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Role of CyclinD1 and CDK4 in the Carcinogenesis Induced by Silica[1]

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Objective To study the role of cyclinD1 and CDK4 in malignant transformation of human fetal lung diploid fibroblast cell line（2BS） induced by silica. **Methods** Recombination vectors with sense and antisense pXJ41-cyclinD1 and pXJ41-CDK4 were constructed, and then transfected into the malignant transformed cells induced by silica, respectively. At the same time, pXJ41-neo was used as the control. **Results** During the progress of the malignant transformation of 2BS cells induced by silica, cyclinD1 and CDK4 were overexpressed. Antisense RNA suppressed cyclinD1 and CDK4 gene expression in the antisense pXJ41-cyclinD1 and pXJ41-CDK4 transfected cells. Antisense RNA led to cell cycle arrest, resulting in lengthened G1 phase (the percentages of cells in the G1 phase changed from 45.1% to 52.7% and 58.0% for cyclinD1 and CDK4 transfected cells, respectively), and eventually attenuated the increase of the proliferation of malignant transformed cells induced by silica. Compared with malignant transformed cells induced by silica, cells transfected with antisense pXJ41-cyclinD1 and pXJ41-CDK4 showed obviously reduced growth rates. On the 8th day, the suppression rates were 58.69 and 77.43% (the growth rate of malignant transformed cells induced by silica was 100%), doubling time changed from 21.0 h to 31.4 h and 21.0 h to 42.7 h, respectively, the growth capacities on soft agar of cells transfected by antisense pXJ41-cyclinD1 and pXJ41-CDK4 decreased obviously. **Conclusion** CyclinD1 and CDK4 play an important role in maintaining transformed phenotype of the cancer cells.

Key words: CyclinD1; CDK4; Antisense RNA; Silica; Carcinogenesis

INTRODUCTION

Silica is one of the most serious occupational hazards in China. Up to 2003, the accumulated cases of pneumoconiosis numbered 581 377. Silicosis accounted for 50% of the cases of pneumoconiosis. In 1996 the International Agency for Research on Cancer (IARC) classified crystalline silica as a human carcinogen (group 1), based on the epidemiological studies and the experimental results across animal species[1-5]. The effects of silica on DNA have observed, including DNA damage induced directly by silica dusts or by reactive oxygen species released by silica dusts, and p53 and TNF gene mutations caused by silica[6-12]. It was reported that silica could transform mammalian cells through binding to deoxyribonucleic acid. Silica could make DNA bind to SiO^- on the surface of silica through hydrogen bond energy, forming DNA-silica compound. Silica can intervene in DNA replication, repairing, gene expression and can interfere with the mitotic spindle formation and the segregation of chromosomes, eventually inducing mutation or carcinogenesis. Although there are many hypotheses about the effects of silica, the cell cycle mechanisms of carcinogenesis remain unclear.

The study on cell cycle and cancer has made great progression in resent years. But there are few reports to prove if the malignant transformation caused by silica dusts has the similar change pattern of cell cycle to the non-occupational tumors. In this study, we used the malignant transformed cell model induced by silica and the antisense techniques to study the role of cyclinD1 and CDK4 in the carcinogenesis induced by silica.

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MATERIALS AND METHODS

Plasmids, Reagents and Antibodies

*Eco*RI, *Sma*I, *Bam*HI, *Kpn*I, *Hpa*I, *Hind*III, T4 DNA ligase, CIAP (Alkaline phosphatase, Calf Intestinal), TransFast^{TM} trabsfection reagent, Transfectam reagent for the transfection of eukaryotic cells were purchased from Promega (Madison, WI 53711-5399 USA). Rnase A was from Sigma-Aldrich (PRC. USA). Anti-digxigenin, Fab fragments conjugated with alkaline phosphatase and DIG RNA labelling kit（SP6/T7）were bought from Roche Company, and the kits of immunohistochemical analysis were from Zhongshan Company.

Vectors and Cells

pUC118-cyclinD1 included the full human cyclinD1 cDNA (about 1.33kb). pBluescript$^{+/-}$-CDK4 included the full human CDK4 cDNA (about 1.3kb). pXJ41-neo (6.885kb, G418 resistance gene, Neo) (the eukaryotype expression vector) was provided by Professor Jianli SANG, a professor of Beijing Normal University. 2BS (the human fetal lung diploid fibroblast cell line) was bought from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Malignant transformed human cells induced by silica were from our laboratory[13].

Generation of Stable Transfectants

The 1.33kb human cyclinD1 cDNA containing the entire coding sequence was cut with *Eco*RI from pUC118-cyclinD1, and subcloned into *Eco*RI site of the eukaryotype expression vector pXJ41-neo. About 400 bp human CDK4 cDNA including the entire CDK4 open reading frame was cut with *Bam*HI and *Kpn*I from pBluescript$^{+/-}$-CDK4 and then inserted in its antisense orientation into *Bam*HI and *Kpn*I sites of the eukaryotype expression vector pXJ41-neo. The resulting plasmids were designated as sense and antisense pXJ-cyclinD1 and antisense pXJ-CDK4, respectively.

Construction of pSPT18-cyclinD1 and pSPT18-CDK4 Plasmids

pSPT18 plasmid used for transcription probe *in vitro*, contained SP6 or T7 RNA polymerase promotor. CyclinD1 cDNA was cut with *Eco*RI and *Hind*III from pUC118-cyclinD1, and the resulting fragment (185 bp) was subcloned into *Eco*RI and *Hind*III sites of the pSPT18 plasmid. CDK4 cDNA was cut with *Kpn*I and *Bam*HI from pBluescript$^{+/-}$-CDK4, and the resulting CDK4 cDNA fragment (200 bp) was subcloned into *Kpn*I and *Bam*HI sites of the pSPT18 plasmid. The resulting plasmids were designated as pSPT18-cyclinD1 and pSPT18-CDK4 and used for immunohistochemistry.

Cell Transfection

The malignant transformed cells induced by silica (were named as transformed cells) were grown in RPMI1640 medium (GIBCO) plus 10% fetal calf serum in a 6-well plate for 24 hours, and the medium for the cell line was replaced by 200 μg/mL to 1 300 μL/mL G418. The cultures were maintained for 14 days. The minimal concentration was identified according to the cell death rate (200 μg/mL).The sense and antisense pXJ-cyclinD1, antisense pXJ-CDK4 and vector empty pXJ41-neo were transfected into the malignant transformed cells induced by silica following the protocol of transfectam reagent for the transtection of eukaryotic cells. The cells were plated the day before the transfection experiment. The plating density for any particular cell line depended upon the growth rate. Adherent cells should be 50%-70% confluent on the day of transfection. The reagent volumes in this experiment were based on the use of 6-well plates. Ten μg of plasmid DNA was added to 500 μL of serum-free medium in a sterile tube and vortex (solution A), and 10 μL of Transfectam® reagent was added to 500 μL of serum-free medium in a sterile tube and mixed (Solution B). Solutions A and B were immediately mixed and added directly to the cells prepared above. The final volume per well was 1500 μL. The cells and solution were incubated for 2 hours，and at the end of the incubation period, and the cells were gently over-laid with 4 mL of the complete RPMI 1640 medium plus 10% FCS (37℃). It was not necessary to remove the transfection medium containing the Transfectam® reagent/DNA mixture. The cells were returned to the incubator and the incubation was continued. After 48-hour incubation, the medium for the transfected cells was replaced with complete RPMI1640 medium containing a certain concentration (200 μg/mL) of G418 for 2-3 weeks. A number of individual drug (G418) resistant clones were randomly picked and digested with 0.25% trypsin. The cell suspensions were plated into 10 mL culture flasks and cultured for the following study. Alternatively, plates containing many G418 resistant clones were trypsinized to yield a “pool” of cells expanded and frozen as seed stocks.

Probe Preparation and RNA in situ Hybridization

Following the procedure of DIG RNA labelling

kits, RNA probes were made. Firstly, the pSPT18-cyclinD1 and pSPT18-CDK4 plasmids were linearized with appropriate enzymes. The pSPT18-cyclinD1 digested by *Eco*R1 was used as the template, and SP6 RNA polymerase promoter was used to produce the antisense probes of the cyclinD1 mRNA. When *Hind*III and T7 RNA polymerase promoter were used, the sense probes of the cyclinD1 mRNA were got. For pSPT18-CDK4, *Bam*HI and T7 polymerase promoter were used to make the sense probes, and *Kpn*I and SP6 polymerase promoter were used to produce antisense probes. The labelled sense and antisense RNA probes were hybridized with the treated cells, as described by XUE Qingshan[14]. The system of Pharmacia LKB Ultrascan XL was used for analyzing the semi-quantify results.

Immunohistochemical Analysis of CyclinD1 and CDK4

Cells were cultured in RPMI1640 medium on glass slides for 48 h. The glass slides covered with cells were washed 3 times with ice-cold phoshate-buffered saline (PBS) and fixed in acetone on ice for 10 min. Then the slides were washed with ice-cold PBS and blocked in normal goat serum at room temperature. After the goat serum was discarded, the cells were incubated with rabbit anti-human cyclinD1 polyclonal antibody or with rabbit anti-human CDK4 monoclonal antibody diluted properly. After overnight incubation with the primary antibodies in wet box at 4℃, slides were washed in PBS and then exposed to the secondary antibodies, anti-rabbit, for 15 min at room temperature. After that, they were washed with PBS and incubated with the S-A/HRP complex for 40 min at room temperature. Finally, the slides were washed with flowing water for 3 min, and counter-stained with Mayer hematoxylin for 5 min. Normal rabbit IgG at the same concentration as the primary antibodies and PBS served as negative controls. The system of Pharmacia LKB Ultrascan XL was used for analyzing the semi-quantified results, according to the intensity of staining and the percentage of positive cells.

Cell Growth and Proliferation

Cells were seeded in triplicate at a density of 1×10^4 per well in 24-well dishes in complete medium at 37℃, 5% CO_2 conditions. The number of cells per well was counted every day for the subsequent 8-10 days. In all experiments, cell culture medium was changed every 3 days. Doubling times were calculated from logarithmic growth curves.

Flow Cytometric Analysis

Exponentially proliferating cells were harvested by trypsinisation, washed in ice-cold PBS, and fixed in ice-cold 70% ethanol for overnight at 4℃. The cells were then washed two times in PBS and treated with RNaseA (1 mg/mL) for 30 min at 37℃, followed by 50 μL propidium iodide (1 mg/mL), and diluted by PBS to 0.5 mL final volume, stained for 40 min in ice without light. An Ortho Cytofluorograf 50H was used to analyse the cell cycle distribution. Approximately 1 000 000 cells were examined for each sample. The percentages of cells within the G1, S, and G2/M phases of cell cycle were determined by computer analysis. All experiments were repeated three times.

Soft Agar Assay

Cells grown in RPMI1640 medium for 36 h were harvested by trypsinisation, and 2×RPMI1640 medium was used to dilute the cells to a final concentration of 200 cells/mL at 37℃. RPMI1640 medium containing 0.6% agar was added into a 6-well plate for about 30 min. After agar concretion, cell suspension (100 cells/mL) containing 0.3% agar was plated in triplicate in 6-well plates containing 0.6% agar above. The cell suspension (100 cells/mL) was also plated in triplicate in 6-well plates without agar. After two weeks of growth, the cell colonies were counted under microscope. All experiments were repeated twice and gave the similar results.

RESULTS

Construction of Antisense CyclinD1 and CDK4 Expression Plasmids

Antisense pXJ-cyclinD1 and pXJ-CDK4 were successfully constructed, and could be used to transfect the malignant transformed cells induced by silica. According to the multiple cloning sites of pXJ-cyclinD1, sense pXJ-cyclinD1 could be cut by *Hind*Ⅲ into 229 bp, 427 bp and 7 554 bp, and antisense pXJ-cyclinD1 could be cut by *Hind*Ⅲ into 427 bp, 1 184 bp and 6 599 bp. The electrophoresis results showed that the plasmids in line 3 were sense pXJ-cyclinD1, and the plasmids in line 4 were antisense pXJ-cyclinD1 (Fig. 1 A). pXJ-CDK4 could be cut by *Bam*HI and *Hpa*I into 2 633 bp and 4 567 bp. The electrophoresis results showed that the plasmids in line 2 were pXJ-CDK4 (Fig. 1 B).

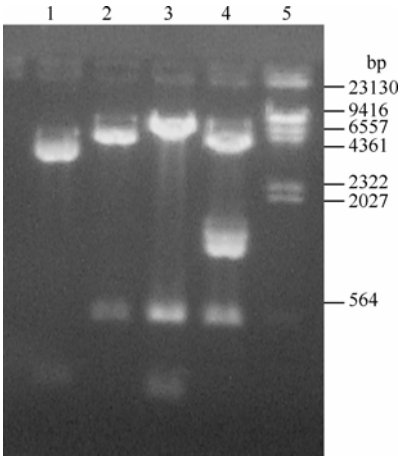

FIG. 1 A. Electrophoresis results of sense and antisense pXJ41-cyclin D1 digested with *Hind*Ⅲ. Lane 1: pUC118-cyclin D1 with *Hind*III, lane 2: pXJ41-neo with *Hind*III; lanes 3, 4: pXJ41-cyclin D1 with *Hind*III; lane 5: λ DNA/*Hind*III Marker.

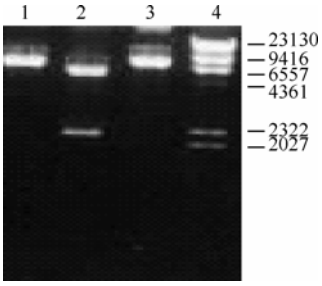

FIG. 1 B. Electrophoresis results of antisense pXJ41-CDK4 digested with *Kpn*I and *Hpa*I. Lanes 1,3: pXJ41-CDK4; lane 2: pXJ41- CDK4 with *Kpn*I and *Hpa*I; lane 4: λ DNA/*Hind*III Marker.

Construction of pSPT18-cyclinD1 and pSPT18-CDK4 Plasmids

According to the multiple cloning sites of pSPT18-cyclinD1, pSPT18-cyclinD1 could be cut with *Eco*RI and *Hind*III into 3100 bp and 185 bp. The results showed that the plasmids in lines 3-5 were digested with *Eco*RI and *Hind*III into expected sizes of fragments, 3100 bp and 185 bp, indicating that the plasmid pSPT18-cyclinD1 was successfully constructed (Fig. 2). So was the pSPT18-CDK4 (Fig. 3).

Expression of Antisense CyclinD1 and CDK4 RNA in Malignant Transformed Cells Induced by Silica

We succeeded in introducing antisense cyclinD1 and CDK4 cDNA sequences respectively into the malignant transformed cells induced by silica. Sense pXJ-cyclinD1 and empty vector pXJ-neo transfected cells were the controls. The results of *in situ* hybridiz-ation suggested that antisense pXJ-cyclinD1

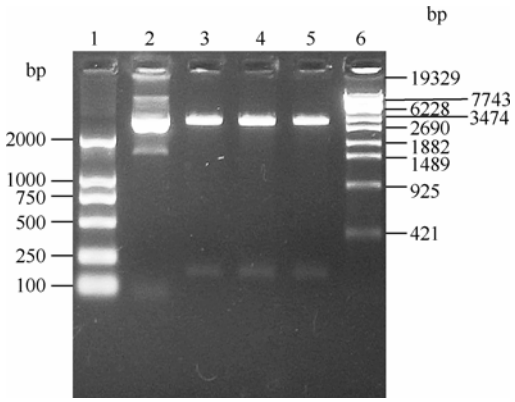

FIG. 2. Electrophoresis results of pSPT18-cyclinD1 digested with *Eco*RI and *Hind*III. Lane 1: DL2000 DNA Marker; lanes 2-5: pSPT18-cyclinD1 with *Eco*RI and *Hind*III; lane 6: λ DNA/*Eco*T 14 I Marker.

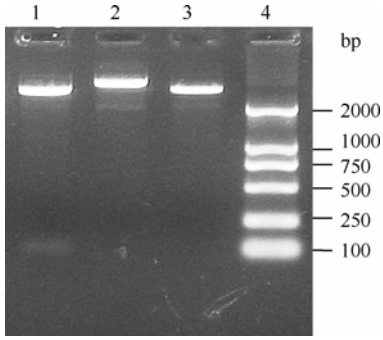

FIG. 3. Electrophoresis results of pSPT18-CDK4 digested with *Bam*HI and *Nco*I. Lanes 1-3: pSPT18-CDK4 with *Bam*HI and *Nco*I; lane 4: DL2 000 DNA Marker.

and pXJ-CDK4, sense pXJ-cyclinD1 and pXJ-neo were transfected successfully into the malignant transformed cells induced by silica. The silica transformed cells transfected by antisense pXJ-cyclinD1 and pXJ-CDK4 as were named as AS-D1 and, AS-K4 cells, those transfected by sense pXJ-cyclinD1 and pXJ-neo were named as S-D1 and, as pXJ cells.

Changes in Gene Expression Levels of CyclinD1 and CDK4

CyclinD1 and CDK4 mRNA were detected in the normal cells, the malignant transformed cells and transfected cells by antisense RNA. The expression of cyclinD1 and CDK4 mRNA increased in malignant transformed cells, and significantly decreased in all the transfected cells. No difference in cyclinD1 and CDK4 mRNA levels was found between the malignant transformed cells and the cells transfected by empty vector pXJ-neo. CyclinD1 and CDK4 expression in most normal 2BS cells was not observed (Figs. 4 and 5).

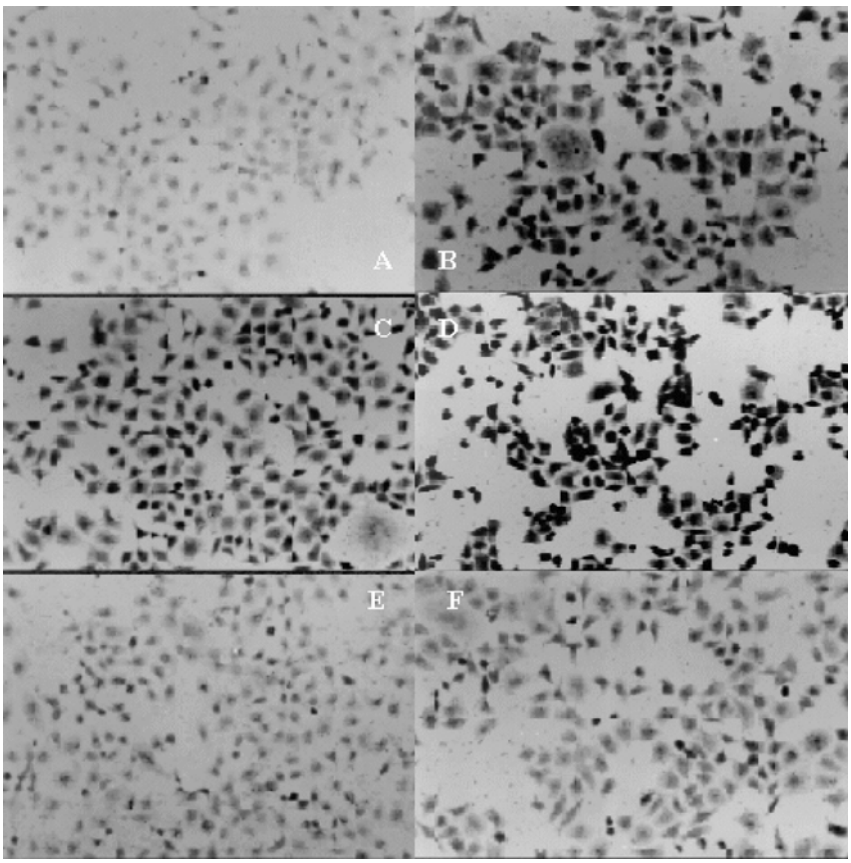

FIG. 4. Expression of cyclinD1 mRNA detected by *in situ* hybridization (200×). A. normal 2BS cells; B. malignant transformed cells; C. empty vector pXJ transfected cells; D. cyclinD1 sense transfected cells; E. cyclinD1 antisense transfected cells; F. CDK4 antisense transfected cells.

Immunohistochemistry observation revealed that cyclinD1 and CDK4 proteins were overexpressed in the malignant transformed cells. The overexpression was attenuated by sense and antisense cyclinD1 and antisense CDK4 transfection, whereas no difference in cyclinD1 and CDK4 protein expression was found between the malignant transformed cells and cells transfected by empty vector pXJ-neo (Figs. 6 and 7). These results indicated that the levels of cyclinD1 and CDK4 mRNA and proteins in the malignant transformed cells increased and antisense RNA could inhibit the expression of the endogenous cyclinD1 and CDK4 genes in cells transfected by antisense cyclinD1 and CDK4.

Semi-quantitative Analysis of Expression of CyclinD1 and CDK4 Genes

The system of Pharmacia LKB Ultrascan XL was used for analyzing the semi-quantified results of the expression of cyclinD1 and CDK4. Representative data are shown in Table 1. Overexpression of cyclinD1 and CDK4 occurred in malignant transformed cells ($P<0.05$), and expression of cyclinD1 and CDK4 decreased after transfected by antisense cyclinD1 and CDK4 ($P<0.05$). No difference in expression of cyclinD1 and CDK4 genes between the cells transfected by control vector pXJ-neo and the malignant transformed cells was found. Compared with the malignant transformed cells, cyclinD1 mRNA expression in S-D1 cells increased, but cyclinD1 protein decreased ($P<0.05$).

Biological Characteristics of Malignant Transformed Cells and Antisense RNA Expression Cells

Cells growth rate The results presented in Fig. 8 demonstrated that transformed cells grew faster than the normal 2BS cells, while the growth of the AS-D1 and AS-K4 cells was inhibited obviously by antisense RNA. After cells grew for 8 days, the inhibition rates of the AS-D1 and AS-K4 cells were about 58.69% and 77.43%, respectively (transformed cell growth rate was 100%).

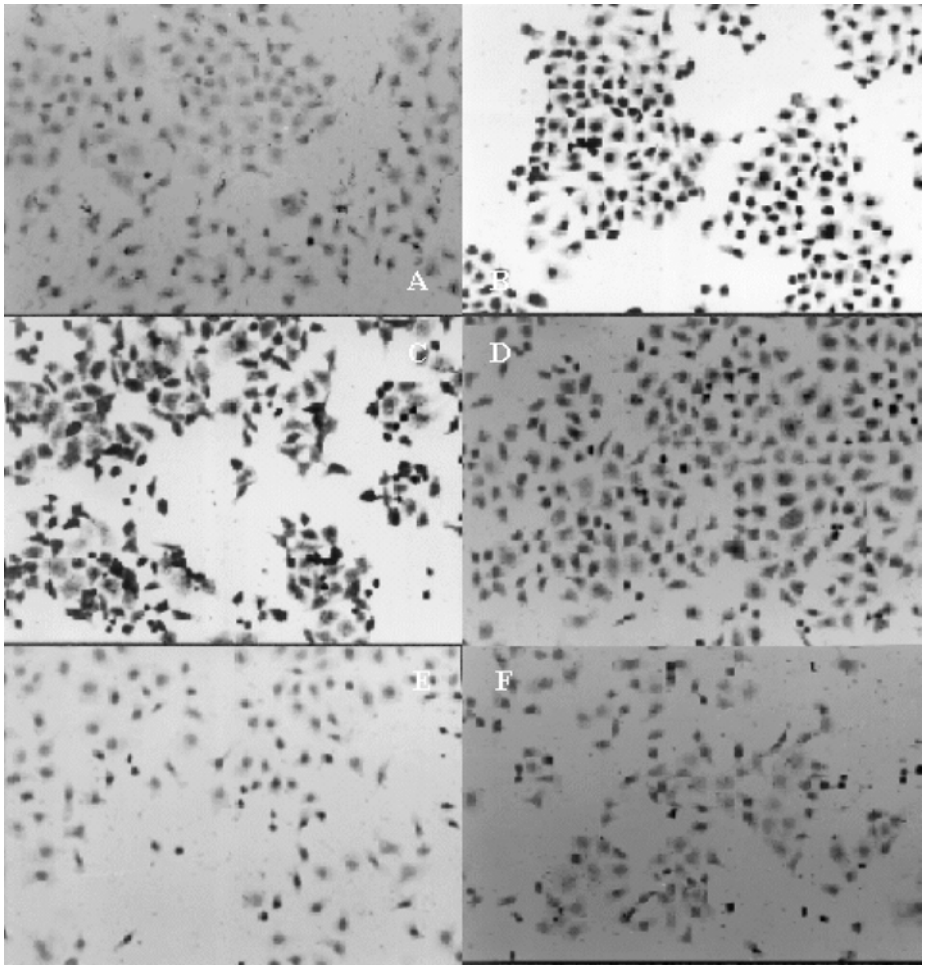

FIG. 5. Expression of CDK4 mRNA detected by *in situ* hybridization (200×). A. 2BS cells; B. Transformed cells; C. pXJ cells; D. S-D1 cells; E. AS-K4 cells; F. AS-D1 cells. Note: See Fig.4 for abbreviations.

TABLE 1

mRNA and Protein Levels of CyclinD1 and CDK4 in Normal 2BS, Transformed Cells, and Transfected Cells ($\bar{x} \pm s$)

	CyclinD1		CDK4	
	mRNA	Protein	mRNA	Protein
2BS Cells	13.99±2.18	26.57±8.69	26.58±4.78	28.37±6.09
Transformed Cells	258.27±58.72[a]	263.54±84[a]	303.47±72.39[a]	255.82±28.33[a]
pXJ Cells	207.13±63.89[b]	237.74±18.46[b]	215.76±33.71[b]	201.39±52.72[b]
S-D1 Cells	420.44±86.91[c]	163.21±18.63[c]	176.47±44.01[c]	186.06±16.50[c]
AS-D1 Cells	44.39±11.50[d]	53.82±11.18[d]	89.27±12.37[d]	80.92±9.87[d]
AS-K4 Cells	140.97±25.42[d]	117.32±13.32[d]	43.73±10.12[d]	41.92±3.25[d]

Note. [a] $P<0.05$ compared with 2BS cells; [b] $P<0.05$, compared with 2BS cells, $P>0.05$ compared with malignant transformed cells by silica; [c] $P<0.05$ compared with 2BS cells and transformed cells; [d] $P<0.05$ compared with transformed cells.

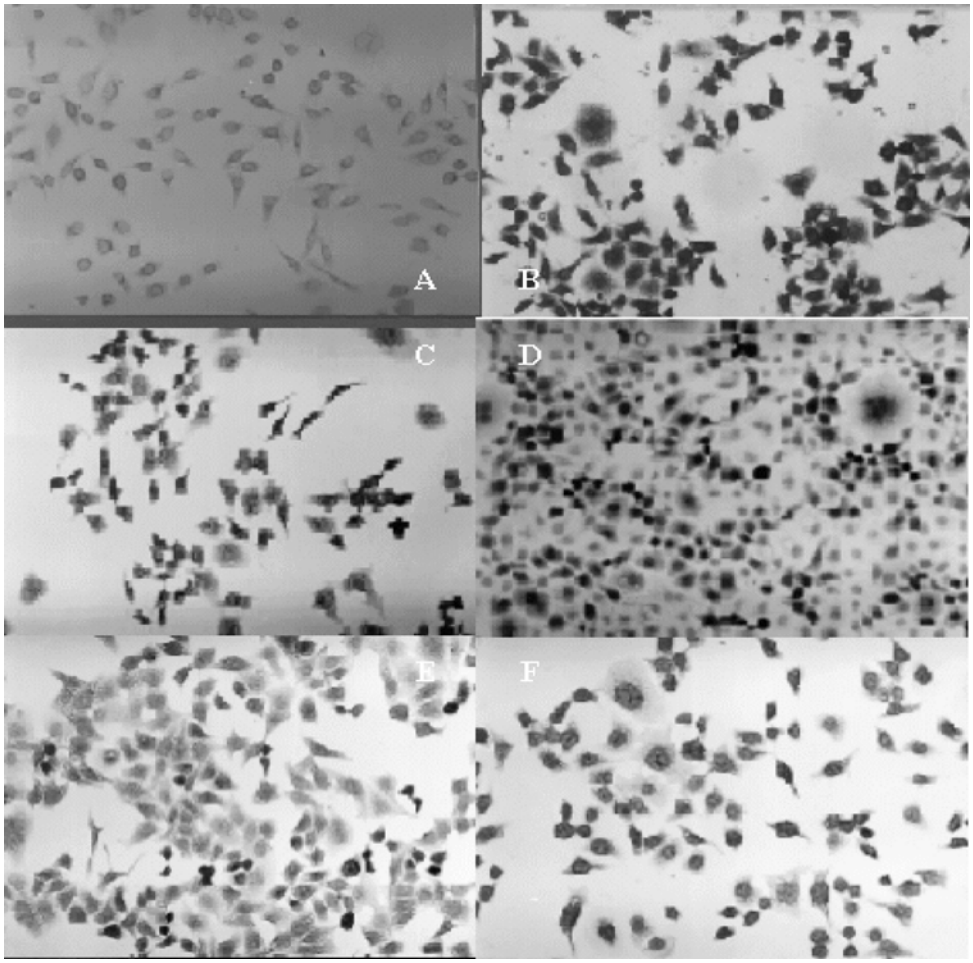

FIG. 6. Expression of cyclinD1 protein detected by immunohistochemistry (200×). A. 2BS cells; B. Transformed cells; C. pXJ cells; D. S-D1 cells; E. AS-D1 cells; F. AS-K4 cells. Note: See Fig. 4 for abbreviations.

Effect of expression level of cyclin D1 and gene on cell doubling time, cell cycle distribution and ability to live in soft agar Compared with the normal 2BS cells, transformed cells had higher growth rate, shorter doubling time of 21.0 h, lower percentage of cells in the G1 phage (45.1%). The transformed cells got the capability of living in soft agar. The growth rate of the antisense transfected transformed cells was significantly lower than that of the untransfected transformed cells or the empty vector transfected cells. The cells transfected by antisense cyclinD1 and CDK4 had longer doubling time of 31.4 h and 42.7 h, higher percentage of cells in the G1 phase of 52.7% and 58.0%, respectively. The ability to live on soft agar of the cells transfected by antisense cyclinD1 and CDK4 decreased. The cyclinD1 and CDK4 of the transfected cells exhibited decreased expression (Table 2).

DISCUSSION

There are many checkpoints in the cell cycle. One such important checkpoint is the G1-S checkpoint in late G1 phase. Cyclin and its partner CDK are important regulating genes in this checkpoint. Both cyclin and CDK are rate-limiting for cells to enter into S phase. CyclinD1-CDK4 complex can enhance phosphorylation of the Rb protein, which functions as a growth inhibitor in the late G1 phase of the cell cycle. CyclinD1-CDK4 plays an important role in many types of cancer. Amplification and overexpression of the cyclinD1 gene has been reported in many types of cancer, including breast, bladder, head and neck, lung and larynx carcinomas[15-22].

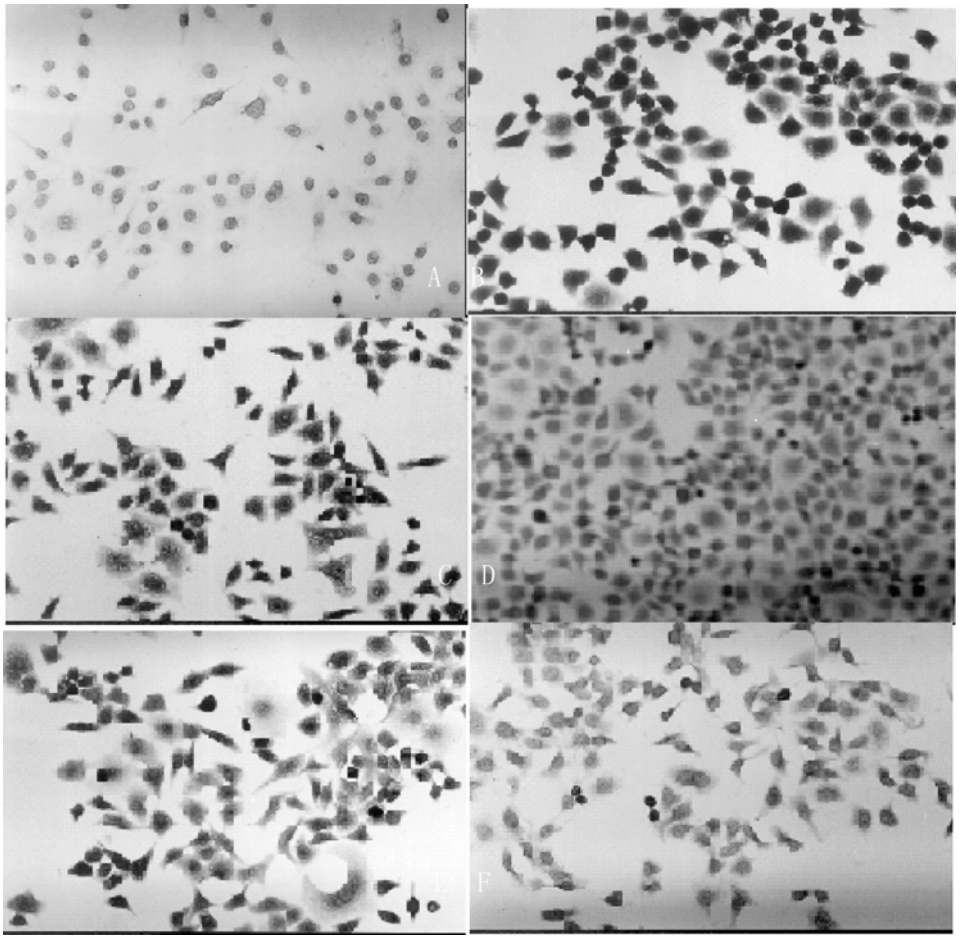

FIG. 7. Expression of CDK4 protein detected by immunohistochemistry (200×). A. 2BS cells; B. Transformed cells; C. pXJ cells; D. S-D1 cells; E. AS-D1 cells; F. AS-K4 cells. (See Fig. 4 for abbreviations)

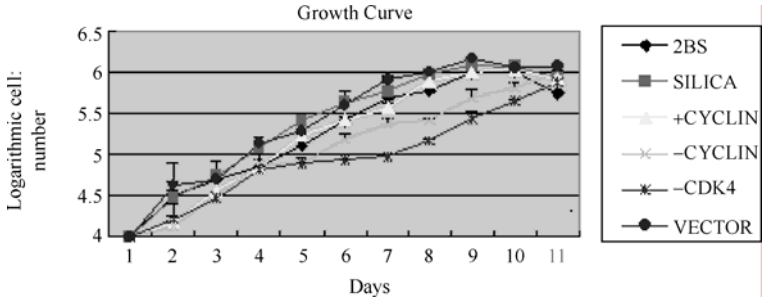

FIG. 8. Reduced growth rate of cyclinD1 and CDK4 antisense transfected cells. 2BS: normal human fetal lung diploid fibroblast cell line; SILICA: silica transformed cells; +CYCLIN: S-D1 cells; -CYCLIN: AS-D1; -CDK4: AS-K4 cells; VECTOR: pXJ cells.

The expression of cyclinD1 gene is fluctuant in cell cycle, and CDK4 gene is relatively invariable. Attention has been focused on the role and change of cyclinD1 in cell cycle, but many studies indicate that CDK4 also plays an important role in carcinomas[23-24]. It has been reported that the amplification and overexpression of CDK4 localized at chromosome band 12q13, contribute to uncontrolled growth in some sarcomas[24]. The present study has provided the evidence that the overexpression and amplification of cyclinD1 and CDK4 are often associated with the survival in some cancer, the increased risk of recurrence in breast cancer and the poorly differentiated tumors in squamous carcinomas of the head and neck and the lung. These data suggest that cyclinD1 and CDK4 play an essential role in some human tumors.

TABLE 2

Effects of CyclinD1 and CDK4 on Cell Proliferation

	Doubling Time (h)	Cell Cycle Distribution (%)			Clone Rate in Soft Agar （%）
		G1	S	G2/M	
2BS Cells	25.7	58.8	27.6	13.6	0.0
Transformed Cells	21.0	45.1	40.3	14.6	81.3
pXJ Cells	21.3	45.2	41.2	13.4	80.2
S-D1 Cells	24.9	51.0	39.2	9.8	82.6
AS-D1 Cells	31.4	52.7	33.1	14.2	65.6
AS-K4 Cells	42.7	58.0	30.0	12.0	61.1

In our study, the expression of mRNA and protein of cyclinD1 and CDK4 in the malignant transformed cells induced by silica was higher than that in normal 2BS cells,, which is in agreement with previous experimental results showing that the overexpression and amplification of cyclinD1 and CDK4 are found in some human tumors[15-24]. Our findings indicate that during the course of malignant transformation of 2BS cells induced by silica, the changes in cyclinD1 and CDK4 expression as well as in cell cycle occurred.

Along with the widespread intensive studies of the nature of antisense RNA, antisense technique plays an important role in gene therapy, in cancer and virosis research, and in cell cycle control research. It has been reported that antisense RNA leads to protein inhibition and cell cycle arrest, and results in delayed tumor growth and causes tumor shrinkage and even apoptosis.

In our experiments, antisense technique was used to study the role of cyclinD1 and CDK4 in the carcinogensis caused by silica. The cell models steadily expressing the antisense RNA were established and the expression of cyclinD1 and CDK4 genes was detected by *in situ* hybridization and immunohistochemistry, suggesting that the expression of cyclinD1 and CDK4 gene is inhibited by antisense cyclinD1 RNA and antisense CDK4 RNA, respectively.

In the cyclinD1 and CDK4 antisense transfected cells, the decrease in cyclinD1 and CDK4 expression could be attributed directly to the presence of abundant cyclinD1 and CDK4 antisense RNA in the stably transfected cells, leading to inhibition of translation of endogenous cyclinD1 and CDK4. Decreased cyclinD1 and CDK4 expression may control the expression of other cell cycle-related proteins, such as CDK4 and cyclinD1 through feedback mechanisms. Alternatively, the changes in proteins may be a consequence of the retarded cell growth resulting from decreased CDK4 and cyclinD1 expression. Although the exact mechanism is difficult to establish from the current experiments, the negative effect of cyclinD1 and CDK4 antisense expression on tumor cell growth is clear.

There are some reports on the relationship between cyclinD1 and CDK4 genes. Yu *et al.*[25] suggested that antisense cyclinD1 RNA could result in a 55% decrease in cyclinD1 mRNA, a 58% decrease in cyclinD1 protein level and a 40%-70% decrease in CDK4 protein level[25]. The results in this study are consistent with the previous studies. Correlation between cyclinD1 and CDK4 expression levels suggests a concurrent role of both genes in growth control of tumor cells.

Because overexpression of cyclinD1 can to shorten the duration of G1[26-28] and result in shorter population doubling time relative to control time, decreased cyclinD1 expression might be expected to lengthen G1 phase of the cycle and to increase the doubling time. In this study, the change of the cell characteristics was observed when the expression of cyclinD1 or CDK4 gene was inhibited showing that the length of G1 and the doubling time are somewhat longer in cyclinD1 and CDK4 antisense transfected cells than in the untransfected cells. The antisense transfected cells showed expression inhibition of cyclinD1 and CDK4 genes, cell growth prohibition and decreased capability of living in soft agar indicating that cell growth suppression and reversion in the tumorigenic properities of the malignant transformed cells induced by silica are mediated by

the direct transfection of antisense pXJ-cyclinD1 and pXJ-CDK4.

It is interesting in our research that the sense cyclinD1 RNA, one of the controls, showed inhibition effect on the malignant transformed cells induced by silica. This phenomenon is conflicting with the traditional theory but is in agreement with the findings discovered by Dr. GUO in 1995, who found that sense RNA is as effective as antisense RNA in suppressing gene expression in worms. Fire called this phenomenon RNA interference (RNAi)[29], which is widely used as a tool in many research fields, including human and animal cell research.

In conclusion, cyclinD1 and CDK4 play an important role in the carcinogenesis induced by silica. The striking effect of cyclinD1 and CDK4 antisense expression on the malignant transformation in human cells suggests a potential approach for therapies against rapidly growing cells in an accessible region of the body.

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