Antitumor bioactivity of adenovirus-mediated p27mt in colorectal cancer cell line SW480

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Abstract

AIM: To explore the antitumor bioactivity of adenovirus-mediated mutant type p27kip1 gene in a colorectal cancer cell line SW480.

METHODS: We constructed recombinant adenovirus vector expressing a mutant type p27kip1 gene (ad-p27mt), with mutation of Thr-187/Pro-188 (ACGCC) to Met-187/Ile-188 (ATGATC), and transduced into SW480 cells. Then we detected expression of p27, Bcl-2 and Bax protein in the transductants by Western blotting, cell cycle of transductants by a digital flow cytometric system, migrating potential with Boyden chamber and SW480 tumor cell growth inhibition in vivo and in vitro.

RESULTS: We found that a recombinant adenovirus vector of expressing ad-p27mt, with mutation of Thr-187/Pro-188 (ACGCC) to Met-187/Ile-188 (ATGATC) has potent inhibition of SW480 tumor cell growth in vitro and in vivo. Furthermore, ad-p27mt induced cell apoptosis via regulating bax and bcl-2 expressions, and G1/S arrest in SW480 cells and inhibited cell migration.

CONCLUSION: ad-p27mt has a strong anti-tumor bioactivity and has the potential to develop into new therapeutic agents for colorectal cancer.

Key words: p27; Adenovirus; Antitumor bioactivity; Colorectal cancer

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INTRODUCTION

p27kip1, a member of the Kip/Cip family of cyclin-dependent kinases inhibitors (CKIs), is a putative tumor suppressor gene, and promoter of apoptosis that has been demonstrated in cancer cells as well as in normal cells. Simultaneously, p27kip1 acts as a safeguard inflammatory injury and plays a role in cell differentiation. Furthermore, p27kip1 was identified as an inhibitor of cyclin E/CDK2 in cells arrested in the G1 phase by lovastatin, transforming growth factor-beta (TGF-β), serum deprivation and contact inhibition.

Over the past years, p27kip1 protein has attracted our attention as an important prognostic factor in various malignancies. In short, lately, it has been reported that expression of p27kip1 protein is associated with poor prognosis in several types of malignancies, including breast, lung, gastric carcinoma and colorectal adenocarcinoma.

The reduced expression of p27kip1 in cancer cells due to an increase in the rate of its degradation. It is thought that the amount of p27kip1 protein is regulated by a posttranscriptional mechanism rather than p27kip1 gene aberrations because p27kip1 gene mutation seems to be uncommon in human malignancies. It has been demonstrated that p27kip1 is poly-ubiquitinated both in vivo and in vitro, and p27kip1 ubiquitination requires its phosphorylation on threonine residue 187 (T187) both in vivo and in vitro.

Gene therapy is a promising approach to restore p27 expression using adenoviral vectors. These agents have induced cell-cycle arrest and loss of cyclinE-
CDK2 activity in cell lines and xenograft models and have triggered apoptosis in cancer cells\(^{[2,19,21-24]}\). The concentration of \(p27\) is thought to be regulated predominantly by the ubiquitin-dependent proteolytic pathway\(^{[26]}\). Degradation of \(p27\) triggered by its phosphorylation on Thr187 is required for the binding of \(p27\) to Skp2, the F-box protein component of an SCF ubiquitin ligase (E3) complex, and such interaction in turn results in the poly-ubiquitylation and degradation of \(p27\)\(^{[16,25]}\). Reduction of \(p27\) levels in various types of malignant tumors results from accelerated proteolytic degradation by this pathway\(^{[26]}\).

However, so far no report has been published on the effects of adenovirus-mediated mutant type \(p27^{hp}\) gene on colorectal cancer cell. Thus, we constructed recombinant adenovirus vector expressing a mutant type \(p27^{hp}\) gene (ad-p27mt), with mutation of Thr187/Pro-188 (ACGGCC) to Met-187/Ile-188 (ATGATC), which will inhibit degradation of \(p27\) protein\(^{[11,16,24,25]}\), and explored its antitumor bioactivity in a colorectal cancer cell line SW480.

**MATERIALS AND METHODS**

**Cell and cell culture**

Human colorectal cancer cell line SW480 was purchased from Shanghai Cell Lines Bank (Shanghai, China). Cells were maintained in RPMI 1640 (Beyotime, China) supplemented with 10% fetal bovine serum (FBS; Beyotime, China).

**Preparation of ad-p27mt**

Ad-p27mt was constructed in the Institute of Clinical Medicine of Yunyang Medical College (Hubei, China). Briefly, the cDNA of human \(p27\) gene was digested from plasmid of pORF9-hp27mt (Invitrogen) and subcloned into the plasmid of pBluescript SK (+) (Stratagene) and formed plasmid of pBluescript-hp27mt. Then human \(p27\) gene was digested from pBluescript-hp27mt and subcloned into shuttle vector pBluescript-CMV (Stratagene) and gained shuttle plasmid of pShuttle-CMV-hp27mt. Adenovirus genomic DNA plasmid of pAdEasy-1 (Stratagene) was transformed into Bju5183 bacteria (Stratagene) and prepared competent Bju5183 bacterium containing pAdEasy-1. pShuttle-CMV-hp27mt was linearized with Pmel (New England Bio labs) and transformed into competent Bju5183 bacterium containing pAdEasy-1 and positive clone of homologous recombination was selected. Identified recombinant adenovirus plasmid of pAd-p27mt was digested with Pael and transfected into HEK293 cells (Stratagene) with liposome polyFect (Qiagen) to package adenovirus particles. HEK293 cells were maintained in DMEM with 10% FBS until the onset of the cytopathic effect. PCR technique was used to detect target gene and the titer of the recombinant adenovirus was determined by measuring the absorbance at 260 nm and 280 nm. Ad-p27mt was propagated in HEK293 cells, purified by two cesium chloride density centrifugations, titered and stored at -70°C. Recombinant adenovirus expressing \(\beta\)-galactosidase (ad-\(\beta\)-gal) without any therapeutic gene was used as the control virus in all experiments.

**Transduction of SW480 cells with ad-p27mt**

Exponential growing of SW480 cells were transduced with ad-p27mt or ad-\(\beta\)-gal at 20 multiplicity of infection for 1 h with gentle frequent shaking and then incubated with complete media for the experiment.

**P27mt sequencing assay of transductant**

Briefly, transductants were schizolysed and DNA was extracted using phenol/chloroform, and then dehydrated alcohol precipitated DNA. Sequence of DNA was assayed by Shanghai Sangon Company.

**Western blotting**

Cells lysates were prepared from transductants of ad-p27mt (SW480-ad-p27mt), transductants of ad-\(\beta\)-gal (SW480-ad-\(\beta\)-gal) and parental cells (SW480) as follows: cells were cultured for 96 h, washed three times with 100 mmol/L phosphate-buffered saline (PBS; Beyotime, China), lysed with RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.1% SDS; Beyotime, China]. The protein concentration of the samples was determined by Bio-Rad protein assay (BioRad, Amersham). Fifty micrograms of protein samples were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to PVDF membrane (Sigma), and membrane was incubated with a 1:1000 dilution of the monoclonal anti-p27 antibody (Beyotime, China), anti-Bax antibody (Beyotime, China), and anti-Bcl-2 antibody (Beyotime, China), respectively. The blots were developed using the enhanced chemiluminescence (ECL) Western blotting system and protocol (Amersham). In all immunoblotting experiments, blots were reprobed with an anti-\(\beta\)-actin antibody (Sigma) for internal control.

**MTT assay**

Cells were seeded on 96-well plates (Beyotime, China) at 4 \(\times\) 10\(^4\) cells per well in RPMI 1640 supplemented with 10% FBS. After 2 d, 4 d and 6 d, the number of cells was quantitated by an assay in which MTT; 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was used.

**Cell cycle analysis**

Cells were seeded in 75 cm\(^2\) culture flask (Beyotime, China) in RPMI 1640 supplemented with 10% FBS. After 96 h, the stuck and floating cells were collected in conical tubes (Beyotime, China). Then, the cells were fixed with 70% cold ethanol and washed with PBS. After treatment with 0.1 mg/mL RNase A (Sigma), the cells were stained with 40 \(\mu\)g/mL propidium iodine (Sigma), and the cell cycle was analyzed by a digital flow cytometric system (Beckman Coulter EPICS-XL).

**Migration assay with Boyden chamber**

Migrative potential was evaluated in the Boyden chamber apparatus (Kylin-bell, China). This assay was developed...
to facilitate analysis of aspects of cancer invasion and metastasis. Briefly, subconfluence cells were starved for 24 h and harvested with 0.05% trypsin (Sigma) containing 0.02% EDTA (Sigma), washed twice with PBS, and resuspended to a final concentration of 5 × 10^5/mL in serum-free medium with 0.1% fraction V bovine serum albumin. PVP filters (Kylin-bell, China) of 8 μm pore size were precoated with gelatin, rinsed in sterile water, and used for assay. Bottom wells of the chamber were filled with 25 μL of RPMI 1640 supplemented with 10% FBS per well and covered with a gelatin coated membrane, and then 50 μL of cells suspension was added to the top wells. After 24 h of incubation, the membranes were stained with Giemsa solution. Cells on the upper surface of the filter were carefully removed with a cotton swab, and the cells that had migrated through the membrane to the lower surface were counted in six different fields under a light microscope at 400 magnification. Each experiment was performed in triplicate wells and repeated 3 times.

**In vivo growth inhibition assay**
BALB/c nude mice were purchased from Animal experiment center of Hubei (Hubei, China; Qualification number: SCXK: Hubei 2003-0005). All animal experimental procedures were conducted and approved by the Institutional Animal Care and Use Committee of Yunyang Medical College (Approval number: SYXK: Hubei 2004-0021). Briefly, SW480 cells were cultured in RPMI 1640 supplemented with 10% FBS. Cells were harvested through two consecutive trypsinizations, centrifuged at 300 × g for 5 min, washed twice, and resuspended in sterile PBS. Cells (1 × 10^6) in 0.2 mL were injected subcutaneously into a 6-week-old nude mouse between the scapulae of each nude mouse. After 14 days, tumors reached a mean size of 200 mm^3 in the mice’s bodies. To test in vivo growth suppressive potential of ad-p27mt on nude mouse xenografts of SW480, intratumoral injections of ad-p27mt, ad-β-gal or PBS were made every other day for total 3 times, respectively. There were ten mice in each group. Twenty-eight days after inoculation, mice were sacrificed by cervical dislocation, and tumor specimens were taken, photographed and weighed.

**Statistical analysis**
Data are mean of at least 3 independent experiments ± SD. Results were compared by one-way analysis of variance (ANOVA). A two-tailed P < 0.05 was regarded as statistically significant. All calculations were performed using the SPSS for Windows version 13.0 statistical program on a personal computer.

**RESULTS**

**p27mt sequencing assay of transductant**
As shown in Figure 1, sequencing result was consistent with p27mt sequence in gene bank.

**Expression of p27, Bax and Bcl-2 protein in the transductants**
As shown in Figure 2, expression of p27 and Bax protein was increased significantly and Bcl-2 protein level was decreased in the SW480-ad-p27mt when compared with that in SW480-ad-β-gal and SW480. The expression of β-actin as an internal control was approximately the same in all of the cells.

**Growth of cells in vitro**
Relative cell number was evaluated by comparing the absorbance in each cell at day 2, day 4 and day 6. As shown in Figure 3, the growth of SW480-ad-p27mt was markedly inhibited compared with SW480-ad-β-gal and SW480.

**Cells cycle analysis**
To further determine whether up-regulation of p27 protein can induce apoptosis and or cell cycle arrest, flow cytometric analysis was performed on each transductant. It is suggested that cells containing a sub-G1 content of DNA reflect the extent to which apoptosis is occurring. Flow cytometric analysis demonstrated that p27mt gene transduced into SW480 induced G1/S arrest and apoptosis. A marked sub-G1 peak and decreased percentage of cells in S phase were detected in SW480-ad-p27mt. Decreased percentage of cells in S phase suggested G1/S arrest (Figure 4 and Table 1).

**In vitro migration assay**
SW480-ad-p27mt significantly showed the poor ability of migration when compared with that of SW480-ad-β-gal and SW480 (Figure 5).

**In vivo growth inhibition assay**
Twenty-eight days after inoculation, ten mice in each
Table 1  Cell cycle analysis of transductant by flow cytometry

|          | Sub-G1 (%) | G0/G1 (%) | S (%) | G2/M (%) |
|----------|------------|-----------|-------|-----------|
| SW480    | 3.88 ± 1.85| 73.17 ± 5.10| 11.42 ± 2.93| 11.21 ± 4.70 |
| SW480-ad-β-gal | 4.30 ± 1.02| 68.27 ± 3.15| 11.1 ± 0.96| 15.43 ± 1.98 |
| SW480-ad-p27mt | 39.13 ± 1.84| 49.40 ± 2.70| 7.31 ± 0.70| 3.92 ± 0.96 |

The percentage of cells of transductants in each phase. The values are mean of 3 independent experiments ± SD. *P = 0.001, vs control cells by one-way ANOVA.

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In this study, we found that transduction of ad-p27mt into SW480 cells resulted in induction of overexpression of p27 protein, which suggests that the approach to restore p27 expression using adenoviral vectors is available and p27mt protein may be resistant to degradation by ubiquitin and more stable. Other studies also have shown that the p27 (T187A) mutant is not ubiquitinated, and the control of p27 protein levels is affected by ubiquitin-dependent degradation, in a ubiquitin-independent and Skp2-independent manner at G01 [10,32], and by Jab1-dependent degradation [33].

We also observed that ad-p27mt induced growth suppression, apoptosis, and G0/S arrest in SW480 cells in vitro as well as the transplanted tumor growth inhibition in vivo, as was expected from the nature of p27 as a CDKIs. These findings are consistent with those of another study in which ad-p27 (T187A) had a greater effect on cell cycle arrest and apoptosis induction because of its resistance to degradation, and suppressed the growth of established lung cancer xenografts [24]. In several studies, intratumoral injections of ad-p27 have been shown to partially suppress tumor formation in animal models [18,23,34]. In other experiments, ad-p27 was the most potent of several cyclin kinase inhibitors in terms of inducing cell-cycle arrest, apoptosis and inhibiting tumorigenesis [18,23,27,34]. The effect of p27 on the cell cycle is regulated mainly by its stability [15,36], but recent studies have shown that the function of p27 is also associated with its subcellular localization [13,39].

Besides Thr187, there are three phosphorylation sites Ser10, Thr157 and Thr198 that are involved in cellular localization [40,41]. Phosphorylation at Ser10 stabilizes p27 protein in G1 [41]. Phosphorylation at Thr157 by protein kinase B/Akt impairs the nuclear import of p27 but does not affect its stability in breast cancer and other cells [37,38]. Chu et al [41] indicating that the oncogenic kinase Src regulates p27 stability through phosphorylation of p27 at tyrosine 74 and tyrosine 88. Src inhibitors increase cellular p27 stability, and Src overexpression accelerates p27 proteolysis.

The relationship between p27 and induction of apoptosis is still unclear. Katayose et al [3] and Naruse et al [4] have suggested that the growth-inhibitory effect and apoptosis induction by overexpression of p27 requires expression of pRb. The pRb-dependent checkpoint in G1 phase is an important apoptotic checkpoint. CyclinE-CDK2 is the primary complex that phosphorylates pRb, which prevents interactions of it with the E2F transcription factor. To further understand the mechanism of ad-p27mt induced apoptosis in SW480 cells, we examined the expression of apoptosis-related genes Bax and Bcl-2 in each transductant. Results showed that ad-p27mt resulted in a marked increase in protein expression of Bax, a pro-apoptotic factor, and a marked decrease in protein expression of Bcl-2, an anti-apoptotic factor that binds to Bax and antagonizes its function. These results suggest that ad-p27mt-induced apoptosis in SW480 cells involves in induction of Bax.

Cell migration is an essential process involved in tumor invasion and metastasis. In this study, we tested the ability of cell migration in each transductant. We found that ad-p27mt resulted in strong migration.
inhibition. Supriatno et al. have reported a similar result in oral cancer cell line. However, its mechanisms remain unclear and need further investigations. We speculate that it may be directly associated with decreased cell proliferation or/and alterations of structural proteins.

In conclusion, ad-p27mt shows a strong anti-tumor bioactivity in a colorectal cancer cell line SW480 and has the potential to develop into new therapeutic agents for colorectal cancer.

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COMMENTS

Background

The incidence of colorectal cancer is increasing all over the world. However it is short of effective therapeutic approach. Gene therapy to restore p27 expressing has been promising, furthermore, a mutant type p27 gene, with mutant of Thr-187/Pro-188 to Met-187/Ile-188, can inhibit degradation of p27 protein by ubiquitin-mediated pathway.

Research frontiers

p27, as a cyclin-dependent kinases inhibitor, tumor suppressor gene, and promoter of apoptosis, has been widely investigated. Furthermore, antitumor activity of p27 has been demonstrated in breast, lung, and oral cancer. But the antitumor bioactivity of p27 mt has not been studied on colorectal cancer.

Innovations and breakthroughs

The study indicates that ad-p27mt has a strong anti-tumor bioactivity in a colorectal cancer cell line SW480.

Applications

This will develop into new therapeutic agents for colorectal cancer.

Peer review

This is an interesting manuscript on the antitumor activity of the adenovirus mediated mutant p27kip gene in a colorectal cancer (CRC) cell line. Major finding of the study was that the tumor cell growth was inhibited both in vitro and in vivo by the gene transfer.
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