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ORIGINAL ARTICLE

Inhibition of lung adenocarcinoma cells by insulin-like growth factor-I receptor and Kirsten rat sarcoma mutations: A mutation analysis with antisense oligodeoxynucleotide

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Keywords
A549; antisense oligodeoxynucleotide; insulin-like growth factor-I receptor; KRAS; lung adenocarcinoma.

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Abstract

Background: Kirsten rat sarcoma (KRAS) mutations are widespread in lung adenocarcinoma patients. The combined utilization of KRAS antisense oligodeoxynucleotide (ASODN) and insulin-like growth factor-I receptor (IGF-IR) may inhibit the proliferation of A549 cell lines of lung adenocarcinoma.

Methods: Point mutations of the KRAS gene in A549 cells were detected by polymerase chain reaction with special sequence primers (PCR-SSP) and gene sequence analysis; ASODN was designed and synthesized according to the mutation specialty of KRAS; and the correlation of gene mutations and clinicopathological features were analyzed. Inhibition on the proliferation and morphostructure change were measured by methyl thiazolyl tetrazolium and colony-forming unit assays. Flow cytometry was used to evaluate the expression of KRAS and IGF-IR proteins and cell apoptosis and reverse transcriptase-polymerase chain reaction were used to detect the expression of KRAS and IGF-IR messenger ribonucleic acid (mRNA). Male nude mice were used to form the mice-human lung cancer model and show the inhibition of KRAS ASODN on A549 cells.

Results: PCR-SSP and gene sequence analysis results showed that the codon 12 of KRAS had changed from GGT to GTT. KRAS ASODN or IGF-IR ASODN could inhibit cell proliferation and promote apoptosis of A549 cells. However, the combined utilization of KRAS ASODN and IGF-IR ASODN could inhibit cell proliferation and promote apoptosis more powerfully than exclusive use of KRAS ASODN or IGF-IR ASODN.

Conclusion: The two ASODNs can inhibit the proliferation of lung adenocarcinoma cells through decreasing the expression of KRAS and IGF-IR mRNA and protein.

Introduction

The traditional treatment of lung adenocarcinoma includes surgery, radiotherapy, chemotherapy, immunotherapy, and endocrinotherapy.[1] But these treatments have a weak effect on lung adenocarcinoma, and, subsequently, the death rate is almost the same as incidence of the disease.[2] With the development of research on molecular biology, it has become clearer that the occurrence and development of lung adenocarcinoma is a complex process, including many processes and the activation of many genes. The activation of other genes can inhibit the process of cancer.[3,4] So far, antisense gene treatment of tumors is the hot point in gene treatment, but most experiments choose only one gene. Some evidence has proven that mutations of codon 12 in the Kirsten rat sarcoma (KRAS) gene can change the expression of protein in lung adenocarcinoma.[5] In this study, polymerase chain reaction with special sequence primers (PCR-SSP) and gene sequence analysis were used to detect the mutation and changing style of codon 12 of the KRAS gene. KRAS antisense

oligodeoxynucleotide (ASODN) was designed and synthesized according to the change style of codon 12 of KRAS. A549 cells were transfected by KRAS ASODN and insulin-like growth factor-I receptor (IGF-IR) ASODN *in vivo* and *in vitro* to show their influence on cell proliferation, cell apoptosis, and expression of the target genes.

Materials and methods

A Trizol Plus RNA Purification Kit was purchased from Gibco (Life Technologies, Langley, OK, USA). A synthesize kit for the first strand of complementary deoxyribonucleic acid (cDNA) of the Moloney-murine leukemia virus (M-MLV) was purchased from Fermentas (Pittburgh, PA, USA). PCR ampliation reaction kits and Annexin V fluorescein/propidium iodide (FITC/PI) double marked *in situ* Cell Apoptosis Detection kits were purchased from the Shanghai Jingmei Lamps and Lanterns Co. Ltd. (Shanghai China). K-ras mAb and IGF-IR mAb were sourced from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Second cross-linking sheep anti-mouse Ab marked and FITC were sourced from Immunotech (Swanton, VT, USA). Liposome transfection was sourced from Invitrogen (Lipofectamine, Life Technologies, Langley, OK, USA).

The up premier of KRAS was 5′-CGCGGATCCATGACT GAATATAAACTTGTG-3′, the down premier was 5′-CGCA AGCTTTTACATAATTACACACTTTGT-3′, and the length of the PCR product was 585 bp. The upper premier of IGF-IR was 5′-CCAAAACTGAAGCCGAGAAG-3′, the down premier was 5′-TGCAGCTGTGGATATCGATG-3′, and the length of the PCR product was 300 bp. The up premier of the inside reference β-actin was 5′-GGACCTGACTGACT ACCTC-3′, the down premier was 5semierceTCCATGACTG AATAT, and the length of the PCR product was 540 bp. All of the above premiers were made by Shanghai Kehua Bioengineering Company (China). The A549 cells were previously stored in our laboratory. Three to four month old nude mice (Certificate of Quality No: 0033298) weighing 10-12g were bought from the Shanghai Animal Laboratory Center of the Chinese Academy of Science. All of the mice were kept in a sterile room with free access to food and water.

A549 cells in logarithm growth period for PCR-SSP and gene sequence analysis were obtained by growing in culture medium (10% RPMI-1640 calf serum) for two to three days. The genomic DNA of A549 cells was extracted according to the design of the special order premiers and according to the order of cDNA in KRAS and the three mutation methods (CGT, GTT and CAT) of codon 12. The premiers were 5′-GGTAGTTGGAGCTC-3′ (R1), 5′-GTAGTTGGAGCTG T-3′ (R2), 5′-GTAGTTGGAGCTGA-3′ (R3), and 5′-CTATT GTTGGATCATATTCG-3′ (R4). The premiers were paired as follows: R1 and R4 were paired for the amplification of CGT mutation and the length of the fragment was 89 bp; R2 and R4 were paired for the amplification of GTT mutation and the length of the fragment was 88 bp; and R3 and R4 were paired for the amplification of GAT mutation and the length of the fragment was 88 bp. PCR-SSP was used to detect the mutation character of A549 according to the model of genomic DNA of A549 cells. Reverse transcriptase (RT)-PCR was used for detecting the amplification of the KRAS gene in A549 cells; 10 μL PCR product for agarose gel electrophoresis (15 g/L) was taken out after the completion of the amplification. The remaining PCR products were sent to Invitrogen to test the DNA sequence.

KRAS ASODN and IGF-IR ASODN were added to the A549 cells, respectively, after transfection by the lipidosome.[6] Two–32 mg/L of KRAS and IGF-IR were added to the 1 × 105/mL cell suspension, including A549 cells which were taken in the logarithm growth period after inoculation in 96-well plates. The same amount of culture medium was added to the cell suspension in the control group and three wells for each concentration. Cell suspension was cultured for 24, 48, 72, and 96 hours, respectively. Methyl thiazolyl tetrazolium (MTT) 20 μL (5 g/L) was added, then the supernatant was removed and 200 μL dimethyl sulfoxide was added four hours before the conclusion of the experiment. After reacting for 15 minutes at 37°C, enzyme-linked immunosorbent assay analyzer was used to detect the absorbency (A) in 570 nm wavelength and the inhibition rate of proliferation was calculated: (1 − A of experimental group/A of the control group) × 100%.

KRAS ASODN and IGF-IR ASODN were simultaneously added to the A549 cells. Inoculation was completed as per the above method, and then the A549 cells were transfected as follows: (i) 16 mg/L concentration of KRAS ASODN; (ii) 16 mg/L concentration of IGF-IR ASODN; (iii) 16 mg/L concentration of KRAS ASODN and IGF-IR ASODN. After culturing for 24, 48, 72, and 96 hours, MTT was used to detect absorbency and calculate the proliferation inhibition rate.

A549 cells taken in the logarithmic phase period were used to form single cell suspension.[7] The cell number was calculated; cell suspension was diluted into 20 cells/mL suspend and added into 6-well plates, 2 mL for each well. Each group had three parallel wells. The same amount of culture fluid was added to the control group. The cell suspensions were kept in 37°C and 5% CO_2 for two to three weeks. Clone forming efficiency was calculated according to the formula: clone forming efficiency = cell number of clone/cell number of inoculation × 100%.

Cell apoptosis was detected by the Annexin V FITC/PI double marking method. After A549 cells taken in the logarithmic phase period were inoculated in 24-well plates according to the standard of 2 mL and 1 × 105/m L cell density for each well, the groups were divided. Transfection was completed in the clone forming experiment and a negative control group was set. Cell suspension of each group was

added in three wells. The cell was isolated by centrifugation 48 hours later and washed with 4°C prepared phosphate-buffered saline (PBS). Cells were resuspended with 250 μL buffer and adjusted to 1 × 109/mL concentration. Then, 100 μL cell suspensions were taken out and added with 5 μL Annexin V-FITC and 10 μL PI into the pipe, keeping the pipe at room temperature for 15 minutes; 400 μL PBS was added to the pipe and flow cytometry was used to detect cell apoptosis.

The cell inoculation and group division followed the same process. Cells were isolated by centrifugation and washed twice with PBS. Then, 0.5 μg murine monoclonal antibody K-ras mAb and IGF-IR mAb was added to the cells, respectively. When detecting the expression of KRAS, the k-ras mAb of 1 mL/L Triton X-100/PBS should be added five minutes later. The cell was kept at room temperature for 30 minutes and washed twice again. The cell was immediately detected by flow cytometry and 1×10^4 cells were calculated. The positive critical value is designed according to negative control group results.

The expressions of KRAS and IGF-IR messenger ribonucleic acid (mRNA) were detected by RT-PCR. Trizol was used to isolate total RNA and 2 μg RNA was taken as a sample. Using OligodT18 as a premier, the first strand of cDNA was synthesized under the influence of M-MLV. For the PCR reaction, 5 μL cDNA was extracted and KRAS, IGF-IR, and β-actin genes, respectively, were amplified. Once the amplification was completed, 10 μL of PCR products were extracted for agarose gel electrophoresis (15 g/L, including 0.5 mg/L ethedium bromide). A gel image analyzer was used to detect the brand intensity level. The ratio of brand intensity level of KRAS and β-actin and the ratio of the length of IGF-IR and β-actin were made as mRNA relative contents in KRAS and IGF-IR, respectively.

Transmission electron microscope detection was then completed.[8] Cells taken after transfection by KRAS ASODN and IGF-IR ASODN with 2.5% glutaric dialdehyde and 1% osmic acid were fixed. Radiant elution was completed once acetone and resin embedded sections were made. The cells were observed by transmission electron microscope after staining by uranyl acetate and lead citrate.

Sixteen three to four week old BALB/c nude mice were injected into the right armpit with 1×10^7 A549 cells taken in the logarithmic growth phase, 0.1 mL for each nude mouse. The mice were divided into random groups 14 days later. ASODNs were injected into the tumors of the experimental group mice; 100 μL saline solutions were injected into the tumors of control group mice. The mice were injected every 48 hours, for a total of five times. The max transverse diameter (a) and max vertical diameter (b) of the tumor were measured by venier caliper every seven days after the injection. The volume of the tumor = $0.5 \times a \times b^2$. The tumor growth curvature was drawn according to the result.

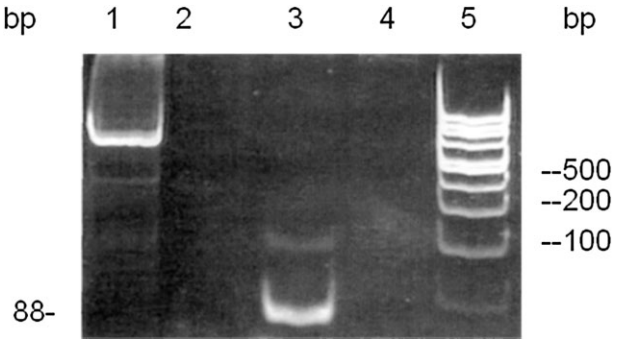

Figure 1 Detection of Kirsten rat sarcoma (KRAS) gene point mutation at codon 12 in A549 cells by polymerase chain reaction with special sequence primers. Lane 1: β-actin; Lane 2: R1-R4 group (CGT mutation); Lane 3: R2-R4 group (GTT mutation); Lane 4: R3-R4 group (GAT mutation); Lane M: 100 bpDNA marker.

The analysis of variance was calculated using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). Data was recorded in the form of $\bar{x} \pm s$ and statistical significance was evaluated by a standard of $\alpha = 0.05$.

Results

DNA of A549 was proliferated by PCR and electrophoresis by non-denaturing polyacrylamide gel and observed under an ultraviolet transmitting analyzer. After electrophoresis, R1 and R4 (CGT mutation) and R3 and R4 (GAT mutation) pairs did not show any strands, while the R2 and R4 pair showed a bright strand of 88 bp in length. The molecular weight of the PCR product met the design. This proved that GGT changes to GTT in codon 12 of the KRAS gene in A549 cells, as shown in Figures 1 and 2.

At a concentration of 2–32 mg/L, KRAS ASODN can prohibit the proliferation of A549 cells. With a higher concentration and longer duration, the inhibition effects become stronger. An obvious inhibition effect resulted when the concentration was increased to 16 mg/L and reached a peak when the concentration was increased to 32 mg/L; however, no sta-

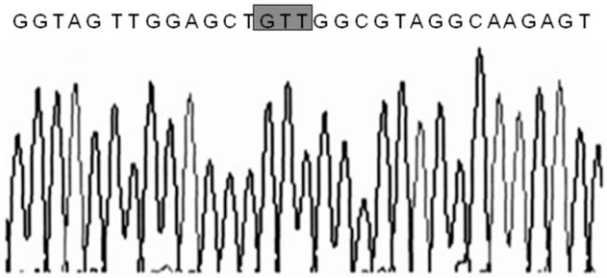

Figure 2 Sequence analysis of Kirsten rat sarcoma (KRAS) gene in A549 cells. The sequence in the box indicates the mutation of KRAS geneat codon 12 in A549 cells and the mutation style is GGT to GTT.

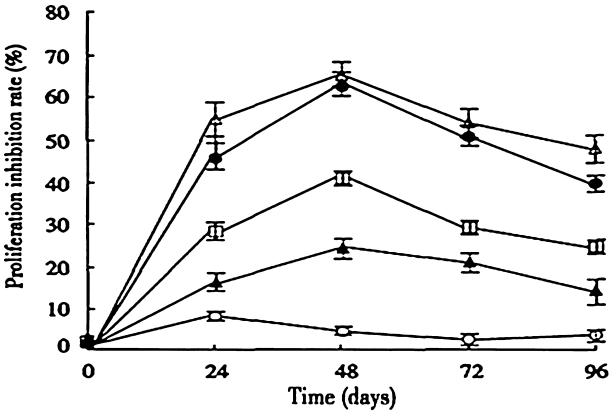

Figure 3 Proliferation inhibition rates of A549 cells after treatment of Kirsten rat sarcoma (KRAS) antisense oligodeoxynucleotide (ASODN). —○—, 2 mg/L K-ras ASODN; —▲—, 4 mg/L K-ras ASODN; —□—, 8 mg/L K-ras ASODN; —●—, 16 mg/L K-ras ASODN; —△—, 32 mg/L K-ras ASODN.

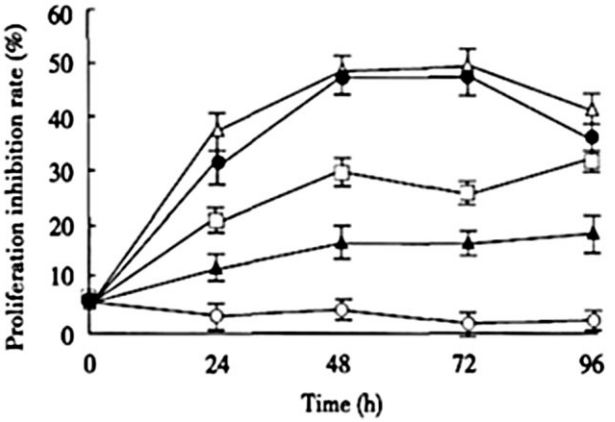

Figure 4 Proliferation inhibition rates of A549 cells after treatment of insulin-like growth factor-I receptor (IGF-IR) antisense oligodeoxynucleotide (ASODN). —○—, 2 mg/L IGF-IR ASODN; —▲—, 4 mg/L IGF-IR ASODN; —□—, 8 mg/L IGF-IR ASODN; —●—, 16 mg/L IGF-IR ASODN; —△—, 32 mg/L IGF-IR ASODN.

tistical significance was reached ($P > 0.05$). The inhibition effect of KRAS, which was 63.3% higher than the control group, also reached a peak level in the 48th hour; after which the inhibition effect became weaker and the cells began to proliferate. When used singly in all concentrations except 2 mg/L, the resulting difference between the KRAS and control groups had statistical significance ($P < 0.01$). KRAS ASODN showed almost no function when the concentration was 2 mg/L, but did inhibit the proliferation of cancer cells when the concentration was increased to 4 mg/L, 8 mg/L, 16 mg/L and 32 mg/L. At a concentration of 2–32 mg/L, IGF-IR ASODN also inhibited the proliferation of A549 cells and the inhibition effect became stronger as the concentration and duration increased.

At a concentration of 16 mg/L and 32 mg/L, the inhibition effect was obvious; however, there was no statistical difference between the results ($P > 0.05$). When the concentration was doubled from 16 g/L to 32 g/L, the effect reached a plateau at 16 mg/L. When used combined for 14, 48, 72, and 96 hours, the inhibition effect of KRAS ASODN and IGF-IR ASODN at a concentration of 16 mg/L was more obvious and showed a statistically significant difference compared with KRAS ASODN at 16 g/L concentration or IGF-IR ASODN at 16 g/L concentration separately ($P < 0.05$). When used combined, KRAS ASODN and IGF-IR ASODN at 16 mg/L reached a peak level inhibition effect on the expression of protein and RNA of KRAS and IGF-IR and the proliferation of A549 cells Figures 3–5.

The colony formation success rates of the control, KRAS ASODN, IGF-IR ASODN, and combined groups were (94 ± 3)%, (55 ± 6)%, (60 ± 3)%, and (15 ± 2)%, respectively. The success rate and the volume of the colony formation of the combined group were lower than the KRAS ASODN and IGF-IR ASODN groups. The difference was statistically significant ($P < 0.01$).

Cells marked by Annexin V FITC/PI were detected by flow cytometry. After adding the cells suspended for 48 hours, the cell apoptosis rates of the combined, KRAS ASODN, IGF-IR ASODN, and control groups were (21.5 ± 0.8)%, (12.8 ± 0.5)%, (8.4 ± 0.9)% and (1.6 ± 0.2)%, respectively. The result of the combined group compared with the other three groups showed a statistically significant difference ($P < 0.01$), while the results of the KRAS ASODN and

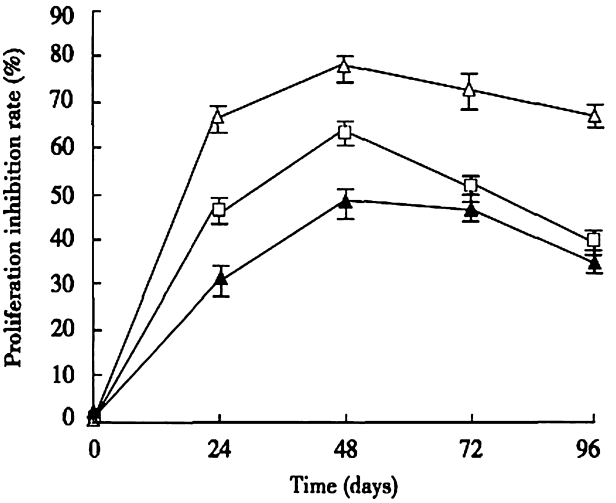

Figure 5 Proliferation inhibition rates of A549 cells after treatment of the combination group. —□—, 16 mg/L K-ras antisense oligodeoxynucleotide (ASODN); —▲—, 16 mg/L insulin-like growth factor-I receptor (IGF-IR) ASODN; —△—, combination.

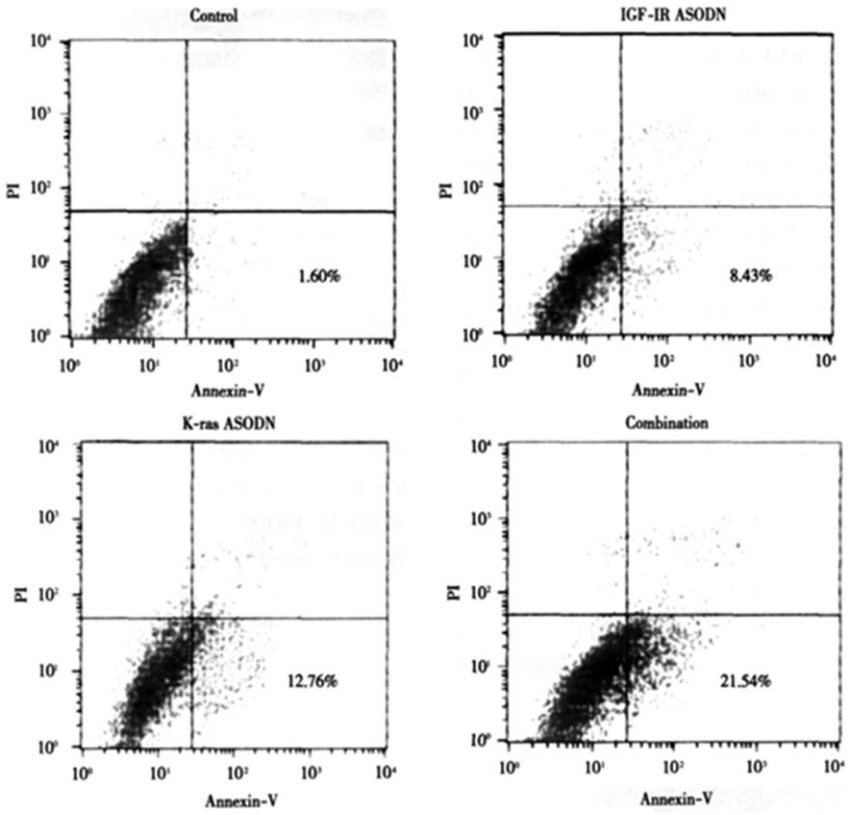

Figure 6 Apoptosis rates of A549 cells analyzed by flow cytometry.

IGF-IR ASODN groups showed no statistical significance ($P > 0.05$, Fig 6).

The expression of KRAS and IGF-IR proteins detected by flow cytometry showed that in the 96th hour at a concentration of 32 mg/L, the positive KRAS protein expression rate of the control and IGF-IR ASODN groups were 76.15% and 69.18%, respectively. The difference had no statistical significance ($P > 0.05$). In the 96th hour, with a concentration of 32 mg/L, the positive KRAS protein expression rate of the KRAS ASODN and combined groups was 25.95% and 19.69%, respectively. The KRAS protein rate expression in the KRAS ASODN and combined groups were obviously lower. The combined group showed a lower expression than the KRAS ASODN group and the difference was statistically significant ($P < 0.01$). The expression of IGF-IR protein in the 96th hour at a concentration of 32 mg/L, resulted in an IGF-IR protein positive rate expression in the control and KRAS ASODN groups of 91.53% and 85.25%, respectively; however, the difference had no statistical significance ($P > 0.05$). In the 96th hour at a concentration of 32 mg/L, the expression of IGF-IR protein positive rate of the combined and IGF-IR ASODN groups were 40.78% and 38.25%, respectively. The difference was statistically significant ($P < 0.01$). These results show that ASODN can inhibit the expression of related protein, but KRAS ASODN does not have a significant affect on the expression of IGF-IR protein and vice versa. The inhibition effect was stronger as the concentration and duration increased (Table 1).

The expression of KRAS mRNA after electrophoresis is shown in Figure 7. The average K-ras/β-actin mRNA in the IGF-IR ASODN, combined, KRAS ASODN, and control groups were 0.389 ± 0.010, 0.213 ± 0.004, 0.275 ± 0.007, and 0.391 ± 0.002, respectively. IGF-IR ASODN did not significantly influence the expression of mRNA of KRAS, while the combined utilization of KRAS ASODN and IGF-IR ASODN and the single use of KRAS ASODN had obvious inhibition effects on the expression of KRAS mRNA. The combined and single KRAS ASODN groups had statistically significant differences when compared with the results of the control group ($P < 0.05$). The combined group was 45.52% lower than the control group. The expression of IGF-IR mRNA after electrophoresis is shown in Figure 8. The average IGF-IR/β-actin mRNA of the KRAS ASODN, combined, IGF-IR ASODN, and control groups were 0.642 ± 0.007, 0.355 ± 0.006, 0.387 ± 0.005, and 0.630 ± 0.019, respectively. KRAS ASODN did not significantly influence the expression of IGF-IR mRNA, while the combined utilization of KRAS ASODN and IGF-IR ASODN and the single use of IGF-IR ASODN had obvious

Table 1 Protein expression of KRAS and IGF-IR in cells by flow cytometry (%)

		Time							
		24 hours		48 hours		72 hours		96 hours	
Concentrate	Group	KRAS protein positive rate (%)	IGF-IR protein positive rate (%)	KRAS protein positive rate (%)	IGF-IR protein positive rate (%)	KRAS protein positive rate (%)	IGF-IR protein positive rate (%)	KRAS protein positive rate (%)	IGF-IR protein positive rate (%)
2g/L	Control	74.42 ± 1.47	91.45 ± 1.82	75.21 ± 1.24	90.27 ± 1.67	76.08 ± 1.51	92.55 ± 1.61	76.05 ± 1.72	91.44 ± 1.85
	KRAS ASODN	68.42 ± 0.12	82.28 ± 1.57	67.91 ± 0.61	82.09 ± 1.54	67.22 ± 0.54	83.01 ± 1.25	65.42 ± 0.44	82.34 ± 1.58
	IGF-IR ASODN	69.62 ± 0.95	72.26 ± 1.54	68.93 ± 0.95	71.74 ± 1.64	68.24 ± 0.78	71.62 ± 1.55	68.43 ± 0.92	70.99 ± 1.85
	Combination	62.41 ± 0.54	68.72 ± 1.54	62.15 ± 0.95	68.37 ± 1.57	62.11 ± 0.65	67.44 ± 1.85	62.07 ± 0.98	68.16 ± 1.48
4 g/L	Control	76.41 ± 1.42	91.55 ± 1.85	76.28 ± 1.24	91.27 ± 1.45	76.18 ± 1.52	91.55 ± 1.66	76.21 ± 1.57	91.54 ± 1.52
	KRAS ASODN	62.42 ± 0.61	84.26 ± 1.54	58.99 ± 0.62	85.07 ± 1.57	52.72 ± 0.54	85.04 ± 1.51	41.45 ± 0.45	82.22 ± 1.54
	IGF-IR ASODN	68.66 ± 0.81	65.27 ± 1.52	68.44 ± 0.72	62.44 ± 1.45	68.17 ± 0.92	57.42 ± 1.52	68.23 ± 0.97	50.72 ± 1.48
	Combination	58.42 ± 0.41	63.75 ± 1.27	46.85 ± 0.85	59.42 ± 1.73	43.51 ± 0.69	52.42 ± 1.99	32.67 ± 0.95	45.26 ± 1.85
8 g/L	Control	76.27 ± 1.44	91.52 ± 1.91	76.24 ± 1.24	91.24 ± 1.73	76.13 ± 1.61	91.55 ± 1.61	76.22 ± 1.58	91.51 ± 1.72
	KRAS ASODN	58.42 ± 0.62	85.22 ± 1.58	55.91 ± 0.64	85.27 ± 1.54	48.72 ± 0.65	85.14 ± 1.54	28.45 ± 0.62	85.21 ± 1.52
	IGF-IR ASODN	68.21 ± 0.82	65.54 ± 1.54	68.42 ± 0.84	58.46 ± 1.51	68.27 ± 0.99	52.41 ± 1.62	67.53 ± 0.92	45.71 ± 1.42
	Combination	54.42 ± 0.92	61.55 ± 1.43	43.15 ± 0.82	55.13 ± 1.77	39.21 ± 0.57	48.45 ± 1.97	23.63 ± 0.84	42.25 ± 1.97
16 g/L	Control	76.22 ± 1.41	91.54 ± 1.93	76.21 ± 1.25	91.25 ± 1.71	76.14 ± 1.62	91.51 ± 1.61	76.18 ± 1.62	91.52 ± 1.71
	KRAS ASODN	58.42 ± 0.62	85.22 ± 1.58	48.93 ± 0.62	85.27 ± 1.54	38.72 ± 0.67	85.14 ± 1.54	24.45 ± 0.65	85.21 ± 1.52
	IGF-IR ASODN	69.22 ± 0.84	63.44 ± 1.57	69.17 ± 0.82	55.46 ± 1.58	69.22 ± 0.98	48.41 ± 1.62	69.13 ± 0.94	42.72 ± 1.55
	Combination	52.42 ± 0.94	60.41 ± 1.42	41.15 ± 0.82	53.13 ± 1.78	36.61 ± 0.88	46.45 ± 1.96	21.61 ± 0.82	40.22 ± 1.95
32 g/L	Control	76.25 ± 1.42	91.57 ± 1.92	76.18 ± 1.25	91.24 ± 1.72	76.11 ± 1.62	91.52 ± 1.66	76.15 ± 1.65	91.53 ± 1.78
	KRAS ASODN	56.92 ± 0.61	85.27 ± 1.58	42.95 ± 0.71	85.26 ± 1.58	32.15 ± 0.67	85.24 ± 1.58	25.95 ± 0.65	85.25 ± 1.56
	IGF-IR ASODN	69.21 ± 0.89	62.42 ± 1.59	69.19 ± 0.87	52.48 ± 1.55	69.19 ± 0.94	45.42 ± 1.59	69.18 ± 0.95	40.78 ± 1.58
	Combination	51.44 ± 0.94	58.41 ± 1.97	40.94 ± 0.84	52.15 ± 1.92	35.69 ± 0.81	45.25 ± 1.95	19.69 ± 0.87	38.25 ± 1.95

All values of three experiments are presented as mean ± standard deviation (SD). ASODN, antisense oligodeoxynucleotide; IGF-IR, insulin-like growth factor-I receptor; KRAS, Kirsten rat sarcoma.

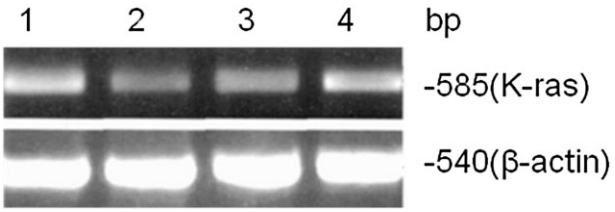

Figure 7 Expression of Kirsten rat sarcoma (KRAS) messenger ribonucleic acid in A549 cells detected by reverse transcription-polymerase chain reaction. Lane 1: A549 cells transfected with insulin-like growth factor-I receptor (IGF-IR); Lane 2: A549 cells transfected by KRAS antisense oligodeoxynucleotide (ASODN) and IGF-IR ASODN; Lane 3: A549 cells transfected by KRAS ASODN; Lane 4: A549 cells in the control group.

inhibition effects on the expression of mRNA of IGF-IR. The combined and single IGF-IR ASODN groups had statistically significant differences when compared with the results of the control group ($P < 0.05$). The combined group was 43.65% lower than the control group.

All of the mice developed a tumor within seven to 10 days and survived the experiment. The inoculation point was not swollen or had any ulceration. The weight of the mice did not significantly change once they developed a tumor, compared to their weight pre-tumor ($P > 0.05$). The tumors in the control group became larger during treatment. The maximum tumor was 1.0 cm diameter and (2230.0 ± 65.6) mm^3 volume, 49 days after inoculation. The growth of the tumor was inhibited in the KRAS ASODN, IGF-IR ASODN, and combined groups, with statistically significant differences ($P < 0.01$). The inhibition effect of the combined group was stronger than the KRAS ASODN and IGF-IR ASODN groups. The difference between the KRAS ASODN and IGF-IR ASODN groups had no statistical significance ($P > 0.05$). The tumor growth curve is shown in Figure 9.

Discussion

The development of molecular biology in recent years has led the study of tumors to the mutation of genes.[9,10] The wide-

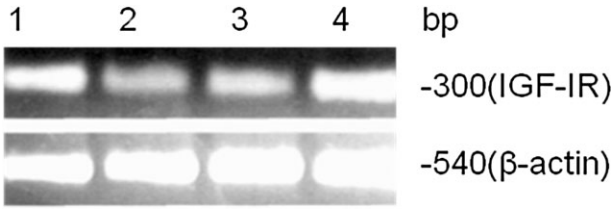

Figure 8 Expression of insulin-like growth factor-I receptor (IGF-IR) messenger ribonucleic acid in A549 cells detected by reverse transcription-polymerase chain reaction. Lane 1: A549 cells transfected with Kirsten rat sarcoma (KRAS) antisense oligodeoxynucleotide (ASODN); Lane 2: A549 cells transfected by KRAS ASODN and IGF-IR ASODN; Lane 3: A549 cells transfected by IGF-IR ASODN; Lane 4: A549 cells in the control group.

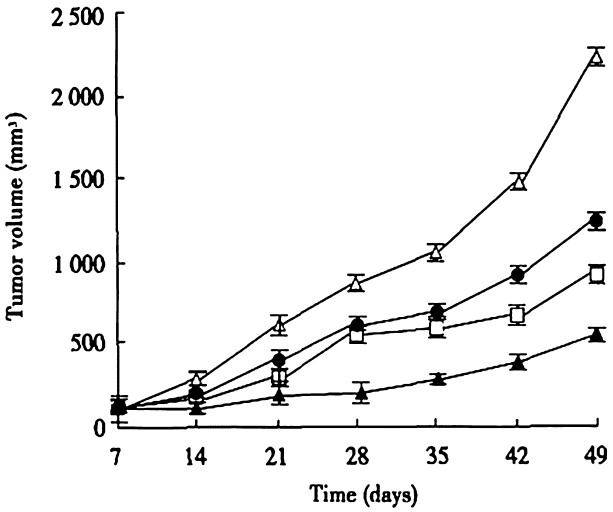

Figure 9 The inhibition effect of Kirsten rat sarcoma (KRAS) antisense oligodeoxynucleotide (ASODN) and insulin-like growth factor-I receptor (IGF-IR) ASODN in combination on tumor growth in the four groups of nude mice. —△—, control; —□—, 16 mg/L K-ras ASODN; —●—, 16 mg/L IGF-IR ASODN; —▲—, combination group.

spread and powerful effects of lung adenocarcinoma are related to gene mutation.[11] Many previous studies have proven that lung adenocarcinoma is a disease with a complex process and a united function of many genes.[12] Treatment of lung adenocarcinoma using gene therapy is promising; however, the use of only one gene may not realize the aim of treatment.[13] Theoretically, it is more effective to treat the disease with two or more genes combined.

In lung adenocarcinoma patients, 85–95% have KRAS gene mutations; most of these mutations occur on codon 12, and more than 95% of the mutations are GAT, GTT, and CGT mutations.[14] The codon 12 mutation occurs early in lung cancer patients and detection of the mutation of codon 12 of KRAS could be a method to assist in the diagnosis of lung adenocarcinoma.[15] The KRAS gene can destroy the protein activation of GTP enzymes, so that the protein remains in an active phase without developing GTP enzymes.[16] If the KRAS protein remains in the GTP enzyme binding stage, the nucleus is activated to grow steadily, possibly causing tumorigenesis.[17] Recently, scholars in China have tried to transect cancer cells with ASODN synthesized according to the KRAS gene and found that ASODN can inhibit the expression of the KRAS gene and the synthesis of the KRAS protein, thereby inhibiting growth and increasing the apoptosis of cancer cells.[18,19] These studies also report that the single use of ASODN cannot completely kill all cancer cells.[20] In search of a more effective treatment method, scholars in foreign countries have attempted to use two or more kinds of ASODN combined, such as mda-7 and IGF-I, to kill cancer cells.[21] They found that combined utilization can gain a more effective reaction than single use.[22]

IGF-IR is a trans-membrane protein with tyrosine activation and is expressed in many kinds of tumor cells.[23] The over expression of the IGF-IR can lead to and sustain the malignant phenotype of the cells and increase anti-apoptosis function, mitosis and proliferation of the tumor cell, and the invasiveness of the tumor.[24,25] Previous studies have shown that IGF-IR is over expressed in lung adenocarcinoma and IGF-IR can inhibit the growth of the tumor both *in vivo* and *in vitro* and promote the process of cell apoptosis. However, the inhibition rate is 45.13%, which is quite low.[22]

In this study, we have mainly focused on the role the mutation of codon 12 of KRAS plays in the occurrence of lung adenocarcinoma and the enhancing effect of IGF-IR in the formation of a tumor, both *in vivo* and *in vitro*. Our research used KRAS ASODN and IGF-IR SODN to transfect the A549 cells to observe their influence on the proliferation and apoptosis of cancer cells and the expression of the target gene. The results show that the two ASODNs influence the cells and genes in different concentrations and the combined utilization of the two ASODNs can cause a stronger effect. Our experiments have shown the same results. The inhibition effect of KRAS ASODN is stronger than that of IGF-IR ASODN. The ras protein is an important avenue for message transactions of IGF-IR and many other items, such as vascular endothelial growth factor; therefore, the ras protein may expand the message coming from IGF-IR and other items so as to inhibit apoptosis and prevent the growth of tumor vessels and proliferation of cancer cells. IGF-IR ASODN can only inhibit one message path of the growth factors, while KRAS ASODN can inhibit various message paths; therefore the inhibition of proliferation effect of KRAS ASODN is stronger than IGF-IR ASODN. The combined utilization of the two ASODNs can inhibit message pathways of KRAS ASODN and IGF-IR ASODN, and, thus, inhibit proliferation and kill cancer cells. KRAS ASODN and IGF-IR ASODN may be delivered to cells by a vector and suppress the activation of growth factors by certain proteins in order to inhibit the growth of tumors. Under the electron scope, there was some cell apoptosis and some of the cells even expanded, were reshaped, and broken. The two ASODNs can not only inhibit the proliferation of cells, but also kill the cancer cells directly. The mechanism of killing the cells directly is worthy of further research.

Conclusion

In brief, the study found that the combined utilization of KRAS ASODN and IGF-IR ASODN *in vivo* and *in vitro* could obviously inhibit the proliferation and apoptosis of A549 cells. The combined utilization of KRAS ASODN and IGF-IR ASODN can inhibit the expression of KRAS and IGF-IR and the expression of mRNA of KRAS and IGF-IR. Using the two ASODNs combined may be a new gene treatment method for lung adenocarcinoma. Whether they are used separately or combined, the two ASODNs cannot inhibit the growth of the cancer cell completely and the rate of tumor growth may even increase in the later stages of treatment. Apart from inhibiting the proliferation of cells, the two ASODNs can also kill cancer cells directly. More attention should be paid to the relationship between the dose and duration *versus* the effect. Various genes influence lung adenocarcinoma; therefore, tumor growth can only be inhibited by gaining control of two kinds of genes.

Disclosure

No authors report any conflict of interest.

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