Inhibition Effects of C-erbB-2 and C-raf-1 ASODN Combined Transfection on Human Ovarian Carcinoma Transplanted Subcutaneously in Nude Mice

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Abstract **Objective** To investigate the inhibition effects of c-erbB-2 and c-raf-1 antisense oligodeoxynucleotides (ASODN) combined transfection on the human ovarian epithelial cancer transplanted subcutaneously in nude mice. **Methods** There were 7 groups: normal control group, c-erbB-2 sense observed group, c-raf-1 sense observed group, c-erbB-2 antisense observed group, c-raf-1 antisense observed group, whole dose combined group, half dose combined group. Human ovarian epithelial cancer cells SKOV3 were treated by different oligodeoxynucleotides, then transplanted subcutaneously in nude mice, respectively. The weight nude mice tumor volume were measured and the tumor growth inhibitory rate was calculated. **Results** There was no difference in weight between sense observed groups and normal control group. There was a larger growth inhibitory rate in whole-dose combined group and half-dose combined group, the first time of tumor formation was 13.7 days and 15.2 days, and the maximum tumor growth inhibitory rates were 61.1% and 71.3%, respectively. **Conclusions** The results suggested that ASODN combined transfection can inhibit the tumorigenesis of ovarian epithelial cancer cells in nude mice, it may be a more useful gene therapy for the ovarian epithelial carcinoma.

Key Words Antisense; Oligonucleotides; C–erbB–2; C–raf–1; Ovarian Carcinoma; Neoplasm transplantation

With the development of study on molecular biology, people begin to understand the development of tumor is a complex biological process effected by multiple steps and genes, this process involve in activation of many oncogenes and inactivation of many anti-oncogenes [1]. At present antisense gene therapy on tumor becomes a hot point in gene therapy[2]. Most of the antisense gene therapies in the past are limited in single gene and in vitro cell level, however, we chose c-erbB-2 and c-raf-1 ASODN combined transfection on human ovarian carcinoma cell line SKOV3, and then transplanted subcutaneously in nude mice. We observed the inhibit effects on tumor development and probe into the best dose of combined transfection.

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MATERIALS AND METHODS

Materials

SKOV3 cell lines are purchased from Tumor Study Lab of Chongqing Medicine University and lipofectin is purchased from Sigma Company. BALB/C nude mice are purchased from Sichuan Antibiotics Research Institute. The sequence of C-erbB-2 sense oligodeoxynucleotide (SODN) is 5′-GGTTCACACGTGGCC-3'; sequence of c-erbB-2 ASODN is 5′-CCAAGTGTGCACCGG-3'[3]; sequence of c-raf-1 SODN is 5′-AATGCATGTCACAGGCGGGA-3' and sequence of c-raf-1 ASODN is 5′-TCCCGCCTGTGACATGCATT-3'[4]. All of these SODN and ASODN are constructed and phosphorothioate repair by Shanghai Biological Industry Company. RPMI-1640 culture medium and calf serum are products of Hyclone Company.

Methods

Cell culture and lipofectin transfection: SKOV3 cell lines are cultured in RPMI-1640 culture medium containing 10% calf serum at 37℃, 5% CO_2. Digest cells in logarithmic period by 0.25% pancreatin and then inoculate into 100ml glass culture

flask with 6×10^5 cells respectively. Culture 20 h after adds 4 ml ordinary culture liquid. Wipe off the culture liquid when 50% at confluence and rinse the surface of cells twice by 3ml culture liquid without serum and antibiotic (tansfection liquid). Dissolution the lipofectin into transfection liquid and keep stillness at room temperature for 45 min and then combined with oligodeoxynucleotide at room temperature for more than 15 min. Transfection according to the following groups: group Ⅰ (normal control), lipofectin 1.5 μg + transfection fluid with total volume 2000 μl; group Ⅱ, c-erbB-2 SODN 6 μg + lipofectin 1.5 μg+ transfection fluid with total volume 2000 μl; group Ⅲ, c-raf-1 SODN 6 μg + lipofectin 1.5 μg + transfection fluid with total volume 2000 μl; group Ⅳ, c-erbB-2 ASODN 6 μg + lipofectin 1.5 μg + transfection fluid with total volume 2000 μl; group Ⅴ, c-raf-1 ASODN 6 μg+ lipofectin 1.5 μg + transfection fluid with total volume 2000 μl; group Ⅵ, whole-dose combined group, c-raf-1 ASODN 6 μg + c-erbB-2 ASODN 6 μg + lipofectin 3 μg+ transfection fluid with total volume 2000 μl; group Ⅶ, half-dose combined group, c-raf-1 ASODN 3 μg + c-erbB-2 ASODN 3 μg + lipofectin 1.5 μg+ transfection fluid with total volume 2000μl. Wipe off the transfection liquid after 18 h, then add 6ml ordinary culture liquid and continue culture 48 h. Replace 4ml fresh ordinary culture liquid and culture 2 h. Digest cells by 0.25% pancreatin and then make 2×10^7/ml suspend cell liquid.

Observation of transplanted subcutaneously tumor in nude mice: Our research use 35 BALB/C female nude mice with age ranged from four to six months and weight ranged from 17.5 g to 22.5 g. Five mice as a group and each mouse have two inoculate points located in back limb at backside. Inoculate 3.0×10^6 cells in each point and observe if there are red swell or dehiscence in inject point after inoculation. Measure the volume of tumors and weight of nude mice at regulate time.

Measure of tumor volume and weight of nude mice: Measure the longest diameter (a) and the shortest diameter (b) with slide gaud after subcutaneously inoculate 10 days, 15 days, 20 days, 25 days, 30days. Weight the nude mice when measure their tumor volume. The volume of tumor (V, mm^3)=$\pi ab^2/6$, tumor inhibitory rate=(Vc−Vo)/Vc×100%[5]. Use Vc for volume of normal control, Vo for volume of observed groups.

Statistical analysis

Adopt the methods of t-test and chi-square test to analysis.

RESULTS

The time of the tumor formation in each group: all of the 35 nude mice survive and there are tumors in each inoculate point without red swell or dehiscence. Mean time of the tumor formation is diagramed in Fig.1. The difference between the group Ⅵ, Ⅶ and group Ⅳ, Ⅴ was significant in statistics (P<0.05). Group Ⅶ is the latest group to form tumor.

The volume of tumor in each group: tumors grow rapidly in group Ⅰ to Ⅲ; tumor can not be

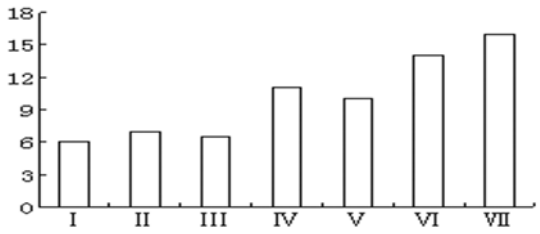

Fig. 1 Mean time of the tumor formation in different groups.

Tab. 1 Comparison of tumor volume of different time after transplanted subcutaneously(mm^3, $\bar{x}\pm S$).

Groups	Tumor volume				
	10d	15d	20d	25d	30d
Ⅰ	41±6	103±22	151±31	205±51	301±67
Ⅱ	35±5	96±21	14±31	199±50	297±60
Ⅲ	37±4	100±16	146±28	203±58	300±65
Ⅳ	0±0	71±9	90±20	146±32	254±50
Ⅴ	0±0	76±10	98±17	153±34	270±55
Ⅵ	0±0	45±8	59±11	122±27	226±46
Ⅶ	0±0	38±10	43±10	107±21	199±43

Tab. 2 Comparison of nude mice weight in different time after transplanted subcutaneously(g, $\bar{x}\pm S$).

Groups	Weight of nude mice		
	10d	20d	30d
Ⅰ	20.6±1.3	21.2±1.1	21.0±1.3
Ⅱ	19.2±1.2	20.7±1.2	20.3±1.5
Ⅲ	20.7±1.5	20.6±1.1	21.5±1.2
Ⅳ	19.6±1.6	19.6±0.7	21.1±1.2
Ⅴ	19.5±1.4	19.8±1.1	21.2±1.3
Ⅵ	20.4±1.3	20.9±0.9	21.5±1.4
Ⅶ	19.8±1.2	19.9±1.3	21.6±1.3

seen in group Ⅳ to Ⅶ 10 days after inoculate and tumor grow slowly 15 days after inoculation, especially in group Ⅶ. Tumor inhibitory rate reach tiptops 20 days after inoculation and then tumor inhibitory rate declined (Tab. 1).

Weight change of nude mice in each group: The changes in weight of nude mice in different time after inoculation were not significant in statisties (P<0.05) (Tab. 2).

DISCUSSION

The Choice of antisense target and application of antisense technology

Researches showed there are high expression of EGFR in many malignant tumor and have close relationship with the development, metastasis and prognosis of tumor[6]. By antisense control technology, we can seal the harmful gene and make it low expression or no expression at all. In this way, we can interfere with uncommon signal transduction in the signal transduction system mediated by EGFR and inverse tumor malignancy phenotype. Then the development of tumor will be inhibited. c-raf-1 can be activated by ras gene, tyrosine kinase, protein kinase and protein kinase activated by ceramide. c-raf-1 is one of the key genes in the signal transduction system mediated by EGFR[4], c-erbB-2 gene is also an important member of EGFR family[6], and both c-erbB-2 and c-raf-1 are ideal targets. Our researches aim directly at the two genes and adopt antisense control technology to observe the inhibiting tumor effect of ASODN combined transfection on human ovarian carcinoma cell line SKOV3. Ribozyme stability of oligodeoxynucleotide will be enhanced after phosphorothioate repair. Lipofectin combined with oligodeoxynucleotide by suction force of static electricity, liposome import oligodeoxynucleotide into cell by effect of cell confluence and endophagocytosis and quite increase density of oligodeoxynucleotide in cell. Combined with liposome avoid direct contact of oligodeoxynucleotide with ribozyme and increase the stability of oligodeoxynucleotide.

Action mechanism of ASODN

Maybe ASODN act in three aspects[7]: (1) ASODN hybridize with initiation site of translation and form stably doublestranded structure; (2) ASODN form RNA-DNA doublestrand with RNA by base complementation and its RNA part can be degradation by enzyme; (3) unspecific effect and noantisense effect. In our research, the c-erbB-2 ASODN we chose complementation with 5′–end coding region of c-erbB-2 mRNA[3], the c-raf-1 ASODN we chose complementation with 3′-end region did not coding of c-raf-1 mRNA[4].

Effect of ASODN transfection on tumor formation

Our results show SKOV3 have different tumor formation ability on nude mice after transfection with ASODN, especially group Ⅵ and Ⅶ. Mainly displayed in different mean time of the first time tumor can be eyeballing and tumor inhibitory rate. Their inhibitory effect has relationship with time, the difference on tumor volume increased in 20 days after transplantation, and then the difference decreased 20 days after transplantation. This maybe caused by the degradation of ASODN in cells. With the devitalization of ASODN, oncogenes recover activity. This result show antisense therapy can achieve best effect only with successive medication or append with other therapy.

Our data reveal there are difference in the time of tumor formation and tumor inhibitory rate, and inhibitory effect in group Ⅶ is better than in group Ⅵ effect. Whether there is receptor mediation or other mechanism in the process of oligodeoxynucleotide enter cell need further study. SODN has light inhibit effect on transplanted subcutaneously tumor; this is the effect of unspecific effect and noantisense effect of SODN[7].

Reliability of ASODN therapy

ASODN inhibit the uncommon gene expression which only high expression in tumor cell, so antisense gene therapy has character of target. Furthermore, low dose of liposome has little toxic effect and side effect on cells. So ASODN therapy has its reliability. The weights of nude mice have no significant lighten after treatment, this also manifest ASODN therapy has reliability.

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