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Adenovirus expressing p27^{kip1} suppresses growth of established esophageal carcinoma xenografts

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Abstract

AIM: To investigate the growth suppression of adenovirus expressing p27^{kip1} on established esophageal tumors in nude mice.

METHODS: Esophageal carcinoma xenografts in nude mice were established by tumor tissue mass transplantation. The successfully constructed recombinant adenoviral vectors carrying p27^{kip1} gene (Ad-p27^{kip1}) were directly injected into the esophageal tumors in nude mice. Compared to control group, the growth curve of tumor was drawn and the growth inhibition rate of tumor was calculated. The histology of tumors was examined by hematoxylin and eosin (H&E) staining. The expression of p27^{kip1} and survivin was detected in tumors by immunohistochemical technique.

RESULTS: The growth of tumors in gene therapy group with Ad-p27^{kip1} was obviously suppressed compared to control group (0.42±0.08 g *vs* 1.17±0.30 g, t=6.39, P<0.01), the inhibition rate of tumor growth reached 64.1%. Pathological detection showed that the tumors in nude mice were poorly differentiated esophageal squamous carcinoma. In addition, the expression of p27^{kip1} was increased, while the expression of survivin was decreased in tumors after being transfected with Ad-p27^{kip1}.

CONCLUSION: p27^{kip1} gene therapy mediated by adenovirus vector has a significant inhibitory effect on esophageal carcinoma *in vivo*. Up-regulated p27^{kip1} expression and down-regulated survivin expression may be its important mechanisms.

Key words: p27^{kip1} gene; Esophageal carcinoma; Xenograft; Nude mice; Survivin gene

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INTRODUCTION

p27^{kip1} is an anti-oncogene with the function of negative regulation of cell cycle[1], and is also involved in the inhibitory reaction of cytokines, induction of cell differentiation and apoptosis, increase of cell adherence and regulation of resistance to drugs for noumenal tumors[2-6]. Our earlier investigation indicates that p27^{kip1} gene transfer mediated by adenovirus can obviously inhibit the growth of esophageal carcinoma cells[7]. Whether this gene therapy has the same effectiveness *in vivo* is worth further investigation. In this study, we explored the growth suppression of adenovirus expressing p27^{kip1} on established esophageal tumor in nude mice in order to find a new strategy for esophageal carcinoma therapy.

MATERIALS AND METHODS

Materials

The esophageal carcinoma cell strain EC109 and 4-week-old nude mice (Balb/C) of both sexes bred under specific pathogen-free conditions were purchased from Cancer Institute, Chinese Academy of Medical Sciences. pCMV5p27^{kip1} was presented by Dr. Gang Wang, Urinary Surgery Research Institute of the First Hospital of Beijing Medical University. pAACCMVpLpA and pJM17 were presented by academician Zu-Ze Wu, No. 2 Research Institute of Academy of Military Medical Sciences. DH$_{5a}$ was presented by Dr. Xu Peng, Heart Disease Department of the First Hospital of Beijing Medical University. Recombinant adenovirus was constructed by Molecular Biology Laboratory of Taihe Hospital. p27^{kip1}cDNA and adenovirus PCR primer were designed and synthesized by Saibaisheng Biological Company (Beijing, China). RPMI 1640 medium was purchased from Gibco BRL (NY, USA). Polyclonal goat antibody of survivin was purchased from Santa Cruz Biotechnology (CA, USA). Monoclonal mouse antibody of p27^{kip1}, ultra sensitive S-P kit, and 3,3-diaminobenzidine (DAB) kit were purchased from Fuzhou Maixin Biotechnology Co. Ltd (Fuzhou, China).

Construction of recombinant adenovirus Ad-p27^{kip1}

The process was the same as described in our previous work[7].

Cell culture

Human esophageal carcinoma cell strain EC109 was maintained in RPMI 1640 medium supplemented with 100 mL/L fetal calf serum (FCS), 100 kU/L penicillin, 100 mg/L streptomycin, 2 mmol/L L-glutamine, and 50 mL/L CO_2 in a humidified incubator at 37 ℃. The medium was changed every 2-3 d.

Establishment of esophageal carcinoma xenografts

EC109 cells growing exponentially were selected. The final concentration was adjusted to 10^7 cells/mL. Nude mice (Balb/C) of 4 wk old received injections into the dorsal midline in a 100 mL volume to establish tumors. The transplanted tumors were reproduced among the animals continually when the original grafts were growing well. Then esophageal carcinoma xenografts were established by transplanting the tumor tissue mass into the subcutaneous tissue of 36 nude mice. They were ready for use when the tumor diameter reached about 0.7 cm.

Therapeutic effect of intratumoral injection of Ad-p27^{kip1} into established tumors

The animals were randomized into three groups, and each group had seven mice with comparable tumor size within and among the groups. Intratumoral injection of Ad-p27^{kip1}, Ad-LacZ (1.0×10^{10} pfu) or PBS was made every other day for totally four times. The growth curve of tumor was drawn and the growth inhibitory rate of tumor was calculated after the animals were killed at wk 4. Tumor sizes were calculated by the formula: tumor volume = 1/2 ×length×width^2. The growth inhibitory rate of tumor was calculated by the formula: inhibitory rate = (tumor mass of control group–tumor mass of experimental group)/ tumor mass of control group.

Histology

The tumor tissues were fixed in 10% neutral formalin and embedded in paraffin. Sections of 5 μm thickness were used for morphological and immunohistochemical examinations. Paraffin sections were stained with hematoxylin and eosin (H&E) to demonstrate esophageal carcinoma tissue components.

Expression of p27^{kip1}

The paraffin sections were washed with phosphate-buffered saline (PBS, pH 7.4) and incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase. After being heated for 10 min in 0.01 mol/L citrate buffer (pH 6.0) using a microwave oven, the sections were incubated with normal animal serum for 10 min and then with monoclonal mouse antibody of p27^{kip1} overnight at 4 ℃. Biotinylated antimouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were subsequently applied. Finally, DAB was used for color development, and hematoxylin was used for counterstaining. As a negative control, the sections were processed in the absence of primary antibody. A scoring method was used to quantitate the p27^{kip1} expression in samples examined. A mean percentage of positive tumor cells was determined in at least five areas at 400-fold magnification. Samples with scores less than 50% were defined as low expression, otherwise as high expression[8]. These scorings were performed in a blinded fashion.

Expression of survivin

The sections carrying survivin protein were stained according to SP immunohistochemical staining method as aforementioned. The primary antibody was polyclonal goat antibody of survivin (dilution 1:200). The mean percentage of positive cells for the expression of survivin was determined in at least five areas at 400-fold magnification, and the samples with less than 10% positively stained cells were defined as negative. Samples with 10-29% positively stained cells were defined as +, 30-59% as ++, and 60% or more than 60% as +++[9].

Statistical analysis

The data were expressed as mean±SD. The difference between each group was analyzed by *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Growth suppression of established esophageal carcinoma xenografts by intratumoral injection of Ad-p27^{kip1}

Intratumoral injection of Ad-p27^{kip1} into established tumors induced partial growth suppression. The growth of tumors in gene therapy group with p27^{kip1} was obviously suppressed, being significantly different from that in control group and Ad-LacZ group ($P<0.01$). The growth inhibitory rate (IR) of tumor reached 64.1% (Figures 1 and 2, Table 1).

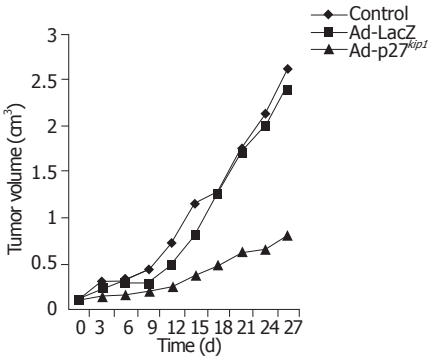

Figure 1 The growth curves of tumor.

Histological evaluation

The result of hematoxylin and eosin staining showed poorly differentiated esophageal squamous carcinoma (Figure 3).

Table 1 Tumor mass of three groups treated with Ad-p27^{kip1}, Ad-LacZ (1.0×10^{10} pfu) or PBS at wk 4

Groups (%)	Tumor mass (g)								IR
	1	2	3	4	5	6	7	mean±SD	
Control	1.02	0.93	1.31	1.47	0.86	1.63	0.95	1.17±0.30	
Ad-LacZ	0.72	1.36	1.12	0.87	0.92	1.23	0.85	1.00±0.23	
Ad-p27^{kip1}	0.32	0.46	0.35	0.43	0.57	0.38	0.40	0.42±0.08^{b}	64.1

$t = 6.39$, $^{b}P<0.01$ *vs* control group. IR: inhibition rate.

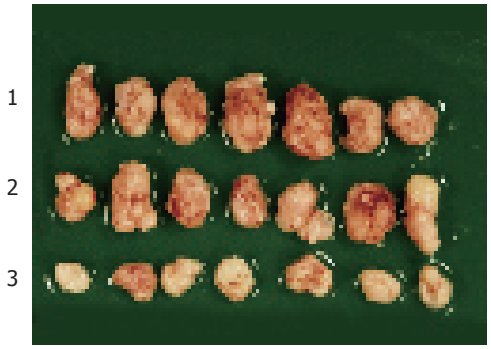

Figure 2 Growth suppression of Ad-p27^{kip1} on established esophageal carcinoma xenografts. 1: control group; 2: Ad-LacZ group; 3: Ad-p27^{kip1} group.

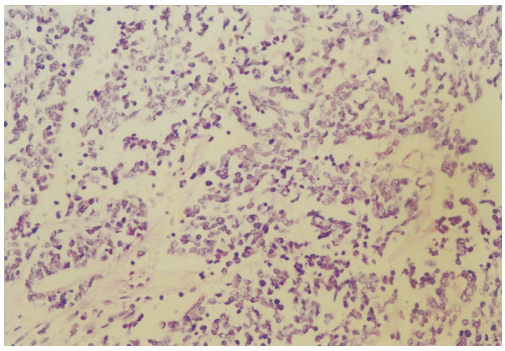

Figure 3 Hematoxylin and eosin (H&E) staining of established esophageal carcinoma xenografts (×200).

Expression of p27^{kip1}

Immunohistochemical staining showed that the expression of p27^{kip1} was increased in established esophageal carcinoma xenografts after being transfected with Ad-p27^{kip1} (Figure 4).

Expression of survivin

Survivin was prominently found in control group by immunohistochemistry and decreased in established esophageal carcinoma xenografts after being transfected with Ad-p27^{kip1} (Figure 5).

DISCUSSION

p27^{kip1} has been mapped to the short arm of chromosome 12 at the 12p12-12p13.1 boundary containing two introns and two exons[10]. Mutations of gene p27^{kip1} occur rarely in human tumors and the decrease in p27^{kip1} expression in tumor tissues is due to post-transcriptional degradation[11]. p27^{kip1} protein belongs to the family of proteins called cyclin-dependent kinase inhibitors (CDKIs). These proteins play an important role as negative regulators of cell cycle-dependent kinases during the progression of cell cycle. p27^{kip1} regulates the progression from G_1 into S phase by binding to and inhibiting the cyclin E/Cdk2 complex, which is required for entry into the S phase. It also interacts with various other cyclin complexes and is therefore designated as a universal CDKI[12-16]. p27^{kip1} expression decreases in esophageal cancer and may correlate with the histologic differentiation. Reduction of p27^{kip1} is considered to be an independent prognostic indicator of esophageal cancer[17-20].

In this study, we found that the growth of established esophageal carcinoma xenografts was obviously depressed and the inhibitory rate reached 64.1% after transfection with Ad-p27^{kip1}. The result of immunohistochemical staining demonstrated that Ad-p27^{kip1} could efficiently express p27^{kip1} in esophageal carcinoma. Ad-p27^{kip1} constructed in the present study is a kind of replication defective adenoviral vector, which has only one opportunity for infection in target cells without any dup-lication ability to fulfill the functions of adenoviral carrier, thus avoiding damage of adenovirus itself to target cells and reaching gene conversion.

We also observed that the expression of survivin was decreased in tumors of nude mice, indicating that p27^{kip1} might downregulate survivin expression. Survivin is a newly identified gene in inhibitors of apoptosis protein (IAP) family and is characterized by a unique structure with a single baculovirus IAP repeat and no zinc-binding domain known as Ring finger[21-23]. Survivin is an oncogene, which has been implicated in inhibition of apoptosis and control of mitotic progression. It is not usually detectable in normal adult tissues, but is prominently expressed in almost all common human cancers and most transformed cell lines. It is a short-lived protein degraded by ubiquitin proteasome pathway and interferes with the activation of caspases, called "cell death executioners"[24-28]. Disruption of survivin-microtubule interactions results in loss of survivin's anti-apoptosis function and increase of caspase-3 activity, a mechanism involved in cell death during mitosis. Survivin functions as a dimer and is regulated in a cell-cycle-dependent manner, peaking at G_2/M, nearly not detectable at G_1, and is associated with the mitotic spindle, centromeres, and the midbody in dividing cells[29-31]. p27^{kip1}, a down-regulating survivin may be associated with G1 blocking of p27^{kip1}, which has been identified in our previous studies[7].

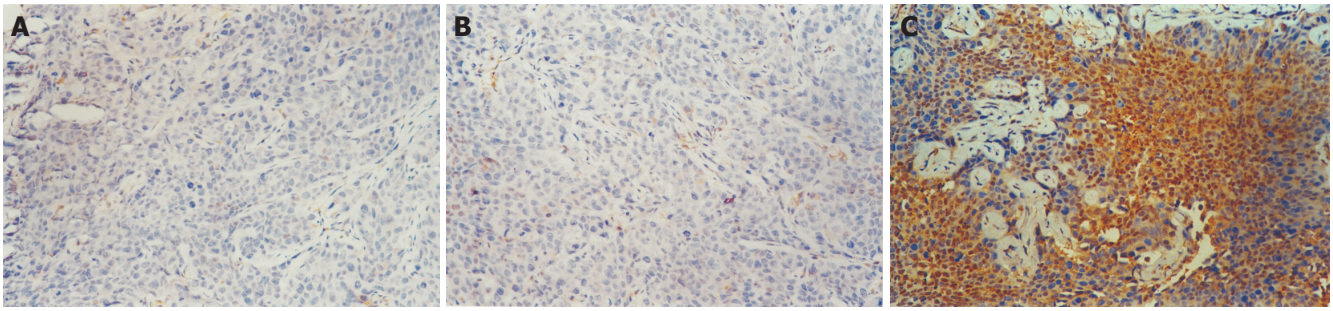

Figure 4 Result of immunohistochemical staining of tumors in nude mice. **A:** control group. p27^{kip1} protein located in cytoplasm of a few cells showing low expression; **B:** Ad-LacZ group. The result showed low expression also; **C:** Ad-p27^{kip1} group. p27^{kip1} protein located in cytoplasm and nucleus showing high expression (×200).

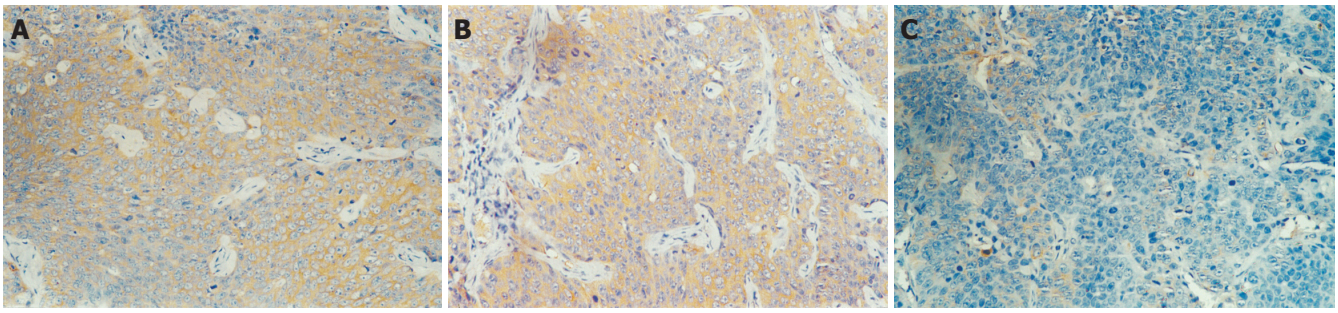

Figure 5 Result of immunohistochemical staining of tumors in nude mice. **A:** control group. Survivin protein located in cytoplasm showing high expression; **B:** Ad-LacZ group. The result showed high expression also; **C:** Ad-p27^{kip1} group. Survivin protein located in cytoplasm of a few cells showing low expression (×200).

In conclusion, p27^{kip1} gene therapy mediated by adenovirus vector has significant inhibitory effect on esophageal carcinoma *in vivo*. Upregulated p27^{kip1} expression and downregulated survivin expression may be its important mechanisms.

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