Antisense Oligonecleotides of CT120 Inhibited the Growth of Lung Adenocarcinoma Cell Line A549

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Abstract **Objective** To clarify the relationship between CT120, a novel human plasma membrane-associated gene, and prolifetation of lung adenocarcinoma cell line A549. **Methods** Expression vector (pcDNA3.1) containing antisense oligonucleotides of CT120 was constructed and transfected into the lung adenocarcinoma cell line A549. RT-PCR and Western blot detected the expression of CT120. Meantime, flow cytometry and soft agarose colony formation analyzed the cell proliferation, and p53, CyclinD1 and cdk4 were detected by RT-PCR. **Results** pcDNA3.1 containing antisense oligonucleotides of CT120 was constructed successfully and it could inhibit the expression of CT120 and suppress of the cell growth effectively. The expression of p53 was up-regulated and the expression of CyclinD1,cdk4 were down-regulated in CT120-pcDNA3.1-A549 cells. **Conclusion** CT120 was a new cancer associated gene, the down-regulation of CT120 expression by antisense oligonucleotides technique may be a new candidate of drug target for treatment of lung cancer.

Key words Antisense oligonucleotides; Lung cancer; RT-PCR; Western blot; Flow cytometry

CT120, a novel human plasma membrane associated gene, was isolated from chromosome 17p13.3 by using positional cloning and RACE (rapid amplification of cDNA ends) by National Laboratory for Oncogenes and Related Genes (GeneBank accession NO. AF477201)[1]. Some research indicated CT120 gene has important role in fetation, cell proliferation and cell differentiation. CT120 may express in various tumor cell lines and its expression was remarkably higher in lung cancer than in noncancerous tissues as well as normal lung tissues. Ectopic expression of CT120 by cDNA transfection could promote the malignant transformation of NIH3T3 cells and overexpression of CT120 in the A549 cells enhanced tumorigenicity in nude mice [2], which indicated CT120 might be a cancer associated gene that related closely to carcinogenesis and cancer progression[3].

Antisense oligonucleotides (ASON) may inhibit expression of a gene by binding to a targetsequence in the mRNA through base complementation. This regulatory effect of ASON has been useful in research and has been explored as a potential therapeutic utility for many diseases [4]. In our present study, we designed ASON of CT120 and ligased it with pcDNA3.1 successfully. We transfected the vector into the A549 cells, and obtained stable clone named. Flow cytometry and soft agarose colony formation indicated the suppression of the cell growth.

MATERIALS AND METHODS

Cell lines and cell culture

The human non-small cell lung cancer cell line A549 was servered by Biochemistry Lab of Medical school, Shandong Universty. Cells were maintained in RPME1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin.

pcDNA3.1 plasmid containing antisense oligonu-

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cleotides of CT120 constructs

The fragment of 408 bp cDNA from A549 cells corresponding to nucleotides 362−770 of CT120 mRNA was amplified using the synthetic primers (F: 5′TGTACCTCTGTGAATGGTGCCGAA3′; R: 5′GCATGGCCACGTTGCAGTAGAAT3′). The 408 bp fragment was directly ligated into the plasmid pUCm−T, and the positive clone was obtained by alpha complementation. With the help of EcoR Ⅰ and BamH Ⅰ, the PCR products were reversly ligated to the vector and the positive clone containing antisense oligonucleotides named pcDNA3.1−CT120 was successfully obtained. The antisense orientation was verified by automatic sequencing.

Stable transfection

A549 cells were grown to 60%−80% confluency in 6cm diameter Petri dishes and then transfected with 10μg pcDNA3.1−CT120 plasmid with LipofectAMINE reagent. Stable trsansfectants were selected for neomycin resistance in the medium containing 1.0mg/ml G418 and later maintained in the medium with 0.4mg/ml G418.

RT−PCR to detect the expression of targeted genes

RNA was extracted with Trizol kit(Gibco Co.).Total RNA was reverse transcribed with moloney murine leukemia virus reverse transcriptase (MuLV from MBI Co.). cDNA were amplified by PCR with primer set 5′AGGTTGGCTCTGACTGT3′ plus 5′TTGACGTGGTGAGGCTC3′, (546bp; for p53) or 5′TCATGCGGTCATTCTCCTTGTCCT3′plus 5′TCCCAGGAGCATTTAGCCATCCTT3′ (317bp; for CT120) or 5′CTGTCGCTGGAGCCCGTGAAAAAG3′plus 5′GAAGTTGTTGGGGCTCCTCAGGTT3′(300bp;for CyclinD1)or 5′CATGTAGACCAGGACCTAAGG3′ plus 5′AACTGGCGCATCACATCCTAG3′ (206bp; for Cdk4) or 5′GTGGGGCGCCCCAGGCACCA3′ plus 5′CTCCTTAATGTCACGCACGATTT3′(500bp; for β−actin1) or 5′GCGGATGTCCACGTCACACT3′ plus 5′CCACTGGCATCGTGATGGAC3′(428bp; for β−actin2) in reaction mixtures containing 10μM deoxynucleoside tripphosphates.

Western blotting

Cell extracts (CE)from cultures of A549 cells and transfected cells were collected by adding 0.05% SDS and equal amounts of total protein were analysed by western blotting. After determining the protein concentration using the Bradford−assay, constant amount of CE proteins were subjected to 15% SDS−PAGE and then transferred onto a nitrocellulos membrane. Detection was performed by using and ECL system. The chicken anti−CT120 antibody (as a expensive gift from National Laboratory for Oncogenes and Related Genes) was prepared by immunization of chicken with synthesized C−terminal 15−mer oligopeptide (CRKAVRLFD TPQAKK) of CT120 from amino acid 241−255. Anti−β−antibody was purchased from Biotop Biotechnology.

Soft agarose colony formation assay

Measurement of colony formation on soft agarose was employed as follows. A549 cells were transfected as described above and cultured at 37℃ under air/CO_2 (19:1) for 24h. An equal volume mixture of 0.8% low−melting−point agarose (in doubly deionized water) and 2×RPMI 1640 containing 20%(v/v) FBS was added to cells and seeded in a six−well plate to solidify. The plate was cooled to 4℃ for 5 min and incubated for 15 days. Colonies containing more than 20 cells were scored as positive.

Table 1 The colony formation results of different cells

	A549 cells	pcDNA3.1−A549 cells	CT120−pcDNA3.1−A549 cells
Numbers of inoculated cells	500	500	500
Numbers of colony formation	90	87	12
Inhibition ratio of colony formation(%)	0	3.33	86.67

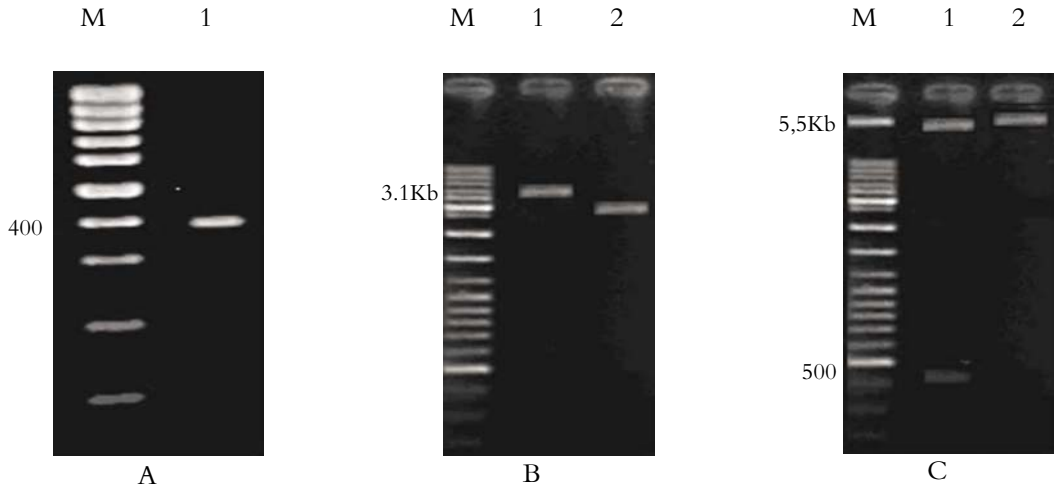

Fig.1 The process of vector construct.

A. Delegated the 408bp target gene.

B. Delegated the process of target gene ligated into pUCm−T vector, and strip1 delegated ligated vector and strip2 delegated control vector.

C Delegated the process of target gene ligated into pcDNA3.1 and strip1 was the result that pcDNA3.1−CT120 was incised by EcoR Ⅰ and BamH Ⅰ, and strip2 was the pcDNA3.1−CT120.

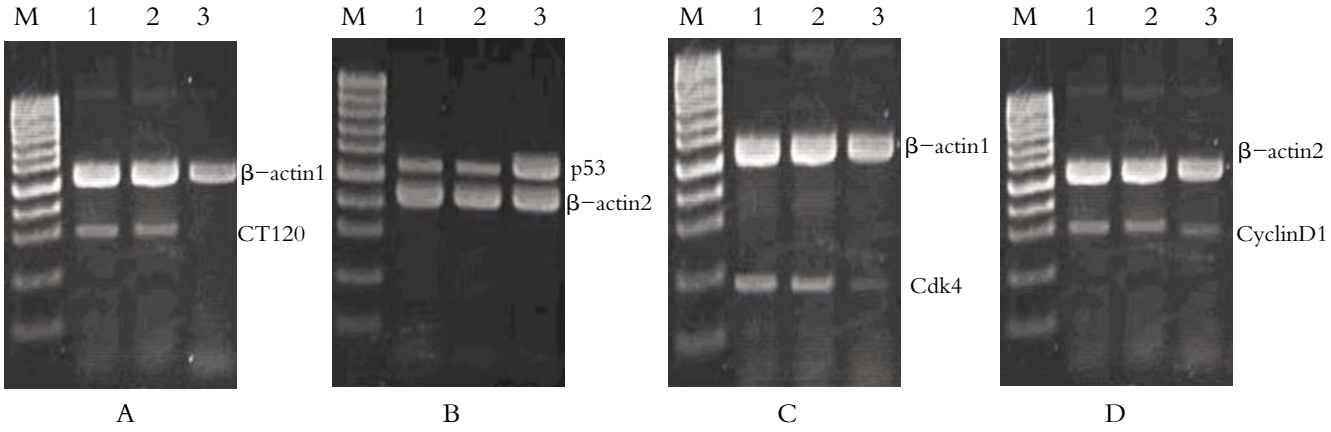

Fig. 2 The expression of CT120, p53, cdk4 and CyclingD1.

M: marker; 1: A549 cells; 2: pcDNA3.1−A549 cells; 3: CT120−pcDNA3.1−A549 cells.

Flow cytometry assay

A549 Cells was plated at 2.5×10^5 cells/75cm^3 flask. When the cells were grown to 80%, we detected the cell cycle by flow cytometry.

RESULTS

The construct of pcDNA3.1−CT120

Target gene (the fragment of 408bp) was obtained by PCR(Fig.1A). The 408bp fragment was ligated into the pUCm−T vector, and the electrophoresis showed the accurate strip (Fig.1B).There were two strips in 2.7kb and 3.1kb, which indicated that the 408bp fragment was ligated into the T vector(2.7kb). Fig.1C was the process of construction of pcDNA3.1 −CT120. Used EcoR Ⅰ and BamH Ⅰ, we cut the target gene from T vector, and successfully ligated it into the pcDNA3.1.

RT−PCR detected the expression of CT120, p53, cdk4 and CyclingD1

We detected the expression of CT120, p53, cdk4 and CyclingD1(Fig.2). The expression of CT120, cdk4 and CyclinD1 in CT120−pcDNA3.1−A549 cells was significantly lower than A549 cells and pcDNA3.1−A549 cells (Fig.2A,C,D). The expression of p53 in

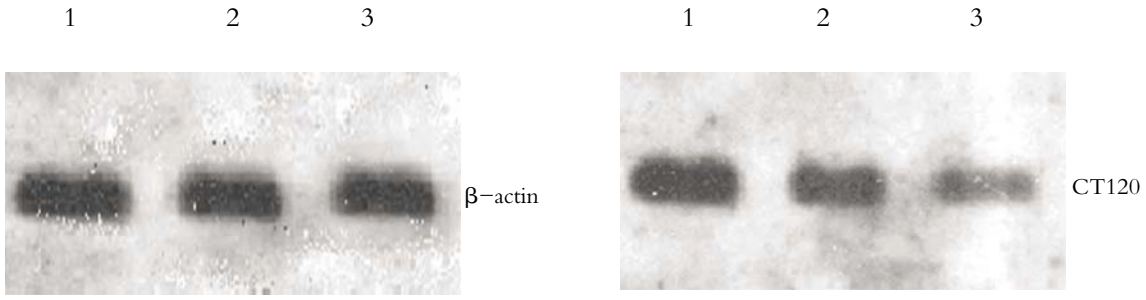

Fig. 3 The expression of CT120 protein.
1: A549 cells; 2: pcDNA3.1−A549 cells; 3: CT120−pcDNA3.1−A549 cells.

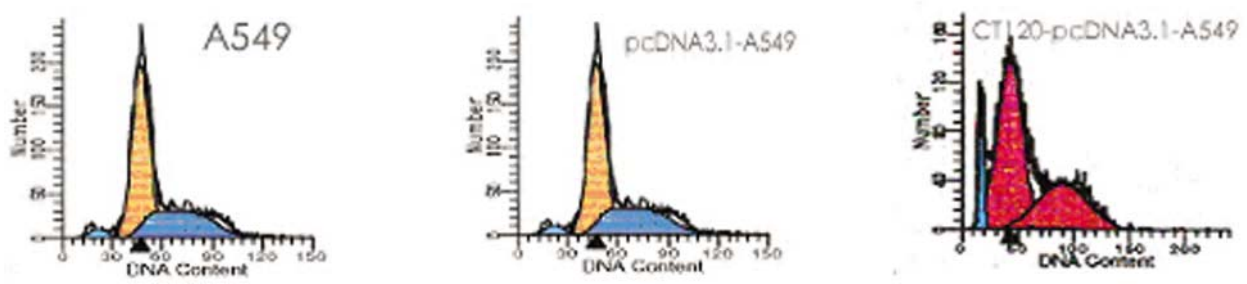

Fig. 4 The cell apoptosis analysis in different groups

CT120−pcDNA3.1−A549 cells was significantly higher than A549 cells and pcDNA3.1−A549 cells(Fig.2B).

Western blot detected the expression of CT120 protein

CT120 protein was significantly reduced in CT120−pcDNA3.1−A549 cells(Fig.3).

Soft agarose colony formation assay

The colony formation ability of CT120 −pcDNA3.1−A549 cells was significantly descended than A549 cells and pcDNA3.1−A549 cells. The inhibition ratio of colony formation was 86.67%(Table1).

Flow cytometry assay

We can discern the prominent apoptosis peak in CT120 −pcDNA3.1 −A549 cells groups, and the cell cycle was normal in A549 cells group and pcDNA3.1−A549 cells groups(Fig.4).

DISCUSSION

The lung cancer is the first resulting death in the western world and in China (according to the recent report of Ministry of Health of The People´s Republic of China), but five year survival rate of the disease has not been improved, so to find out the novel therapy drug is essential for lung cancer [5]. ASON can inhibit the expression of target gene by base complementation and arrive at the target of suppression of tumor growth. CT120 gene has been proved as a novel tumor related gene and was especially expressed in lung tumor [2]. In our present study, we successfully constructed the expression vector of CT120 named CT120 −pcDNA3.1 (Fig.1) and transfected the vector into A549 cells. Finally, we obtained the positive clone named CT120−pcDNA3.1−A549 by G418 selection that stable inhibited the expression of CT120(Fig.2A, Fig.3). The results of soft agarose colony formation and Flow cytometry indicated that the cell growth has been inhibited and the cells appeared apoptosis by silencing the CT120 gene, which indicated that CT120 was a tumor related gene and silencing CT120 gene can inhibit the tumor cell growth.

In order to explore the mechanism of suppression of cell growth by silencing CT120 gene, we detected the expression of p53, CyclinD1 and cdk4. The results of RT −PCR indicated that the expression of p53 gene

was significantly increased in CT120-pcDNA3.1-A549 cells group. p53 as an anti-oncogene has been certificated by all researchers [6]. We conferred that the cell growth suppression by silencing CT120 was related to the enhancement of p53 expression. The detail signal transduction need to further study. We also detected the expression of CyclinD1 and cdk4. RT-PCR results indicated that silencing CT120 gene could reduce the expression of CyclinD1 and cdk4. CyclinD1 and cdk4 were cell cycle regulatory gene and their expression products could promote cell transmition from G1 phase to M phase. CyclinD1-cdk4 complex could make Rb (a transcriptional factor)phosphated, then phosphated Rb released E2F that was a transcriptional factor and could combine DNA to promote cell cycle conversion[7~9]. The descend of the expression of CyclinD1and cdk4 resulted in the suppression of cell growth by above pathway. In short, silencing CT120 could enhance the expression of p53 and reduce the expression of CyclinD1and cdk4, which resulted in G1 phase arrest and apoptosis.

In present study, our results support the notion that CT120 is essential for maintenance of malignant phenotypes of lung cancer, silencing CT120 gene can inhibit the cell growth, which indicated that CT120 may be a new candidate of drug target for treatment of lung cancer.

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