Yongzhong Wu[1], Qinglan Ren[2], Shaolin Li[2]

[1] Department of oncology, The first Affiliated Hospital, Chongqing Medical University, Chongqing, 400016, P.R. China
[2] Department of Nuclear Medicine, Basic Medical College, Chongqing Medical University, Chongqing, 400016, P.R. China

Our research was designed to investigate the effects of combined C–erbB–2 and C–raf–1 antisense oligode–oxynucleotide (ASODN) on the treatment of ovarian carcinoma xenograft in nude mice. The model of xenografts derived from ovarian epithelial cancer $SKOV_3$ cells was established in Balb/C nude mice, then they were randomly divided into a negative control group and 6 experimental groups [intraperitoneal injection of (1) liposome–C–erbB–2–SODN, (2) liposome–C–raf–1–SODN, (3) liposome–C–erbB–2–ASODN, (4) liposome–C–raf–1–ASODN, (5) whole–dose combined ASODN, (6) half–dose combined ASODN]. The weight of nude mice and tumor volume were measured. The tumor growth inhibitory rates and the tumor volume decreased rates were calculated. Combined C–erbB–2 and c–raf–1 ASODN exhibited potent tumor growth inhibition. The tumor volume inhibitory rates were 72.5% and 78.4%; the tumor weight inhibitory rates were 70.7% and 75.3%; the tumor volume decreased rates were 29.7% and 41.6% for whole–dose combined group and half–dose combined group post–experiment, respectively. Of the 7 groups, there was no significant difference on nude mice weight post–experiment and therefore the toxicity was endurable. Combined C–erbB–2 and C–raf–1 ASODN showed potent tumor growth inhibition in vivo.

Antisense oligodeoxyuncleotide (ASODN); C–erbB–2; C–raf–1; Ovarian epithelial carcinoma; xenograft

Ovarian carcinoma is a kind of malignant tumor which threaten the health of women seriously. According to the latest report, there are about 23000 new cases been found in America every year [1]. Furthermore, their five years survival rate below 30% [2]. Some researcher point out that biological therapy will play a key role in the future therapy of ovarian carcinoma [3]. Studies showed the development of tumor is a complex biological process effect by multiple steps and genes, this process involve in activation of many oncogenes and inactivation of many anti–oncogenes [4]. These studies establish foundation for theory of genes combined therapy and antisense gene therapy on tumor become a hot point [5,6]. Most of the antisense gene therapies in the past are limited in single gene and in vitro cell level. C–erbB–2 and C–raf–1 are two key genes in the tumor signal transduction system mediated by epidermal growth factor receptor (EGFR). We observe the effects of C–erbB–2 and C–raf–1 gene antisense oligodeoxynucleotide (ASODN) combined on ovarian carcinoma with high expression of EGFR[7].

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Correspondence to: Wu Yong–Zhong
Tel: 86–23–69012246
Fax:86–23–68811487
E–mail: cqmdwyz@ctta.cq.cn

MATERIALS AND METHODS

$SKOV_3$ cell lines were purchased from Tumor Study Lab of our Medicine School and lipofectin is purchased from Sigma Company. BALB/c nude mice were purchased from Sichuan Antibiotics Research Institute and breeding by Laboratory Animal Centre of chong qing medical university. The sequence of C–erbB–2 sense oligodeoxynucleotide (SODN) is 5/–GGTTCACACGTGGCC–3´, sequence of C–erbB–2 ASODN is 5/–CCAAGTGTGCACCGG–3´[8]; The sequence of C–raf–1 SODN is 5/–AATGCATGTCACAGGCGGGA–3´ and sequence of C–raf–1 ASODN is 5/–TCCCGCCTGTGACATG-

CATT-3´[9]. All of these SODN and ASODN were constructed and phosphorothioate repair by Shanghai Biological Industry Company. RPMI-1640 culture medium and calf serum are products of Hyclone Company.

$SKOV_3$ cell lines were cultured in RPMI-1640 culture medium containing 10% calf serum at 37℃, 5% CO_2. Cells in logarithmic period were digested by 0.25% pancreatin and then made 2×10^7/ml suspend cell liquid. Our research use 35 BALB/c female nude mice with age range from four to six weeks and weight range from 17.5g to 22.5g. 4.0×10^6 $SKOV_3$ cells were injected to the back of nude mice near left hindlimb, and Observed if there are red swell or dehiscence in inject point after inoculation.

These nude mice were classed into 7 groups 15 days after inoculation at subcutaneous, 5 nude mice with tumor in each group. Measured the weight and tumor volume, then intraperitoneal injection according to their group: group Ⅰ is used as normal control, lipofectin 10 μg+transfection fluid 600μl (culture fluid without serum or antibiotic); group Ⅱ, C-erbB-2 SODN 40μg+lipofectin 10 μg+transfection fluid 600μl; group Ⅲ, C-raf-1 SODN 40μg+lipofectin 10 μg+transfection fluid 600μl; group Ⅳ, C-erbB-2 ASODN 40μg+lipofectin 10 μg+transfection fluid 600μl; group Ⅴ, C-raf-1 ASODN 40μg +lipofectin 10 μg +transfection fluid 600μl; group Ⅵ, whole-dose combined group, C-raf-1 ASODN 40μg+C-erbB-2 ASODN 40μg+lipofectin 20 μg+transfection fluid 1200μl; group Ⅶ, half-dose combined group, C-raf-1 ASODN 20μg+C-erbB-2 ASODN 20μg +lipofectin 10μg +transfection fluid 600μl. Before intraperitoneal injection, first resolute lipofectin in transfection fluid for 45 min then combined with ASODN for 15min at room temperature. Inject every 5-day, 3 in number. Weight the nude mice and measure their tumor volume after injection. Put these nude mice to death after 15 days of the first injection and take out of the tumor for measurement.

Measure the longest diameter (a) and the shortest diameter (b) by slide gaud. The volume of tumor (V, mm^3)$=\pi ab^2/6$, tumor volume inhibitory rate=$(Vc-Vo)/Vc\times100\%$[10]. Use Vc for volume of normal control, Vo for volume of observed groups. Tumor volume decreased rate=$(Vb-Va)/Vb\times100\%$. Use Vb for volume before treatment and Va for volume after treatment. The method to calculate tumor weight inhibitory rate is same as the way to calculate tumor volume decreased rate.

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

RESULTS

All of the 35 nude mice were alive and tumor were formed in all mice. The inject point had no red swell or dehiscence. And there were no statistical difference in the weight and tumor volume of these nude mice each group before treatment.

But days after injection, there were difference in tumor volume and tumor inhibitory rate among each group. The tumor volumes increased in the first 3 groups, however, in the rest 4 groups tumor volumes decreased with time elongation see tab.1, We observed that the group with the best effect was half-dose combined group, but there were decrease of tumor volumes in both whole-dose combined group and half-dose combined group ($P<0.05$) (Tab.1-5).

Comparison of Tumor Volume in Different Treatment Time (mm^3, $\bar{x}\pm s$)

Groups	0days	5days	10days	15days
Ⅰ	123.0±24.5	160.7±40.8	225.1±71.1	321.4±67.1
Ⅱ	116.3±20.6	146.5±39.2[c]	199.0±66.3[c]	297.1±59.8[c]
Ⅲ	128.5±16.3	151.6±37.6[c]	203.2±78.1[c]	299.7±65.1[c]
Ⅳ	119.7±19.1	117.3±35.6[a]	112.3±21.5[b]	92.5±39.8[b]
Ⅴ	122.8±25.9	120.5±41.9[a]	110.4±24.1[b]	102.6±34.7[b]
Ⅵ	125.9±27.7	122.7±31.2[a]	100.7±26.7[b]	88.5±35.9[b]
Ⅶ	118.7±19.9	102.2±29.7[b]	98.1±21.1[b]	69.3±23.2[b]

Compared with normal control group [a]$P<0.05$ [b]$P<0.01$ [c]$P>0.05$. After treatment 15 days: compared group Ⅶ with group Ⅵ, $P<0.05$; compared group Ⅶ with group Ⅵ and group Ⅴ, $P<0.01$.

DISCUSSION

Researches showed that there are high expression of EGFR in many malignant tumor and have close correlation with the development, metastasis and prognosis of tumor [7,11]. 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 uncommon signal transduction in the signal transduction system mediated by EGFR and inversio tumor malignancy phenotype. Then the development of tumor will be inhibited. C−raf−1 can be activated by ras gene, tyrosine kinase src, JAK1, protein kinase Cα and protein kinase activated by ceramide. C−raf−1 is one of the key genes in the signal transduction system mediated by EGFR, so both C−erbB−2 and C−raf−1 are ideal target [9]. Our researches aim directly at these

Comparison of the Tumor Volume Inhibitory Rates in Different Treatment Time (%)

Groups	5days	10days	15days
Ⅰ	−	−	−
Ⅱ	8.8	11.6	7.6
Ⅲ	5.7	9.7	6.8
Ⅳ	27.0	50.1	71.2
Ⅴ	25.0	51.0	68.1
Ⅵ	23.6	55.6	72.5
Ⅶ	36.4	56.4	78.4

Comparison of the Tumor Weight Inhibitory Rates Treated after 15 days

Groups	Tumor weight (mg, x±s)	Tumor weight inhibitory (%)
Ⅰ	352±69	−
Ⅱ	337±72	4.3
Ⅲ	311±63	11.6
Ⅳ	138±45	60.8
Ⅴ	125±38	64.5
Ⅵ	103±33	70.7
Ⅶ	87±37	75.3

Comparison of Tumor Volume Decreased Rates in Experimental Groups before and after Treatment

Groups	Volume (mm^3, $\bar{x}$±s)		Tumor volume decreased rate (%)
	Before treatment	After treatment	
Ⅳ	119.7±19.1	92.5±39.8	22.7
Ⅴ	122.8±25.9	102.6±34.7	16.7
Ⅵ	125.9±27.7	88.5±35.9	29.7
Ⅶ	118.7±19.9	69.3±23.2	41.6

1. Compared group Ⅶ with group Ⅵ, $P<0.05$
2. Compared group Ⅶ with group Ⅵ and group Ⅴ, $P<0.01$

Comparison of Nude Mice Weight before and after treatment

Groups	Nude mice weight (g, $\bar{x}$±s)	
	Before treatment	After treatment
Ⅰ	20.1±1.2	21.5±1.3
Ⅱ	19.9±1.1	21.3±1.3
Ⅲ	19.7±1.0	20.9±1.4
Ⅳ	18.6±0.9	20.1±1.1
Ⅴ	19.0±1.3	20.5±1.3
Ⅵ	20.3±1.1	21.9±1.2
Ⅶ	19.4±1.0	21.0±1.2

two genes and adopt antisense control technology to observe the effect of ASODN combined treatment on human ovarian carcinoma transplanted subcutaneously in nude mice. Ribozyme stability of oligodeoxynucleotide (ODN) will be enhanced after phosphorothioate repair. Lipofectin combined with ODN by suction force of static electricity, liposome import ODN into cell by effect of cell confluence and endophagocytosis and quite increase density of ODN in cell. Combined with liposome avoid direct contact of ODN with ribozyme and increase the stability of ODN. These research results establish foundation for our experiment.

Action mechanism of ASODN is still not clear completely, maybe it act in three aspects: ASODN form stably doublestranded structure with initiation site of translation and hinder the initiation of translation; ASODN from RNA-DNA doublestrand with RNA by base complementation and its RNA part can be degradation by RNaseH enzyme; unspecific effect and noantisense effect [12]. In our research, the C-erbB-2 ASODN complementation with 5´-end coding region of C-erbB-2 mRNA [8], the C-raf-1 ASODN complementation with 3´-end region did not coding of C-raf-1 mRNA[9]. Results of our experiment showed combined ASODN groups have significant better effect than single ASODN groups on transplanted subcutaneously tumor, and half-dose combined group showed better effect than whole-dose group, which suggested that there was a best-dose combined on the technology of combined ASODN. Whether this is caused by receptor mediation or other mechanism need further study. In our experiment, SODN has light effect on transplanted subcutaneously tumor, this is the effect of unspecific effect and noantisense effect of ODN [12].

In our research, combined ASODN technology has better effect than signal ASODN and this is similar to some report[13]. Now there are many studies limited in signal gene or in vitro cell level. Although they get some effects but these effects are still unsatisfactory. Combined gene therapy will be the trend of study in future and the ideal dose of combined therapy will be the hot point and question point. The ratio of lipofectin with ODN is 1:4 in our experiment, this is the result of many experiment of us, and this ratio match with report of Kim et al.[14].

ASODN inhibitory the uncommon gene expression which only high expression in tumor cell, so antisense gene therapy has character of target. Low dose of liposome has little toxic effect and side effect on cells. In order to remove the interference of liposome, we add liposome to control group with same dose. The weights of nude mice have no significant lighten after treatment, which manifested ASODN therapy to be reliability.

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