BRIEF ARTICLES

Effect of \( p27^{mt} \) gene on apoptosis of the colorectal cancer cell line Lovo

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AIM: To construct \( p27^{mt} \) recombinant adenovirus, transfect the colorectal cell line Lovo and observe the effects of \( p27^{mt} \) on Lovo cell apoptosis and cell cycle inhibition.

METHODS: We constructed recombinant adenovirus containing \( p27^{mt} \) by homologous recombination in bacteria. The colorectal cancer cell line Lovo was infected with recombinant replication-defective adenovirus Ad\( p27^{mt} \), and expression of \( p27^{mt} \) was determined by Western blotting; the inhibitory effect of \( p27^{mt} \) on Lovo cells was detected by cytometry. Cell cycle was determined by flow cytometry. DNA fragment analysis,PI staining and flow cytometry showed that 77.96% of colorectal cancer cells were inhibited in phase G\(_1/G_0\), while in the Ad-LacZ group and blank control group, 27.57% and 25.29% cells were inhibited in the same phase, respectively. DNA fragment analysis, flow cytometry and TUNEL assay demonstrated that \( p27^{mt} \) is able to induce apoptosis in colorectal cancer cells.

CONCLUSION: \( p27^{mt} \) has an obvious blocking effect on colorectal cancer cell cycle, and most cells were inhibited in phase G\(_1/G_0\). Therefore, \( p27^{mt} \) can induce apoptosis in colorectal cells.

Abstract

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RESULTS: The recombinant adenovirus which already contained \( p27^{mt} \) target gene was successfully constructed. When multiplicity of infection was \( \geq 50 \), the infection efficiency was 100%. After transfection of Lovo cells with Ad\( p27^{mt} \) the cells had high \( p27 \) expression which was identified by immunoblotting assay. PI staining and flow cytometry showed that 77.96% of colorectal cancer cells were inhibited in phase G\(_1/G_0\), while in the Ad-LacZ group and blank control group, 27.57% and 25.29% cells were inhibited in the same phase, respectively. DNA fragment analysis, flow cytometry and TUNEL assay demonstrated that \( p27^{mt} \) is able to induce apoptosis in colorectal cancer cells.

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Key words: Apoptosis; Cell cycle; Colorectal cancer; \( p27^{mt} \); Recombinant adenovirus

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INTRODUCTION

\( p27^{Kip1} \) (\( p27 \)) is a cyclin dependent kinase inhibitor (CDKI), whose specific late G\(_1\) destruction allows progression of the cell across the G\(_1/S\) boundary. The protein was ubiquitinated by S-phase kinase-interacting protein-2 (Skp2) following its specific phosphorylation, and was subsequently degraded by the 26S proteasome[1]. There was a direct relationship between the low level of \( p27 \) and rapid proliferation occurring in several benign states and in many malignancies. It has been reported that \( p27 \) levels were markedly reduced in several malignancies, such as those of the skin[2], liver[3], bladder[4], thyroid[5], breast[6], prostate[7] and endometrium[8]. In some of the tumors studied, a strong correlation was found between the low level of \( p27 \), the aggressiveness of the disease and poor prognosis of the patients[9]. Interestingly, \( p27 \) in all these tumors was of the wild-type species (\( p27^{wt} \)), and its regulation has been attributed to phosphorylation of Thr-187 and subsequent
ubiquitination\(^{30}\). Overexpression of p27 via adenoviral gene transfer could suppress cancer cell growth regardless of p27 mutation\(^{19}\). Montagno et al\(^{31}\) showed that the ubiquitination of p27 did not occur in p27mt with Thr-187 to Ala [p27 (T187A)]. Sheaff et al\(^{32}\) showed that the transfection of p27 (T187A) plasmid caused a G1 block, which was both resistant to and not modulated by cyclin E/Cdk2. On the basis of these observations of p27 regulation and the nature of the p27 tumor suppressor gene, we constructed Adenovirus expressing p27mt (Thr-187/Pro-188 to Met-187/Ile-188) to infect the colorectal cancer cell line Lovo, and then investigated its expression and functional significance in the cell proliferation and apoptosis of Lovo cells, by which we aimed to discuss novel methods of gene therapy in colorectal cancer.

**MATERIALS AND METHODS**

**Main reagents**

The restriction endonucleases such as Age I, Nhe I, Kpn I, Pci I and Pme I were purchased from New England Biolabs Co. HindIII, EcoRI, λDNA HindIII marker, 200 bp DNA ladder, dNTP, Tag enzyme and T4 DNA ligase were purchased from Huamei Biological Co. (China). The Western blotting kit was purchased from KPL Co. (USA). The rat anti-human p27kip1 multi-antibody was purchased from Santa Cruz Co. (USA). The horseradish peroxidase (HRP) labeled sheep anti-rat IgG monoclonal antibody was purchase from Zhongshan Co. (China). The p27mt primer was designed and synthesized by Beijing SaiBaisheng Biological Co. (China). The fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. (China). The liposome (polycy) was purchased from Qiagen Co. (USA). Trypsin, DMEM culture medium, Hepes and Cscl were purchased from Sigma Co. (USA).

**Plasmid, strain, adenovirus and cell lines**

The pORF9-p27mt plasmid was purchased from Invigen (USA). The pAdeasy-1 plasmid, pBluescript II sk (+), Ad293 cell, E. coli BJ5183 and XL10-gold were purchased from Stratagene (USA). The LacZ recombinant adenovirus (Ad-LacZ with titer 7.15 × 10\(^7\) /L)\(^{33}\) and DH5α were gratefully provided by Doctor Wang Jianing, Clinical Research Institute, Yuyang Medical College. The Lovo cell line was purchased from Type Culture Collection Center, Wuhan University.

**Main equipment**

This equipment included a high speed freezing centrifuge (Universal 32R, Germany), the ultraspeed freezing centrifuge (Tokyo Cp80max, Japan), inverted phase contrast microscope (Nikon TE2000-u, Japan), CO\(_2\) culture box (CB150#00-1761, wrb-binder), PCR machine (Biometra. Germany), ultraviolet spectrophotometer (Aurid CE2401, UK), Coulter Epics XL flow cytometer (Beckman Co., USA), high speed table-top centrifuge and a water bath shaking table (China).

**Construction and identification of p27mt recombinant adenovirus**

After pORF9-p27mt was digested by Age I and Nhe I enzymes, the 619 bp fragment was recycled and subcloned into pBluescript II SK (+) which was digested by Xma I and Xba I enzymes, thus obtaining pBluescript-p27mt. Then pBluescript-p27mt was digested by Not I and Kpn I enzymes, and the 699 bp fragment was recycled and inserted into the shuttle plasmid vector pShuttle-CMV which was digested by the same enzymes, thus the transfer plasmid pShuttle-CMV-p27mt was obtained. The competent E. coli was transformed by the adenoviral framework plasmid pAdeasy-1. According to the ampicillin-resistant gene, the BJ5183 containing pAdeasy-1 was picked out and prepared into the ultra-competence BJ5183 containing pAdeasy-1. Then, the ultra-competence BJ5183 was transformed by transfer plasmid pShuttle-CMV-p27mt which was digested by Pme I enzyme and dephosphorylated by alkaline phosphatase. A little DNA from the transformed clone bacterial plasmid was taken out and the suspect DNA of the recombinant adenovirus plasmid was chosen according to the size of the plasmid in agarose electrophoresis. If the chosen DNA was identified as the correct DNA by digestion of Pci I enzyme, then the recombinant adenovirus plasmid pAdeasy-1-p27mt could be prepared. Recombinant adenovirus plasmid DNA was excised by Pci I, then transfected by Ad293 via liposome polycy mediation, where the change in cells at different times after transfection was determined. When it appeared that 90% had cell lesions, scratch 293 cells from the culture bottle were vortexed three times at -80°C to +37°C to lyse the cells, then centrifuged and the supernatant containing the virus was collected, the 293 cells were reinfected with the above virus and proliferation of the virus occurred at a large scale. Purification of recombinant adenovirus was similar to the method of Cortin et al\(^{34}\). After purification the adenovirus underwent dialysis, test virus titers were detected using an ultraviolet spectrophotometer. Fifty microliter of purified adenovirus liquid, 100 g/L SDS 20 μL, PBS 430 μL, were assayed at absorbance values of \(A_260\) and \(A_280\), then the granule numbers and purity of adenovirus were determined. If \(A_260 = 10^3\) pufr/L and \(A_{260}/A_{280} > 1.3\) this indicated that the purity was relatively high when virus titers (pufr/L) = \(A_{280} \times \text{dilution} \times 10^3\). PCR identification of recombinant adenovirus Ad-p27mt was carried out. Recombinant adenovirus genome DNA from the high titer virus storage liquid served as a template. Using primer toward the reporter gene p27mt, the PCR reaction parameters were: pre-denaturation at 95°C for 5 min, denaturation at 94°C for 20 s, annealing at 56°C for 30 s, elongate at 72°C for 30 s, 30 cycles, elongate at 72°C for 10 min. Primer 1: 5’CCTAGAGGCCCAGTGAGTG3’; Primer 2: 5’GAAGATCTCGGTTGACGGTGC3’.

**Detection of p27mt gene expression**

Lovo cells were incubated in 100 mL/L FCS and RPMI-1640 culture medium at 37°C in a 50 mL/L CO\(_2\) culture box until the cells spread to 70%-80% of the area and were used in the following experiment. Lovo cells taken from the 15 cm culture flask were infected with Ad-LacZ according to an Multiplicity of infection (MOI) of 20, 40, 50 and 100 and then incubated for another 48 h. The cells were then fixed by 5 mL/L glutar for 15 min and washed three times with PBS,
X-gal staining solution (20:1) was added. The cells were then incubated at 37℃ in the 50 mL/L CO₂ culture box for 4-24 h. The blue-stained cells, i.e. the positive cells in which the LacZ gene was expressed, were observed under the microscope and the percentage of positive cells was calculated.

Lovo cells taken from the 75 cm² culture flask were infected with Ad-p27mt (MOI 50) and Ad-LacZ (MOI 50), respectively. After incubation in the same conditions for 48 h, the cells were digested by 0.5 g/L trypsin, collected and washed twice with PBS. After lysis by 500 μL 1 × SDS-PAGE cell lysis solution and boiling for 5 min, the cells were centrifuged, the supernatant was collected and then detected by Western blotting.

**Cycle detection and apoptosis of cells infected with Ad-p27mt by flow cytometry**

Lovo cells cultured in the 75 cm² culture flask were infected with Ad-p27mt (MOI 50). After incubation for 48 h, the cells were digested by 0.5 g/L trypsin, collected and then washed twice with PBS. The cell concentration was adjusted to 10⁷/L with PBS. One hundred microliter of cell suspension was taken out and mixed with 200 μL DNA-PREP™ LPR and placed at room temperature in the dark for 3 min. The cell suspension was then mixed with 1000 μL DNA-PREP stain (PI staining). The cell cycle phase and apoptosis were detected by a Coulter Epics XL flow cytometer 15 min later. Ad-LacZ (MOI 50) group and normal controls (Lovo cells cultivated without adenovirus) were used as control groups.

**Apoptosis by DNA fragment analysis**

The cells in the three groups (Ad-p27mt and Ad-LacZ for 48 h and the normal control), were collected and centrifuged at 1000 r/min for 5 min. The supernatant was discarded, and 500 μL of cell lysis solution [1% Np40, 20 mmol/L EDTA, 50 mmol/L Tris-HCl (pH7.5)] and 10 μL protease K were added to the cell sediment. Following incubation in a water bath (56℃) for 1-2 h and extraction with phenol and chloroform, DNA was precipitated by dehydrated alcohol. After washing once with 700 mL/L alcohol, 200 μL TE was added to lyse the DNA. Then RNase (final concentration 50 μg/mL) was added and placed at 37℃ for one night. The DNA was electrophoresed in 10 g/L agarose gel and the results were observed under an ultraviolet lamp.

**Detection of cell apoptosis by the TUNEL method**

1 × 10⁶ cell suspension was inoculated into a 60 mm dish with a cover glass (washed and high-pressure sterilized). Each of the Ad-p27mt group and the normal control were inoculated into 6 glasses and incubated for 24 h. The glasses were then taken out and washed twice with 1 × PBS and fixed with methanol and freezing acetic acid (3:1) for 30 min. The next procedure was carried out according to the kit instructions. One thousand cells were counted on each glass and the average number of apoptotic cells was determined. Then the apoptotic index (AI), i.e. the number of apoptotic cells in every 100 cancer cells, was calculated.

**X-gal chemical staining**

After Lovo cells were infected with Ad-LacZ, the adenovirus-mediated gene transfer rate was evaluated by X-gal staining. The results showed that the infection efficiency could reach 100% when MOI was larger than 50, which indicated that recombinant adenovirus could effectively transfer the gene to Lovo cells in vitro (Figure 2).

**The expression of p27 protein was evaluated after Lovo cells were infected with human mutant p27 recombinant adenovirus in vitro**

After Lovo cells were infected with Ad-p27mt (MOI 50) for 24 h, these cells were collected and lysed using 1 × SDS × PAGE cell lysis solution. After boiling at 100℃ for 5 min, the cells were centrifuged. The supernatant was collected and the protein was detected by the TMB system Western blotting kit (KPL, USA). After staining with TMB stain, a high expression of 27 KD protein was observed in the Ad-p27mt group while only slight expression (endogenous expression) was observed in the Ad-LacZ group and the normal control group. This showed that the p27mt recombinant adenovirus constructed in the present study could express p27mt gene in Lovo cells and the protein could also be expressed at a high level in Lovo cells (Figure 3).
Apoptosis of colorectal cancer cells induced by Ad-p27mt

After Lovo cells were treated with Ad-p27mt, Ad-LacZ and without virus for 24 h, apoptosis was observed by flow cytometry and was repeated six times. The average value of hypodiploid cells was: 41.0%, 4.67% and 1.96%, respectively. After statistical analysis, there was a significant difference among the three groups, ($P < 0.01$) (Figure 4).

Detection of DNA fragments

The results of DNA electrophoresis showed that the gene bands were intact in the Ad-LacZ and normal control group, while there were obvious 180-200 bp diploid “trapezia” bands in the Ad-p27mt infected group, which was in concordance with the characteristic changes of apoptosis (Figure 5).

Detection of cell apoptosis by the TUNEL method

The nuclei of apoptotic cells were dark stained, the cytoplasm was concentrated and the cells had shrunk. The AI of the Ad-p27mt and the control group were (82.6% ± 3.2%) and (5.0% ± 3.5%), respectively and showed a significant difference ($P < 0.05$). This demonstrated that Ad-p27mt could obviously induce apoptosis in colorectal cancer cells (Figure 6).
The effect of exogenous p27mt on the cell cycle (Table 1)

The cell cycle of Lovo cells after the various treatments are shown in Table 1. It can be seen that in the Ad-LacZ and blank control groups, the number of cells in the G0/G1 phase decreased gradually and the percentage of cells in the S phase increased, which indicated that the transition time of the cell cycle was shortened and cell proliferation was active. However, the percentage of cells in the G0/G1 phase decreased and the percentage of cells in the S phase increased and the cell cycle was arrested in the G0/G1 phase in the Ad-p27mt group, which was significantly different from the blank and Ad-LacZ groups (P < 0.01).

Table 1  The effect of Ad-p27mt on the cell cycle of Lovo cells (mean ± SD)

| Group           | G0/G1   | S     | G2/M   |
|-----------------|---------|-------|--------|
| Blank group     | 25.29 ± 1.04 | 41.12 ± 1.19 | 33.34 ± 1.55 |
| Ad-LacZ group   | 27.57 ± 0.45  | 38.21 ± 0.44  | 34.22 ± 0.92  |
| Ad-p27mt group  | 77.96 ± 2.20 | 8.98 ± 0.17  | 13.06 ± 2.35  |

Table 1: The effect of Ad-p27mt on the cell cycle of Lovo cells (mean ± SD).

The concentration of cyclin/CDK is the key factor for cells passing the G1-S threshold. Cell cycle analysis showed that the cleavage of tumor cells was stopped at the G1 stage by p27mt suppressing the activity of the cyclin/CDK kinase. Hurteau et al reported that the accumulation of p27 played a role in the cell cycle arrest mechanism at the initiation of cell differentiation. A related report in China showed that the p27 gene suppressed DNA replication and protein synthesis and reduced cell mitosis division and inhibited cell generation.

In our study, apoptosis of colorectal carcinoma cells was successfully induced by the application of the mutant gene p27. The apoptosis rate was significantly higher than that of wild-type p27 reported in our previous study, which serves as a very useful experimental support for tumor suppression function reconstruction in the gene therapy of colorectal carcinoma. The efficacy of this method in vivo and the mechanism of apoptosis should be determined in future studies.

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COMMENTS

Background

Along with the improvement in living standards and a change in diet, there has been a gradual increase in the incidence of colorectal cancer in China. However, no effective therapeutic modalities are available for this condition.

Gene therapy for the restoration of colorectal carcinoma. The efficacy of this therapy of colorectal carcinoma should be examined.

Innovations and breakthroughs

The study indicates that Ad-p27mt has a strong apoptosis inducing bioactivity as well as a cell cycle inhibitory effect in colorectal cancer in vitro.

Peer review

This is a nice article. This in vitro effect of p27mt should be examined in vivo to determine the safety and efficacy.

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