[Article ID] 1000 - 4718(2007)11 - 2185 - 06

Effect of RNA interference on Polo - like kinase - 1 in A549 cells

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[**ABSTRACT**] **AIM**: To investigate whether RNA interference (RNAi) induced by small interference RNA (siRNA) could suppress Polo - like kinase - 1 (Plk 1) expression and its effects in A549 cells. **METHODS**: A recombinant plasmid containing siRNA targeting Plk1 (psiRNA - hH1 - Plk1) was transfected into A549 cells with Lipofectamine 2000. Expressions of Plk1, cyclin B1 and p53 protein were detected by Western blotting. Cell proliferation was evaluated by direct cell counting, while cell cycle and apoptosis were examined by flow cytometry, and expression of α - tubulin was detected by immunofluorescence. **RESULTS**: The results demonstrated that sequence specific siRNA targeting Plk1 was capable of suppressing Plk1 expression, and reflecting in lower kinase activity in A549 cells. The level of Plk1 protein was reduced by at least 70% after 48 h of psiRNA - hH1 - Plk1 treatment relative to controls. Expressions of cyclin B1 and p53 were increased greatly after Plk1 depletion, and cells showed absence of microtubule polymerization and spindle abnormalities in staining for α - tubulin. Growth inhibition, G_2/M arrest and apoptosis were observed in psiRNA - hH1 - Plk1 transfected group. **CONCLUSION**: All these data suggest that siRNA targeted against human Plk1 may be a valuable tool in cancer therapy.

[**KEY WORDS**] RNA interference; Polo - like kinase - 1; Carcinoma, non - small - cell lung

[**CLC number**] R734.2 [**Document code**] A

The Polo - like kinase - 1 (Plk1), a mammalian serine/threonine protein kinase, has been shown to be an important regulator at multiple mitotic stages, involving regulation of entry into mitosis, centrosome maturation, bipolar spindle formation, regulation of anaphase - promotion complex/cyclosome (APC/C), execution of cytokinesis, and the signal transduction in DNA damage checkpoint and spindle checkpoint[1,2]. In addition, a close correlation between mammalian Plk1 expression and carcinogenesis was documented. The elevated expression of Plk1 is common in many types of cancer, and an increasing body of evidence from clinical research suggests that the level of Plk1 expression is directly related to proliferation, matastasis and resistance to chemotherapy and radiation in non - small - cell lung carcinomas (NSCLC)[3]. Therefore, inhibition of Plk1 function may be an important application in cancer therapy.

RNA interference (RNAi) is a process of sequence - specific post - transcriptional gene silencing via double - stranded RNA (dsRNA), which is widely used to analyze gene function nowadays[4]. Here we took advantage of DNA - based siRNA technology to deplete Plk1 in A549 cells to determine the role of Plk1 in tumorigenesis. Western blotting was used to determine whether siRNA targeted to Plk1 could suppress Plk1 function. Proliferation and apoptosis were also assayed in transfected cells.

MATERIALS AND METHODS

1 Reagents

psiRNA - hH1 and Lipofectamine™ 2000 were from InvicoGen. RPMI - 1640 culture medium and newborn calf serum were from Hyclone. Anti Plk1, cyclin B1 and anti p53 antibodies, Horseradish peroxidase - conjugated mouse IgG and enhanced chemiluminescence (ECL) kit were from Santa Cruz. Anti α - tubulin was from NeoMarker. BCA protein quantification kit was from Pierce. *Bbs*I, *Ase*I and T4 DNA ligase were from MBI. Plasmid extracting kits was from Promega. A549 cells were from China Center for Type Culture Collection (CCTCC).

[**Received date**] 2006 - 01 - 17 [**Accepted**] 2006 - 04 - 10

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2 Vector construction

psiRNA – hHl contains the H1 RNA pol III promoter, which can be transcribed into a short dsRNA with a hairpin structure (short hairpin RNA, shRNA). The targeting sequence of human Plk1 (GenBank accession No. NM_005030) is 5' – ACCTCCGGATCAAGAAGAA – 3', corresponding to the coding region 778 – 796 relative to the first nucleotide of the start codon. Two complementary oligonucleotides were designed. The forward and reverse oligonucleotides were 5' – TCCCAACCTCCGGATCAAGAAGAACCACCTTCTTCTT- GATCCGGAGGTTT – 3', and 5' – CAAAAAACCTCCGGATCAAGAAGGTGGTTCTTCTTGATCCGGAGGTT – 3', respectively. They were annealed and inserted into psiRNA – hH1 according to the manufacturer's instruction. After white/blue selection, the recombinant plasmid (psiRNA – hH1 – Plk1) was confirmed by digesting with *Ase*I. Another oligonucleotides was synthesized as negative control but having two point mutations within the targeting sequence (5' – ACCTCCGGATCGGGAAGAA – 3'). The sequence was detected against EST libraries to ensure there was no gene targeted in human cells. Then psiRNA – hHl – nonspecific was constructed as described above. All the plasmids were confirmed by DNA sequencing (Bioasia Co., China).

3 Cell culture and transfection

A549 cells were maintained in RPMI – 1640 medium supplemented with 10% calf serum, 1×10^5 U/L penicillin and 100 mg/L streptomycin at 37 ℃ in a water – saturated atmosphere of 5% CO_2 in air. Cells were divided into four groups: (1) control: control cells were incubated with Opti – MEM alone; (2) psiRNA – hHl: cells were transfected with psiRNA – hHl; (3) psiRNA – non: cells were transfected with psiRNA – nonspecific; (4) psiRNA – hHl – Plk1: cells were transfected with psiRNA – hHl – Plk1. Transient transfection was carried out using Lipofectamine 2000 according to the manufacturer' instructions. Cells were seeded, without antibiotics, at 5×10^4 cells/cm^2 in 6 – cm – diameter culture dish, corresponding to a density of 90% at the time of transfection. A549 cells were transfected with 6 μg psiRNA – hH1 – Plk1 or psiRNA – hHl – nonspecific. After 6 h of incubation at 37 ℃, the plasmid – containing medium was replaced with normal cell culture medium, and the cells were incubated for additional 48 h before harvested.

4 Western blotting

A549 cells were washed with phosphate – buffered saline (PBS), collected by 0.25% trypsinization and lysed in buffer[50 mmol/L Tris – HC1 (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.25% sodium deoxycholate, 1% Triton X – 100, 0.1% SDS, 1 mmol/L sodium fluoride(NaF), 1 mmol/L Na_3VO_4], plus protease inhibitors (10 mg/L aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride) to obtain whole cell protein. Lysates were cleared by centrifugation and protein concentration determined by BCA kit. 40 μg of protein was subjected to SDS – polyacrylamide gel electrophoresis (12%), transferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TBS – T [50 mmol/L Tris – HCl (pH 7.6), 150 mmol/L NaCl, 0.1% Tween 20] for 2 h at room temperature (25 ℃), and subsequently incubated with antibodies in blocking buffer (Plk1, 1:200; cyclinBl, 1:500; p53, 1:200; β – actin, 1:500) at 4 ℃ overnight. Folllowing washed with TBS – T, the membranes were incubated with horseradish peroxidase (HRP) – conjugated rabbit anti – goat secondary antibody (1:10 000) for 1h at room temperature (25 ℃). The membranes were washed with TBS – T and protein bands were visualized by ECL according to the manufacturer' instructions and exposed to X – ray films(Fuji, Japan).

5 Cell counting

Cells were seeded at 5×10^4 cells/cm^2 density in six – well plate for about 24 h and transfected with 3 μg of plasmids. At 48h post transfection, cells were washed with PBS twice, incubated with 0.5 mL 0.25% trypsin for 15 min at 37 ℃. The digestion solution was mixed with 1.5 mL PBS and 0.1% trypan blue, and aspirated repeatedly to make single cell suspension. Cell counting was performed using a hemocyte counting chamber and cell viability was assessed by trypan blue staining($n = 2$ per group, samples counted in triplicate). The sum of cell number of transfected groups

was normalized by that of control group, then the result of transfected cell was further expressed as the percentage relative to control(100%).

6 Flow cytometry

Cells seeded in culture plate were transfected with plasmids as described above. After incubation for 48 h, cells were harvested by trypsinization and centrifugation, washed with cold PBS, and fixed overnight with 80% ethanol at −20 ℃. Before measurement, cells were washed, collected, and resuspended in PBS containing 10 mg/L propidium iodide and 100 mg/L RNase A, then incubated at 4 ℃ for at least 30 min. Subsequent analyses of cell cycle distribution and apoptosis were performed using FACSCalibur (Becton Dickinson).

7 Immunofluorescence

Cells grown on glass slides in six-well plate were transfected as described above. The slides was washed with cold PBS prior to fix with cold methanol and 4 mmol/L of EGTA at −20 ℃ for 5 min. Slides were then washed with PBS, blocked with 5% bovine serum albumin and 0.2% Triton X-100 for 30 min before anti-α-tubulin monoclonal antibody (1∶100) in blocking butter were applied and incubated at 4 ℃ overnight. The slides was washed with PBS, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG for 1 h at room temperature (25 ℃). The slides were washed again, then incubated with 1 mg/L Hoechst 33258 in PBS for 15 min to counterstain nuclei, and mounted with glycerol-PBS. Cells were visualized and photographed using X70 Olympus fluorescence microscopy.

8 Statistical analysis

Data were expressed as $\bar{x} \pm s$ for the indicated number of separate experiment, and analyzed with SPSS 11.5 software. F analysis was performed to consider random effects of different treatments, q analysis was used to consider difference between two means.

RESULTS

1 Specific inhibition of Plk1 protein expression by siRNA

Levels of Plk1 protein were normalized to the levels of β-actin protein, then the normalized Plk1 protein levels were presented relative to those in control group for different plasmid-related effects. The value of psiRNA-hHl, psiRNA-non and psiRNA-hHl-Plk1 were 0.85 ± 0.30, 0.77 ± 0.26, 0.35 ± 0.12 respectively when the value of Plk1 protein of control group was determined as 1 (Fig 1).

2 Plk1 depletion prevents cyclin B1 degradation and stabilizes p53

To determine whether the lower Plk1 protein levels in siRNA-transfected cells would be reflected in lower kinase activity, we measured the cyclinB1 level and found an increased level of cyclin B1 relative to controls (Fig 2). Thus, Plk1 depletion is associated with lower kinase activity, and that prevents APC activation and cyclinB degradation. p53 plays an important role in apoptosis and cell-cycle arrest after various stress stimuli. Consequently, we tested whether the p53 pathway was activated in Plk1-depleted cells. The results showed that Plk1 depletion dramatically increased the stability of p53 whereas the p53 level was in low expression in other groups(Fig 3).

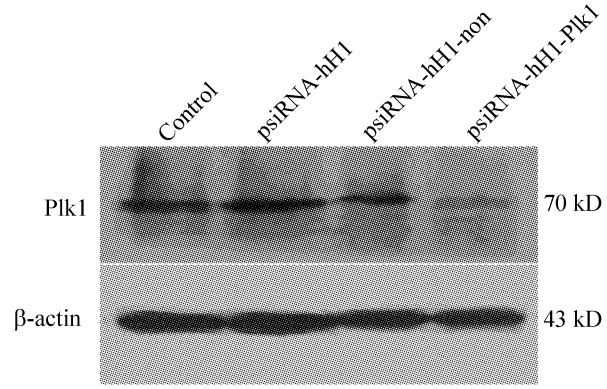

Fig 1 Effect of small interfering RNA(siRNA) transfection on Polo-like kinase-1(Plk1) protein levels in A549 cells.

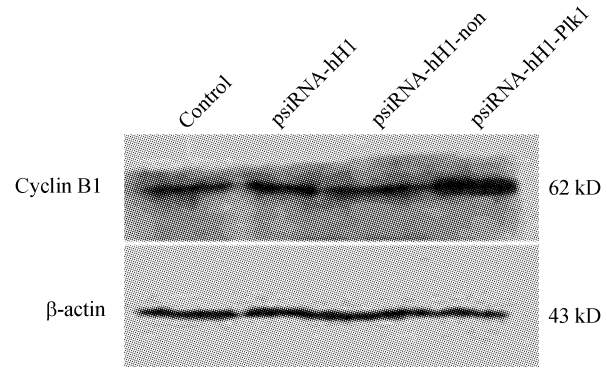

Fig 2 Effect of siRNA transfection on cyclin B1 protein levels in A549 cells.

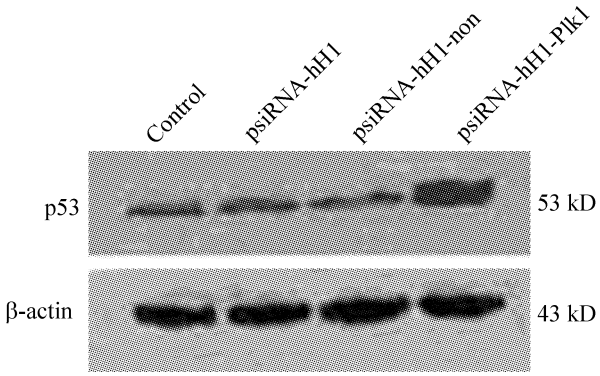

Fig 3 Effect of siRNA transfection on p53 protein levels in A549 cells.

3 Antiproliferative effect in A549 cells *in vitro* associated with reduced levels of Plk1 protein

Under phase contrast microscopy, by 48 h post transfection, many cells in psiRNA – hHl – Plk1 had developed a round phenotype, lost substrate adhesion; some cells in other groups also appeared thus but pro portionately less than that of psiRNA – hH 1 – Plk 1 when the toxicity of transfection reagent was considered. The number of viable A549 cells in psiRNA – hHl – Plk1 was strongly reduced 48 h after transfection, and no more than 20.52% relative to control ($P<0.01$). On the other hand, no statistical differences were observed among the other three groups(Fig 4).

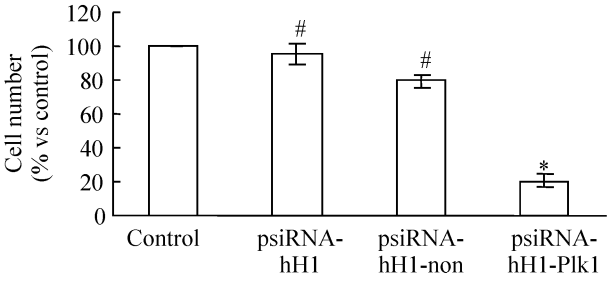

Fig 4 Effect of siRNA transfection on proliferation in A549 cells. $\bar{x}\pm s$. $n=3$. $^{*}P<0.05$, $^{\#}P>0.05$ *vs* control.

4 Cell cycle arrest and apoptosis with reduced levels of Plk1 protein

Flow cytometry was performed to examine whether the Plk1 depletion was associated with arrest at particular stages of the cell cycle. At 48 h after transfection, the percentage of cells in G_2/M of psiRNA – hHl – Plk1 group was increased 60.80% relative to control ($P<0.01$), and 33.26% relative to psiRNA – hH1 – non ($P<0.05$). Moreover, the cells with sub – G_1 DNA content also increased dramatically in psiRNA – hH1 – Plk1 transfected cells, suggesting the increasing of apoptosis in Plk1 – depleted cells (Tab 1).

Tab 1 Effect of siRNA transfection on cell cycle distribution in A549 cells($\bar{x}\pm s$. $n=3$)

A549 cells	Apoptosis rate	G_0/G_1	S	G_2/M
Control	0.55±0.22	51.38±3.52	29.45±2.41	19.16±2.40
psiRNA – hH1	0.42±0.25#	50.31±4.15	28.42±3.63#	20.41±3.28#
psiRNA – hH1 – non	0.71±0.19#	48.24±3.39	28.18±1.92#	23.12±2.84#
psiRNA – hH1 – Plk1	20.36±2.27*	39.66±4.30	9.53±2.73*	30.81±3.29*

*$P<0.05$ *vs* control; #$P>0.05$ *vs* control at 48 h after transfection.

5 Abrogation of spindle formation associated with reduced levels of Plk1 protein(Fig 5)

Antibodies directed against α – tubulin to visualize the spindle apparatus. Part of cells in psiRNA – hH1 – Plk1 were in mitosis characterized by condensed chromosomes. Metaphase and telophase chromosomal arrangements were less identified. A part of nucleus of M phase were devoid of microtubule connection or monoastral microtubule arrays. By contrast, cells in control, psiRNA – hHl and psiRNA – hHl – non showed much microtubule assembly around prophase cells, bipolar spindle formation and condensed chromosomes.

For Hoechst 33258 staining, the highly condensed, particles – like and blue fluorescence were seen in cells transfected with psiRNA – hHl – Plk1, which was the typical phenotype changes of apoptosis.

DISCUSSION

The biochemical antagonistic effect to RNAi has been reported[5]. It showed that RNAi was inhibited when the dsRNA molecule was first deaminated by A-DAR2, a human adenosine deaminase. Researchers predicted tumor cells may use this defense mechanism and upregulate such enzyme in response to therapeutic interventions by dsRNA. So the intrinsic ability to use the RNAi machinery in cancer gene therapy is doubtful. In the present study, a siRNA expression vector targeting Plk1 was engineered, and the results showed that specific inhibition of Plk1 protein expression and inducing cytotoxicity in A549 cells after transfected with psiRNA – hH1 – Plk1. Thus, siRNA targeted against human Plk1 may be a very useful approach for lung cancer treatment.

Plk1 plays an important role at G_2/M transition. CyclinB/Cdc2, which is a trigger for initiation of mito –

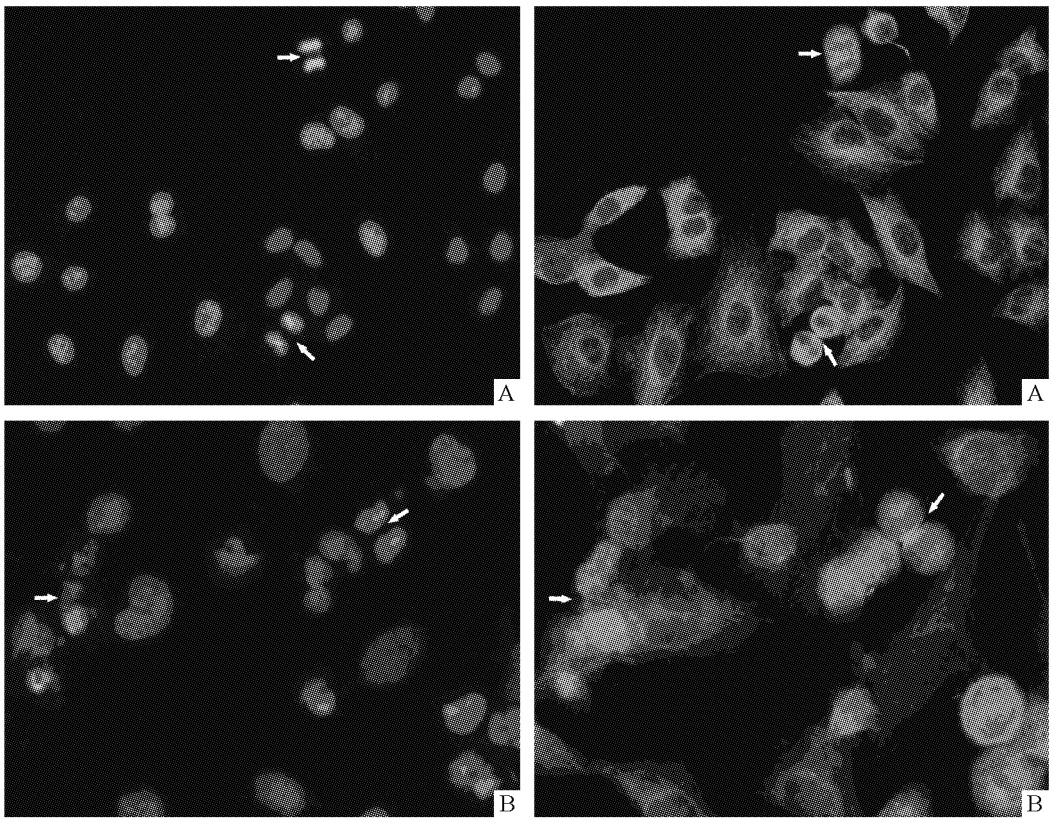

Fig 5 Effect of siRNA transfection on spindle formation and apoptosis of A549 cells for DNA by Hoechst33258 staining (blue), for α – tubulin by immunostaining (green). A: normal A549 cells showed normal spindle formation (×200); B: psiRNA – Hhl – Plk1 transfection of A549 cells resulted in abnormal microtubule array and abrogated spindle formation. Arrows (left) point to apoptosis nucleus (×200).

sis, is dephosphorylated and activated by Cdc25C[6]. Plk1 can directly phosphorylate Cdc25C and promote the translocation of Cdc25C into nucleus for playing its function[7]. The signaling pathways could not be activated after Plk1 depletion. We also found that the expression of p53 protein was increased significantly in A549 cells after transfected with psiRNA – hHl – Plk1, indicating the depletion of Plk1 dramatically increasing the stability of p53. In addition to the effects on the levels of p53 expression, Plk1 has been implicated in abroga – ting p53 function. Ando et al[8] reported that the over-expression of Plk1 was co – localized with the endogenous p53 in mammalian cell nucleus. Plk1 greatly decreased the p53 – mediated transcription from the p53 – responsive $p21^{WAF1}$, *MDM2*, *Apaf – l*, *BAX* promoters, and, then, abrogated p53 function. Consequently, the p53 function was improved after Plk1 depletion, and then, p53 regulates p21, an inhibitor of many cyclin/cyclin – dependent kinase (CDK) complexes, results in cell growth arrest in both G_1/S and G_2/M[9]. At the same time, increased level of p53 activates *CD95*, *TRAIL*, *Apaf – l*, *Bax*, the key molecules in signaling system of apoptosis, and induced apoptosis[10]. As showed in FACS profiles, the percentage of cells in G_2/M was increased dramatically in A549 cells transfected with psiRNA – hH1 – Plk1. Cells with sub – G_1 DNA content also increased significantly, suggesting the increasing of apoptosis in Plk1 – depleted cells.

Plk1 also takes part in the centrosome maturation and spindle assembly in mitosis[11]. Centrosomes play a critical role in regulating quantities, stabilities and polarity of microtubules in interphase, and formation of bipolar spindle in mitosis. Plks transiently localize to centrosome at G_2/M, and directly phosphorylate the centrosorne protein asp (abnormal spindle protein), to promote the maturity and separation of centrosome[12]. The effort of many laboratories[13] have found that in different cancer cells, Plk1 depletion led to smaller centrosome, or multiple centrosomes, or separated centrosome without microtubule interaction, but the disturbed chro-

mosome segregation and polyploidy were seen in common. In our test, part of the cells transfected with psiRNA – hHl – Plk1 revealed devoid of microtubule connection or asymmetric microtubule arrays, or even monopolar spindle formation. Dumbbell – like nuclei indicated that chromatid did not be separated.

To date, several techniques and compounds to inhibit Plk1 have been identified and appear to be promising. Given the effects of siRNA targeting Plk1 that we observed in culture cancer cells, future experiments examining the effects described above in xenograft experiment are of obvious importance.

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RNA 干扰技术抑制 Polo – like 激酶 1 表达对 A549 细胞的影响

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[**摘 要**] **目的**: 观察 RNA 干扰技术能否有效抑制非小细胞肺癌细胞株 A549 细胞中 Polo – like 激酶 1(Plk1)的表达水平,以及抑制后对 A549 细胞生长的影响。**方法**: 运用脂质体法,以 Plk1 为靶点,构建能产生 siRNA 的质粒载体 psiRNA – hH1 – Plk1 并转入 A549 细胞。RT – PCR 检测 Plk1 mRNA 表达的变化、Western blotting 检测 Plk1、cyclin B1、p53 蛋白的表达变化、细胞计数分析细胞增殖、流式细胞术分析细胞周期变化和凋亡、免疫荧光染色检测 α 微管蛋白的表达。**结果**: psiRNA – hH1 – Plk1 质粒能特异地抑制 Plk1 基因的表达并使其活性下降,致使 cyclin B1 及 p53 蛋白的表达水平升高,微管聚集障碍或形成单极的纺锤体; A549 细胞增殖减慢,出现 G_2/M 期阻滞和凋亡。**结论**: 上述结果提示针对 Plk1 基因的 RNA 干扰有望用于肿瘤的基因治疗。

[**关键词**] RNA 干扰; Polo – like 激酶 1; 癌,非小细胞肺

[**中图分类号**] R734.2 [**文献标识码**] A