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Construction of MiRNA Eukaryotic Expression Vector and its Stable Expression in Human Liver Cancer Cells

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Abstract

Recent research indicates that miR-26a is involved in various biological processes including cell cycle, apoptosis and Hepatocellular carcinoma (HCC). In present report, we demonstrate the construction of recombinant plasmid miR-26a expression vector and its stable expression of miR-26a in transfected human liver cancer cells (HepG2). The double strands oligo encoding the miR-26a was designed and synthesized to generate the mature miR-26a expression plasmid. Use of the vectors in mammalian cells permits visual detection of cells expressing the pre-miRNA through co-cistronic expression of EGFP. The positive clones were screened by restriction enzyme digestion and sequenced. The new expression vector of miR-26a was named pHsa-miR-26a. PHsa-miR-26a and its controls were transfected to HepG2 cells. Fluorescence detection displayed that fluorescence intensity of GFP was highest during 48 and 72 hours post-transfection, and qRT-PCR showed a significantly increase in miR-26a expression in the vector transfected cells compared with the expression in its controls. The recombinant plasmid expression vector of miR-26a was constructed successfully, which may facilitate further study of its function in the process of cell cycle and HCC.

Keywords: miR-26a, HepG2, vector

1. Introduction

MicroRNAs (miRNAs) are a class of small single stranded RNAs, about 22nt in length, and play a

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pivot role in gene expression regulation. Increasing evidence has demonstrated that miRNAs are involved in the process of many diseases, for example, cancer, diabetes, cardiovascular disease, neurodegenerative disease. MiRNAs are generally down-regulated in many cancer types; although they have been found aberrantly over-expressed. Recent studies have reported that miR-26a downregulation in hepatocellular carcinoma[1], a finding associated with overall survival and response to interferon therapy for these patients[2]. Since miR-26a downregulation has been associated with cancer properties, large scale functional studies of miR-26a regulation in human liver cancer cells will contribute to the understanding and treatment of relative pathophysiologic conditions such as tumor progression/ regression, response to treatment, etc.

Hepatocellular carcinoma (HCC) is the third-leading cause of death from cancer and the fifth most common malignancy worldwide. HepG2 cells is a valuable model in study Pathogenesis and treatment of human hepatocellular carcinoma. Gene therapy vectors are particularly promising, Plasmid-based vectors are capable of achieving a stable and efficient gene expression in target cells in vitro and it has been used in the miRNA expression vector construction. In the present report, we demonstrate that a recombinant plasmid expression vector of miR-26a was constructed and could be effectively transfected into HepG2 cells. Construction of expression vector establish the basis for the future research.

2. Materials and methods

2.1. Plasmid and reagents

The pPG-eGFP-miR Vectors and *E.coli* DH5*a* were obtained from shanghai genepharm Co., Ltd; Taq enzyme and T4 ligase, restriction enzyme, marker, plasmid exacting kit were purchased from TaKaRa Company. Transfection reagent (QickShuttle) were purchased from Beijing KangBiQuan biotech Co., Ltd; primer was designed and synthesized by shanghai sangon biotech Co., Ltd.

2.2. Cell cultures

The cell lines used in this study were preserved in our lab. HepG2 cells were grown in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA). Cells were maintained at 37^0C and 5% CO_2 during all experiments.

2.3. Construction of miR-26a expression vectors

The pPG-eGFP-miR Vectors used in this study are based on knockdown cassettes driven by RNA Polymerase II promoters that allow expression of engineered miRNA sequences. The prototypical miRNA is a 22-mer single strand RNA molecule. A more efficient expression vector is to express a single RNA that is about 22-mer hairpin with a loop (Fig 1a). When expressed in mammalian cells, the hairpin structure is apparently recognized by Dicer, the nuclease responsible for a functional miRNA[3].

Use of the vectors for RNAi analysis of target gene in mammalian cells permits visual detection of cells expressing the pre-miRNA through co-cistronic expression of EmGFP. The double strands oligo (sense strand: 5'- TGCTGTTCAAGTAATCCAGGATAGGCTGTTTTGGCCACTGACTGACAGCCTATCGGATTACTTGAA-3'; antisense strand:5'-CCTGTTCAAGTAATCCGATAGGCTGTCAGTCAGTGGCCAAAACAGCCTATCCTGGATTACTTGAAC-3') encoding the miR-26a was synthesized by shanghai sangon (Shanghai, China) and cloned into the BamH I and Xho I-digested site of eukaryotic expression vector. The construct was transformed into competent *E. coli* that were cultured in the selecting medium with 250 ug of spectinomycin/ml. The positive clones of the

selected plasmid were confirmed by DNA sequencing technology[4]. The pPG-eGFP-miR-neg control plasmid contains an insert (the sequence without 5'overhangs: 5'-TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACAT TT-3') that can form a hairpin structure which is processed into mature miRNA, but is predicted not to target any known sequences in the human, mouse, and rat genomes.

2.4. Gene transfer and miR-26a expression assay

Human liver cancer cell line HepG2 was transfected with plasmid encoding the miRNA expression vectors described above using the PEI-based polycation (KangBiQuan Co., Beijing, China). Two days after transfection, cells harboring an expressing integrant were viewed by fluorescenc microscopy based on GFP. The cells were transfected for 72 h, harvested and extracted total RNA.

Gene-specific primers and reverse transcriptase were used to convert mature miRNA to cDNA [5]; DNase-treated total RNA (20 ul total volume) was incubated with 1ul 10 mM of reverse transcription primers listed in the table 1. The reaction was heated to 80°C for 5 min to denature the RNA, and the reactions were cooled to room temperature quickly, then the remaining reagents (5× buffer, dNTPs, DTT, RNase inhibitor, primescript RTase) were added as specified according to the manufacturer's protocol. The reaction proceeded for 45 min at 42°C followed by 5 min incubation at 85°C to inactivate the Reverse transcriptase. cDNA may be stored indefinitely at -20°C or -80 °C.

Then Real-time quantitative PCR was performed to evaluate miR-26a expression in HepG2 cells post 3 days transfection using standard protocols on an Applied Biosystems 7500 Sequence Detection System. Briefly, 1.25ul of cDNA was added to 10 ul of the 2×SYBR green PCR master mix (TaKaRa, Dalian, China), 200 nM of each primer and water to 20ul. The reactions were amplified for 15 s at 95°C and 1 min at 60°C for 40 cycles. The thermal denaturation protocol was run at the end of the PCR to determine the number of products that were present in the reaction. Reactions are typically run in triple. The cycle number at which the reaction crossed an arbitrarily placed threshold (Ct) was determined for each gene and the relative amount of the miRNA. Target gene expression was normalized to the expression of the housekeeping gene U6 for each sample. Data were analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method [6].

Table 1 gene specific Primers used to amplify the miRNAs

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
RT-miR-26a	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT TGAGAGCCTATC	
miR-26a	ACACTCCAGCTGGGTTCAAGTAATCCAGGA	TGGTGTCGTGGAGTC
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

3. Statistical Analysis

Data were expressed as mean ± standard deviation (SD) and Statistical significance was calculated by either two-tailed unpaired Student's t test or analysis of variance (ANOVA) as appropriate. p values less than 0.05 was considered as statistically significant.

4. Results

4.1. Construction of recombinant miR-26a

Compared with the chemical synthesis, construction of the eukaryotic expression vector should be

more economic, and applied in long-term research of its biological function. Both mature siRNA and miRNA are precursor sequences cutting by RNase III enzyme DICER to form～22 nt small molecule RNA. This process is very similar between siRNA and miRNA. So siRNA expression vectors were current extensively used in expression miRNA molecules. The construction process was shown by schematic map (Fig1 a). After annealing of sense strand and antisense strand, the fragment of double strands oligo was directly cloned into pPG-eGFP-miR vector. The new expression vector of miR-26a was named pHsa-miR-26a. In the successfully constructed vector pHsa-miR-26a, restriction enzyme digestion products were submitted to agarose gel (Fig 1b). The positive clones sequenced by shanghai sangon biotech Co., Ltd. were confirmed containing target gene which demonstrated that the inserted fragment were correct.

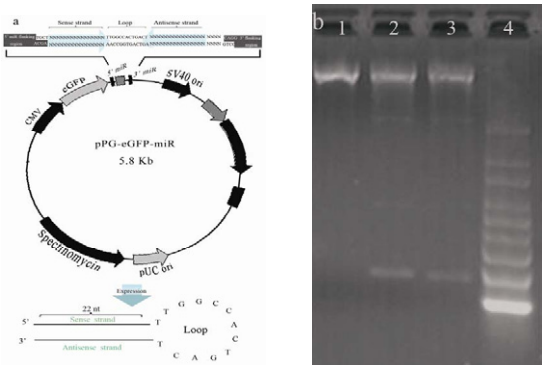

Fig 1. The construction of pHsa-m-26a vector. (a) Schematic map of pHsa-miR-26a vector construction. (b) Lane 1. Vector without restriction enzyme digestion; Lane 2, 3. pHsa-miR-26a and pHsa-miR-NC by double restriction endonuclease digestion with BamH I and Xho I respectively. The size of the small fragment was about 200 bp; Lane 4. DNA ladder

4.2. Efficiency of gene transfection

After transfection, HepG2 cells were monitored for GFP by fluorescence microscopy. Twenty four hours post- transfection of pHsa-miR-26a, fluorescence microscopy analysis showed that fluorescence intensity of GFP in pHsa-miR-26a transfected group was obviously enhanced than that of controls. After 5 days monitoring, the result suggested that the GFP expression was highest between 48 and 72 hours post-transfection (Fig. 2).

4.3. miR-26a expression in HepG2 cells

Forty eight hours after transfection. HepG2 cells were harvested and total RNA was extracted for monitoring miRNA expression. The results of realtime quantitative RT-PCR showed that the miR-26a level of cells transfected recombination plasmid vector was increased by 8.96-fold compared with untransfected cells ($p< 0.05$); in contrast, the expression level of miR-26a showed no obvious change in cells transfected empty vector ($p> 0.05$).

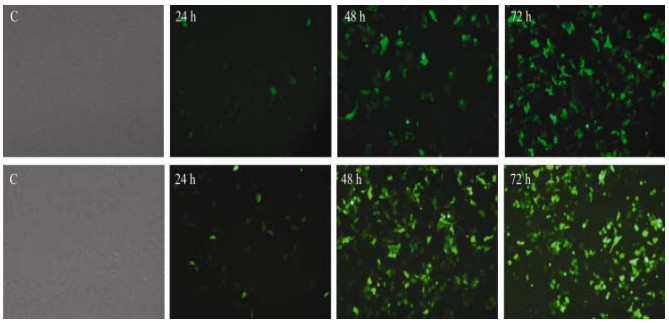

Fig 2. GFP expression in HepG2 cells. C: untransfected cells control; 24h, 48h and 72h represent different time points post-transfection respectively. Top panel: cells transfected with negative control (miR-NC vector). Bottom panel: cells transfected with pHsa-miR-26a vector.

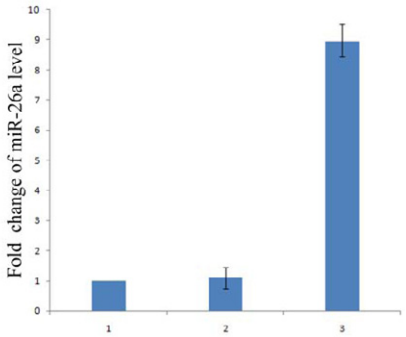

Fig 3. Relative expression change of miR-26a level. 1: untransfected group; 2: negative control group; 3: pHsa-miR-26a vector transfected group. $P< 0.05$ compared with groups 3 and 1.

5. Discussion

Since the first miRNAs was found in Caenorhabditis elegans in 1993[7]. Thousands of miRNAs have been found in all plant and animal and other eukaryotes, more and more reports indicate that miRNAs are correlated with cancer or virus infection diseases [8,9]. The liver serves as an ideal site for the study of miRNAs. MiR-26a was down-regulated in the majority of HCCs and in all examined HCC-derived cell lines. The high frequency of aberrant regulation of these miRNAs in HCC versus normal liver suggests that they might play an important role in hepatocarcinogenesis. In addition, miRNAs represent a vital component of the innate antiviral immune response [10]. Some investigators found that IFN-B down-regulates the expression of liver-specific miR26a[2]. Therefore, it indicated miRNAs attack viral infections through the interferon system. The relationship between miR-26a and liver immunity remained elusive. As a powerful tool for gene expression regulation, miRNA provide us a more in-depth understanding of the complex mechanisms.

The method of obtaining miRNAs in mammalian cells by an expression vector showed inexpensive and could be used in long-term experiments. The miRNA expression vector includes an antibiotic resistance gene that provides a mechanism to select for transfected cells that expression the introduced DNA. It also includes an enhanced green fluorescent protein expression cassette which could coexpress with miRNA. The pPG-eGFP-miR based vectors developed in the present study provide an effective mechanism for introduction of the miRNAs into cultured cells. It provides a mammalian expression

vector that directs intracellular synthesis of small RNA.

6. Conclusions

In this report, the recombinant plasmid miR-26a vector was successfully constructed by molecular cloning technology, and the results also showed that the miR-26a can be efficiently expressed in HepG2 cells via polycation-mediated gene transfer. Combining the properties of recombinant plasmid vector with the pivot role of miR-26a in the regulation of the cell cycle process, construction of eukaryotic expression vector of miR-26a may facilitate further study in HCC. This method of construction and identification may have broad application in other miRNAs research.

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