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Impact of *p27mt* gene on transplantation model of human colorectal cancer in nude mice

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Abstract

AIM: To investigate the inhibitory and anti-metastatic effect of mutant p27 gene (*p27mt*) on the growth of colorectal cancer xenografts in nude mice and its underlying mechanism.

METHODS: Inhibitory effect of *p27mt* gene on the growth of colorectal cancer xenografts was determined by measurement of tumor size before and after direct intra-tumoral injection of Ad-p27mt in a pre-established transplantation model of human colorectal cancer in nude mice. Cell cycle and apoptosis were detected by flow cytometry performed on single-cell suspension from an isolated tumor. Expression of MMP-9 in tumor tissue was detected by immunohistochemistry.

RESULTS: The average sizes of transplantation tumors were 1.94 ± 0.67 cm^3, 2.75 ± 0.83 cm^3 and 3.01 ± 0.76 cm^3 in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.05$). The average proliferation rates were 37.34% ± 1.45%, 53.16% ± 3.27% and 54.48% ± 2.43%, in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.05$). The average apoptosis rates were 19.79% ± 3.32%, 6.38% ± 4.91% and 7.25% ± 5.20% in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.01$). The average MMP-9 expression rates were 20%, 75% and 66.7% in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.01$).

CONCLUSION: *p27mt* inhibits the growth of transplanted tumor by blocking the proliferation of cancer xenografts and by promoting apoptosis of transplantated tumor cells, as well as decrease transplanted tumor metastasis.

Key words: Colorectal cancer; *p27mt* gene; Nude mice; MMP-9

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INTRODUCTION

Along with the improvement of people's living standard and change in diet, there has been a gradual increase in the incidence of colorectal cancer[1]. None of the current treatment modalities for colorectal cancer, including surgery, radiotherapy and chemotherapy, is effective. With the advent of post-genomic era, the function of genes has become a priority research area and brought the dawn in gene therapy for tumor. Since *p27* is an anti-oncogene, this study was to evaluate the inhibitory and anti-metastatic effect of *p27mt* gene on the growth of colorectal cancer xenografts in nude mice and its underlying mechanism and to provide the theoretical basis for the use of *p27* in clinical treatment of colorectal cancer.

MATERIALS AND METHODS

Cell line and adenovirus

Lovo cell line, purchased from the Center for Type Culture Collection of Wuhan University, was cultured in RPMI 1640 medium. Working density of Levo cells

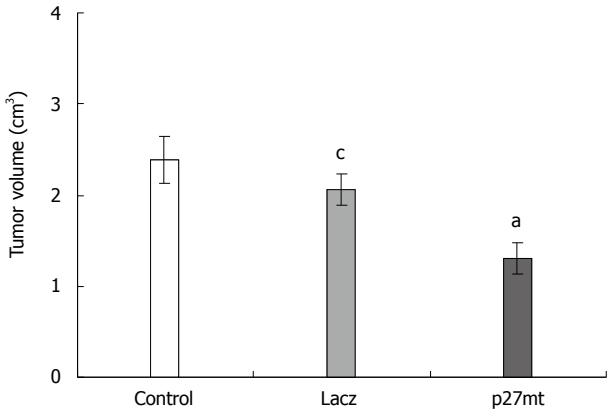

Figure 1 Comparison of transplanted tumor volume among different groups (cm^3). $^aP < 0.050$ *vs* Ad-Lacz, $^cP > 0.05$ *vs* control.

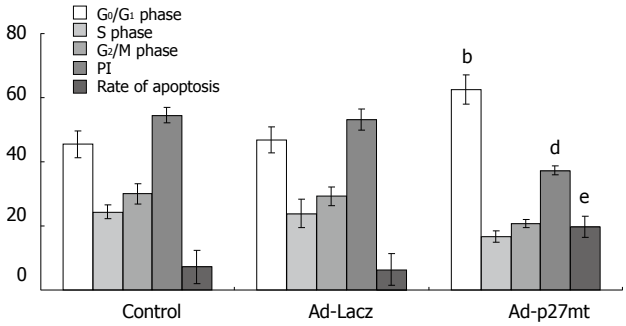

Figure 2 Comparison of status of cell cycle, PI and rate of apoptosis between different groups. $^bP < 0.01$ *vs* Ad-LacZ, $^dP < 0.01$ *vs* Ad-LacZ, $^eP < 0.01$ *vs* Ad-LacZ.

was 1×10^8/mL, with living cell count by trypan blue > 99%. Ad-LacZ was constructed and presented by Wang *et al*[2]. Ad-p27mt was self-constructed[3].

Establishment of transplantation model of colorectal cancer in nude mice and grouping

Thirty-six BALB/C nude mice, 4-6 wk old, and weighing 18-25 g, were purchased from the Laboratory Animal Management Center of Hubei Province. Lovo cell suspension (0.2 mL) was inoculated subcutaneously at the right back skin of each nude mouse. Upon tumor development, 27 nude mice whose tumor size was 0.5-1.5 cm in diameter were randomly assigned to control group, Ad-LacZ group or Ad-p27mt group. PBS (0.1 mL), Ad-LacZ (0.1 mL) with a virus density of 10^{10} pfu/mL, or Ad-p27mt (0.1 mL) with a virus density of 10^{10} pfu/mL was directly injected into the tumor of nude mice in the three groups, respectively, once every 3 d, for 28 d.

Determination of transplanted tumor size

Transplanted tumor size was calculated according to the following formula: $V = ab^2/2$, where a and b represent the length and width of the xenograft, respectively.

Flow cytometry

The 28th day after virus injection into the transplanted tumor, all mice were sacrificed with their tumors removed, weighed and photographed. Tumor tissue (15 g) was used in preparation of single cell suspension. Two hundred μL DNA-PREP™ LPR was mixed with 100 μL single cell suspension, and 1000 μL DNA-PREP stain was added into the mixture 3 min after the mixture was set at room temperature and protected from light. Fifteen min later, cell cycle and apoptosis were determined with a Coulter Epics XL flow cytometer. Proliferation index (PI) was calculated according to the following formula[4]:

$$PI = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$$

Immunohistochemical detection of MMP-9 expression

Anti-human mouse MMP-9 monoclonal antibody, S-P staining kit and DAB developer were obtained from Beijing Zhongshan Biotechnology, Co, Ltd. Since MMP-9 appears to be brown granules in cytoplasm, total cell number and the number of MMP-9 positive cells were counted in 5 visual fields of the matrix area around the tumor nest under microscope. Based on the scope and extent of staining, immunohistochemical results were logged according to the following criteria: "-" - no positively stained cells; "+" - cells lightly stained or < 10% cells stained; "++" - moderately stained or 10%-25% cells stained; "+++" - darkly stained or more than 50% cells stained, where - represents negative expression, +/++ stands for weakly positive expression, and + + + stands for strong expression.

Statistical analysis

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

RESULTS

Comparison of transplanted tumor size between different groups

The average size of transplanted tumor in the Ad-p27mt group (1.94 ± 0.67 cm^3) was significantly smaller than that in the control group (3.01 ± 0.76 cm^3) ($P < 0.05$), no statistical significance was found in the average size of transplanted tumor between the two groups (3.01 ± 0.76 cm^3 *vs* 2.75 ± 0.83 cm^3) (Figure 1).

Comparison of cell cycle, PI and apoptosis between different groups

More cells at G_0/G_1 phase and less cells at S and G_2/M phase were observed in the Ad-p27mt group than the other two groups ($P < 0.05$). However, the difference between the two groups was insignificant ($P < 0.05$, Figure 2).

MMP-9 expression in transplanted tumor

Since MMP-9 is mainly found in the matrix area around tumor nest, brown stained granules were observed in cytoplasm of cancer cells (Figure 3). MMP-9 expression rate for the Ad-p27mt group was significantly decreased compared with control group (Table 1).

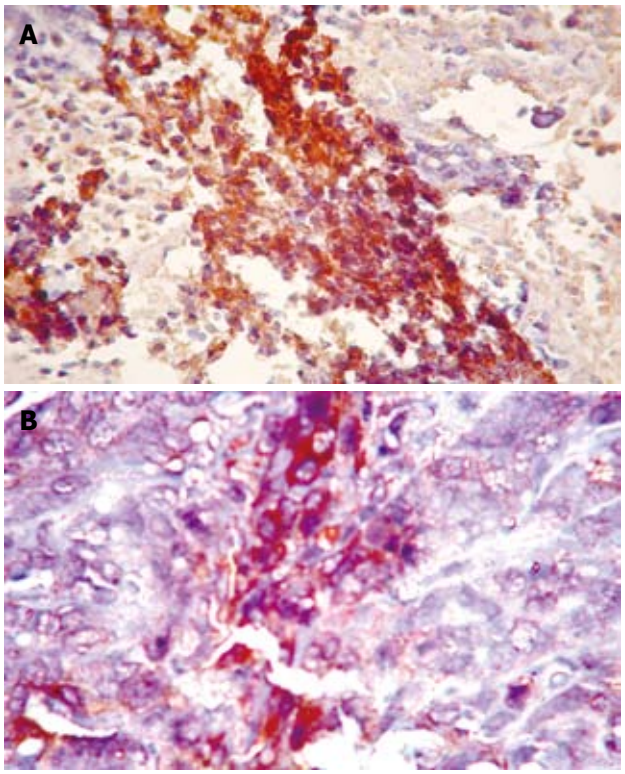

Figure 3 Expression of MMP-9 in the transplanted tumor. A: control group; B: Ad-p27mt group.

DISCUSSION

While *p27* is a negative regulator of cell cycle and a tumor suppressor[5], tumor may develop when abnormal (missing or decreased) expression of *p27* and attenuated inhibition on cell cycle lead to uncontrolled cell growth and carcinogenesis[6]. The results of studies demonstrate that decreased *p27* expression was associated with ubiquitin-mediated proteasome phosphorylation and abnormal activity of *p27*[7,8]. We investigated the *in vivo* inhibitory effect of *p27* on transplanted tumor by intratumoral injection of mutated *p27mt* adenovirus.

Park *et al*[9] found that inhibition of mutant *p27* (*p27mt*) on tumor cells seem stronger than that of wild type *p27* (*p27wt*) as demonstrated in cells arrested in G_0/G_1 phase, and that the apoptosis promoting activity of *p27mt* is also stronger. Another study revealed that the half-life of *p27mt* is over 12 h, much longer than that of *p27wt* (2 h)[10]. Through determination of the size of transplanted tumor, this study displayed that *p27mt* gene significantly inhibited the growth of colorectal cancer by inhibiting cell proliferation and by promoting cell apoptosis, suggesting that *p27mt* can evidently suppress cell proliferation at G_0/G_1 phase. The apoptosis promoting activity of *p27mt* was more obvious in control group, while the apoptosis rate of *p27wt* was up to 37.9% ± 3.32%.

It was reported in our preliminary study that the expression level of *p27* in colorectal cancer tissue is quite low[11]. In this study, the expression of MMP-9 was significantly decreased in *p27mt* group. MMP-9, in the form of proenzyme in cytoplasm, when released under physiological condition, may degrade extracellular matrix and is involved in development of human body and multiple physiological processes including tissue repair[12]. When disturbance of MMP-9 gene occurs, increased proenzyme leads to escalated degradation of extracellular components, including Ⅳ and Ⅴ collagen and laminin, and undermined integrity of basement membrane. Therefore, MMP-9 plays a very important role in the process of tumor metastasis[13]. In this study, a reduced MMP-9 expression was observed after *p27mt* was injected. No tumor metastasis was found with in 28 d after transplantation of the tumor.

In conclusion, *p27mt* inhibits the growth of colorectal cancer by inhibiting cancer cell proliferation and promoting cell apoptosis as well as metastasis of colorectal cancer.

Table 1 Comparison of MMP-9 positive rate between different groups

Group	-	+	++	+++	Positive rate (%)
Control	12	4	11	9	66.7
Ad-LacZ	9	2	14	11	75[a]
Ad-p27mt	30	2	4	0	20[c]

[a]$P > 0.05$ *vs* control group; [c]$P < 0.05$ *vs* Ad-LacZ group and control group.

COMMENTS

Background

Along with the improvement in people's living standard and changes in diet, there has been a gradual increase in the incidence of colorectal cancer in China. However, no effective therapeutic modalities are available for it. Gene therapy for restoration of p27 expression is a promising therapy for it. A mutant type of p27 gene, with mutant of Thr-187/Pro-188 to Met-187/Ile-188, can inhibit degradation of *p27* protein through the ubiquitin-mediated pathway. The inhibitory effect of mutant *p27 (p27mt)* seems stronger than that of wild type *p27 (p27wt)* on tumor cells, as demonstrated by cells arrested in the G_0/G_1 phase. The apoptosis promoting activity of *p27mt* is also stronger. However, no study about its effect on colorectal cancer is available.

Research frontiers

p27, a cyclin-dependent kinases inhibitor, a tumor suppressor gene, and a promoter of apoptosis, has been widely investigated. Anti-tumor activity of *p27* has been demonstrated in breast, lung, and oral cancer. However, the anti-tumor bioactivity of *p27mt* has not been studied on colorectal cancer.

Innovations and breakthroughs

The results of this study indicate that *p27mt* gene has a strong anti-tumor bioactivity on colorectal cancer *in vivo* and *in vitro*.

Applications

This gene may be developed into a new therapeutic agent for colorectal cancer.

Peer review

This study showed the effect of over-expression of a mutant form of *p27* on colorectal cancer growth in a xenotransplantation model. The results are largely descriptive and the effect of *p27mt* seems modest on tumor growth. Further study is needed to show the expression of transfected *p27mt* gene.

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