

BRIEF ARTICLES

Effect of *p27mt* gene on apoptosis of the colorectal cancer cell line Lovo

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Supported by The Natural Science Foundation of Hubei Province, No. 2003ABA193; Bureau of Science and Technology of Shiyan City, No. 2005ZD036

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Received: April 13, 2009 Revised: May 11, 2009

Accepted: May 18, 2009

Published online: June 14, 2009

immunoblotting assay. PI staining and flow cytometry showed that 77.96% of colorectal cancer cells were inhibited in phase G₀/G₁, 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 *p27mt* is able to induce apoptosis in colorectal cancer cells.

CONCLUSION: *p27mt* has an obvious blocking effect on colorectal cancer cell cycle, and most cells were inhibited in phase G₀/G₁. Therefore, *p27mt* can induce apoptosis in colorectal cells.

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Key words: Apoptosis; Cell cycle; Colorectal cancer; *p27mt*; Recombinant adenovirus

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Chen J, Ding WH, Xu SY, Wang JN, Huang YZ, Deng CS. Effect of *p27mt* gene on apoptosis of the colorectal cancer cell line Lovo. *World J Gastroenterol* 2009; 15(22): 2794-2799 Available from: URL: <http://www.wjgnet.com/1007-9327/15/2794.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.2794>

Abstract

AIM: To construct *p27mt* recombinant adenovirus, transfect the colorectal cell line Lovo and observe the effects of *p27mt* on Lovo cell apoptosis and cell cycle inhibition.

METHODS: We constructed recombinant adenovirus containing *p27mt* by homologous recombination in bacteria. The colorectal cancer cell line Lovo was infected with recombinant replication-defective adenovirus Ad-*p27mt*, and expression of *p27mt* was determined by Western blotting; the inhibitory effect of *p27mt* on Lovo cells was detected by cytometry. Cell cycle was determined by flow cytometry. DNA fragment analysis identified the occurrence of apoptosis.

RESULTS: The recombinant adenovirus which already contained *p27mt* target gene was successfully constructed. When multiplicity of infection was ≥ 50 , the infection efficiency was 100%. After transfection of Lovo cells with Ad-*p27mt* the cells had high *p27* expression which was identified by

INTRODUCTION

p27Kip1 (*p27*) is a cyclin dependent kinase inhibitor (CDKI), whose specific late G₁ destruction allows progression of the cell across the G₁/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^[6]. Interestingly, *p27* in all these tumors was of the wild-type species (*p27mt*), and its regulation has been attributed to phosphorylation of Thr-187 and subsequent

ubiquitination^[9]. Overexpression of *p27* via adenoviral gene transfer could suppress cancer cell growth regardless of *p27* mutation^[10]. Montagnoli *et al*^[11] showed that the ubiquitination of *p27* did not occur in *p27mt* with Thr-187 to Ala [*p27* (T187A)]. Sheaff *et al*^[12] 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, *Pac* I and *Pme* I were purchased from New England Biolabs Co. *Hind* III, *Eco*RI, λ DNA *Hind* III 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 purchased 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 (polyfect) 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 Invivogen (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^{15} /L)^[13] and DH5 α were gratefully provided by Doctor Wang Jianing, Clinical Research Institute, Yongyang 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), CO₂ culture box (CB150#00-17611, wtb-binder), PCR machine (Biometra, Germany), ultraviolet spectrophotometer (Auriud 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 *Pac* I enzyme, then the recombinant adenovirus plasmid pAdeasy-1-*p27mt* could be prepared. Recombinant adenovirus plasmid DNA was excised by *Pac* I, then transfected by Ad293 via liposome polyfect 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*^[14]. 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 μ L, PBS 430 μ L, were assayed at absorbance values of A_{260} and A_{280} , then the granule numbers and purity of adenovirus were determined. If $A_{260} = 10^{15}$ pfu/L and $A_{260}/A_{280} > 1.3$ this indicated that the purity was relatively high when virus titers (pfu/L) = $A_{260} \times \text{dilution} \times 10^{15}$. 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'CCTAGAGGGCAAGTACGAGTG3'; Primer 2: 5'GAAGAATCGTTCGGTTGCAGGTCGCT3'.

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 CO₂ 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 glutaral for 15 min and washed three times with PBS,

X-gal staining solution (20:1) was added. The cells were then incubated at 37°C 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 cm 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 μ L 1 \times 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 cm 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 10⁹/L with PBS. One hundred microliter of cell suspension was taken out and mixed with 200 μ L DNA-PREP™ LPR and placed at room temperature in the dark for 3 min. The cell suspension was then mixed with 1000 μ L 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 μ L of cell lysis solution [1% NP40, 20 mmol/L EDTA, 50 mmol/L Tris-HCl (pH7.5)] and 10 μ L protease K were added to the cell sediment. Following incubation in a water bath (56°C) 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 μ L TE was added to lyse the DNA. Then RNase (final concentration 50 μ g/mL) was added and placed at 37°C 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 \times 10⁴ 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 \times 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.

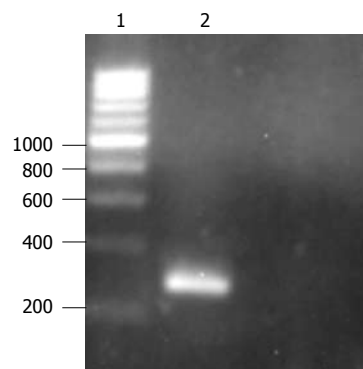


Figure 1 Identification of Ad-p27mt by PCR. 1: 200bp marker; 2: Ad-p27mt.

Statistical analysis

One way-ANOVA was used in processing the measured data, which were expressed as mean \pm SD. χ^2 test was adopted in the calculation of enumeration data.

RESULTS

PCR identification of the recombinant adenovirus Ad-p27mt

The pathologically changed 293 cells and the culture fluid were collected, frozen and thawed three times and centrifuged. Five millilitre of the supernatant was taken out and added to 1 mg protease K, 2 mL 1% SDS, 10 mmol/L EDTA and 20 mmol/L Tris-HCl for 2 h. After centrifugation, the supernatant was extracted with phenol and chloroform. After precipitation with dehydrated alcohol, the viral DNA was collected. Then forward and reverse primers were added and the PCR reaction was carried out. The 275 bp target gene was amplified, which showed that the recombinant adenovirus already contained the *p27mt* target gene (Figure 1).

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 \times SDS \times PAGE cell lysis solution. After boiling at 100°C 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).

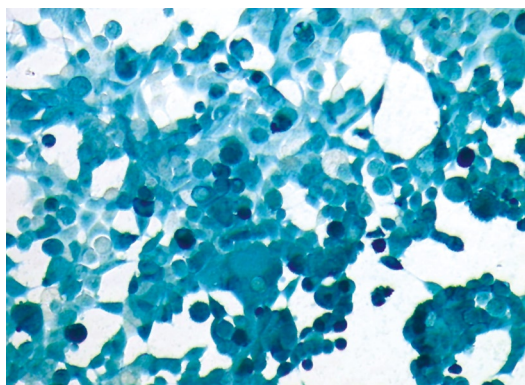


Figure 2 X-gal chemical staining detected the infection efficiency of Ad-p27mt (MOI 50).

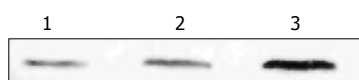


Figure 3 Western blotting of expressed p27 protein. 1: Non-infected Lovo cells; 2: Ad-lacZ infected Lovo cells; 3: Ad-p27mt infected Lovo cells.

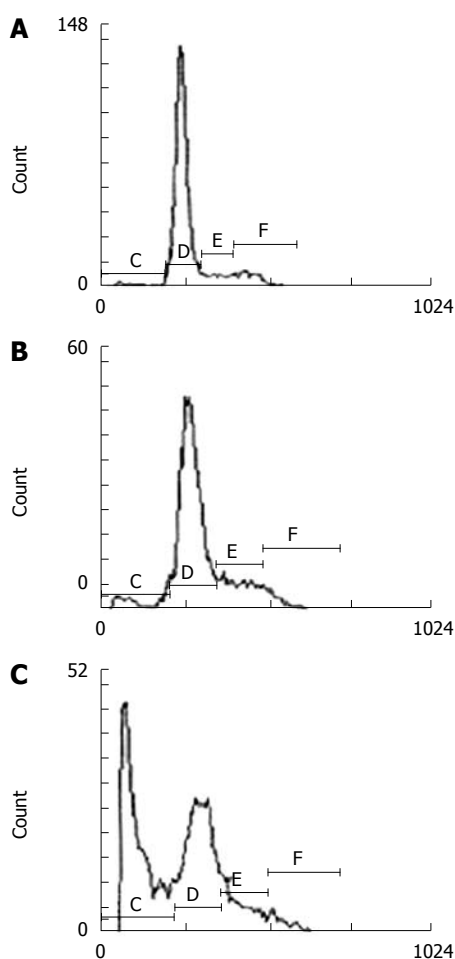


Figure 4 Determination of cell apoptosis by Flow cytometry. A: Non-infected Lovo cells; B: Ad-lacZ infected Lovo cells; C: Ad-p27mt infected Lovo cells.

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

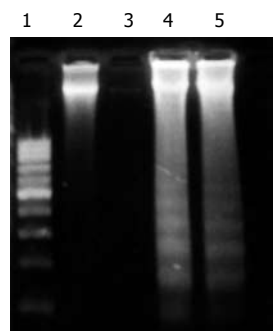


Figure 5 Determination of cell apoptosis by DNA fragment analysis. 1: Marker; 2: Blank group; 3: Ad-lacZ group; 4, 5: Ad-p27mt group.

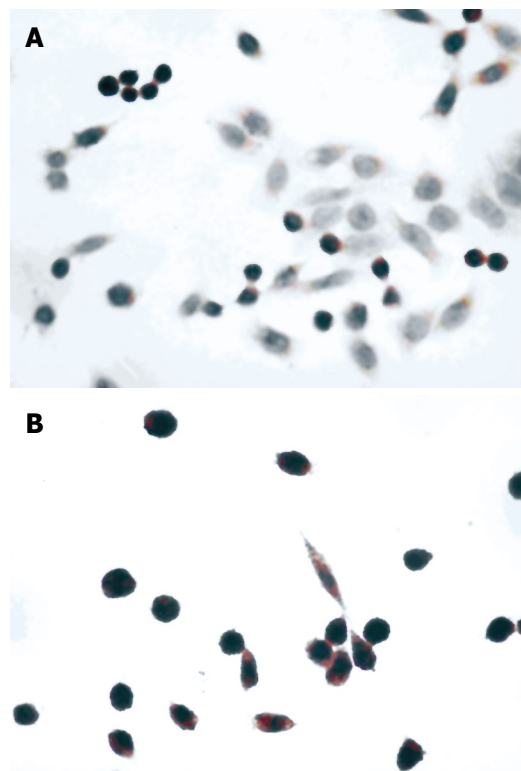


Figure 6 Detection of cell apoptosis by the TUNEL method. A: Blank group; B: Ad-p27mt.

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 ($82.6\% \pm 3.2\%$) and ($5.0\% \pm 3.5\%$), respectively and showed a significant difference ($P < 0.05$). This demonstrated that Ad-p27mt could obviously induce apoptosis in colorectal cancer cells (Figure 6).

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

| Group | G ₀ /G ₁ | S | G ₂ /M |
|----------------|--------------------------------|-------------------------------|-------------------------------|
| Blank group | 25.29 \pm 1.04 | 41.12 \pm 1.19 | 33.34 \pm 1.55 |
| Ad-LacZ group | 27.57 \pm 0.45 ^b | 38.21 \pm 0.44 ^b | 34.22 \pm 0.92 ^b |
| Ad-p27mt group | 77.96 \pm 2.20 ^d | 8.98 \pm 0.17 ^d | 13.06 \pm 2.35 ^d |

Hypodiploid cells were not included (^b $P < 0.01$ vs Ad-LacZ group, ^d $P < 0.01$ vs Blank group).

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 ($P < 0.01$).

DISCUSSION

Currently, functional reconstruction of anti-oncogenes is a reasonable strategy for tumor gene therapy. p27 protein degradation is mainly caused by phosphorylation of the 187th threonine of p27 which is mediated by ubiquitin^[15-17]. Park *et al*^[18,19] replaced the 187th threonine and the 188th proline of the wild-type p27 with methionine and isoleucine, respectively, by which the target phosphorylation site of CDK would be protected from phosphorylation, and thus the mutant p27 was prepared. These authors constructed a replication-deficient recombinant adenovirus which carried p27mt and p27wt, respectively and proved that the inhibition and apoptotic effects in cancer cells was more obvious in the mutant p27 (p27mt) than in the wild-type p27 (p27wt).

This study constructed a replication-deficient recombinant adenovirus which successfully carried p27mt (Ad-p27mt). Ad-p27mt was then transfected into Lovo cells. When the MOI ≥ 50 , the infection efficiency was 100%. Using Western blotting, high expression of p27 was observed, and using FACS, the rate of apoptosis was up to 41.0% in the Ad-p27mt group which was significantly different compared to the control group ($P < 0.01$). DNA analysis showed a 180-200 bp DNA ladder, and using the TUNEL technique, the apoptotic index was sharply upregulated to 82.6% which was significantly different compared to the control group ($P < 0.05$). These results showed that p27mt is an important gene and is related to the incidence of colorectal carcinoma. The downregulation of p27 may be the main cause of cell differentiation dysfunction and apoptosis dysfunction. Mutant p27 promoted the expression of p27 in Lovo cells, which led to apoptosis in tumor cells. This may be a potential new therapy for colorectal carcinoma.

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*^[20] reported that the accumulation of p27 played a role in the cell cycle arrest mechanism at the initiation of cell differentiation. A related report^[21] 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^[21], 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.

ACKNOWLEDGMENTS

We thank Dr. Xiao-Jun Yang for his excellent technical assistance.

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 p27 expression is a promising therapy. A mutant type p27 gene, with a mutation of Thr-187/Pro-188 to Met-187/Ile-188, can inhibit degradation of p27 protein by the ubiquitin-mediated pathway. Inhibition of mutant p27 (p27mt) in tumor cells was stronger than that in wild-type p27 (p27wt), which was demonstrated by cells arresting in the G₀/G₁ phase. In addition, the apoptosis promoting activity of p27mt was also stronger. However, few studies on the p27 gene in colorectal cancer have been reported.

Research frontiers

p27, as a cyclin-dependent kinase inhibitor, tumor suppressor gene, and promoter of apoptosis, has been widely investigated in malignancies such as skin, liver, bladder, thyroid, breast, prostate and endometrium cancer. However, the apoptotic bioactivity of p27mt has not been studied in colorectal cancer.

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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