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The effects of the same target on malignant proliferation of human lung cancer cells with different expression levels of COX-2 protein

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Abstract ***Objective:*** The aim of the study was to explore the effects of the same target (si-10) on lung cancer cells with different expression levels of cyclooxygenase-2 (COX-2) protein by RNAi and malignant proliferation of these cells. ***Methods:*** COX-2 was selected as the target and one siRNA expression vector with the best effect was selected and thought as the subject from three COX-2 siRNA expression vectors with human U6 promoter. The siRNA expression vector (psi-10) and the vacant vector (pEGFP) were transfected into these cells with different COX-2 expression states (801D, A549 and LTEP-A2) with lipofectamine respectively and the transfected cell strains were constructed. The change of COX-2 expression levels was examined by Western blot and RT-PCR. The effects on the proliferation of lung cancer cells were studied by cell growth curve and clonogenic assay. ***Results:*** The siRNA and U6 promoter were validated by PCR, restriction endonucleases identification and DNA sequencing and BLAST alignment and cloned into the pEGFP vector. The cell strains transfected that 801D was used as maternal line were named as 801D-p and 801D-10 respectively. The cell strains transfected that A549 was used as maternal line were named as A549-p and A549-10 respectively. The cell strains transfected that LTEP-A2 was used as maternal line were named as LTEP-A2-p and LTEP-A2-10 respectively. These cells transfected pEGFP (801D-p, A549-p and LTEP-A2-p) had the expression of GFP and 801D-10, A549-10 and LTEP-A2-10 cells had not in 24, 48 and 72 hours after transfected. The results of RT-PCR and Western blot showed the siRNA expression vector produced marked effects in two cells (A549 and LTEP-A2) expressing COX-2 and the expression of COX-2 was inhibited. But the inhibited effects were different and the expression of COX-2 was more inhibited obviously in LTEP-A2 cells than in A549 cells though the expression of COX-2 was also inhibited obviously in A549 cells. In contract to their maternal line, the levels of COX-2 mRNA of LTEP-A2-10 and A549-10 cells reduced 64.2% and 61.2% respectively; the levels of COX-2 protein reduced 60.2% and 56.2% respectively. But the levels of COX-2 mRNA and protein had not change in 801D cells not expressing COX-2. The results of cell growth curve and clonogenic assay showed the growth of LTEP-A2-10 cells slowed and the clonal formation rate reduced and the size of the colonies became small; the growth of A549-10 cells showed slow and more obviously in the cell growth curve especially. But the growth of 801D-10 cells had not obvious change. ***Conclusion:*** The si-10 target of COX-2 has different inhibition effects on lung cancer cells with different COX-2 expression levels and the different inhibition effects have different effects on cells malignant proliferation.

Key words cyclooxygenase-2 (COX-2); lung cancer cells; RNAi; malignant proliferation

Cyclooxygenase (COX) is a complete membrane binding protein. It is a rate-limiting enzyme to produce prostanoids and plays a key role in the beginning step of arachidonic acid metabolism. At present, three isozymes (COX-1, COX-2 and COX-3) of COX are found. COX-2 is an early stress gene and hardly expresses in most tissues and organs of normal organism. But it is activated by cytokines, growth factors, phorbol ester, oncogenes and chemical carcinogens and its expression increases obviously. Recent researches [1–9] show COX-2 has an important effect in tumorigenesis, development and metastasis. RNAi, a fast and efficient technique of quenching gene expression, is a powerful tool for the investigation of gene therapy in infectious diseases and tumors. In the study, RNAi technique was used to explore the interfering effect of human lung cancer cells with different expression levels of COX-2 protein and the effects on malignant proliferation of these cells *in vitro* so that we laid the founds of the study on function and tumor treatments.

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Materials and methods

Main reagents

The PCR products purification kit and relative enzymes were bought from Promega (USA). The reagents of cells culture and transfection were from Invitrogen (USA). The protein extraction kit was from Pierce (USA). All antibodies and relative reagents were from Beijing Zhongshan Biology Limited Company (China). The HRP-GAPDH control standard was bought from Shanghai Kangcheng Biology Company (China).

Cell lines

801D cells were sponsored by the Chinese People's Liberation Arm 301 Hospital. A549 cells were bought from Beijing Union Medical University Cell Centre (China). LTEP-A2 cell line was a human lung adenocarcinoma cell line constructed by our laboratory (Beijing Tuberculosis and Thoracic Tumor Research Institute, China).

Primers and the design and synthesis of COX-2 special target sites

The primers of human U6 promoter were following: P1: 5'-CGGAATTCAAGGTCGGGCAGGAAGAGGGCCTA-3'; P2: 5'-GGGGTACCTAGTATATGTGCTGCCGAAGCGAGCAC-3'. According to siRNA design principle [10, 11], three 19-nt sequences were selected from COX-2 mRNA. Three primers (P3, P4, and P5) with three different targets respectively were synthesized by Shanghai Shenggong Company (China). Every primer had a Kpnl site, transcript end signal (TTTTT) and two reverse complement arrangement 19-nt separated by 9-NT. The primers were synthesized by Shanghai Shenggong Biology Company (China).

Plasmid construction and screening the good target

The protocols of the expressed plasmids (psi-3, psi-7 and psi-10) construction and screening good target (si-10) were seen by reference 12 [12].

Selecting lung cancer cells with different expression levels of COX-2 protein

The COX-2 protein expression was examined by Western blot. The protocol was the same of following Western blot analysis.

Cell culture, plasmids transfection and construction of transfected cell strains

Genomic DNA was extracted from human peripheral blood. P1 and P2 were used to amplify human U6 promoter by PCR. U6 promoter and pEGFP plasmid were connected and transformed into E. Coli JM109. Bacterial colonies were pooled and plasmid DNA was extracted. The inserted sequence was confirmed by PCR, restriction endonucleases and DNA sequencing. The resulting plasmid was named as pU6. The pU6 plasmid was used as the model. Three couple primers were composed of P1, P3, P4, and P5 respectively. Three sequences with U6 promoter and target sites were acquired PCR and cloned into pEGFP plasmid. The resulting plasmids were identified and designated as psi-3, psi-7 and psi-10. The culture medium was RPMI 1640 (Gibco, USA) supplied with 10% fetal bovine serum (Gibco, USA), 100 U/mL of penicillin and 100 U/mL of streptomycin. A total of 3×10^5 cells were seeded into 35 mm culture plate and cultured 24 h in a humidified 37 ℃ incubator with 5% CO_2. The next day (when the cells were 70%–80% confluent), cells were transfected respectively with the plasmids (pEGFP, psi-3, psi-7 and psi-10) by lipofectamine (Invitrogen, USA) in accordance with the manufacturer's protocol. 10% RPMI 1640 with G418 (the end concentration was 800 μg/mL) was used after transfection. The cells were not cultured until the clones against G418 appeared. The cell strains were named as 801D-p, 801D-10, A549-p, A549-10, LTEP-A2-p and LTEP-A2-10 respectively.

Green fluorescence protein (GFP) expression

GFP expression was observed after pEGFP and psi-10 were transfected into three maternities (801D, A549 and LTEP-A2) 24 h, 48 h and 72 h in fluorescence microscope, respectively.

RT-PCR analysis

Total RNA of three maternities and the transfected cell strains was extracted. The concentration and purity of RNA was examined. The reactions were performed as follows: 50 ℃ 30 min, 95 ℃ 15 min, 35 cycles of denaturation (94 ℃/30 s), annealing (60 ℃/1 min), and extension (72 ℃/10 min). The primers used were COX-2-forward, 5'-AGTACCGCAAACGCTTTATGC-3'; COX-2-reverse, 5'-AACTTGCATTGATGGTGACT-3'; β-actin-forward, 5'-TGAGCGCGGCTACAGCTT-3'; β-actin-reverse, 5'-TCCTTAATGTCACGCACGATTT-3'; β-actin was used as internal standard. The predicted sizes of PCR products for COX-2 and β-actin were 389 bp and 621 bp respectively. PCR products were separated on a 1.5% agarose gel and analyzed by image acquisition and analysis system. The ratio of COX-2/β-actin was the relative expression level.

Western blot analysis

Three maternities and the transfected cell strains were harvested and lysed in mammalian cell lysis buffer. Western blot analysis was performed with the use of conventional protocols. In brief, the protein concentration was determined with a bicinchoninic acid kit with bovine serums album as a standard (Pierce, USA). Fifty microgramme total proteins were separated on 12% polyacyl-

amide gels by using standard sodium dodecyl sulfate-pdyacryamide gel electoresis (SDS-PAGE) techniques, then transfected to nitrocellulose for immunoblotting. GAPDH was used as an internal control. ECL (Pierce, USA) was adopted for detection. The ratio of COX-2/GAPDH was the protein relative expression level.

Cell growth curve assay

All cells were seeded at a density of 5×10^4 /bottle in triplicate respectively. The cell numbers were quantified every day and recorded for seven days in all. Then the cell growth curve was drawn.

Colony forming assays

Three maternities and the transfected cell strains were seeded in 35 mm dishes at 300 cells/dish. After 14 days incubation, the colonies were stained with Giemsa and manually counted. Colonies containing more than 50 cells were scored. Every group was in triplicate. The colony forming rate (= median colony number / cell number seeded × 100%) and the colony forming inhibited rate [= (colony forming rate of control group – colony forming rate of trail group) / colony forming rate of control group × 100%] were counted.

Flow cytometry assay

801D, 801D-10, A549, A549-10, LTEP-A2 and LTEP-A2-10 cells were harvested and washed once in PBS and stained with propidium iodide. The apoptotic cells and change of cell cycle were assessed by flow cytometric detection.

Statistical analysis

SPSS 10.0 software was used to analyze the data. One way ANOVA was used to compare the statistical of the differences in data from the more than two groups. Difference was considered to be significant at $P \leq 0.05$. The protein expression was analyzed with a Gel EDAS analysis system and Gel-pro Analyzer 3.1 software.

Results

Screening the good target

The inserted exogenous genes were validated by PCR, restriction endonucleares, DNA sequencing and BLAST in NCBI data. There were not base mutation, deletion and transposition to appear. The si-10 had the best effect and was selected as the target by RT-PCR and Western blot analysis.

Selecting human lung cancer cells with different expression levels of COX-2 protein

COX-2 had no expression in 801D cells, and had down expression in A549 cells and high expression in LTEP-A2 cells by Western blot assays (Fig. 1). These cells were selected as the subjects in the study.

GFP expression

Green fluorescence was seen in 801D, A549 and LTEP-A2 cells at 24 h after pEGFP plasmid was transfected. There were 12–15 cells to express green fluorescence protein in every scope. The transfected rate was 10%. The amount of GFP increased two folds at 48 h and not any more at 72 h (Fig. 2). Green fluorescence of full views was seen in LTEP-A2-p cells screened by G418 (Fig. 3). The other groups had not green fluorescence to see at 24, 48 and 72 h after transfection. The GFP expression of LTEP-

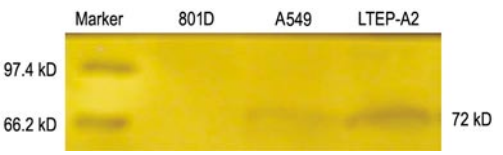

Fig. 1 The expressions of COX-2 protein in three cell lines

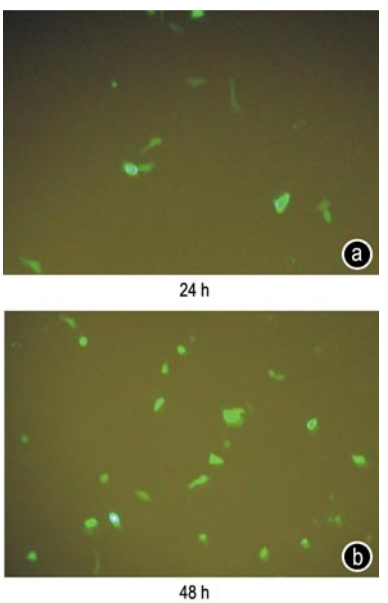

Fig. 2 The expressions of GFP after LTEP-A2 was transfected into pEGFP for 24 and 48 h

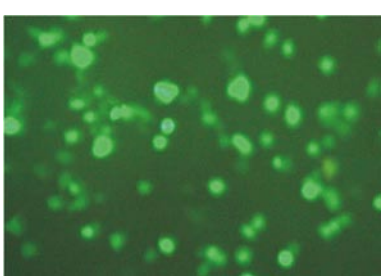

Fig. 3 The GFP expression of the transfected cell strain (LTEP-A2-10) screened by G418

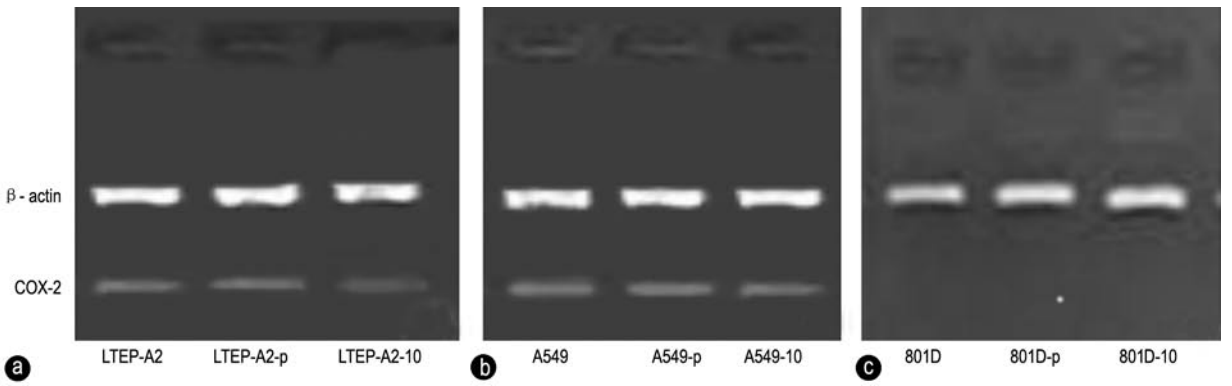

Fig. 4 The expression changes of COX-2 mRNA examined by RT-PCR

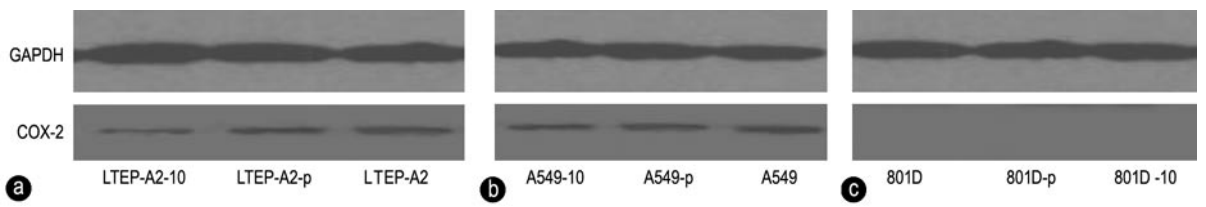

Fig. 5 The expression changes of COX-2 protein examined by Western blot

A2-p cells was only shown here. The GFP expressions of 801D and A549-p cells were similar to that of LTEP-A2-p and were not shown here in order to avoid repeat.

The changes of COX-2 mRNA by RNAi

The β-actin levels were corresponding in LTEP-A2, LTEP-A2-p and LTEP-A2-10 cells. The COX-2 mRNA levels were corresponding in the controls (LTEP-A2 and LTEP-A2-p cells). In contrast to the controls, the expression levels of COX-2 mRNA reduced in LTEP-A2-10 cells (Fig. 4a). Fig. 4b showed the β-actin levels were corresponding in A549, A549-p and A549-10 cells. The COX-2 mRNA levels were corresponding in the controls (A549 and A549-p cells). In 801D, 801D-p and 801D-10 cells, the β-actin levels were corresponding and the COX-2 mRNA expression had no changes in other words there were COX-2 mRNA expressions in three cells (Fig. 4c). The results of quantitative analysis showed the COX-2/β-actin values of LTEP-A2-10 and A549-10 cells were 0.358 and 0.388 if those of LTEP-A2 and A549 cells were defined 1 respectively. The relative levels of COX-2 mRNA reduced 64.2% and 61.2% respectively in LTEP-A2-10 and A549-10 cells.

The changes of COX-2 protein by RNAi

The GAPDH levels were corresponding in LTEP-A2, LTEP-A2-p and LTEP-A2-10 cells. The COX-2 mRNA levels were corresponding in the controls (LTEP-A2 and LTEP-A2-p cells). In contrast to the controls, the expression levels of COX-2 protein reduced in LTEP-A2-10 cells (Fig. 5a). Fig. 5b showed the GAPDH levels were corresponding in A549, A549-p and A549-10 cells. The COX-2 protein levels were corresponding in the controls (A549 and A549-p cells). In 801D, 801D-p and 801D-10 cells, the GAPDH levels were corresponding and the COX-2 protein expression had no changes in other words there were COX-2 protein expressions in three cells (Fig. 5c). The results of quantitative analysis showed the COX-2/GAPDH values of LTEP-A2-10 and A549-10 cells were 0.398 and 0.438 if those of LTEP-A2 and A549 cells were defined 1 respectively. The relative levels of COX-2 protein reduced 60.2% and 56.2% respectively in LTEP-A2-10 and A549-10 cells.

Decreased levels of COX-2 significantly altered the growth speeds of lung cancer cells

Fig. 6 showed the growth speeds of LTEP-A2 and LTEP-A2-p were corresponding in seven days. The growth speed of LTEP-A2-10 was very slow from the first day to the seventh day and had a gently increased trend. Fig. 7 showed the growth speeds of A549 and A549-p were corresponding in seven days. The growth of A549-10 cells had a gently in creased tread from the first day to the seventh day. The results of statistical analysis showed LTEP-A2-10 was different with LTEP-A2 and LTEP-A2-p ($P < 0.05$). But the growth speeds of 801D, 801D-p and 801D-10 cells were not different ($P > 0.05$; Fig. 8).

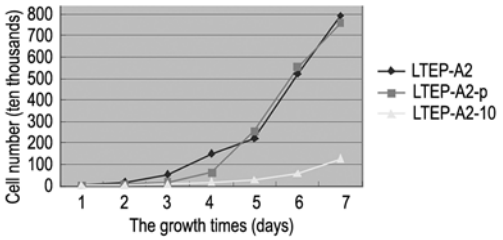

Fig. 6 The growth states of LTEP-A2, LTEP-A2-p and LTEP-A2-10

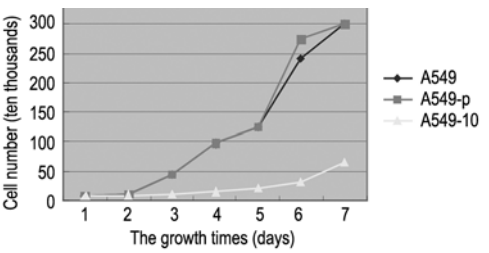

Fig. 7 The growth states of A549, A549-p and A549-10

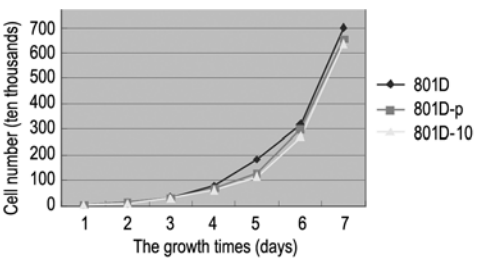

Fig. 8 The growth states of 801D, 801D-p and 801D-10

Decreases of COX-2 proteins inhibit colony formation

Table 1 and Fig. 9a showed the colony numbers of LTEP-A2 and LTEP-A2-p were corresponding and that of LTEP-A2-10 reduced obviously ($P < 0.05$) and the colony sizes of LTEP-A2-10 became small in contrast to the controls. Table 2 and Fig. 9b showed the colony numbers of two controls (A549 and A549-p) were corresponding and

Table 1 The colony formation of LTEP-A2, LTEP-A2-p and LTEP-A2-10

Groups	The colony numbers ($\bar{\chi} \pm s$)	The colony formation rate (%)	The colony formation inhibited rate (%)
LTEP-A2	108 ± 17	36.0	
LTEP-A2-p	120 ± 20	40.0	
LTEP-A2-10	30 ± 13*	10.0*	73.7*

* $P = 0.001$, LTEP-A2-10 was different with two controls (LTEP-A2 and LTEP-A2-p)

Table 2 The colony formation of A549, A549-p and A549-10

Groups	The colony numbers ($\bar{\chi} \pm s$)	The colony formation rate (%)	The colony formation inhibited rate (%)
A549	108 ± 37	36.0	
A549-p	120 ± 40	40.0	
A549-10	45 ± 28*	15.0*	60.5*

* $P = 0.003$, A549-10 was different with two controls (A549 and A549-p)

Table 3 The colony formation of 801D, 801D-p and 801D-10

Groups	The colony numbers ($\bar{\chi} \pm s$)	The colony formation rate (%)	The colony formation inhibited rate (%)
801D	146 ± 20	48.6	
801D-p	152 ± 36	50.6	
801D-10	126 ± 48	42.0	15.3

$P > 0.05$, 801D-10 was not different with two controls (801D and 801D-p)

that of A549-10 reduced obviously ($P < 0.05$). There were not significant differences in 801D, 801D-p and 801D-10 ($P > 0.05$; Table 3 and Fig. 9c). Because the colony numbers of the maternities were not different with those of their transfected cell strains with vain vectors, the colony numbers of LTEP-A2 and LTEP-A2-p and these of A549 and A549-p were averaged respectively as the colony numbers of their respective. The colony forming rates of two controls were 38.0% and 37.0% respectively. The colony formation inhibited rates of LTEP-A2-10 and A549-10 were 73.7% and 60.5% respectively.

Cell growth curve and colony formation assays showed

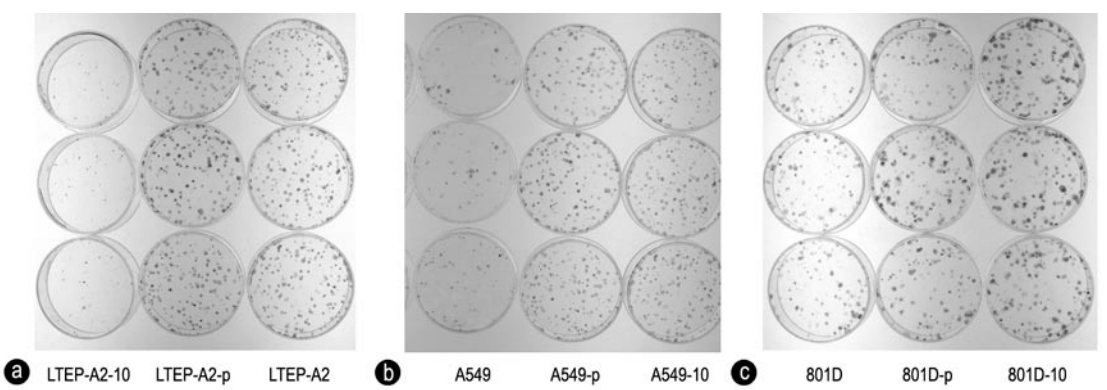

Fig. 9 The colony formation of three maternities and the transfected cell strains

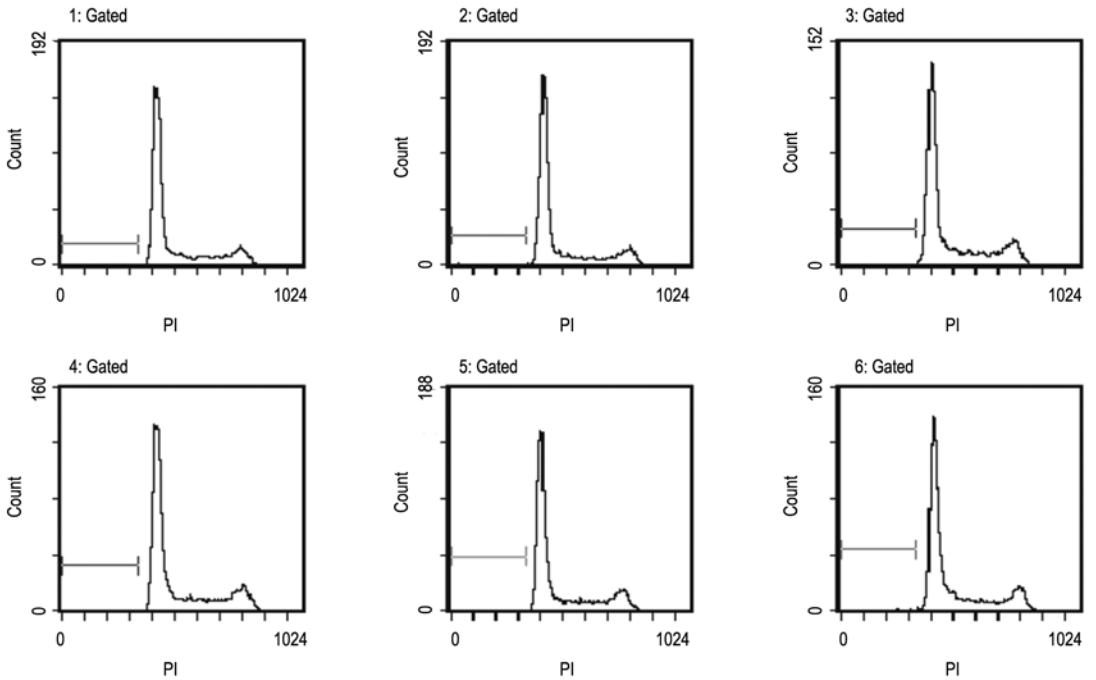

Fig. 10 The cell cycles and cell apoptosis of LTEP-A2 and LTEP-A2-10 cells. Above three figures were LTEP-A2 cells cultured 24, 48 and 72 hours respectively; bellow three figures were LTEP-A2-10 cells cultured 24, 48 and 72 hours respectively

the cell growth became slow and the colony formation reduced and the colony sizes became small in LTEP-A2-10 cells in which the effect of quenching COX-2 expression was best. The cell growth slowed also and the colony formation reduced in A549-10 cells in which the effect of quenching COX-2 expression was better. In contrast to LTEP-A2-10, the effect on malignant proliferation of A549-10 cells was next. The target had not effect on the growth of 801D cells.

No induction of apoptosis by RNAi depletion of COX-2

The results of flow cytometry showed there were not cell cycle arrest and apoptosis cells to appear in A549-10 and LTEP-A2-10 cells (Fig. 10). The results of LTEP-A2 and LTEP-A2-10 cells were only shown here in order to avoid repeat because the results of A549-10 and LTEP-A2-10 cells were similar.

Discussion

COX-2 is involved in tumorigenesis and development except for involving inflammatory. The expression of COX-2 is very high in primary tumors and higher in metastases tissues. Overexpression of COX-2 is a usual phenomenon in tumor tissues. The activity of COX-2 can be an early event [13–21]. In human, COX-2 expression is upregulated in about one-third of atypical adenomatoly hyperplasias and carcinoma *in situ* specimens obtained from lung and in 70%–90% of invasive adenocarcinomas of the lung. The proportion of adenocarcinomas cells with increased COX-2 expression is much greater in lymph node metastases than in the corresponding primary tumors, which suggests COX-2 can promote location invasion and distant metastasis of tumor tissues. The inhibitor of COX-2 could reduce tumor cell motion, adherence and invasion *in vivo* and *in vitro*. The special and non-special inhibitors of COX-2 have a very good effect on preventing colon cancer [22–26] so it is regarded more.

In this study, three sequences were selected as the interfering targets from COX-2 cDNA according to siRNA designed principle and the interfering effect of si-10 was best by a series experiments. In the study, si-10 was selected as interfering target of COX-2. Whether the si-10 target had different effects on human lung cancer cells with different expression levels of COX-2 protein and whether the changes of COX-2 expression had effects on malignant proliferation of these cells further were explored.

After the three maternities (801D, A549 and LTEP-A2) were transfected into the vain vector pEGFP, green fluorescence could be seen. But green fluorescence could not be seen after the maternities were transfected into the psi-10 plasmid. The reason is pEGFP can express green fluorescence protein. But in the psi-10 plasmid, U6 promoter and target sequence were inserted into MCS of pEGFP.

Because target sequence contains the transcriptional end signal of U6 promoter which lies in the upstream of GFP gene, which makes GFP can not be transcribed. Of course, green fluorescence could not be seen in A549-10, 801D-10 and LTEP-A2-10 cells.

From protein and mRNA levels, the effects that si-10 interfered with COX-2 expression of 801D, A549 and LTEP-A2 cells were examined. The results showed the effects were different: 801D cells did not express COX-2 protein and si-10 had no effect on COX-2 and COX-2 had not expression, A549 cells downexpressed COX-2 protein and si-10 had more obvious effect on COX-2 and the expression of COX-2 reduced 56.2%; LTEP-A2 cells highly expressed COX-2 protein and si-10 had most obvious effect on COX-2 and the expression of COX-2 reduced 60.2%. How to explain these? For 801D cells, the explanation is easy. RNAi is a special interfering technique and that siRNA recognizes, binds and degrades target mRNA is special and siRNA does not play a role in other mRNA, so the expression of COX-2 has no change in 801D cells. The same target has different effects on A549 and LTEP-A2 cells. The effect is good in LTEP-A2 highly expressing COX-2 and second place in A549 down expressing COX-2. The different effects are relative to the difference of COX-2 expression or the difference of cell types needs to certify further.

Whether can the different interfering effects affect cell malignant proliferation? The results are certain that si-10 target has different effect on 801D, A549 and LTEP-A2 cells. The effect is most obvious in LTEP-A2, more obvious in A549 and not obvious in 801D cells. This can be relative to the degrees of COX-2 reduction. It suggests COX-2 is relative to malignant proliferation of tumor cells. At the same time, it suggests COX-2 is involving in tumorigenesis and development. In addition, it is noted worthy that the cell growth slows and the colony formation reduces and the colony sizes become small in LTEP-A2 cells. The cell growth curve reflects the growth state of cell population and the colony formation reflects the growth state of cell individual. So the study suggests obvious reduction of COX-2 expression can affect not only the growth of cell population but also that of cell individual.

In addition, A549 and LTEP-A2–10 cells do not appear cell cycle arrest and apoptosis cells by flow cytometry analysis. About the relation between COX-2 and apoptosis, some think COX-2 can elevate Bcl-2/Bax protein levels and inhibit apoptosis. Charames [27] uses RNAi technique to inhibit COX-2 expression. The COX-2 protein reduces 57% in contrast to the control, but the Bcl-2 expression and Caspase-3 activity have not change. This is corresponding with our study results. It is well known RNAi can not completely quench target gene expression so the retained COX-2 can take act constantly. In the study, quenching COX-2 gene by RNAi can lead to the malignant proliferation of LTEP-A2 cells slows and the malignant phenotype reduces but there is not cell cycle arrest and apoptosis cells to appear. So the mechanism that quenching COX-2 gene inhibits tumor cells' growth needs to study further.

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