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Construction and Identification of a Vector Expressing RNA Interference Aimed at the Human CyclinD1 Gene and its Expression in Vitro

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OBJECTIVE To construct a eukaryotic expression vector for RNA interference of the human cyclinD1 gene, and to detect its interference effect in human ovarian cancer cells (HO-8910).

METHODS Four target gene segments were synthesized and cloned into the pSUPER vector respectively to construct four recombinant eukaryotic expression vectors, pSUPER-C1~4. The four recombinant vectors were identified by enzyme digestion analysis and DNA sequencing. Then HO-8910 cells were transfected with the pSUPER-C1~4 vectors and subjected to G418 selection. In G418-resistant cells, the interference effect was detected by RT-PCR.

RESULTS Enzyme digestion analysis and DNA sequencing showed that the target segments were cloned into the pSUPER vector. The four recombinant vectors inhibited transcription of the cyclinD1 gene. The pSUPER-C2 vector had a better interference effect.

CONCLUSION The sequence-specific siRNA effectively interfered with expression of the cyclinD1 gene that was selected. The transcription and expression of the cyclinD1 gene were inhibited effectively by the constructed RNAi eukaryotic expression vectors in the ovarian cancer cells. These results indicate that it is possible to search for a new tumor gene therapy method.

KEYWORDS: RNA interference, siRNA, cyclinD1, cell cycle, gene therapy.

INTRODUCTION

CyclinD1 is a key regulatory protein in cell cycle progression. Expression of this protein can accelerate the cell cycle from G0/G1 and increase tumor cell proliferation. CyclinD1 is regarded as a type of oncogene. For example, it is amplified in a number of primary cancers, suggesting that it can serve as a new treatment target[1]. Therapy with RNA interference (RNAi) has the characteristics of high efficiency, high specificity and low toxicity, thus, siRNA has now become a powerful tool for studies on gene function and cancer therapy[2]. In this study, we devised the design and constructed the siRNA plasmid expression vector. Its transcript can form a short hairpin RNA (shRNA) with inverted, repeated sequence separated by a short loop sequence. The shRNA then was processed into functional siRNA to degradate target cyclinD1 mRNA and silence its expression. This technique makes it possible to search for new gene therapy methods for tumors.

MATERIALS AND METHODS

Materials

The pSUPER plasmid was acquired from the OligoEng Corp. RPMI 1640 medium was purchased from the Gibco Co., fetal calf

serum (FBS) was obtained from the HyClone Co. A reverse transcription system kit, restriction enzymes and T4 DNA ligase were purchased from the Promega Co. Trizol reagents, transfection reagent LipofectamineTM2000 and *E.coli.*DH5α were purchased from the Invitrogen Corp. The PCR primers for cyclinD1and β-actin were designed by Primers 5.0 software and were synthesized by the Shanghai Biotech Co.

Cell line and culture

The tumor line HO-8910, came from the cell bank of the Shanghai Biology Institute of the Chinese Science and Technology Academy. This cell line is a highly metastatic ovarian tumor. The HO-8910 cells were cultured in RPMI 1640 medium containing 10% FBS in a humidified atmosphere of 95% air, 5% CO_2 at 37 ℃. All the media were supplemented with 2 mmol/L L-glutamine, 100 mg/L penicillin and 100 kU/ L streptomycin.

Construction of the siRNA plasmid expression vectors

SiRNA-expressing plasmids were constructed by cloning siRNA sequences into pSUPER via Bgl II and Hind III sites. SiRNAs targeted against cyclinD1 (NM-053056) were designed by a program available online (http//www.ambion.com/techlib/misc/siRNA-design.htm) and the top-ranked gene sequences were chosen for this study[3,4]. The siRNA sequences were further subjected to a BLAST search against the human genome and EST databases to ensure that no human genes were targeted. The target sequence of the cyclin D1 gene and four DNA chains were synthesized as shown in Table 1. The structure of the DNA chains is Bgl II+sense chain+loop+antisense chain+termination signal+Hind III.

Two equal amounts of DNA chains were annealed and phosphorylated.To insert the targeting sequence, DNA oligos were designed and cloned into the Bgl II/ Hind III sites of the pSUPER vector. The directional cloning was accomplished with the aid of T4 DNA ligase. The ligated plasmid DNA was transfected into competent DH5a cells which were then seeded on solid LB medium containing 0.05 g/L ampicillin and cultured at 37℃ overnight. Three monoclonal colonies were picked out and seeded in 3 ml LB culture fluid containing 0.05 g/L ampicillin and cultured at 37 ℃ overnight in a rocking bed.

The plasmids were extracted following the manufacturer's instruction. Several colonies were then selected and minipreped until a positive clone was located by digesting primarily with EcoR I and Hind III. Finally, sequencing was carried out for further identification. The new plasmids were named pSUPER-C1~4. The oligonucleotides were synthesized by the Shanghai Sangon Co. and the concentration and purity of the plasmids were detected by ultraviolet spectrophotometry. The plasmids were stored at -20 ℃ for subsequent experiments.

Transfection by the plasmids

Transfection was performed following the manufacturer's instructions. HO-8910 cells were seeded on 6-well culture plates and grown to 80%~90% confluence before the transfection. Only LipofectamineTM 2000 was used for the transfection in the blank control group. Plasmid pSUPER was used for the transfection in the control group and the plasmids pSUPER-C1~4 were used for the transfection in the experimental groups. The common complete medium was replaced by antibiotic-free medium containing serum. Six hours after transfection, the medium was replaced by the common complete medium. At 24, 48 and 72 h following the transfection, expression of EGFP in the HO-8910 cells was observed under an inverse fluorescence microscope. Forty-eight hours later, G-4l8 was added into the medium at a final concentration of 600 ug/m1. After two-weeks, 4 clones were individually selected and expanded.

Expression of cyclinD1 mRNA detected by semi-quantitive RT-PCR

Table 1. Oligonucleotides corresponding to the target sequence and the control sequence

SiRNA	Sequence	Location
C1	5'-GATCCCCAAACAGATCATCCGCAAATTCAAGAGATTTGCGGATGATCTGTTTGTTTTTTGGAAA-3' 5'-AGCTTTTCCAAAAAACAA ACAGATCATCCGCAAATCTCTTGAATTTGCGGATGATCTGTTTGGGG-3'	496-514
C2	5'-GATCCCACACGCGCAGACCTTCGAGTTCAAGAGATAACGAAGGTCTGCGCGTGTTTTTTGGAAA-3' 5'-AGCTTTTCCAAAAAAACACGCGCAGACCTTCGTTTCTCTTGAATCCGAAGGTCTGCGCGTGTGGG-3'	576-594
C3	5'-GATCCCGCATGTTCGTGGCCTCTAATTCAAGAGATTAGAGGCCACGAACATGCTTTTTTGGAAA-3' 5'-AGCTTTTCCAAAAAAGCATGTTCGTGGCCTCTAATCTCTTGAAGTAGAGGCCACGAACATGCGGG-3'	729-747
C4	5'-GATCCCGCATGGTGATAGGAAACAGTTCAAGAGATTAGAGGCCACGAACATGCTTTTTTGGAAA-3' 5'-AGCTTTTCCAAAAAAGCATGTTCGTGGCCTCTACTCTCTTGAATAGAGGCCACGAACATGACGGG-3'	934-952

Groups of 1.0×10^6 cells were collected and total RNA was extracted using the Trizol reagent following the manufacturer's instructions. The concentration and purity of the total RNA was detected by ultraviolet spectrophotometry. RT-PCR was performed by a two-step method. Synthesis of cDNA was conducted using the reverse transcription system kit following the manufacturer's instructions. Amplification of human β-actin served as an internal standard. The forward primers used were 5'-CCC AGC ACA ATG AAG ATC AAG ATC AT-3' and for the reverse 5' - ATC TGC TGG AAG GTG GAC AGC GA-3', and the amplification product was 586 bp. The primers of cyclinD1 were 5'-CTT TCT CAA GGA CCA CCG-3' and reverse 5'-GCA CTT TCT CCG CAG TTT-3' and the amplification product was 478 bp. Thirty cycles of PCR amplification were performed with denaturing at 94℃ for 30 s, annealing at 58℃ for 40 s, and extension at 72℃ for 30 s. In addition, there was pre-denaturing at 94℃ for 4 min which was extended at 72℃ for 10 min The PCR products were separated in 1.5% agarose gels and visualized by staining with ethidium bromide. Semi-quantitative analysis was performed with the Gel Works software. The expression intensity of cyclinD1 was determined by noting the ratio of the photodensity of the RT-PCR products of cyclinD1 to β-actin. The inhibition ratio of cyclinD1 expression was calculated by the following formula: inhibition ratio of cyclinD1 expression (1-the expression intensity of cyclinD1 in the observation group/the expression intensity of cyclinD1 in the blank control group)×100%.

Statistical analysis

Data were expressed as the mean±standard deviation (SD). The *t* test was used to analyze the results by SPSS10.0 software. *P* values less than 0.05 were judged statistically significant.

RESULTS

Identification of recombinant plasmids

The recombinant extracted plasmids were double digested with EcoR I and Hind III, then checked by 0.5% agarose gel electrophoresis. The recombinant plasmids produced two bands at 5,202 bp and 281 bp, while the empty vector was observed to have two bands at 5,202 bp and 227 bp (Fig.1). Eventually the recombinant plasmid was identiflied to have a correct sequence by DNA sequencing analysis, and the resulting sequencing confirmed that the DNA chains had been ligated to the vector.

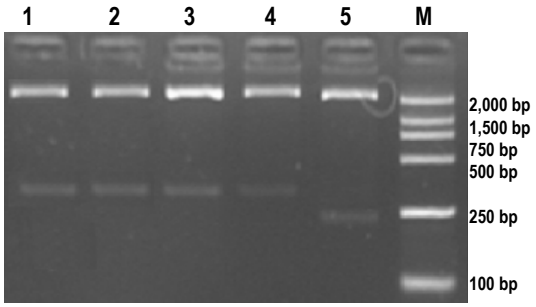

Fig.1. Identification of recombinant plasmids by restriction endonuclease digestion. M: DL2000 marker; 1~4:pSUPER-C1~C4; 5: pSUPER cut with EcoR I/Hind III,respectively.

Microscopic observation

Control cells showed similar morphologleal character: HO-8910 cells were markedly atypical, polygonal, and round, with deeply dyed karyomegaly, cytoplasmic ratio enlargement, active mitosis, and binuclear and multinuclear giant cells. The HO8910 cells were in a mass and sheet arrangement (Fig.2A). At 48 h after transfection with pSUPER-C2~C4 the cells showed slower growth and alteration of cell shape. Some of them were apoptotic, not well-stacked and shrunken with partial fragments compared with normal cells (Fig.2B). However the pSUPER-C1 cells showed no difference compared to the control cells. EGFP expression was detected under phase contrast fluorescence microscopy at 48 h. This EGFP carries a mutation in its chromophore, which shifts the excitation peak to 488 nm and enhances its fluorescence intensity. The rate of transfection ranged from 42±3.8% (Fig.2C).

Inhibition of cyclinD1 mRNA expression

RT-PCR was used for analysis of cyclinD1 mRNA expression in the HO-8910 cells. The amplified DNA fragments were fractionated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. We examined three siRNA-expressing vector pSUPER-C which target human cyclinD1 as shown in Fig.3. The RT-PCR products showed a band of 478 bp, and the β-actin product was located at 583 bp. The HO-8910 cells transfected with pSUPER-C2, C3 and C4, were noticeably weaker in the cyclinD1 band compared with the control cells (86.3%, 63.5%, and 57.6%, $P<0.05$), while in the pSUPER and pSUPER-C1 transfected cells showed no difference compared to the contro1s. Of the four siRNA-expressing vectors, pSUPER-C2 effectively suppressed the synthesis of cyclinD1 mRNA in the HO-8910 cells. RT-PCR product quantification showed that the pSUPER-C2 suppressed cyclinD1 mRNA produc-

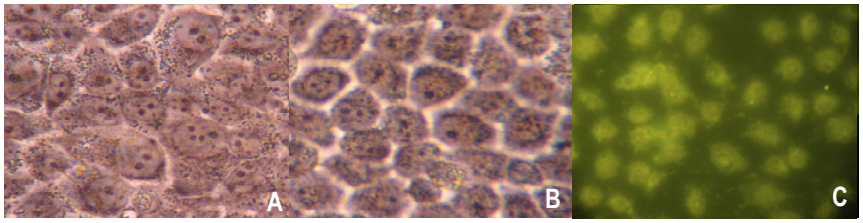

Fig.2. Morphology and expression of EGFP in HO-8910 cells after transfection (200×).A: control cells; B: Transfected with pSUPER-C2; C: Transfected with pSUPER-C2 (by fluorescence microscope).

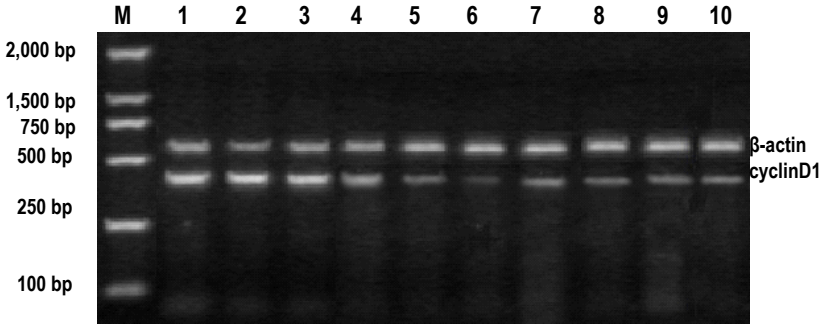

Fig.3. Expression of cyclinD1 mRNA in HO-8910 cells transfected with pSUPER-C detected by RT-PCR(β-actin 583 bp product was used as a control). M:DL2000 marker; 1:Control cells;2: Transfected with pSUPER; 3~4: Transfected with pSUPER-C1; 5~6: Transfected with pSUPER-C2; 7~8: Transfected with pSUPER-C3; 9~10: Transfected with pSUPER-C4 HO-8910 cells.

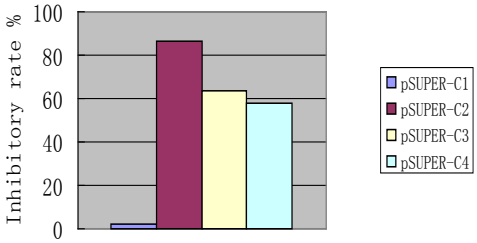

Fig.4. Quantitation of the inhibitory percentage of cyclinD1 mRNA in transfected HO-8910 cells. Each level of PCR product of cyclinD1 gene was quantified and normalized to the level of β-actin. The inhibitory rate was calculated by comparing to the control cells. The results were expressed as mean±SD from independent experiments.

tion to 86.3% of that in the control cells. Selection of an effective target sequence is the key point of RNA interference (Fig.4). On the basis of these results, we selected pSUPER-C2 as the most highly functional siRNA expressing vector in further studies.

DISCUSSION

RNA interference(RNAi) using double-strand RNA that targets homologous mRNA for degradation, effectively blocks gene expression at the post-transcriptional level. This inhibition can be sufficiently initiated by introducing 21~23 nucleotide small interfering RNA (siRNA) duplexes into human cells, causing effective, highly specific inhibition of both endogenous and exogenous genes[5].

This reduction in gene expression is transient, which severely restricts its application in mammalian cells. Brummelkamp and colleagues[6] have created a new vector, pSUPER that generates siRNAs in mammalian cells. This vector brings about sustained gene inactivation without cytotoxicity, and loss of functional phenotypes which will be assayed in the future. pSUPER has several key features that equip it for the job. It contains an RNA-polymerase-III promoter, a well-defined transcription start site and a termination signal that consists of five Ts. The pSUPER vector was constructed by inserting a reporter gene that enhansed a green fluorescent protein (EGFP) into a pSUPER vector. This EGFP carries a mutation in its chromophore, which shifts the excitation peak to 488 nm and enhances its fluorescence intensity. EGFP provides an opportunity to visualize only cells expressing this protein at a wavelength of 488 nm. Therefore, the pSUPER vector is a new and powerful system to analyze gene function in a variety of mammalian cell types. siRNA generating vectors could be designed to inactivate disease-associated transcripts that contain point mutations and leave unaffected the expression of the remaining wild-type transcript. These vectors could also be used in high-throughput in vitro screens for loss of functional phenotypes[7,8].

To target a specific mRNA for degradation, a portion of the mRNA target sequence must be known, and a segment of the target mRNA must be chosen that will be used for targeting by the cognate siRNA duplex. This process can be conducted as follows:

①Select the target region from the open reading frame of a desired cDNA sequence, preferably 50~100 nucleotides downstream from the start codon.

②Search for sequences 5'-AA (N19) UU, where N is any nucleotide, in the mRNA sequence, and ideally choose those with 50% G/C content. Nevertheless, 32~79% G/C content has also worked well. Highly G-rich sequences should be avoided because they tend to form G-quartet structures.

③Perform a BLAST search (www.ncbi.nlm.nih.gov/BLAST) using the selected siRNA sequences as the input against libraries or mRNA sequences of the respective organism to ensure that only a single gene is targeted.

④Synthesize several siRNA duplexes to control for the specificity of the knockdown experiments. Furthermore, a nonspecific siRNA duplex may be needed as a control[9,10].

So, we selected four oligonucleotides of cyclinD1 mRNA. Structure of a single target sequence itself is apt to form a hairpin by self-folding when performing the annealing reaction, which needs step-cooling or an ideal temperature gradient. This study, demonstrated that this step is helpful in cloning the ectogenesis fragment into the plasmid vector. Before agitation in bacteria transformation, incubating with gently shaking back and forth from time to time may increase the number of the colonies in plates more than a static incubation[11].

Until recently, siRNAs for gene targeting experiments have only been introduced into cells via classic gene transfer methods, such as liposome-mediated, electro-poration or microinjection. LipofectamineTM 2000 has been used successfully to transfect siRNA into HO8910 cells for RNAi studies. As EGFP provides an opportunity to visualize only the cells expressing this protein, the transfected cells were regarded as fluorescence-positive cells, which (green) can been detected by fluorescence microscopy. The control cells used were fluorescence-negative cells, which cannot be detected by fluorescence microscopy[12,13].

Normal operation of the cell cycle is determined by a regulatory system composed of cyclin, cyclin-dependent kinases(CDKs) and a cyclin-dependent kinases inhibitor(CDKI)[14]. CyclinD1 is a key regulatory protein in cell cycle progression, which can accelerate the cell cycle from G1 to S and increase tumor cell proliferation. CyclinD1 is regarded as a type of oncogene that can promote progression of the cell cycle G1 to S by cyclinD1-dependent kinase (CDK4/CDK6)-mediated phosphorylation of the retinoblastoma(Rb) protein. This results in inhibition of E2F and motivates the synthesis of DNA, making the cells transit from G1 to S causing accelerated proliferation. The relationship between the abnormal cyclinD1 over-expression and tumor pathogenesis has been the focus of attention in recent years[15,16].

In this study we explored a stable suppression of cyclinD1 expression in HO-8910 cells via RNAi. Four target sites were expressed in HO-8910 cells via online designed software, and recombinant pSUPER-C1~4 were constructed to direct siRNAs transcription in transfected cells. Our preliminary results demonstrated that the expression of cyclinD1 mRNA was significantly suppressed to 86.3% in transfected cells with pSUPER-C2 compared with control cells. The inhibitory effect was shown to be specific because no non-specific down-regulation of gene expression was observed, as demonstrated by the β-actin control.

We reported here an optimal method in constructing a recombinant plasmid, pSUPER-C2, which may serve other plasmid-based RNAi research in the exploration cyclinD1 inhibition via a vector-based RNAi in HO-8910 cells. Although the biological features of cells with down-regulated expression of cycinD1 await further investigation, the issue of delivery and tumor cell-targeting is still a major challenge before RNAi becomes a practical reality.

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