Construction of specific short hairpin RNA targeting Nucleostemin mediated by T7 RNA polymerase promoter *in vitro*

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Abstract

Objective. To explore the silencing efficacy of short hairpin RNA targeting Nucleostemin (shRNA-NS) on acute leukaemia cells. *Methods.* Two shRNAs-NS (shRNA-NS-1, shRNA-NS-2) were designed and synthesized by *in vitro* transcription system. Two candidates of shRNA-NS targeting sequences in Nucleostemin cDNA were screened out from the consensus sequences of three Nucleostemin cDNA variants registered in Genebank. The synthesized shRNAs were quantified by gel electrophoresis. The interfering effect of shRNA-Nucleostemin was determined by investigating the changes in cell morphology and in NS-mRNA expression. *Results.* shRNA-NS-1 and shRNA-NS-2 were successfully constructed without degradation and diffusion. The concentrations of two shRNA-NS were 5.24 μmol/L and 3.35 μmol/L, respectively. Forty-eight hours after transfection shRNA-NS into HL-60 cells, cell densities and aggregated degree were significant declined and cell sizes and shape were quite different compared with the control group. The Nucleostemin mRNA expressions in cells transfected with shRNA-NS were decreased significantly, which the inhibiting rates of shRNA-NS-1 and shRNA-NS-2 were 37.82% and 71.88%, respectively. *Conclusion*. shRNA-NS-2 shows a desireable NS-targeted inhibiting effect, which can be used for further investigation of Nucleostemin roles in acute leukemia cells and the possibility of shRNA-NS as a new gene targeting in acute leukemia therapy. [Life Science Journal. 2007; 4(3): 8 – 12] (ISSN: 1097 – 8135).

Keywords: T7 RNA polymerase promoter; Nucleostemin; *in vitro* construction; DNA template; short hairpin RNA

1 Introduction

RNA interference (RNAi) mediated by double-stranded RNA (dsRNA) is a post-transcriptional gene silencing (PTGS) phenomenon, which is an ancient conserved-mechanism during evolution and responsible for counteractingviral infection, endogenous gene mutation and endogenous transposon shifting[1]. For another RNAi can specifically inhibit the expression of its targeting gene in mammalian cells. Compared with RNA antisense technology, RNAi appears more exquisite specificity, efficiency and endurance of gene-specific silencing, so RNAi may be a powerful tools for targeting validation in biomedical research today, as well as a hopeful approach for cancer therapeutics, especially for those caused by overexpression of certain proteins. Nucleostemin (NS) gene was discovered in 2002, NS protein mainly exists in the cell nucleus which may take part in the regulation of cell cycle, maintenance of self-renewal and indefinite proliferation of stem cells and cancer cells[3]. We had reported that overexpression of NS is found in acute leukemia cells, and NS proteins exists more in cells of early differentiation stage than those of later stage[4]. So in present study, we designed and constructed specific NS targeting short hairpin RNAs (shRNAs-NS) catalyzed by T7 RNA polymerase and mediated by T7 RNA polymerase promoter *in vitro*, then evaluated their efficacy of NS suppression.

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2 Materials and Methods

2.1 Designing of shRNA-NS

Consensus sequences of 1833 bp exist in three complete cDNA clones of human NS in GenBank (Variants, NM014366, NM206825, NM206826). The candidates of shRNA-NS targeting sequences were screened out from the consensus sequences according to the principals of siRNA designing. All the 19 bp sequences followed 2-Adenine in the downstream of the AUG initiation codon, and two of 21 bp sequences are determined as the targeting cDNA sequences due to the blast results in GenBank using the software of Blast Research. Their sequences were as follows:

cDNA-NS-1: 5'-AAGCTGAGCTAAGGAAACAGA-3'
cDNA-NS-2: 5'-AAGCCTAGGAAAGACCCAGGA-3'

The RNA sequences were:

RNA-NS-1: 5'-UCUGUUUCCUUAGCUCAGCUU-3'
RNA-NS-2: 5'-UCCUGGGUCUUUCCUAGGCUU-3'

The designed shRNAs involved 49 bp of nucleotides, comprising 19 bp perfectly matched nucleotides bases pairing, connected by a 9 bp loop [aaguucucu] and ended in a 2-uridine 3'-overhang. The sequences of shRNA-NS-1 and shRNA-NS-2 were as follows:

shRNA-NS-1: 5'-GCUGAGCUAAGGAAACAGA ucucuugaa UCUGUUUCCUUAGCUCAGCUU-3'
shRNA-NS-2: 5'-GCCUAGGAAAGACCCAGGA aaguucucu UCCUGGGUCUUUCCUAGGCUU-3'

2.2 Constructing of DNA template for shRNA-NS

A single DNA template containing two annealed oligonucleotide strands may be used to generate a single hairpin siRNA, which involved T7 promoter sequence, RNAi target sense sequence, loop, RNAi target antisense sequence as well as 2-AA at 5'-end, another strand was its complementary strand. The sequences of T7 promoter was 23 bp, namely 5'-ggatcctaatacgactcactata-3'.

The DNA oligonucleotides of shRNA-NS-1 were: top strand for hairpin: 5'-ggatcctaatacgactcactataGCTGAGCTAAGGAAACAGAttcaagagaTCTGTTTCCTTAGCTCAGC-3'; bottom strand for hairpin: 5'-AAGCTGAGCTAAGGAAACAGAtctcttgaaTCTGTTTCCTTAGCTCAGCtatagtgagtcgtattaggatcc-3'.

The DNA oligonucleotides of shRNA-NS-2 were: top strand for hairpin: 5'-ggatcctaatacgactcactataGCCTAGGAAAGACCCAGGAttcaagagaTCCTGGGTCTTTCCTAGGC-3'; bottom strand for hairpin: 5'-AAGCCTAGGAAAGACCCAGGAtctcttgaaTCCTGGGTCTTTCCTAGGCtatagtgagtcgtattaggatcc-3'

The DNA oligonucleotides were synthesized in Shanghai Sangon Biological Engineering Technology & Services Co. Ltd, and purified by HAP methods.

2.3 Synthesizing of shRNAs-NS

2.3.1 Annealing DNA oligonucleotides. DNA oligonucleotides were resuspended in nuclease-free water to a final concentration of 100 pmol/μl, annealed to generate double DNA templates with the sense strand RNA and antisense strand RNA templates. The volume of each reaction component was 100 μl, involving both the top and bottom oligonucleotide strands for shRNA 10 μl respectively, 2 × oligo annealing buffer 50 μl, nuclease-free water 30 μl. The reaction mixtures were heated to 94 ºC for 4 minutes and cooled to room temperature, thus generated the annealed DNA templates with the final concentration 10 pmol/μl.

2.3.2 Synthesizing of shRNA-NS catalyzed by T7 RNA polymerase. Each 20 μl transcription reaction consisted of annealed DNA template 2 μl, T7 express enzyme, RNase inhibitor, NTP and inorganic pyrophosphatase mix. Incubated for 30 minutes at 37 ºC, added 1 μl RQ1 RNase-free DNase and incubated for 30 minutes at 37 ºC in order to remove the DNA templates, after that heated 10 minutes at 70 ºC and cooled to room temperature, thus shRNAs-NS were obtained.

2.3.3 Purifying shRNA-NS. Added 2 μl of 3 M sodium acetate (pH 5.2) and 20 μl of isopropanol to each 20 μl transcription reaction. Mixed and placed on ice for 5 minutes. Spinned at 12000 g, 4 ºC for 10 minutes, and the pellet was washed with 0.5 ml of cold 70% ethanol twice. Air-dried the pellet and resuspended the RNA sample in nuclease-free water, then stored at – 70 ºC.

2.4 Electrophoresis of shRNA-NS products

1 μl of shRNA-NS products were fractioned in 25 g/L of agarose gel electrophoresis while 1 μl and 0.5 μl of the annealed DNA template were taken as control. After 20 minutes at 80 V the electrophoresis bands were investigated, and scores of gray scale was evaluated by the software of Gene Genius. The concentrations of shRNA-NS were calculated according to the scores of gray scale, concentration of DNA template, the molecule weights of shRNA-NS and DNA template.

2.5 Evaluation of the suppression efficacy of shRNA-NS

The human acute myelocytic leukemia cell line HL-60 was cultured in RPMI1640 supplemented with 100 ml/L fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37 ºC with 5% CO_2. Cells were harvested 24 hours later, adjusted to the

density of 4 × 10^5 /μl with whole medium, aliquoted into 24-well plates for 500 μl per well. In order to evaluate the suppression efficacy of shRNA-NS, cells were grouped to the transfected groups (R1 and R2) and the control groups (C1 and C2). Preparing for the transfection, 2 μl CodeBreaker siRNA transfection agent was added into 125 μl serum free medium; twenty minutes later at room temperature, the R1 group, the R2 group and the C1 group were added into shRNA-NS-1, shRNA-NS-2, and non-related control shRNA with final shRNA concentration 10 nmol/L respectively. C2 group was added transfection agent only. Forty-eight hours after transfection, cell growth morphometry were observed under inverted microscope, the total RNA was extracted using TRIzol. RT-PCR was used to amplify NS gene fragments with the expected sizes of 418 bp. The forward primer was 5'-AAAGCCATTCGGGTTGGAGT-3' and the reverse primer was 5'-ACCACAGCAGTTTGGCAGCAC-3'. β-actin gene with the expected size of 315 bp was taken as internal control, with forward primer 5'-TCCTGTGGCATCCACGAAACT-3' and the reverse primer: 5'-GAAGCATTTGCGGTGGACGAT-3'.

3 Results

3.1 Electrophoretic result of constructed shRNA-NS

The products synthesized by T7 promote polymerase were separated by electrophoresis. Results indicated that the quality of shRNA-NS was satisfying, with only one bands in lane 1 and lane 2 respectively, illstrating no degradation, no diffusion, and the complete removal of DNA templates were removed completely. The molecule sizes of shRNA-NS were 49 bp (Figure 1).

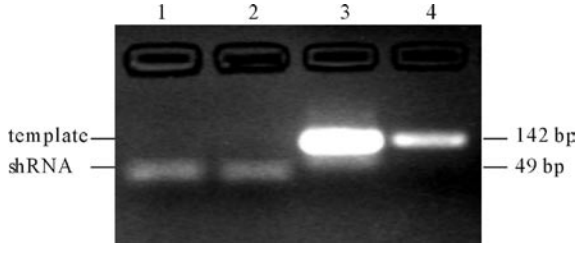

Figure 1. Electrophoretic result of constructed shRNA-NS and DNA template. Lane 1: shRNA-NS-1; Lane 2: shRNA-NS-2; Lane 3: 10 μmol/L of annealed DNA template; Lane 4: 5 μmol/L of annealed DNA template.

3.2 Determination of shRNA concentrations

The scores of scanning gray scale in lane 1, lane 2, lane 3, and lane 4 were 22562, 14663, 118479, 63351, respectively. The related-scores of shRNA-NS-1, shRNA-NS-2 were 0.36, 0.23. Molecular weights of shRNA-NS-1, shRNA-NS-2 and annealed DNA template were 15028, 15030 and 43785 respectively, and shRNA concentrations were calculated as followed:

The concentration of shRNA-NS-1: 43785 × 5 × 0.36 ÷ 15028 = 5.24 μmol/L

The concentration of shRNA-NS-2: 43785 × 5 × 0.23 ÷ 15030 = 3.35 μmol/L

3.3 Effects of shRNA-NS in cell growth morphology

Cells in control group were suspended and aggregated. The cells' sizes were even and the shapes were smooth and glossy. But compared with those in control group, cell densities and aggregative degrees were decreased and the sizes of cells were obviously different in the shRNA-NS group at the 48th hour after transfection, especially the cell shapes in shRNA-NS-2 group changed to fusiform even pseudopod (Figure 2).

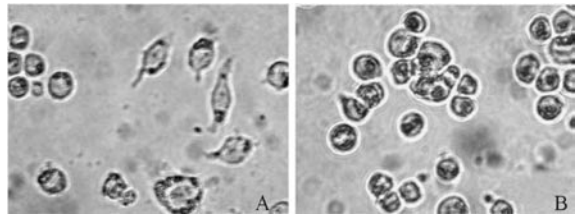

Figure 2. Cell morphology of HL-60 cells treated with shRNA-NS-2 for 48 hours. A: shRNA-NS-2 group; B: C1 group.

3.4 Result of RT-PCR

Cells for RT-PCR were harvested when incubated with shRNA for forty-eight hours. The related contents of NS mRNA to GAPDH mRNA in the C1 group, the C2 group, the R1 group and the R2 group were 0.826, 0.809, 0.503, 0.207 respectively. Compared with the control groups, the expression of NS mRNA was significantly downregulated by shRNA-NS-1, shRNA-NS-2, which the inhibiting rates were 37.82 %, 71.88 %, and the more effective one was shRNA-NS-2 (Figure 3).

4 Discussion

RNAi is a process of sequence-specific post-transcriptional gene silencing mediated by small double-stranded RNA (dsDNA). The RNAi is to cleave dsRNA into 21 – 25 bp of small interfering RNA (siRNA) catalyzed by the ribonuclease III. The siRNA can recognize and degrade the targeting mRNA with homologous sequences. The reaction mediated by siRNA holds the typical feature of biocatalysis so siRNA appears very high efficient[5, 6].

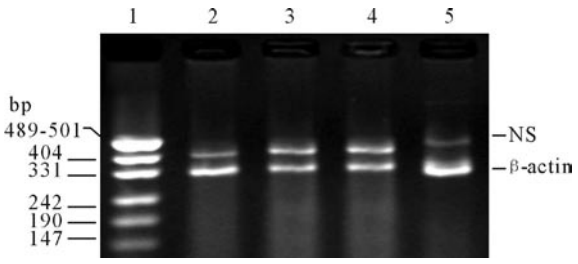

Figure 3. Effects of shRNA-NS on NS mRNA expression in HL-60 cells at the 48th hour after transfection. Lane 1: DNA marker; Lane 2: R1 group; Lane 3: C1 group; Lane 4: C2 group; Lane 5: R2 group.

RNAi, known as a gene-knockout technique with exquisite specificity, high efficiency and endurance, is easily operated and time-saving. The biological phenomena induced by specific gene silencing could be observed soon compared with other gene targeting techniques. RNAi also has the adventage of higher efficacy and less reagents, compared with the antisense nucleotide technique.

The products of NS gene are p53-binding protein with molecule weigh 61 KD. Our previous studies discovered that continuous overexpression of NS protein existed in acute leukemia cells. NS protein is a specific regulatory factor responsible for stem cells or cancer cells acrossing G2/M checkout point, so NS may play a role in cell proliferation, differentiation[1]. It has been reported that downregulation of NS gene could induce cell out of normal cell cycle and result in differentiation[7, 8]. In present research, we designed and constructed specific shRNAs-NS in order to provide a useful tool for exploring NS roles in cell survival, differentiation, apoptosis of leukemia cells and the possibility of NS targeting RNAi as a new approach to anti-leukocytosis. Presently three variants of NS cDNA have been registered in GeneBank which their common sequences are 1833 bp. In order to make the designed NS-shRNA versatile, we took the common sequence as targeting sequences to design NS-shRNA. Results indicated two of the designed shRNA-NS could effectively downregulate the expression of NS mRNA, and the more effective one was shRNA-NS-2 with inhibiting rate 71.88%. For another the suppression of NS gene could result in changes in cellular morphology, some of HL-60 cells treated by shRNA-NS-2 were changed from round to fusiform even pseudopod, indicated new differentiation maybe induced by NS suppression[9, 10].

The full length of our designed shRNAs was 49 bp, comprising 19 bp perfectly matched base pairs which connected by a 9 bp loop, ended in a 2-uridine 3'-overhang[11]. The hairpin shape of shRNA was caused through natural annealing. Through comparing the interfering efficacy of 5 bp, 7 bp and 9 bp of spaced loops in shRNA, Brummelkamp found that shRNA connected by 9 bp loop was most effective for interfering gene[12]. shRNAs are rapidly cleaved into siRNA in cells by Dicer enzyme, which represent RNA duplexes of specific length and structure that finally guide sequence-specific degradation of mRNAs homologous in sequence to the siRNAs[13]. With only one 3'-ending and one 5'-ending in shRNA strand, shRNAs are more stable than siRNA because the opportunities of degradation are decreased in theory.

T7 RNA polymerase is a DNA dependent RNA polymerase. According to T7 RiboMAX™ Express RNAi System, T7 promoter sequences are involved in the double DNA template strands to initiate transcription, and T7 RNA polymerase is necessary to specifically recognize T7 promoter. The shortest sequence of T7 promoter is 5'-TAATACGACTCACTATA-3', but adding 5'-GGATCC-3' to the upstream of promoter could enhance the binding efficacy of T7 polymerase to the promoter, and to yield more RNA products. Besides, the first base following promoter at 5'-end in the DNA template should be designed as nucleotide G because T7 RNA polymerse always takes G as start site of transcription[14]. With accordance to principals listed above, we synthesized shRNA-NS-1, shRNA-NS-2 which their concentrations were 5.24 μmol/L, 3.35 μmol/L, respectively.

Absorbance at 260 nm with UV spectrophotometer doesn't adapt to determine the concentrations of shRNAs synthesized by transcription *in vitro*. Free nucleotides would be generated in the process of removing DNA templates. So we took annealing DNA template with definite concentration as control, calculated the concentrations of NS-shRNAs depending on gray scales, molecule weights of shRNA and annealed DNA template. This method is proven to be easy and rapid.

The shRNAs synthesis mediated by T7 promoter *in vitro* transcription is according to the principles of transcription *in vivo*, so the product is close to natural shRNA, not easy to degrade. It has been reported that the interfering efficacy of shRNA is better than siRNA. The transcriptional synthesis *in vitro* is of lower cost, higher quality, lower toxicity and higher stability compared with chemical synthesis. Only one tenth of shRNAs generated by chemosynthesis could obtain the same interfering efficacy. Furthermore, transfecting shRNA into cells by transcription synthesis is easier than transfecting expressing vectors into cells, and additionally, the quantity of transfecting shRNA is controllable. It's like the therapy pattern *in vivo*.

5 Conclusion

The product of shRNA-NS shows a NS-targeted inhibiting effect, which can be used for further research of NS roles in acute leukemia cells and the possibility of shRNA-NS as a new gene targeting in cancer therapy.

References

1. Sijen T, Fleenor J, Simmer F, *et al*. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 2001; 107: 465 – 76.
2. Gupta S, Schoer RA, Egan JE, *et al*. Inducible, reversible, and stable RNA interference in mammalian cells. Proc Natl Acad Sci USA 2004; 101(7): 1927 – 32.
3. Tsai RYL, Mckay RD. A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Dev 2002; 16(23): 2991 – 3003.
4. Yue BH, Sui L, zhao XQ, *et al*. Expression of nucleostemin gene in human acute leukemic cells. Life Science Journal 2006; 3(2): 12 – 6.
5. Marjori M, Antonius M, Jan K. RNA: Guiding gene silencing. Science 2001; 293: 1080 – 1.
6. Richard W, Lin S. Double-stranded RNA poses puzzle. Nature 2001: 391: 744 – 5.
7. Normile D. Cell proliferation: Common control for cancer, stem cells. Science 2002: 298(5600): 1869 – 70.
8. Bernnardi R, Pandolefi PP. The nucleolus: at the stem of immortality. Nat Med 2003; 9(1): 24 – 5.
9. Huang WG, Su Q, Huang C, *et al*. Construction of differentially expressed cDNA library in human leukemia HL-60 cells induced by diallyl disfuld with suppression subtractive hybridization. Chinese Pharmacol Bulletin 2004; 20(4): 445 – 8.
10. Wang ZH, Lin J. Effects of berberine on the proliferation and differentiation of HL 60 cells. Chinese Pharmacol Bulletin 2004; 20(11): 1305 – 8
11. Donze O, Picard D. RNA interference in mammalian cells using siRNAs synthesized with T7 RNApolymerase. Nuc Acids Res 2002; 30(10): e46 – 9.
12. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002; 296: 550 – 3.
13. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 2001; 15(2): 188 – 200.
14. Milligan JF, Uhlenbeck OC. Synthesis of small RNAs using T7 RNA polymerase. Methods Enzymol 1989; 180: 51 – 62.