Construction of miRNA Interference Lentiviral Vector with Human miR-221 Gene*

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ABSTRACT Objective: To construct anti-miR-221 RNA interference lentiviral vector and seek the effective target sequence, to provide new gene approach for gilma investigation. Methods: To synthetic double-stranded DNA oligo sequence, and it was inserted into the digested vector of RNA interference directly. Then the vector was transplanted into the competent cells Bacterial competent cells. The clone was detected by PCR identification. The positive clones were the purpose of building RNA interference lentiviral vector with a successful gene. The target gene and vector were digested separately. Purified enzyme products were connected or re-orientationed later. The products were translated into bacterial competent cells. The positive clones of PCR were sequenced and analysis and comparison. The right clone will be the success of fusion protein expression vector. Two plasmids were then co-transfected into 293T cells, and effective target sequence was detected by western bolt. Results: Recombinant plasmid was inserted into the plasmid successfully and completly,,the target number PscSI576 was found being the best target for interference after coransfection. Conclusion: The human miR-221 gene RNA interference lentiviral vector was successfully constructed , and an effective target sequence was found.

Key words: RNA interference; Lentiviral; DNA expression vector; miR-221

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Introduction

MicroRNAs(miRNAs) represent a class of short, non-coding, regulatory functions with single-stranded RNA molecules of about 21-25 nucleotides, miRNA can promote mRNA degradation and (or) inhibit the translation process to play a negative regulator of gene expression on target post-transcriptional level [1-3]. miRNA-221 is an integral part of the microRNA family, which has played an important role in melanoma[4], breast cancer[5], prostate cancer [6] and other tumors. Lorimer IA found that the miRNA-221 expression in gliomas was much higher than that of normal tissue via studying of miRNA expression profile in glioblastoma [7]. This result suggested that the high expression of miRNA-221 could make it a marker for gliomas. Recently studies have shown that miRNA-221 was over-expression in tumor tissue, which was closely related with the occurrence of cancer., miRNA-221 can combine with multiple tumor suppressor genes that can inhibit the expression of mRNA [7-9], p27 gene was a downstream regulated gene of miRNA-221[10]。 This atudy was to constructe the RNA interference lentiviral vector and screen an effective target sequence, which provided a new idea for the early diagnosis and treatment of gliomas.

1 Materials

pEGFP-C1 Vector were purchased from Clonetech Inc. (cat. No.#632465) ; Age I and EcoRI enzymes were purchased from New England Biolabs (NEB) Company; reagents primer of PCR (R&F) and dsDNA oligo were purchased from Shanghai GeneChem Co., Ltd.; PCR instrument was purchased from Applied Biosystems Inc.; Regulators electrophoresis was purchased from BioRad Inc.; Gel Imager was purchased from tanon company ;positive clone sequenced by Invitrogen Corporation.

2 Methods

2.1 RNA interference lentiviral vector′s preparation

2.1.1 Construction of siRNA expression vector To found the target gene sequences , siRNA target design software (Invitrogen) was used to designa number of target sites referencing siRNA target design principles [11]. The selected sequences were analyzed by bioinformatics analysis (blast analysis, SNP analysis). Finally, four active sites were selected, which was named PscSI576, PscSI577, PscSI578, PscSI579. An invalid serial number PSCNC were designed (Table 1), Then the best kinetic parameters of target was selected into the following-up testing procedures. The viral vector frame was construction in accordance with (Table 2). The synthetic sense and antisense DNA fragments joined annealing reaction system and blended. Then the efficiency of double-stranded form was detected by 12% PAEG non-denaturing PAGE gel electrophoresis. pGCSIL-GFP vector was digested by *Age* I and *EcoRI.*

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Table 1 siRNA target sequence

Marker	Fragment Name	TargetSeq	StartPos	GC
PscSI576	miRNA-221	ggCATGAACCTGGCATACAAT	20	47.62 %
PscSI577	miRNA-221	aaCCTGGCATACAATGTAGAT	26	38.10 %
PscSI578	miRNA-221	tgGCATACAATGTAGATTTCT	30	33.33 %
PscSI579	miRNA-221	tgGGTTTCAGGCTACCTGGAA	82	52.38 %
PSC-NC		TTCTCCGAACGTGTCACGT		52.63 %

Table 2 viral vector frame

NO.	5'	STEMP	Loop	STEMP	3'
PscSI576-1	Ccgg	ggCATGAACCTGGCATACAAT	CTCGAG	ATTGTATGCCAGGTTCATGcc	TTTTTg
PscSI576-2	aattgaaaaa	ggCATGAACCTGGCATACAAT	CTCGAG	ATTGTATGCCAGGTTCATGcc	
PscSI577-1	Ccgg	aaCCTGGCATACAATGTAGAT	CTCGAG	ATCTACATTGTATGCCAGGtt	TTTTTc
PscSI577-2	aattgaaaaa	aaCCTGGCATACAATGTAGAT	CTCGAG	ATCTACATTGTATGCCAGGtt	
PscSI578-1	Ccgg	tgGCATACAATGTAGATTTCT	CTCGAG	AGAAATCTACATTGTATGCca	TTTTTg
PscSI578-2	aattgaaaaa	tgGCATACAATGTAGATTTCT	CTCGAG	AGAAATCTACATTGTATGCca	
PscSI579-1	Ccgg	tgGGTTTCAGGCTACCTGGAA	CTCGAG	TTCCAGGTAGCCTGAAACCca	TTTTTg
PscSI579-2	aattgaaaaa	tgGGTTTCAGGCTACCTGGAA	CTCGAG	TTCCAGGTAGCCTGAAACCca	
PSCNC-1	T	TTCTCCGAACGTGTCACGT	TTCAAGAGA	ACGTGACACGTTCGGAGAA	TTTTTTC
PSCNC-2	TCGAGAAAAAA	TTCTCCGAACGTGTCACGT	TCTCTTGAA	ACGTGACACGTTCGGAGAA	A

2.1.2 Preparation and transformation of the cloning The competent cells of E. coli were prepared by using calcium chloride, the annealed DNA and linearized vector were transformed on demand. The positive clones were identified after transformed, up primer 5'-CCTATTTCCCATGATTCCTTCATA-3',down primer： 5'-GTAATAC- GGTTATCCACGCG-3'.

2.1.3 DNA sequencing and extraction of positive clones Positive bacteria clones were sequenced by ABI 3730 sequencer of the Invitrogen Corporation. Positive clones which were right from the selecting sequence were extracted plasmid clones by using QIAGEN-tip 500 of the columns in accordance with the kit instructions.

2.2 Construction of anti－miRNA－221 over－expression vector

2.2.1 Linearization of eukaryotic expression vector *Mfe I/Hpa* I restriction enzyme were used to digestEukaryotic expression vector.

2.2.2 Fragment obtained by PCR Primers were synthesized by Shanghai GenChem Technology Co., Ltd.,up primer：ACCGCAATTGTGAACATCCAGGTCTGGGGCATGAACCTGGCATACA ATGTA GATTTCTGTGTTCGTTAGGCAACA,down primer：ACCGGTTAACGAGAACATGTTTCCAGGTAGCCTGA AACCCAGCAG ACAATGTAGCTGTTGCCTAACGAACACAG. After PCR reaction, the fragment was verified by agarose gel electrophoresis. Using *Mfe* I / *Hpa* I to digest, agarose gel electrophoresis was down to verify it.

2.2.3 Preparation of recombinant cloning The PCR product and eukaryotic expression vector were transformation into the fresh competent cells of *E. coli.* Bacteria were amplified by LB medium. The plasmid DNA was extracted by ultra-pure extraction kit of QIAGEN Company.

2.2.4 Identification of positive clones The PCR amplification was accordance with normal reaction system, up primer: CATGGTCCTGCTGGAGTTCGTG,down primer: ATTTGTAACCATTATAAGCTGC. Finally, the products of positive clones were sequenced.

2.3 Selection the effective target of RNA interference

2.3.1 Cotransfection of the over－expression plasmid and the interference vector The over-expression cloned plasmid of target genes and RNAi vector for different interference target of 0.25μg, 0.5μg were co-transfected into the cultured tools cells (293T cells). Empty plasmid (PC), non-interference knockdown plasmid targets (NC) Groups were set up to controls.

2.3.2 Detection of transfection efficiency Transfection efficiency was detected by fluorescence microscope after 24 h (the proportion of the positive- immunofluorescence cells in the microscope). The transfected proteins of extraction were collection after 36-48 h. The expression of protein was detected by Western blot. Cumulative optical density (IOD) of bands was detected by Gel-Pro analyzer 4 software.

3 Results

3.1 Preparation of the target RNA interference lentiviral vector

3.1.1 Detection the formation of double-stranded Electrophoresis results showed that double-stranded DNA oligo synthesized successfully contrasting the position of single-stranded, the double-stranded department (Fig.1).

3.1.2 Construction of vshRNA vector After digested by *Age* I and *EcoRI*, the plasmid without digested had the band around 5 kb significantly, plasmid with digested in the 8 kb band, which showed that vshRNA vector was Construct.

3.1.3 Identification of positive clones by PCR The size of the clones with vshRNA fragment was 340 bp; the size of the positive clones of PCR without vshRNA fragment was 306 bp(Fig.2). Positive clones had a band at 340bp around.

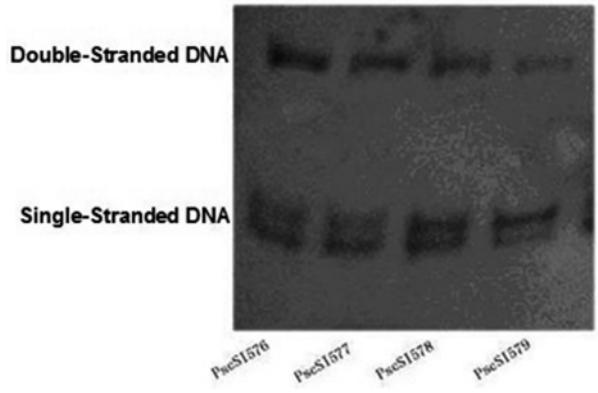

Fig.1 Show the double-stranded has strip

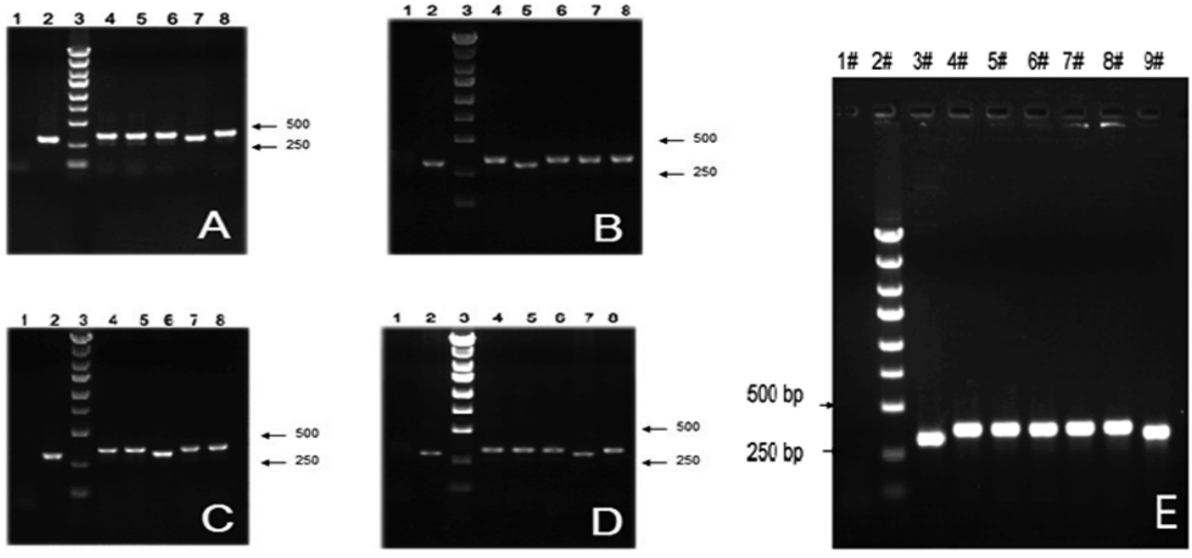

Fig.2 A-D 1:negative control (ddH2O); 2:negative control (no-load group);3 :Marker;A:PscSI576 -1,2,3,4,5 ;B: PscSI577-1,2,3,4,5 ;C: PscSI578-1, 2,3,4,5 ;D :PscSI579-1,2,3,4,5 ;E: positive clones PscNC-1;lane1# :negative control(ddH2O); 2# :Marker; 3# :negative control(no-load group); 4# :positive control (insert group); 5~9#:PSC-NC-1,2,3,4,5

3.2 Construction of target gene over-expression vector

3.2.1 Obtained fragment by PCR The fragment were obtained by PCR, the PCR products size was 130 bp (Fig.3).

3.2.2 Digestion of PCR fragments The target gene were purificated by using Mfe I/HpaI, there was a band at about 130 bp (Fig.4).

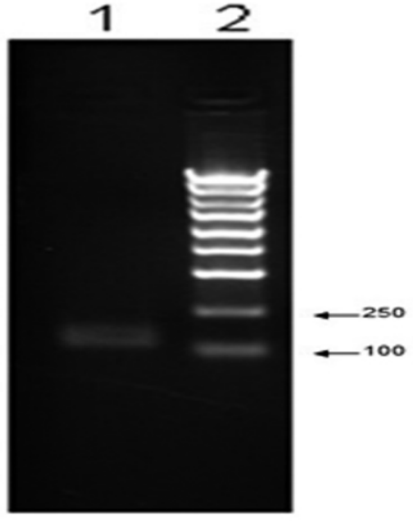

Fig.3 1: DNA Marker; 2: products of PCR

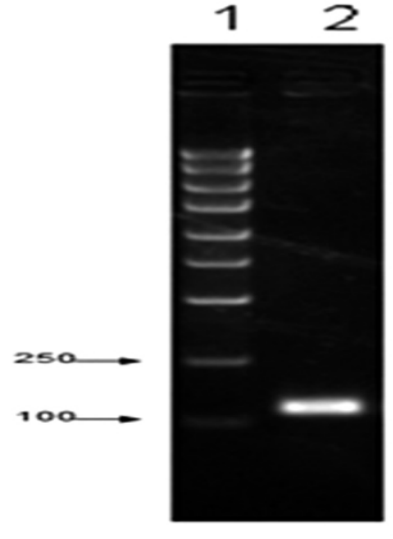

Fig.4 1: Digestion products of PCR fragment; 2: Marker

3.2.3 Identification of PCR-positive clones The size of PCR products were 467 bp, self-ligation was 365 bp (Fig.5). Finally, positive clones were sequenced, which showed that the fragments were cloned into the vector correctly.

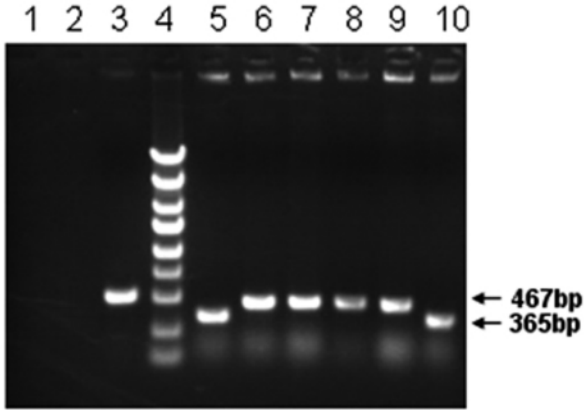

Fig.5 No.1 lane :negative control (ddH_2O) ,No.2 lane :negative control (no-load group) ,No.3 lane :positive control (the vector group with the determined insert) ,No.4 lane :DNA Marker,No.5 lane : pEGFP-C1-3FLAG-MIR221-1,No.6 lane :pEGFP-C1-3FLAG-MIR221-2, No.7 lane :pEGFP-C1-3FLAG-MIR221-3,No.8 lane : pEGFP-C1-3FLAG-MIR221-4,No.9 lane :pEGFP-C1-3FLAG-MIR221-5, No.10 lane :pEGFP-C1-3FLAG-MIR221-6.

3.3 Effective screening of target RNA:

The over-expression plasmid and siRNA vectors were co-transfected into 293T cells successfully. Transfection rate was more than 70 % (Fig.6). Western blot analysis showed that interference plasmid' IOD of 0.25 μg : PC: 561.48, NC: 604.11, PscSI576: 454.43, PscSI577 :566.56, PscSI578: 493.68, PscSI579: 623.01,interference plasmid' IOD of 0.5 μg: PC :694.88, NC: 583.45, PscSI576: 128.06, PscSI577: 158.24, PscSI578: 350.08, PscSI579: 420.16 (Fig.7).the knockdown effect of PscSI576 target was consistent with two concentrations, which was the best target.

4 Discussion

Past of cancer research focused primarily on the relationship between abnormal expression of tumor-associated protein and tumor occurrence and development. Since 2002, Calin GA firstly reported that abnormalities of miRNA associated with tumor, more and more evidence showed that miRNA played an important role in tumor occurrence and development [12]. The miRNA research provided a new way of thinking for a more comprehensive insight into the pathogenesis of tumors.

Human gliomas are common primary intracranial tumor, the molecular mechanism of its occurrence and development remains unclear after years of study. miR-221 showed high expression in the hepatocarcinoma [13], prostate cancer [6], breast cancer [5], melanoma[4] and others, indicating that miR-221 was a cancer gene in these tumors. Chan JA [14] and Ciafre SA [15] were carried out initial research on miRNA on the role of the human brain in 2005 respectively. Ciafre SA found that the expression of miRNA-221 and miRNA-222 tended to increasing in the expression profile of human glioma among which miRNA-221 was the most significant. Therefore they believed that the abnormal expression of miRNA-221 may be the molecular marker of glioblastoma. miRNA-221 would be combined with the 3'UTR of Several tumor suppressor genes to suppressed its expression area, of which the most studied was on p27 gene. Its encoded protein was P27kip1 protein which was considered to be a variety of non-specific, broad-spec-

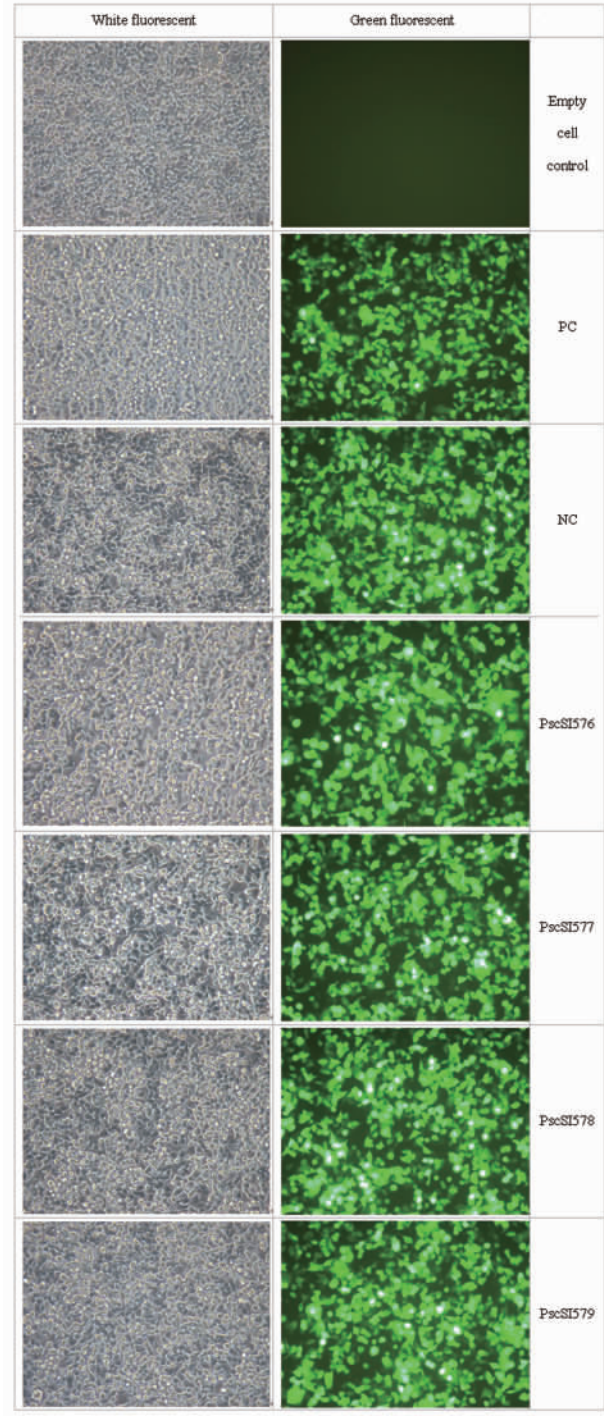

Fig.6 293T cells, expression plasmid 0.5 ug, interference plasmid 0.5 ug, lipofectamine1uL, cotransfection 24 h (× 200). NC: knockdown plasmid without interference target.

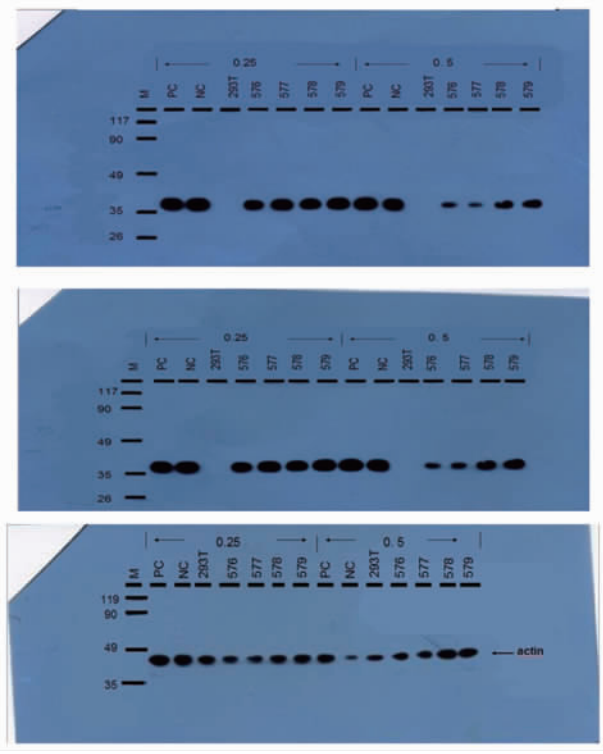

Fig.7 The expression protein after knockdown was detected by western blot

trum cyclin-dependent kinase inhibitor, and P27kip1 protein was a member of the CDK inhibitor family. As a negative regulator of cell cycle, its over-expression might affect cell cycle progression [16], thereby affecting the cell proliferation [17]. Novakova J found that miRNA-221/222 combinated with p27kip1 mRNA in 3'-UTR to reduce the expression of p27kip1 in the brain glioblastoma, so that cell cycle arrested at G1 phase [18]. So they thought that p27kip1 mRNA was a target gene of miRNA-221 by inhibiting the expression of miRNA-221 to restore the expression level of p27kip1 protein and inhibiting the growth of glioblastoma. This showed that abnormal expression of miRNA-221 was closely related to the occurrence and development of glioma, and exploring its role in glioma would furtherly deepen the understanding of the molecular mechanism of occurrence and development, so it would broaden the molecular glioma treatment strategies.

This study designed multiple RNA interference target sequence by using RNA interference sequences in accordance with the common web site design principles, then the best targets of the kinetic parameters were selected for the following-up experiments. The double-stranded DNA oligo of synthetic sequence with interference, which had restricted sites at both ends with sticky, was connected into the digested RNA interference vector directly. The connected good products were connected into the preparation of competent cells, then the grown clones were done of PCR identification, and done of sequencing comparison,the right ones were the construction of the target gene RNA interference lentiviral vector successfully. Then from the cDNA library or a plasmid cloning templates containing the target gene, we did the dual digestion on the purpose of the target gene and vector by using PCR to fishing the target gene. Purification of enzyme products were done directional connection or reorientation, its products were translated into bacterial competent cells, the grown clones were done of PCR identification,its upstream and downstream primers were designed on the carrier and the target gene. The clones identified as positive clones by PCR demonstrated that the target genes had been linked to the purpose of directional vectors. The PCR positive clones were sequenced and analyzed of comparative, the right one would be the fusion protein expression vector of construction successfully. Above of those were consistent with the literature reports. The two plasmid vectors were then cotransfected into 293T cells, finding the best target by western blot. Lastly we successful constructed the human miR-221 gene RNA interference lentiviral vector, and srceen the effective target sequence of interference. This experiment laid the foundation of follow-up study for early detection, classification and prognosis of tumors.

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针对 miR-221 基因干扰慢病毒载体的构建及其有效靶序列的筛选与检测 *

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摘要 **目的**:构建携带人 miR-221 基因的 miRNA 干扰慢病毒载体并寻找其有效靶序列,为胶质瘤的研究提供一种新的方法。**方法**:合成含干扰序列的双链 DNA oligo 直接连入酶切后的 RNA 干扰载体上。将产物转入细菌感受态细胞,对长出的克隆进行 PCR 鉴定,阳性克隆即为目的基因 RNA 干扰慢病毒载体质粒。再将目的基因与目的载体分别进行双酶切,纯化酶切产物后进行定向连接,其产物转入细菌感受态细胞,再对 PCR 鉴定阳性的克隆进行测序和分析比对,比对正确即为融合蛋白过表达质粒载体,然后将两种质粒共转染入 293T 细胞,用 western bolt 法检测其有效敲减靶序列。**结果**:重组质粒经测序鉴定证明各转录模板完整、正确插入到相应质粒中,共转染后发现编号为 PscSI576 的靶点干扰效果最好。**结论**:本实验成功构建了人 miR-221 基因的 RNA 干扰慢病毒载体,并找到了有效的干扰靶序列。

关键词:RNA 干扰;慢病毒;DNA 表达载体;miR-221

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