

# Inhibition of adenovirus-mediated p27kip1 gene on growth of esophageal carcinoma cell strain

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## Abstract

**AIM:** To investigate the inhibition of p27kip1 gene on the growth of esophageal carcinoma cell strain (EC9706).

**METHODS:** Recombinant adenovirus Ad-p27kip1 was constructed and transfected into esophageal carcinoma cell EC-9706, and its effect on p27kip1 expression, the growth of esophageal carcinoma cell, DNA replication, protein synthesis, cell multiplication and apoptosis were explored by means of cell growth count, <sup>3</sup>H-TdR, <sup>3</sup>H-Leucine incorporation, flow cytometry, DNA fragment analysis and TUNEL.

**RESULTS:** Recombinant adenovirus Ad-p27kip1 was successfully constructed with a virus titer of  $1.24 \times 10^{12}$  pfu/ml. p27kip protein expression increased markedly after EC-9706 transfection, while incorporation quantity of <sup>3</sup>H-TdR and <sup>3</sup>H-Leucine decreased significantly. The growth of esophageal carcinoma cell was inhibited obviously. Testing of flow cytometry displayed a typical apoptosis peak, and DNA gel electrophoresis showed a typical apoptosis ladder. TUNEL showed the apoptosis rate of Ad-p27kip1 group and control group to be 37.3 % and 1.26 % ( $P < 0.001$ ) respectively.

**CONCLUSION:** Ad-p27kip1 can inhibit the growth and multiplication of esophageal carcinoma cells and induce apoptosis. Therefore, enhanced p27kip1 expression may be a new way to treat esophageal carcinoma.

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## INTRODUCTION

As a kind of thermostable cell cycle inhibitive protein, p27 (mol. wt=27 KD) can inhibit CDK activity and consequently regulate cell cycle negatively when combined with CyclinE-CDK2 or CyclinE-CDK4<sup>[1]</sup>. The expression level and activity of p27 protein are associated with the formation of tumor. p27 protein quantity in tumor is markedly lower than that in normal cells level and its content is also closely related to the degree of differentiation, action of molecular biology and prognosis of tumor<sup>[2-7]</sup>. In the present study, we aim to give a tentative

answer to whether enhanced expression of p27 protein inhibit the growth of cancer cell?

## MATERIALS AND METHODS

### Materials

**Reagent** p27kip1SP reagent was obtained from Beijing Zhongshan Biology Company. Endoenzyme, KpnI, BamHI and T4 DNA ligase were purchased from Huamei Biological Engineering Company. DMEM, RPMI1640, Lipofectamine were purchased from GIBCO/BRL Company. Low melting-temperature agarose and X-gal were obtained from Promega Company. CsCl was from Sigma Company. And high-grade neogenetic bovine serum from Hangzhou Sijiqing Biological Engineering Material Co. Ltd. p27kip1cDNA and adenovirus PCR primer were designed and synthesized by Saibaisheng Biological Company. And tryPsase from Shanghai Biological Products Company. <sup>3</sup>H-TDR and <sup>3</sup>H-Leucine were provided by Beijing Atomic Power Research Institute. DNA-PREPTMLPR and DNA-PREPTMstain were obtained from America Beckman Coulter Company.

**Plasmid, strain, adenovirus and cell line** PCMV5p27kip1 was presented by Dr. Wang Gang, Urinary Surgery Research Institute of the First Hospital of Beijing Medical University. PAACCMVPLPA and PJM17 were presented by academican Wu Zhuze, No. 2 Research Institute of Military Academy of Medical Science. DH 5a was presented by Dr. Peng Xu, Endocardial Department of the First Hospital of Beijing Medical University. Recombinant adenovirus was constructed by Molecular Biology Laboratory of Taihe Hospital. 293 cell was Hek cell line transferred from the gene of adenovirus E1 region, esophageal carcinoma cell strain EC9706 was presented by professor Wang Mingrong, China Academy of Medical Sciences.

### Methods

**Construction of adenovirus shuttle plasmid carrying p27kip1** Double enzyme cut was performed on pCMV5 p27kip1 and pACCMVPLPA with KpnI and BamHI respectively, and the fragments were separated at low melting-temperature agarose gel electrophoresis. The recovered p27kip1cDNA and adenovirus fragments were connected overnight with T<sub>4</sub>DNA ligase at 4 °C by directional clone, and then transferred into receptive colibacillus DH<sub>5a</sub>. From the selection of colonial amplification, a small quantity of plasmid was extracted and double cut with KpnI and BamHI. The presence of 690 bp and 8 800 bp in agarose gel after electrophoresis indicated that p27kip1cDNA had been inserted into adenovirus shuttle vector, hence successful construction of adenovirus shuttle plasmid pAd-p27kip1 carrying p27kip1. **Transfection of 293 cell by Lipofectamine-mediated pAd-p27kip1 and pJM17, and preparation of adenovirus recombinant Ad-p27kip1** (1) 293 cell was inoculated in a 9 cm plat, cultured in an incubator at 37 °C and 5 % CO<sub>2</sub>, and transfected at 80 % fusion. (2) DNA-lipofectamine compound was dripped in an Eppendorf tube. Plasmid pAd-p27kip1 and pJM17 were diluted in 1 ml DMEM culture fluid, and revolved

for 1 s, then lipofectamine suspension was added uniformly. It was kept at room temperature for 30 minutes. (3) DNA-lipofectamine compound was dripped in culture flasks, and cultured in an incubator at 37 °C and 5 % CO<sub>2</sub>. 0.5 % agarose was added for covering (0.5 % agarose contains 1×DMEM, 10 % Fcs), and cultured in an incubator with 37 °C and 5 % CO<sub>2</sub> after its coagulation, then pathological changes of 293 cell were observed.

#### PCR appraisal of recombinant adenovirus Ad-p27kip1<sup>[8]</sup>

From 293 cell undergone pathologic changes after common transfection, culture supernate 100 µl was drawn, from which cell chip was removed centrifugally, and DNA was extracted and precipitated, then PCR amplification was performed on this DNA model. Primers 1 and 2 were used for amplifying p27kip1 gene, primer 3 and primer 4 for amplifying adenovirus skeleton gene fragments. The product of PCR amplification was appraised with 0.8 % agarose through electrophoresis, and held to be recombinant adenovirus Ad-p27kip1 containing human p27kip1 if gene fragments of 275 bp and 860 bp were both amplified. The sequence of primers was as follows.

Primer 1: 5' CCTAGAGGGCAAAGTACGAGTA 3'

Primer 2: 5' GAAGAATCGTCGGTTGCAGGTCGCT 3'

Primer 3: 5' TCGTTTCTCAGCAAGCTGTTG 3'

Primer 4: 5' CAATCTGAACTCAAAGCGTGG 3'

#### Amplification, purification and titer of recombinant adenovirus Ad-p27kip1

Recombinant adenovirus supernate 500 µl was added to 293 cell with 80 % fusion, and cultured in an incubator at 37 °C and 5 % CO<sub>2</sub>. Ultracentrifugation in CsCl gradient and purification were performed on the adenovirus supernate by Graham method<sup>[8]</sup>. Then purified virus fluid 100 µl, 10 % SDS 20 µl, PBS 880 µl were taken to test light absorption value of OD<sub>260</sub>nm and OD<sub>280</sub> nm of the DNA of virus gene group, and accordingly to calculate the grannule quantity and purity of the virus. 1OD<sub>260</sub>=10<sup>12</sup>pfu/ml, OD<sub>260</sub>/OD<sub>280</sub>>1.3 showed the purity was fairly high, virus titer pfu/ml=A260 ×dilution×10<sup>12</sup>.

**Transduction efficiency test of adenovirus** Recombinant adenovirus Ad-LacZ was used to infect cells of esophageal carcinoma EC-9706 respectively by MOI 25, 50, 100 and 200. When X-gal was dyed, the cells dyed blue under microscope were positive cells expressed by LacZ gene, then the percentage of the cells dyed blue was calculated.

#### Influence of Ad-p27kip1 on growth curve of esophageal carcinoma cell

1×10<sup>4</sup>/ml cell suspension was made of the cultured esophageal carcinoma cells (5 % FcsRPMI1640) after digestion and collection. The cells of esophageal carcinoma were inoculated in 4 pieces of culture boards with 24 holes/ piece according to the quantity of 1 ml/hole, cultured for 24 h. When in 40-50 % fusion, cells were rinsed with RPMI1640, and went on culture for 24 h for synchronization. The test was divided into three groups: experimental group: Ad-p27kip1, negative control groups: Ad-LacZ, blank group: free-virus. The test groups were infected with the cells of esophageal carcinoma (RPMI1640) for 2 h with 100 MOI, during which the culture fluid was shaken every 15 min, and two hours later exchanged with 5 % Fcs RPMI1640 for culture. Prior to the transfection of esophageal carcinoma cells by virus, three holes were taken as cell count to obtain the average value, afterwards daily cell count was collected for 4 days, and the changes of growth curve of esophageal carcinoma cell were recorded.

**Incorporation test of <sup>3</sup>H-TdR and <sup>3</sup>H-leucine** As described above, the experimental group and control group were cultured for 3 h with 5 % Fcs RPMI1640, and then for 12 h with free-Fcs RPMI1640. Each group was added 1 µCi <sup>3</sup>H-TdR or <sup>3</sup>H-leucine, which was rinsed with PBS at the 24th, 48th and 72th hour and fixed with methyl alcohol for 10 min and absolute ethyl alcohol for 10 min. Finally, 0.1M NaOH 200 µl was added. 200 µl of each was taken after blowing, and mixed in 5 ml

scintillation liquid for overnight. On the following day, the CPM of <sup>3</sup>H was tested, three times for each group with three wells.

**Change of p27 protein expression of cell of esophageal carcinoma** p27 protein expression of the three groups was detected by immunohistochemical SP method.

**FCM detection after occurrence Ad-p27kip1 effect on esophageal carcinoma cell** After 48 h effect, cells from the three groups were collected, centrifugated under digestion (1 000 rpm, 5 min). PBS was added for regulating the cell density to 10<sup>6</sup>/ml with supernates removed. 100 µl cell suspension was put in a preparatory tube, and mixed with DNA-PREPTMLPR 200 µl. Half a minute later DNA-PREPTM stain reagent (PI staining) 2 ml was added for mixing. After staying static for half an hour, cell cycle and apoptosis were detected with FCM, and processed by SYSTEM II TM software from Coulter Company.

**Apoptosis detected with DNA fragment analysis and TUNEL method** From the experimental group after the above-mentioned effect, genome DNA was extracted regularly. DNA fragment was analyzed by agarose gel electrophoresis. At the same time, apoptosis was detected by TUNEL method, and contrasted with that in the blank group.

## RESULTS

### Construction of pAd-p27kip1 shuttle plasmid

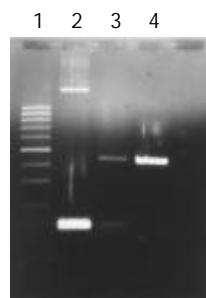
Double enzyme cutting was performed on pCMV5p27kip1 and pACCMVpLpA with KpnI and BamHI, then 690 bp fragment and 8 800 bp fragment were collected respectively at low melting-temperature agarose gel electrophoresis, for the production of plasmid pAd-p27kip1 with coupled reaction. Through transformation, amplification and extraction of colibacillus, enzyme cutting appraisal were conducted to approve that pAd-p27kip1 contained p27kip1 and adenovirus carrier skeleton (Figure 1).



**Figure 1** pAd-p27kip1 appraisal 1 200 bp ladder Marker: 200, 400, 600, 800, 1 000, 1 200, 1 400, 1 600, 1 800, 2 000 bp. 2, pCMV5p27kip1 (kpnI+BamHI): 690 bp segment. 3, pAd-p27kip1(kpnI+BamHI): 690 bp and 8 800 bp segment. 4, pACCMVPLPA (kpnI+BamHI): 8 800 bp segment. 5, Hind III marker: 2 027, 2 322, 4 361, 6 557, 9 416, 23 130 bp.

### Construction, amplification, purification, titer test of Ad-p27kip1 and PCR appraisal

When contransected with the above synthesized pAd-p27kip1 and pJM17, 293 cells underwent pathologic changes and floated as in grape clusters, which suggested that Ad-p27kip1 was produced through homologous recombinant, ultracentrifugation in CsCl gradient and purification. Spectrophotometer detection showed that the virus titer was 1.24×10<sup>12</sup> pfu/ml, OD<sub>260</sub>/OD<sub>280</sub>>1.3, which suggested that both the virus titer and the purity were fairly high. The extracted Ad-p27kip1 adenovirus DNA underwent PCR amplification for a contrast between PCMV5p27kip1 and pACCMVpLpA, with 275 bp and 860 bp as the amplification products (Figure 2).



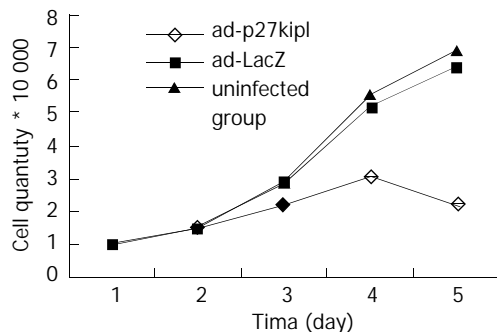
**Figure 2** Ad-p27kip1 PCR appraisal 1 200 bp ladder Marker: 200, 400, 600, 800, 1 000, 1 200, 1 400, 1 600, 1 800, 2 000 bp, 2, pCMV5p27kip1 model, 275 bp gene segment, 3, Ad-p27kip1 model: 275 bp and 860 bp gene segment, 4, PAVVMVPLPA model, 860 bp gene segment.

#### Transfection efficiency of recombinant adenovirus

Cells of esophageal carcinoma were infected with Ad-LacZ by MOI 25, 50, 100, 200 respectively, then stained with X-gal 48 h later. The transfection efficiency (%) was found to be 90, 100, 100, 100 respectively. It was concluded that 100 % transduction efficiency could be achieved with  $\text{MOI} \geq 50$ , hence MOI 100 should be adopted for EC-9706 in the following experiments.

#### Growth curve of esophageal carcinoma cell

Cell growth curve showed that the count of cancer cell in the experimental group declined over time, reached the bottom on the fourth day, but in Ad-LacZ group and blank group, it was just the opposite (Figure 3).



**Figure 3** Growth curve of cell of esophageal carcinoma.

**Table 1**  $^3\text{H}$ -TdR incorporation quantity ( $\bar{x} \pm s$  CPM/Well)

Group	2nd day	3rd day	4th day
Ad-p27kip1	2 013.42 $\pm$ 84.38 <sup>a</sup>	1 546.54 $\pm$ 104.14 <sup>a</sup>	244.22 $\pm$ 39.62 <sup>a</sup>
Ad-LacZ	3 316.81 $\pm$ 96.37 <sup>b</sup>	3 574.21 $\pm$ 68.58 <sup>b</sup>	4 451.38 $\pm$ 154.67 <sup>b</sup>
Blank	3 564.34 $\pm$ 72.82	3 685.72 $\pm$ 122.23	4 468.65 $\pm$ 237.36

<sup>a</sup> $P < 0.001$  vs Blank group, <sup>b</sup> $P > 0.05$  Ad-LacZ group vs Blank group.

**Table 2** Incorporation quantity of  $^3\text{H}$ -Leucine ( $\bar{x} \pm s$  CPM/Well)

Group	2nd day	3rd day	4th day
Ad-p27kip1	595.16 $\pm$ 57.06 <sup>a</sup>	422.82 $\pm$ 45.81 <sup>a</sup>	267.58 $\pm$ 37.56 <sup>a</sup>
Ad-LacZ	886.47 $\pm$ 48.47 <sup>b</sup>	915.44 $\pm$ 93.58 <sup>b</sup>	1 139.46 $\pm$ 89.24 <sup>b</sup>
Blank	895.21 $\pm$ 967.52	967.52 $\pm$ 78.35	1 243.95 $\pm$ 06.72

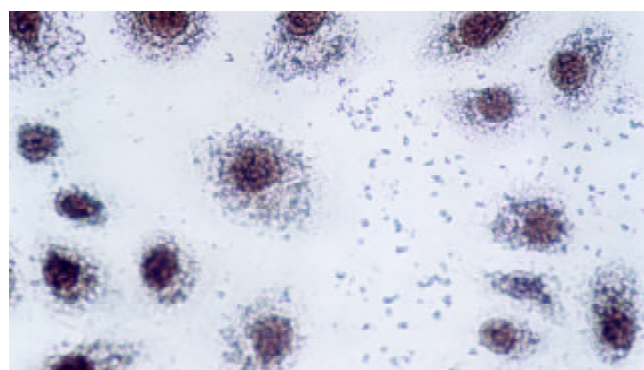
<sup>a</sup> $P < 0.001$  vs Blank group, <sup>b</sup> $P > 0.05$  Ad-LacZ group vs Blank group.

#### Effect of Ad-p27kip1 on incorporation quantity of esophageal carcinoma cells $^3\text{H}$ -TdR and $^3\text{H}$ -leucine

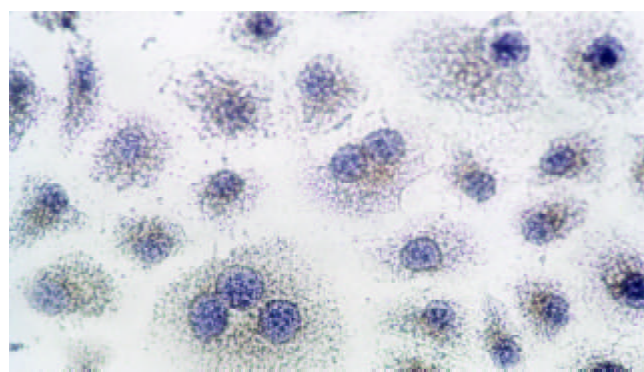
The incorporation quantity of experimental group was markedly lower than that of the control group ( $P < 0.001$ ), declined over time as against the rise in Ad-LacZ group and blank group. There was no statistical difference (Table 1 and Table 2) between them.

#### Influence of Ad-p27kip1 on p27 expression of esophageal carcinoma cell

Immunocytochemical staining after virus transfection of esophageal carcinoma cells for 24 h showed that p27 expression increased clearly in experimental group, whereas there was not any change in control group (Figure 4 and 5).



**Figure 4** p27kip1 overexpression in transfection group  $\times 200$ .



**Figure 5** p27 negative expression in control group  $\times 200$ .

#### Induction and quantitative detection of apoptosis esophageal carcinoma cells by Ad-p27kip1

Forty-eight hours after Ad-p27kip1, Ad-LacZ and blank management for EC-9706 cell, FCM determination showed that apoptotic cells took on an obvious apoptosis peak prior to G0/G1 peak. The apoptosis value of Ad-p27kip1, Ad-LacZ and blank group was 32.7 %, 5.72 % and 0.05 % respectively. Ad-p27kip1 group had the highest rate of apoptosis whereas blank group had the lowest rate.

#### Influence of Ad-p27kip on cell cycle of esophageal carcinoma

Cell cycle from FCM is listed in Table 3. In Ad-LacZ and blank control group, G1/G0 stage ratio decreased gradually whereas S stage increased, which indicated a rapidly changing G1/G0  $\rightarrow$  S procedure and active cell multiplication. However, in the experimental group, G1/G0 stage ratio was fairly high and S stage decreased. There was a significant difference from control group ( $P < 0.05$ ). Cell cycle arrested G1 and inhibited cell multiplication.

**Table 3** Effect of Ad-p27 on EC9706 cell cycle through fluid cell meter

Group	Distribution of cell cycle (%)		
	G0/G1	S	G2/M
Blank	40.39±3.86	49.61±4.27	13.10±1.03
Ad-LacZ	44.17±2.63	39.56±3.39	16.27±2.85
Ad-p27kip1	66.52±5.48 <sup>a,b</sup>	21.15±3.26 <sup>a,b</sup>	12.33±2.72

<sup>a</sup> $P < 0.05$  vs Blank group, <sup>b</sup> $P < 0.05$  vs Ad-LacZ group.

### Analysis of DNA fragment

After cell EC-9706 was processed by Ad-p27kip1, gel electrophoresis of genome DNA displayed a clear ladder band, but no ladder band was found in contrasting group (Figure 6).



**Figure 6** DNA fragment analysis 1 200 bp ladder marker: 200, 400, 600, 800, 1 000, 1 200, 1 400, 1 600, 1 800, 2 000 bp. 2, Ad-p27kip1 group: 2 µg. 3, Ad-p27kip1 group: 1 µg. 4. Blank group.

### Detection of cell apoptosis by TUNEL Method

If brownish yellow color of karyon was found with TUNEL, it showed positive apoptosis, and no color was negative. The apoptosis rate of Ad-p27kip1 group and control group was 37.3 % and 1.26 % respectively, and there was obvious difference  $\chi^2$  testing ( $P < 0.01$ ).

## DISCUSSION

Advances in cellular biology of tumor and molecular biology have found that the occurrence of esophageal carcinoma is a comprehensive pathologic process with multifunction, multistage and multigene variations. Activation of various oncogenes and inactivation of anti-oncogene may be the major factors for normal epithelial canceration. Genetic treatment aimed to import objective genes into gene mutation or lost cells with the use of gene engineering technology, and to have functional expression replacing the original genes in order to recover the functions and effects of original genes and correct genetic distortion or genetic loss resulted from cellular developmental disturbance and realize the treatment<sup>[9]</sup>. p53, p21, Egr-1, FHIT, VEGF, E2F-1 and hIFN-beta have been used as target genes in the treatment of esophagus carcinoma both *in vivo* and *in vitro*, and certain efficacy has been obtained<sup>[10-18]</sup>. Currently the analysis on esophagus carcinoma treatment through gene recombinant adenovirus p53 has entered the clinical stage<sup>[19]</sup>, which will provide a bright future for tumor treatment.

### P27 as candidate objective gene for tumor genetic treatment.

The key to genetic treatment is the correct selection of exogenous objective genes, its import into target cells and steady expression. Koguchi<sup>[20]</sup> used adenovirus carrier to pack p27 gene to transfect astrocytes, and found its overexpression inhibited multiplication activity of astrocytes. Katner<sup>[21]</sup> used

adenovirus carrier containing p27kip1cDNA to transfect human 786-0 renal carcinoma cells, and found that the cell with p27kip1 overexpression lost the growth features of tumor cells, and that CDK activity of transfected cells was inhibited obviously, and that the multiplication time was extended. Patel<sup>[22]</sup> used p27kip gene to transfect human tumor cell line AV-W9 to induce cell death. The study of p27 gene in human breast cancer cells<sup>[23]</sup>, neuroblastoma cells<sup>[24]</sup>, prostate carcinoma cells<sup>[25]</sup> and lymphoma<sup>[26]</sup> showed the similar results, suggesting that p27 gene could be used as an objective gene for genetic treatment of tumor.

### Construction of recombinant adenovirus carrier Ad-p27kip1 and its influence on p27 expression

Adenovirus carrier is of small pathogenicity and low genetic toxicity. With its wide host range, adenovirus can infect not only duplicated cells or cleavage cells, but also resting cells. Its huge package volume allows the insertion of 7.5kb exogenic gene fragment without any active carcinogene or insertion mutation within non-integral host chromosome. The virus can reach a high titer and 100 % infection rate through reproduction and purification. With its stable properties and safety for the human, it was considered as a gene conversion carrier of highly efficient expression<sup>[27]</sup> and most promising for genetic treatment<sup>[28]</sup>.

Ad-p27kip1 constructed in the present study is a kind of replication of E1-deleted adenovirus vector. pJM17, the adenovirus skeleton with E1 region removed, can produce homologous recombinant with shuttle plasmids for producing infective adenovirus. Gene in E1 region is needed for adenovirus duplication, which requires that the duplication of intact adenovirus should be carried out in cell transfected by gene of E1 region. But since 293 cell is the right packaging cell for the transfection, replication of defective adenovirus has only one opportunity for infection in target cell without any duplication ability to fulfil the functions of adenovirus carrier, avoid damage of adenovirus itself to target cells and reach gene conversion.

Ad-p27kip1 containing human p27kip1 can positively connect p27kip1cDNA to CMV promoter of adenovirus. And as appraised by PCR amplification, p27 expression increased markedly after transfection of esophageal carcinoma cell. Contrasting adenovirus Ad-LacZ used in our study achieved 100 % induction efficiency with MOI=50. Ad-p27kip1 virus titer was  $1.24 \times 10^{12}$  pfu/ml,  $OD_{260}/OD_{280} > 1.3$ , virus titer and purity were fairly high. This suggests that high expression of p27 after transfection is related to high induction efficiency and high purity, it can meet the demands of genetic treatment.

### Inhibition of Ad-p27kip1 on growth of esophageal carcinoma

Studies demonstrated that declination of p27kip1 expression was an early event of esophageal carcinoma genesis, and also an independent prognostic factor of esophageal carcinoma<sup>[29-32]</sup>. We imported human p27kip1 gene into esophageal carcinoma cell EC9706 and analyzed its biological properties through cell growth curve, <sup>3</sup>H-TdR and <sup>3</sup>H-Leucine incorporation experiment, FCM and DNA fragment analysis. We found that esophageal carcinoma cell displayed G1 stage block after import of the gene, and that p27 inhibited cell multiplication of esophageal carcinoma obviously, and that from G1 stage to S stage, the self-multiplication ability of the control cell decreased, and the apoptosis rate increased to 32.7 %, a significant difference ( $P < 0.01$ ) compared with that of control group and Ad-LacZ group. It may be concluded p27kip1 gene is an important gene for occurrence of esophageal carcinoma, decreased p27 expression may be a major factor to cell differentiation and death disturbance, while increased of p27 expression can promote

cell death of esophageal carcinoma, which offers a new perspective on treating esophageal carcinoma.

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