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Apoptosis Effects of ODC and AdoMetDC Biantisense Virus on Esophageal Cancer Cell Eca109*

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Abstract Polyamine biosynthesis is controlled primarily by ornithine Decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC). Antisense ODC and AdoMetDC sequences were cloned into an adenoviral vector (Ad-ODC-AdoMetDCas). To study the inhibitory effects of Ad-ODC-AdoMetDCas on polyamine biosynthesis and esophageal cancer cell apoptosis, adenovirus-mediated gene transduction efficiency was assessed with counting GFP-positive cells using MTT. The malignant phenotype of Eca109 cells was assessed by growth curve. Western blot and HPLC were used to detect ODC and AdoMetDC expression and polyamine content in Eca109 cells. TUNEL was used to analyze cell apoptosis. The change of morphology of apoptotic cells was observed by electron microscope. It was demonstrated approximate 70% of Eca109 cells were infected with Ad-ODC-AdoMetDCas when MOI reached 50. The expression of ODC was inhibited in the infected tumor cells. Ad-ODC-AdoMetDCas could inhibit Eca109 cell growth and invasive ability. TUNEL proved that Ad-ODC-AdoMetDCas can lead to cell apoptosis. Characterized morphology was observed by electron microscope (chromatin condensation, nuclear disintegration, formation of apoptotic bodies). It was suggested Ad-ODC-AdoMetDCas has significant inhibitory effects on esophageal cancer cell proliferation , leads to cell apoptosis and bears therapeutic potential for the treatment of esophageal cancer.

Key words ornithine decarboxylase, S-adenosylmethionine decarboxylase, polyamine, esophageal cancer Eca109, gene therapy
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Polyamines are naturally occurring aliphatic polycations found in almost all living organisms. Polyamines include spermidine, spermine and putrescine. Polyamines regulate gene expression by changing the sequence of DNA and regulating signal transduction, so they have critical functions in cell growth and differentiation. It was reported that the content and biosynthesis of polyamines in tumor cells or tissues was obviously advanced [1]. Ornithine decarboxylase (ODC) is the first key enzyme required for polyamine synthesis, decarboxylating ornithine to produce putrescine. The second, rate-limiting enzyme is S-adenosylmethionine decarboxylase (AdoMetDC). It generates the aminopropyl donor, decarboxylated Sadenosylmethionine (dcSAM), by decarboxylating adenosylmethionine. DcSAM donates its propylamine moiety for the formation of spermidine and spermine. Previous work has primarily focused on ODC, but the activity of AdoMetDC was also high in rumor cells. The over expression of AdoMetDC could cause malignant transformation of NIH3T3 cells. So it could be a more powerful transformation inductor than ODC. Inhibition of ODC and AdoMetDC activity might prevent the formation and metastasis of tumor[2]. These findings could help in the design of new therapeutic strategies for the treatment of esophageal cancer in future.

In order to find out the effect of esophageal carcinoma therapy by inhibiting the expression of ODC and AdoMetDC and reducing the intracellular content of polyamine, we constructed an adenovirus containing antisense sequences of both ODC and AdoMetDC (Ad-ODCAdoMetDCas) based on the construction of an adenovirus containing antisense

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sequence of ODC (Ad-ODCas)[1, 3], and studied the effect of Ad-ODCAdoMetDCas on Eca109 cell apoptosis. Then we can offer experiment evidence to gene therapy of esophageal carcinoma.

1 Materials and methods

1.1 Cell culture and reagents

Cell culture: Cell line Eca109 cells, were purchased from Shanghai Cell Bank, the Chinese Academy of Sciences, the adenoviral vector, containing a green fluorescence protein gene, Ad-GFP, Ad-ODCas and Ad-ODC-AdoMetDCas were prepared in Department of Medicine, Medical Molecular Biology Experimental Center, Shandong University[2].

Reagents: DMEM, RPMI1640 medium were purchased from Gibco company, fetal bovine serum were purchased from Hangzhou Sijiqing Biotechnology Company (Hangzhou, China), an MTT antibody and an ODC antibody were purchased from Sigma Company. TUNEL kit were purchased from Beijing Zhongshan Biotechnology Company (Beijing, China), respectively.

1.2 Multiple of infection

Dermination of infected effeciency of recombinant adenovirus (Ad-ODC-AdoMetDCas), with an appropriate incubation, the Eca109 cells were seeded at a density of 5×10^4/ml, 100 μl of erevy well were plated in 96-well tissue culture plates and grown overnight. The next day, tumor cells were infected by Ad-GFP and Ad-ODCAdoMetDCas at different MOI of 100, 50, 25, 10 and 1. Cells in each treatment group were plated in hexapoid. MTT was added(5 g/L) and incubated for 4 h. every wells were 20 μl, incubated for 4 h, then treated with DMSO150 μl, oscillation for 10 min, then the absorbance was measured at 570 nm(BIO-RAD Model 680).

1.3 MTT experiment and cell growth curve

The esophageal carcinoma cells were plated in 96-well tissue culture plates at a density of 5×10^3 cells/well with an incubation at 37℃, containing 5% CO_2. After 24 h, tumor cells were infected by Ad-GFP and Ad-ODCAdoMetDCas at an MOI of 50 and 25, with PBS as a control. Cells in each treatment group were plated in hexapoid. 20 μl MTT (5 g/L) was added in every well after 24, 48, 72 and 96 h. The cells were incubated for 4 h, and then treated with 150 μl of Dimethyl Sulfoxide to dissolve the hyacinthine sediment. Then the absorbance was measured at 570 nm(BIO-RAD Model 680) and draw the growth curve of cells.

1.4 Western blot analysis of the expression of ODC, AdoMetDC protein

After each group of 1×10^6 Eca109 cells had been treated with phosphate-buffered saline (PBS), Ad-GFP, Ad-ODCas and Ad-ODC-AdoMetDCas at an MOI of 50 for 72 h, total cell lysates were prepared in extraction buffer containing 50 mmol/L Tris (pH8.0), 1% NP-40, 1 mg/L aprotinin, 0.1% sodium dodecyl sulfate (SDS), 0.02% sodium azide, 150 mmol/L NaCl and 100 mg/L phenylmethylsulfonyl fluoride. Sample protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay. After SDS-PAGE (12% separatation gel) samples were transferred onto nitrocellulose membranes . After an incubation with 5% nonfat dry milk for 2 h, and an incubation with appropriate first antibodies to stay overnight, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and developed using the SuperSignal West Pico luminol reagent, with β-actin was used as the internal control.

1.5 Measurement of polyamine content (HPLC)

1×10^7 cells were collected and washed by ice cold PBS, then centrifuged by 1 000 r/min for 5 min. The superfluous PBS was scraped with cotton swaps. After 0.5 ml 10% perchloric acid was added, the cell sedimentation were incubated for 30 min in 4℃. The suspension was centrifuged by 12 000 *g* for 3 min. Supernatant could be stored in –20℃. 200 μl dansyl chloride desorbed in acetone, 200 μl supernatant, 50 μl hexane diamine and 50 μl saturated Na_2CO_3 were mixed, and the compound was placed in water bath with the temperature of 50℃ for 30 min. Then the compound was analyzed after application of sample. Mobile phase A and B was methanol and distilled water, respectively. A and B were mixed by 8∶2 to the column at the beginning. Then A increased to 100% in 10 min, while B decreased to 0. It was maintained for 15 min. The flow rate was 1 ml/min. The excitation wavelength of fluorescence was 370 nm, and the emission wave length was 506 nm. The paper rate was 3 mm/min. Temperature of column was 40℃.

1.6 TUNEL assay

After each group of 1×10^6 Eca109 cells had been infected with phosphate-buffered saline (PBS), Ad-GFP, and Ad-ODC-AdoMetDCas at an MOI of 50. The cells, at a density of 5×10^7/ml, were fixed in the hothouse of 4% neutral formalin solution for 10 min,

add a 100 μl cell suspension to the slide and dried, washed with PBS, incubated at room temperature for 30 min in a solution containing 0.3% H_2O_2 methanol, washed wih PBS again, incubated for 2 min with transparent liquid(0.1%TritonX-100 dissoved in 0.1% solution of Sodium Citrates) in icebath, washed with PBS and dried, a 50 μl TUNEL Reaction mixed solution was added, and then incubated in wetbox at 37℃ for 60 min, a 50 μl transforming agent -POD was added and incubated in wetbox at 37℃ for 30 min, washed with PBS, a 100 μl DAB substrate solution was added and incubated at roon temperature for 10 min. Mounting, analyze the result under the light microscope.

1.7 Ultrastrustural changes of cells observed by electron microscope

Cells above-mentioned were harvested, 4℃, fixed with 3% Glutaral, and then fixed with 1% perosmic acid, dehydrated by ethanol and acetone one by one, embedded by epoxy resin, made into ultrathin section, double stained by lead and uranium, observed and photographed using H-600A model transmission electron icroscope1(Hitachi, Japan).

1.8 Statistical analysis

Data are reported as $\bar{x} \pm s$. Statistical differences between control and treated cells were evaluated using Student's t-test (using SPSS Version 12.0).

2 Results

2.1 Recombinant adenovirusAd-ODC-AdoMetDCas inhibit the growth of esophageal cancer cells

Ad-ODC-AdoMetDCas has dose dependent inhibitory effects on esophageal cancer cells (Figure 1). On the basis of experiment result, the infection titer of the Eca109 cells we chose is at 50 MOI (Mutiple of Infection). The Eca109 cells grows fast under normal condition. After infected by Ad-ODC-AdoMetDCas, the cells grows significantly slowly, the curve of growth is lower and more level (Figure 2). The largest growth inhibiting effeciency is to 70%, and without cytotoxicity significantly.

2.2 Ad-ODC-AdoMetDCas inhibits ODC and AdoMetDC gene expression

Eca109 cells were treated with PBS, Ad-GFP, Ad-ODCas and Ad-ODC- AdoMetDCas for 72 h. After that, gene expression of ODC and AdoMetDC was tested with Western blot. The result in Figure 3 shows that gene expression of ODC was significantly decreased in cells treated with Ad-ODC (ODC expression in Eca 109 cells infected with Ad-ODC accounted for 45% of that in cells treated with rAd-GFP), but gene expression of AdoMetDC was not significantly different. While gene expression of both ODC and AdeMetDC was inhibited significantly in cells treated with Ad-ODC- AdoMetDCas. The results analyzed by SmartView software showed that ODC and AdoMetDC expression in Eca 109 cells infected with Ad-ODC- AdoMetDCas accounted for 60% of that in cells treated with rAd-GFP (Figure 3).

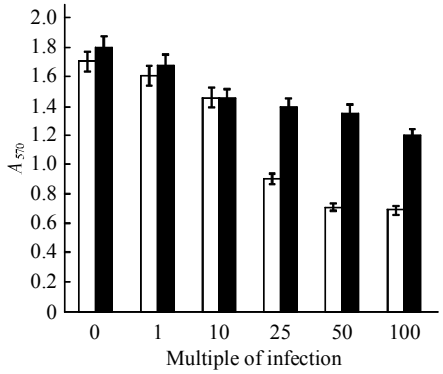

Fig. 1 Effect of different titer Ad-ODC-AdoMetDCas and Ad-GFP on cell proliferation of Eca109 cells

□: Ad-ODC-AdoMetDCas; ■: Ad-GFP.

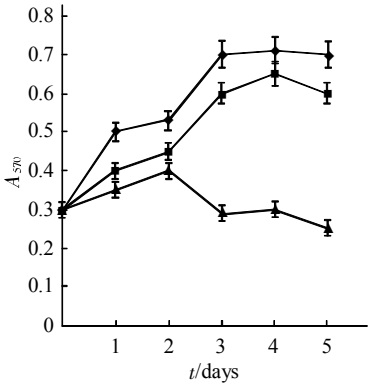

Fig. 2 Effect of different virus on proliferation of Eca109 cells

◆—◆: No virus-treated cells; ■—■: Ad-GFP-infected cells; ▲—▲: Ad-ODC-AdoMetDCas-infected cells.

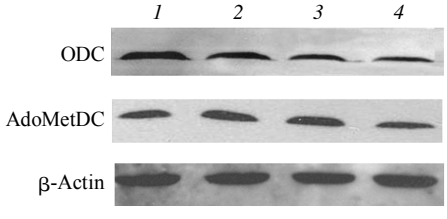

Fig. 3 Effect of adenovirus on expression of ODC and AdoMetDC

1: PBS-infected cells; *2*: Ad-GFP-infected cells; *3*: Ad-ODCas-infected cells; *4*: Ad-ODC-AdoMetDCas-infected cells.

2.3 Ad-ODC- AdoMetDCas decreases polyamine content in Eca109 cells

Ad-ODCas and Ad-ODC- AdoMetDCas decrease the expression of ODC and AdoMetDC. They also decrease polyamine content in Eca109 cells. Table 1 shows that after the incubation with Ad-ODCas for 72 h, the content of putrescine and spermidine in Eca109 cells decreased significantly ($P < 0.05$), while the content of spermine didn't decreased significantly ($P > 0.05$). HPLC also exhibited a decrease of concentrations of the three polyamines, especially of putrescine ($P<0.05$) (Table 1).

Table 1 Effects of adenovirus on polyamine content

Treatment	Polyamine pools/(pmol•mg^{-1})		
	Put	Spd	Spm
Eca109	590	1 560	1 489
+ Ad-GFP	525	1 463	1 672
+ Ad-ODCas	254*	1 189*	1 321
+ Ad-ODC-AdoMetDCas	76*	632*	337*

2.4 TUNEL assay

The Eca109 cells show significantly apoptosis after treated with Ad-ODC-AdoMetDCas ,but the rAd-GFP treated and no virus-treated cells are not significantly different(Figure 4).

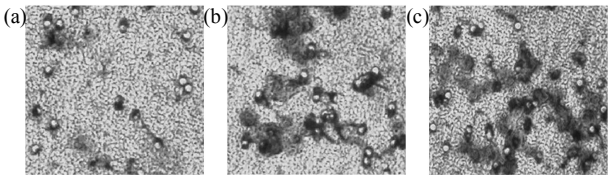

Fig. 4 Effect of Ad2-DC-AdoMetDCas on apoptosis of Eca109 cells (×100)

(a) Ad-ODC-AdoMetDCas-infected cells. (b) Ad-GFP-infected cells. (c) Control group.

2.5 Ultrastrustural changes of cells obersved by electron microscope after treated with Ad-ODC-AdoMetDCas

The Eca109 cells show significantly apoptosis, and cells on late apoptosis are observed, nuclear membrane of cells collapse,volume narrowed, vacuolar in intracytoplasm degenerated, crescent formed or assembled to the nuclear membrane massively (Figure 5).

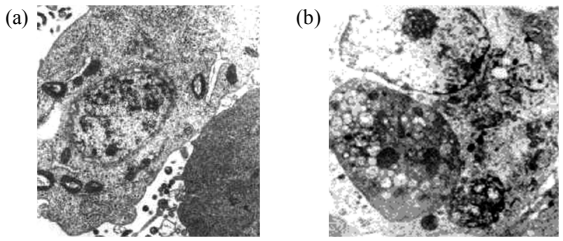

Fig. 5 Changes of cell morphology on electron microscope (×4000)

(a) Control group. (b) Ad-ODC-AdoMetDCas-infected cells(chromatin condensation, nuclear disintegration,formation of apoptotic bodies).

3 Discussion

Previous studies have demonstrated the correlation between lung cancer and aberrant polyamine biosynthesis pathway. In addition, studies in our laboratory [1, 3, 4] have demonstrated that ODC mRNA and ODC protein levels in lung cancer were higher than that were found in the surrounding normal tissues. Therefore, ODC and AdoMetDC become important targets for treating lung cancer. We had constructed an adenovirus expressing antisense ODC RNA and proved it's inhibitory effects on lung cancer growth. However, many previous studies demonstrated that AdoMetDC activity was elevated in feedback due to the single inhibition of ODC, and therefore, spermine content is unaffected while putrescine and spermidine contents are downregulated[1, 3, 4]. Therefore, based on the constructed adenovirus singly expressing antisense ODC, we further constructed an adenovirus which can simultaneously express both antisense ODC and AdoMetDC, valued its inhibitory effects on esophageal cancer proliferation and invasion, and thus provided an alternative gene therapy modality for esophageal cancer.

Gene therapy has become the hot spot of carcinoma therapy. With the continual development of molecular marker, antisense techniques that targeted certain molecule was demonstrated to have great perspective in carcinoma therapy. The carcinoma therapy that targeted ODC, AdoMetDC and metabolism of polyamines has become the hot spot of researches recently. As the key enzymes of polyamine biosynthetic pathway, ODC and AdoMetDC are mainly regulated by positive and regenerative feedback of polyamine concentration. High concentration of polyamine would induce low ODC and AdoMetDC activity, while low concentration of polyamine would

induce high ODC and AdoMetDC activity. Moreover, there are restriction and complementation between the activity of ODC and AdoMetDC. After ODC inhibition, only putrescine and spermidine were shown to be decreased, while spermine was shown to be increased. The reason is ODC inhibition would induce high AdoMetDC activity and high spermine concentration. Similarly, AdoMetDC inhibition would induce high ODC activity and high putrescine concentration. Therefore, the activity of ODC and AdoMetDC should be inhibited simultaneously to decrease the concentration of three polyamines above-mentioned. This is one of the reasons that we apply Ad-ODC- AdoMetDCas.

In our research, we demonstrated that Ad-ODC-AdoMetDCas contribute significantly to inhibiting proliferation of Eca109 cells ($P < 0.05$) and decreasing expression of ODC and AdoMetDC in Eca109 cells ($P < 0.05$). Many researches has proven that inhibition of ODC activity and expression could step down the proliferation of tumor cells [4, 5]. Some recent research about polyamine analogues indicated that polyamine analogues could inhibit proliferation of tumor cells instead of polyamines [6]. Choi, *et al.* [7] reported that suppressor (AzI) of antienzyme (Az) which is the major regulator of ODC was indicated to show high expression in Eca109 cells, by decreasing the expression of AzI and inducing high activity of Az then inhibiting ODC and decreasing the biosynthesis of polyamines. Wallon, *et al*. [8] reported that intracellular polyamine concentration regulated the sensitivity to carcinogen. It was demonstrated in many researches that in various cancers, inhibition of ODC and AdoMetDC could significantly inhibit proliferation of tumor cells, such as prostatic carcinoma, breast carcinoma, colon carcinoma and pancreatic carcinoma[9~12]. The results above-mentioned indicated that inhibition of ODC and AdoMetDC could significantly inhibit tumors, so ODC and AdeMetDC could be perspective target of tumor antisense gene therapy.

Although polyamine levels are intricately controlled by polyamine synthesis, metabolism, uptake and exhaust. Elevated activity by ODC and AdoMetDC might be responsible for increased levels of polyamines in cancer cells compared with those in normal cells. Elevated levels of ODC and AdoMetDC activity were found in various cancers, such as prostate, breast and colorectal cancer [13], and are related to cancer recurrence [14]. Our recent work has proven that inhibition of ODC activity by recombinant antisense ODC adenovirus has had antitumor effects on human lung cancer [1, 3]. This adenovirus, however, did not inhibit AdoMetDC, a critical enzyme that is normally elevated in tumor cells. We speculate that double inhibition of ODC and AdoMetDC might be a more effective way to suppress tumor growth. Our *in vitro* study demonstrated more robust antitumor effects by dual inhibition of both ODC and AdoMetDC activities compared to inhibition of ODC activity alone. Double inhibition by Ad-ODCAdoMetDCas infection significantly reduced ODC and AdoMetDC protein levels more than 50% in Eca109 cells and more than 70% in Eca109 cells compared to controls, and Ad-ODC-AdoMetDCas infection depresses three types of polyamines. In contrast, only putrescine and spermidine were shown to be decreased after Ad-ODCas infection. It might be related with Ad-ODCas only inhibitory effect on expression of ODC and no inhibitory effect on expression of AdoMetDC.

In order to approach the mechanism of ODC and AdoMetDC anti-sense RNA inhibiting the tumors proliferation. TUNEL was used to analyze cell apoptosis. As compared to controls, the Ad-ODC-AdoMetDCas infected cells show significant apoptosis (nuclear collapse, endochylema vacuolar change, karyorrhexis, chromatin condensation). As reported [15], after stimulated by active oxygen molecules, the reduction of the level of polyamines in cells lead to apoptosis and the overexpression of ODC leading to Put increase, can also induce apoptosis. The current study [15] demonstrated that, generous assemble or attenuation to the utmost of polyamines may destroy many functions of cells, thus lead to cell apoptosis. The study therefore concluded that induce to apoptosis is one of mechanisms of ODC and AdoMetDC antisense RNA inhibiting the tumors proliferation. Cell apoptosis ratio was significantly increased by TUNEL assay. Characterized morphology was observed by electron microscope (chromatin condensation, nuclear disintegration, formation of apoptotic bodies).

In summary, we provide evidence that polyamine reduction by antisense techniques that targeted ODC and AdoMetDCas, inhibits the expression of ODC and AdoMetDCas gene, suppresses esophageal cancer cell growth and proliferation *in vitro*, and leads to apoptosis. Synergistic inhibition of both ODC and

AdoMetDC activities by gene therapy approaches therefore might represent a novel treatment option for esophageal cancer and provides evidence for the feasibility of gene therapy approaches to the esophageal cancer.

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ODC 和 AdoMetDC 双反义腺病毒载体对食管癌细胞凋亡影响的研究 *

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摘要 为探讨 ODC 和 AdoMetDC 双反义腺病毒载体(Ad-ODC-AdoMetDCas)对食管癌 Eca109 细胞凋亡作用的影响，应用 MTT 法观察 Ad-ODC-AdoMetDCas 对食管癌 Eca109 细胞生长增殖的影响，采用 Western blot 和 HPLC 的方法分别检测腺病毒载体对食管癌 Eca109 细胞中 ODC 和 AdoMetDC 蛋白表达以及胞内多胺含量的抑制作用,同时应用原位末端标记(TUNEL)法观察 Ad-ODC-AdoMetDCas 对食管癌 Eca109 细胞凋亡作用的影响, 透射电镜进一步观察细胞超微结构的改变. 实验结果显示，应用 MTT 法观察发现 Ad-ODC-AdoMetDCas 对食管癌 Eca109 细胞生长增殖有显著抑制作用. 以 Ad-ODC-AdoMetDCas 感染食管癌 Eca109 细胞，可明显抑制食管癌 Eca109 细胞中 ODC 和 AdoMetDC 基因表达. HPLC 结果显示，食管癌 Eca109 细胞感染 Ad-ODC-AdoMetDCas 后,细胞内 3 种多胺含量都明显降低. TUNEL 标记检测结果显示 Ad-ODC-AdoMetDCas 可明显引起食管癌 Eca109 细胞凋亡. 透射电镜观察到典型的细胞凋亡特征(表现细胞体积缩小，核皱缩、碎裂,染色质呈块状边集等). 实验表明，ODC 和 AdoMetDC 双反义腺病毒载体(Ad-ODC-AdoMetDCas)具有显著抑制食管癌细胞生长增殖,降低细胞多胺合成,促进细胞凋亡，为探讨食管癌基因治疗的可行性提供实验依据.

关键词 鸟氨酸脱羧酶，S- 甲硫氨酸脱羧酶，多胺， 食管癌 Eca109 细胞，基因治疗

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