased. These data indicated that ad-rhLTF induced tumor cell apoptosis and arrested cell cycle in G_0/G_1 phase (Fig. 2).

2.5. Effects of ad-rhLTF on the expression of Bcl-2, Bax and caspase 3 proteins

The expression of Bcl-2, Bax and caspase 3 proteins were examined with the streptavidin peroxidase method and RT-PCR. Compared with the control group, administered with ad-rhLTF (25×10^8 pfu/ml) reduced the expression of Bcl-2 while increased the expression of Bax and caspase 3 proteins. The positive rate of Bcl-2 was 69.4% in the control group, administered with CTX and ad-rhLTF, the positive number of Bcl-2 decreased to 41.8% in CTX group and 25.4% in ad-rhLTF group (Table 2, Fig. 3). The positive rate of Bax was 23.8% in the control group, after administration with CTX and ad-rhLTF, the positive number of Bcl-2 increased significantly to 43.5% and 72.8%, respectively. (Table 2, Fig. 4). Similar to Bax, the positive percentage of caspase 3 was 35.6% in the control group while the treatment of CTX and ad-rhLTF significantly increased the number of caspase 3 positive cells to 62.3% in CTX group and 74.7% in ad-rhLTF group (Table 2, Fig. 5).
The expression of Bcl-2, Bax and caspase 3 mRNA were further detected using RT-PCR. As shown in Fig. 6, the result was in line with that of the immunohistochemical assay, which showed that the expression of Bcl-2 mRNA was reduced while the expression of Bax mRNA and caspase 3 mRNA was increased, and the down-regulation of Bcl-2 and up-regulation of Bax led to a decrease in the ratio of Bcl-2/Bax (Fig. 6).

3. Discussion

Gene therapy of breast cancer is a promising strategy besides surgery, chemotherapy and radiotherapy, which can carry tumor suppressor or other antitumor drugs directly into the tumor site (Zhang et al.2008). Adenovirus vectors are highly efficient for gene delivery *in vivo* and direct *in situ* tumor transduction, and extensively used in the clinical gene therapy of a variety of cancers and experimental studies (Huang et al. 2006). Therefore, recombinant adenovirus vectors expressing human lactoferrin

Table 2: Effect of ad-rhLTF on the expression of Bcl-2, Bax and caspase 3 (x ± s, %)

Groups	n	Treatment	Bcl-2	Bax	Caspase 3
Control	10	Vehicle	69.4 ± 8.47	23.8 ± 4.32	35.6 ± 6.44
CTX	10	25mg/kg	41.8 ± 7.57*	43.5 ± 7.56*	62.3 ± 8.35*
ad-rhLTF	10	25×10^8 pfu/ml	25.4 ± 5.34*	72.8 ± 9.17*	74.7 ± 9.26*

* $p < 0.05$ as compared with control group, values are mean ± SD

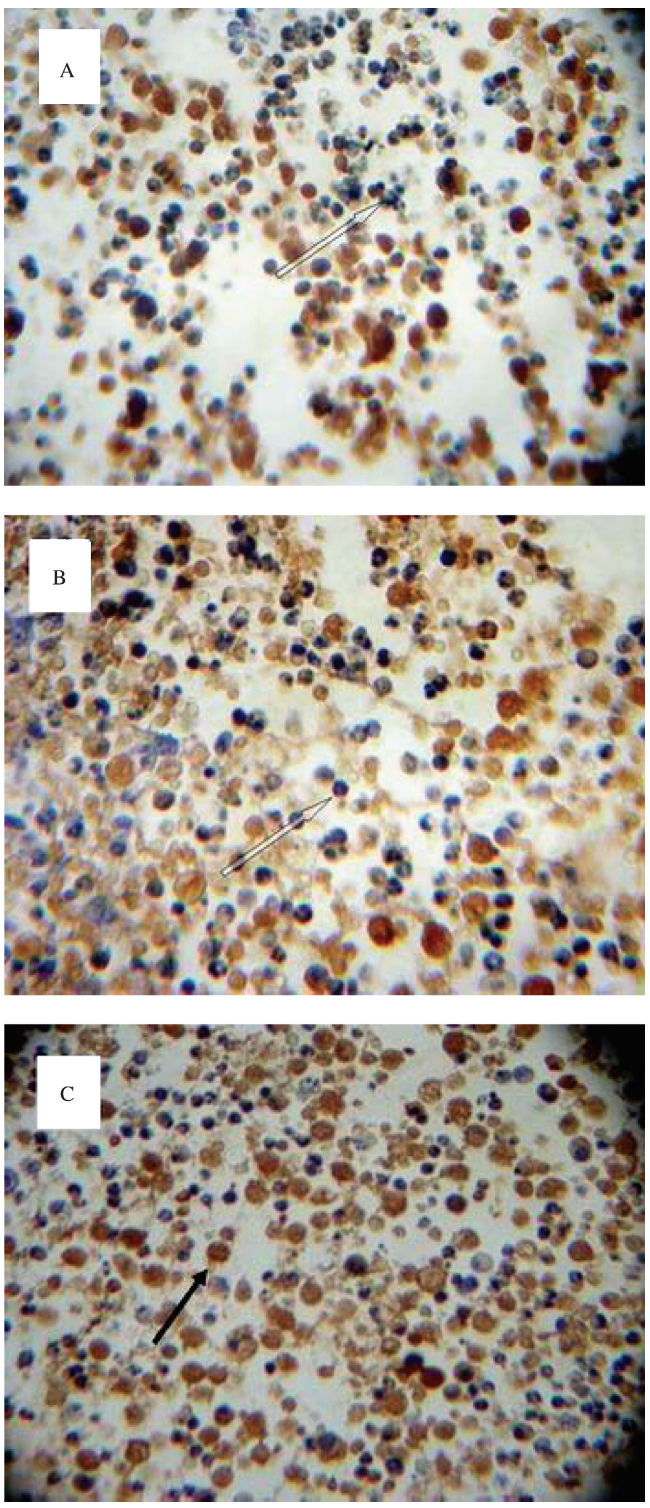


Fig. 5: Effect of ad-rhLTF on the expression of caspase 3 in EMT6 tumor tissues (S-P × 400). Black arrows indicate that nucleus is stained brown, which is a caspase 3 protein positive nucleus; white arrows indicate that nucleus is stained blue, which is a caspase 3 protein negative nucleus. A control group B CTX group C ad-rhLTF group

cDNA were constructed and its therapeutic potential for breast cancer was studied.

The results in the present study demonstrated that ad-rhLTF possessed growth inhibition to EMT6 cells in a dose-dependent manner, induced tumor cell apoptosis and increased the proportion of tumor cells in the G_0/G_1 phase, reduced Bcl-2 expression while increased the Bax and capase 3 expressions. These findings are in accordance with previous studies using bovine LTF which showed G_1 arrest, caspase 3 activation, and PARP cleavage in oral, head and neck and colon cancers (Fujita et al. 2004; Sakai et al. 2004; Xiao et al. 2004).

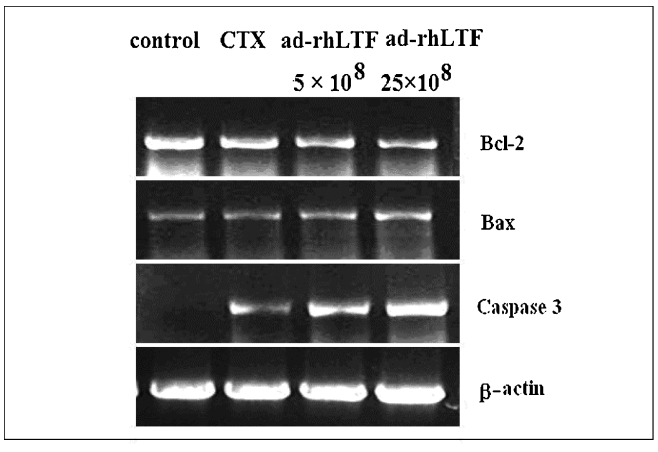
Apoptosis is one of the main types of programmed cell death, and the process is controlled by a number of complex proteins. The Bcl-2 family proteins are key regulators of mitochondria pathway which is an important mechanism of apoptosis (Scorrano and Korsmeyer 2003; Wan et al. 2008). Its effects are dependent on the balance between antiapoptotic Bcl-2 and proapoptotic Bax (Green and Evan 2002; Mehmet 2002). In response to apoptotic stimuli such as DNA damage, Bcl-2 family members regulate mitochondrial release of cytochrome c to cytosol, where it subsequently forms a complex with Apaf-1 and caspase-9, leading to the activation of the caspase-3, which subsequently activates the rest of the caspase cascade and leads to apoptosis (Cheung et al. 2002; Mlejnek 2001; Reed 2000; Scorrano 2003). So in the present study the expression of Bcl-2 family and caspase-3 were examined. The ad-rhLTF could decrease the expression of Bcl-2 and increase the expression of Bax and follow by the activation of caspase-3. The up-regulation of Bax expression and the reduction of Bcl-2 expression in the treated cell lines led to a decrease in the ratio of Bcl-2/Bax, which may be responsible for the drug-induced apoptotic processes. These results suggest that the apoptosis-induction effect of ad-rhLTF might occur through the mitochondria-dependent pathway and associated with activation of caspase-3.

In conclusion, the results obtained in the present study indicated that ad-rhLTF resulted in tumor growth inhibition in EMT6 mice and the antitumor effects of ad-rhLTF might be associated with arresting tumor cells in the G_0/G_1 phase, inducing cell apoptosis and regulation of the expression of Bcl-2, Bax, and activation of caspase 3. Ad-rhLTF might be a promising drug for breast cancer treatment.

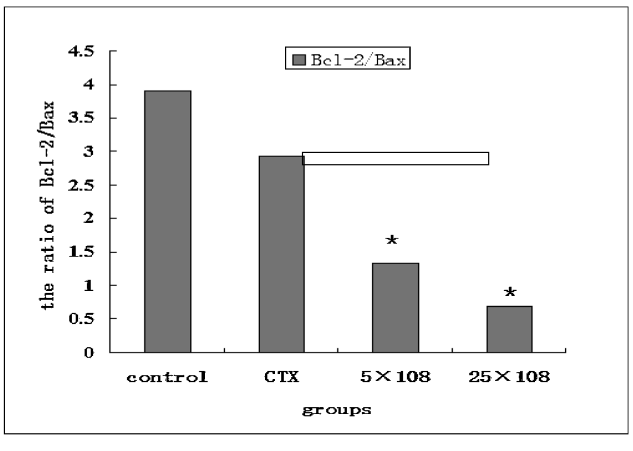
4. Experimental

4.1. Chemicals and instruments

The commercial standard protein (recombinant hLTF) and anti-lactoferrin monoclonal antibody (No. L3262) were obtained from sigma chemical co. (St Louis, MO, USA). Mouse anti-Bcl-2, Bax and caspase 3 monoclonal antibodies and streptavidinbiotin peroxidase immunohistochemical reagent kits were obtained from ZhongShan Bio Inc. (Beijing, China). Trizol was from Gibco Co. (Gaithersburg, MD, USA). Reverse transcriptase cDNA synthesis kit was purchased from Takara Bio. Inc. (Dalian, China). Human embryonic kidney (HEK) 293 cells line and The EMT6 cell line were obtained from the Cancer Institute of Chinese Academy of Medical Sciences. All other chemicals used were of analytical reagent grade.



(A)



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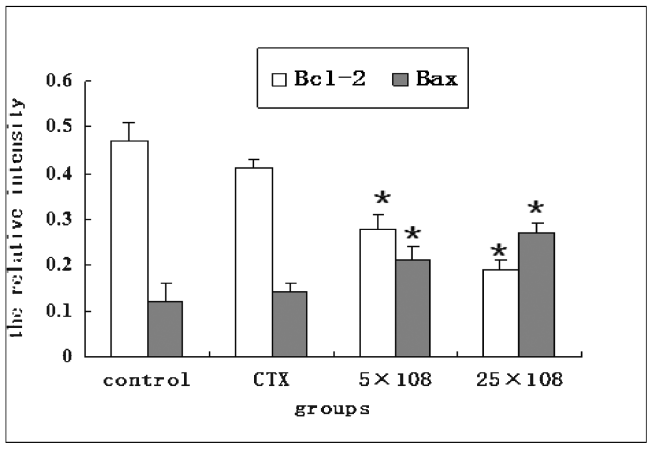

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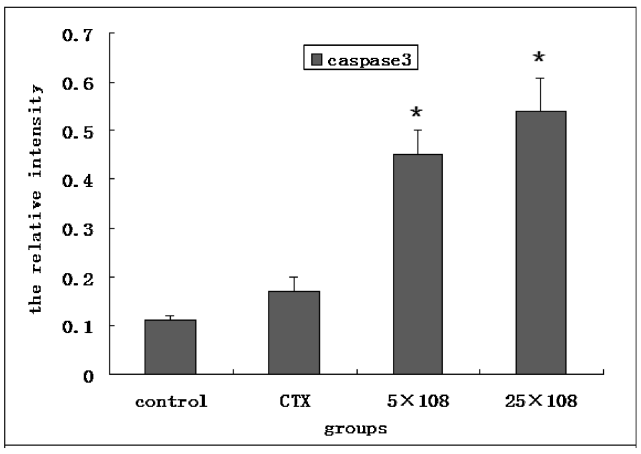

(D)

Fig. 6: The expression of apoptosis related proteins in tumor tissues. A: RT-PCR was performed to determine apoptosis related proteins' mRNA expression. β-actin was used as a control; Data shown is representative of three independent experiments; B: The intensity of Bcl-2 and Bax bands was quantified and was shown as relative expression level after normalized by β-actin (n = 3, means ± S.D). *$P < 0.05$, vs. control group. C: the ratio of Bcl-2/Bax was showed. *P < 0.05 vs. control group. D: The intensity of caspase 3 was quantified and was shown as relative expression level after normalized by β-actin (n = 3, means ± S.D). *P < 0.05, vs. control group.

4.2. Construction of ad-rhLTF containing the human lactoferrin cDNA gene and GFP reported gene

The recombinant human lactoferrin adenoviral vectors containing the human lactoferrin cDNA gene and reported gene GFP were constructed according to the method described previously (Han et al. 2007, 2008). The viral stocks were produced by transient transfection of HEK 293 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The final linearized ad-rhLTF was identified and titrated and stored at −70 °C until use.

4.3. Preparation of viral stock

HEK 293 cells were cultured in DMEM medium containing 10% fetal bovine serum and incubated at 37 °C in atmosphere with 5% CO_2. We added ad-rhLTF 100 μl to HEK 293 cells when cells had grown to 80%. After 48 h, the titer of the adenovirus stock was determined by GFP expression on semi-confluent 293 cells. We collected the 293 cells and obtained the virus through 4 times freezing at Liquid Nitrogen and melting at 37 °C and diluted the virus stock concentration based on the plaque-forming units (pfu)/ml with normal saline to the desired concentration, stored at −70 °C until use.

4.4. Animals

Fifty female Kunming mice (6 week-old) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23; revised 1978 and the number approved by Administrated-Committee of Laboratory Animals was 062310). The mice were randomly divided into five groups. Each group comprised ten animals. One group was used for the preparation of EMT6 tumor cells. The other four groups were used for different treatments. Animals were housed in plastic cages with free access to food and water and maintained on a regulated environment (20 ± 2 °C).

4.5. EMT6 tumor cells

The EMT6 breast cancer cell line (10^7/ml) was injected into the right fore limb in one group of mice (0.2 ml/mouse). We removed solid tumor and made to cell suspension and diluted to concentration of 5×10^6/mL under aseptic conditions in normal saline when tumor size grew to 1 cm.

4.6. Animal model and treatment

Four groups of mice were all injected with 0.2 ml/mouse of 5×10^6/mL EMT6 breast cancer cells in the skin under the right fore limb. Then we began to administrate with various drugs when tumor size reached 0.3–0.5 cm. One group was injected normal saline 100 μl in the tumor site as the control group. One group was given cyclophosphamide (CTX) (25 mg/kg body weight, once/day, i. p.), a standard antitumor reference drug, which was designated as the tumor control group (CTX group). The other two groups received ad-rhLTF 100 μl in tumor sites at a dose of 5×10^8 pfu/ml and 25×10^8 pfu/ml, once every two days, for 7 times in 14 days. After 14 days, all mice were weighed and killed, and then the tumor was removed and weighed. According to the mean weight of tumor, the rate of tumor inhibition was calculated as follows: Rate of inhibition (%) = (mean tumor weight of control group - mean tumor weight of treated group)/mean tumor weight of control group × 100%.

4.7. Expression of ad-rhLTF in tumor tissues by western blotting analysis

The protein samples of tumor tissues from various groups were mixed with equal volumes of loading buffer and boiled for 5 min. After separation in 10% SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride membrane using a semi-dry transfer method. The membrane was blocked with 5% non-fat dried milk in TBS with 0.05% Tween-20 (TBST) for 1 h at room temperature, washed three times with TBST, and incubated with TBST containing 5% of nonfat dried milk and anti-lactoferrin monoclonal antibody for 2 h at room temperature. The membrane was then washed three

times with TBST followed by 1 h incubation with horseradish peroxidase-labeled anti-IgG antibody at room temperature. After repeated washing with TBST, the expression of ad-rhLTF was detected by using the ECL kit and exposed to X-ray film.

4.8. Cell cycle and apoptosis assay by flow cytometry

The tumors which were collected from the control group, CTX group and ad-rhLTF group (25×10^8 pfu/ml) were minced, single cell suspension was prepared with 200 mesh filtering, centrifuged at 956 g for 5 min, washed three times, adjusted cell concentration to 10^6/ml, fixed with 70% ethanol for 30 min at 4 °C, DNA content and cell cycle were analyzed by flow cytometry after treatment with RNase and Propidium Iodide staining for 30 min. The proportion of cells in each cell cycle was calculated. The data were analyzed with CellQuest safeware (Becton Dickinson, USA).

4.9. Immunohistochemical analysis for Bcl-2, Bax, and caspase 3 in tumor tissues

Tumor specimens collected from the control group, CTX group and ad-rhLTF group (25×10^8 pfu/ml) were fixed in 10% (v/v) neutral formalin solution and embedded in paraffin. Tumor sections were prepared and used to examine the expression of Bcl-2, Bax, and caspase 3 proteins. The tumor sections were stained by the standard immunohistochemical streptavidin peroxidase method which was described in the procedure program of streptavidin-peroxidase reagents kit. The distinctly brown cells staining suggested positive cells and blue cells staining indicated negative cells under a microscope. Image was acquired by using Leica Application suite software (Leica Microsystems Ltd. Germany), and the average number of positive cells was counted by using Leica QWin software (Leica Microsystems Ltd. Germeny) in five randomly selected optical fields (200 tumor cells/per field). The average positive rate was calculated as follows: Positive rate = number of positive cells/total number of cells × 100%.

4.10. RNA extraction and reverse transcription PCR

Total RNA in tumor tissues was extracted using Trizol. RT-PCR was performed using a reverse transcriptase cDNA synthesis kit according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed into cDNA and then followed by PCR amplification using specific primers as follows:

Genes	Primers	The length of sequence (bp)
Bcl-2	Sense: 5′-CCTGGCACCTGGCGGATAGC-3′	
	Anti-sense: 5′-CGACTGAAGAGTGAGCCCAGCAGAAC-3′	529
Bax	Sense:5′-GCTCTGAACAGATCATGAAGACAG-3′Anti-sense: 5′-CAATCCAAAGTGGACCTGAGG-3′	746
caspase-3	Sense: 5′-TTTGTTTGTGTGCTTCTGAGCC-3′	
	Anti-sense: 5′-GATGTTCTGGAGAGCCCCG-3′	400
β-actin	Sense: 5′-AATGGGTCAGAAGGACTCCTATGTGG-3′	
	Anti-sense: 5′-CGCCTA GAAGCACTTGCGGTG-3′	994

Bcl-2 or Bax was amplified by 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s in order. Caspase-3 was amplified by 30 cycles at 94 °C for 45 s, 52 °C for 45 s, and 72 °C 1 min. All of them were followed by a 7 min extension at 72 °C. PCR products were electrophoresed on 1.0% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. The intensity of bands was quantified by using LabWork 3.0 UVP software (UVP, USA).

4.11. Statistical analysis

Data were expressed as mean ± S.D. One-way analysis of variance and Duncan's multiple range tests were used for determining differences between groups, and $P < 0.05$ was regarded as statistically significant.

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