

Effects of knocking-down Nucleostemin gene on apoptosis of HL-60 cells *in vitro*

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Abstract: Objective. To explore whether the apoptosis of leukemia cells could be induced by knocking-down Nucleostemin (NS) gene *in vitro*. **Methods.** HL-60 cells were taken as a model, and was directly transfected with Nucleostemin specific short hairpin RNA (NS-shRNA). Sequences that unrelated with NS gene were taken as a control. The blocking effect of NS-shRNA was detected by Reverse Ttranscription PCR (RT-PCR), the morphology changes in culture state were observed under inverted microscope, and the changes of cell shape and nucleus were detected by Wright-Giemsa staining. The amount of apoptotic cells were assayed by flow cytometer (FCM) and Tunel technique, and the positive rate of apoptosis was determined in the meanwhile. **Results.** Two NS-shRNA were synthesized *in vitro*, and the more effective one was selected to be transfected into HL-60 cells. The blocking rate of NS-mRNA reached up to 74.94%. 48 hours after transfection, nuclear fragmentations and “apoptosis bodies” showed in HL-60 cells, observed by Wright-Giemsa staining. The apoptosis rates in transfected groups were (25.32±3.06)% and (27.3±3.21)% respectively, but were only (3.12±0.38)% and (3.30±1.52)% in control group, detected by FCM and Tunel technique. The difference between the treated group and the control group was significant ($P<0.01$). **Conclusion.** The apoptosis of HL-60 leukemia cells can be induced by the silencing of NS gene *in vitro*, which means NS could be a candidate gene for the theoretical therapy principle of leukemia.

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1. Introduction

Nucleostemin(NS) was originally identified by Tsai and McKay in 2002, and abundantly expressed in both embryonic and adult CNS stem cells in culture. It is also present in embryonic stem cells, primitive cells in bone marrow, and cancer cells. Later, it is found that NS also exists in some human solid tumor cells, but not in the differentiated cells. NS protein weights 61kD, it contains a highly basic region in the N terminus, two GTP-binding motifs, a coiled-coil domain and an acidic domain in the carboxyl end. NS locates in the nucleolus, and its expression is abruptly down-regulated during differentiation prior to terminal cell division^[1,2]. NS plays a significant role in the self-renewal and infinite proliferation state of stem cells and malignant tumor cells, also, it probably participate in the G2/M transition as a key regulation factor^[1,8,9]. Previous studies of our research group had certified the high expression of NS gene and NS protein in leukemia cells^[3]. RNA interference(RNAi) technique^[4,5] is used in our present study. In order to

inhibit the expression of NS gene, we synthesized Nucleostemin specific short hairpin RNA (NS-shRNA) *in vitro*, and directly transfected NS-shRNA into HL-60 cells, then we observed the changes of the apoptosis induced in HL-60 cells, and approached the apoptotic state after targetingly knocked down NS gene in leukemia cells. This research will be helpful for us to understand further the function of NS, and also support a new strategy to treat leukemia by using RNAi technique.

2. Materials and Methods

2.1 Cell culturing

The leukemia cell line of HL-60 was kindly provided by Shanghai Institutes for Cell, Chinese Academy of Medical Science, and was conserved by the Department of Histology and Embryology of Zhengzhou University. Cells were cultured in RPMI1640 medium, supplemented with 10% fetal bovine serum (containing 100 U/ml penicillin and 100µg/ml streptomycin) at 37 °C with 5% CO₂. The culture medium was changed on alternate days.

2.2 Designing and synthesizing of NS-shRNA

Three variants (variant, NM014366, NM206825, NM206826) of complete cDNA in human were retrieved from NCBI GenBank Web site, they have a common sequence of 1833bp. Other homologous coding sequences and expressed sequence tag (EST) homologous sequences were discarded according to the principals of siRNA designing. Finally, two of 21 bp sequences were determined as targeting cDNA sequences (below):

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

cDNA-NS-2: 5' -AAGCTAGGAAAGACCCAGGA-3'

The corresponding RNA sequences are shown as follows:

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

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

The shRNA contains 19 fully paired bases. These bases constitutes the sense strand and the antisense strand of interfering RNA, and the two strands are connected by a 9 bp loop [aaguucucu] and ended in a 2-uridine 3'-overhang. The total length of the designed NS-shRNA was 49 bp. The sequences of the two NS-shRNA are listed as follows:

NS-shRNA- 1:

5'-GCUGAGCUAAGGAAACAGA ucucuugaa

UCUGUUUCCUUAGCUCAGCUU-3'

NS-shRNA- 2:

5'-GCCUAGGAAAGACCCAGGA aaguucucu

UCCUGGGUCUUUCCUAGGCUU-3'

To synthesize NS-shRNA, first of all, the complementary single DNA templates for NS-shRNA, which contained T7 promoter sequence, were constructed *in vitro*, and then they were annealed to generate double DNA template. 1 μ l of NS-shRNA products were fractioned in 25g/L of agarose gel electrophoresis while 1 μ l and 0.5 μ l of the annealed DNA template were taken as the control, so as to detect the synthesizing effect of NS-shRNA.

2.3 Transfection of NS-shRNA into HL-60 leukemia cells

Logarithmic growth phase cells were adjusted to the density of $4 \times 10^5/\mu$ l with whole medium, then aliquoted into 6-well plates for 2.5 ml per well. Cells were randomly grouped into the transfected groups (R₁ group were treated by NS-shRNA-1, and R₂ group were treated by NS-shRNA-2) and the control groups (C₁ were only treated by transfection reagent as blank control, and C₂ were treated by unrelated siRNA sequences as negative control). Preparing for the transfection, 7.5 μ l Code Breaker siRNA transfection agent (Promega Corporation, Madison in Wisconsin, USA. <http://www.promega.com>) was added into 625 μ l non-serum medium, the transfected group was added NS-shRNA to the final concentration of 10 nmol/, according to preliminary experiment, while the control

group was added non-related shRNA.

2.4 Blocking effect on NS-mRNA in HL-60 cells after transfection

48 hours after transfection, total RNA was extracted from each group for Reverse Transcription PCR(RT-PCR), and then the image analysis was carried out after electrophoresis, the NS-shRNA blocking effect was calculated by the decrease of NS-mRNA expression. The forward primer of NS gene was 5'-AAAGCCATTCGGGTTGGAGT-3', and the reverse primer was 5'-ACCACAGCAGTTTGGCAGCAC-3', with the expected size of 418 bp. β -actin gene was taken as internal control with the expected size of 315 bp, its forward primer was 5'-TCCTGTGGCATCCACGAAACT-3', and the reverse primer was 5'-GAAGCATTGCGGTGGACGAT-3'.

2.5 Observation of apoptosis in culture state by inverted microscope

After routinely cultured for 24 hours, cell morphology was observed under the inverted microscope in culture state both in the transfected groups and control groups.

2.6 Detection of apoptosis by Wright-Giemsa staining

Cells were harvested 48 hours after transfection, then centrifuged to condense and smeared. The morphology and karyomorphism of HL-60 cells were observed by Wright-Giemsa staining and micrography.

2.7 Observation of apoptosis by flow cytometry (FCM)

After routinely cultured for 24 hours, cells both in transfected groups and control groups were harvested and centrifuged, then treated according to the operating instruction of Annexin V-FITC Kit, and washed twice by PBS which precooled to 4°C, then 250 μ l 1:4 diluted binding buffer was added to reconstitute the cells, and the cells were adjusted to the density of $1 \times 10^6/\text{ml}$. 100 μ l suspension was added into the 5 ml flow tube, then 5 μ l Annexin V/FITC and 10 μ l 20 μ g/L propidium iodide (PI) were added and mixed, the solution was incubated in dark area for 15 minutes, 400 μ l PBS was added into the reaction tube, then detected by flow cytometry (FCM) and analyzed by Cell quest 1.2 software.

2.8 Detection of apoptosis by dUTP nick end labeling (Tunel) technology

48 hours after transfection, cells in each group were harvested and smeared on the anti-run slides that enveloped by APES, and washed three times with PBS,

then incubated with endogenous peroxidase blocking solution (0.3% H₂O₂ methanol solution) for 30 minutes, and again washed 3 times with PBS, then incubated with penetration solution in ice bath for 2 minutes, and then treated according to the instruction manual of Tuel Kit.

2.9 Statistical analysis

All experimental data were processed by SPSS 13.0 software. The experimental variables were determined using ANOVA or *t*-test. Data was reported as $\bar{x} \pm SD$, statistical significance was set *P* less than 0.05.

3. Results

3.1 NS-mRNA and NS protein changes after treated 48 hours with NS-shRNA

3.1.1 NS-mRNA change

Cells were harvested for RT-PCR after incubated with NS-shRNA for 48 hours. According to scanning gray scale analysis, the related score were 0.826, 0.809 in C1 group and C2 group, and 0.503, 0.207 in R1 (NS-shRNA-1) and R2 (NS-shRNA-2) group. Compared with the control groups, the expression of NS-mRNA was significantly down-regulated after transfected with NS-shRNA-1 and NS-shRNA-2, with the inhibiting rates of 39.10% and 74.94%, and the more effective one was NS-shRNA-2, so it was selected for further experiments (Figure 1).

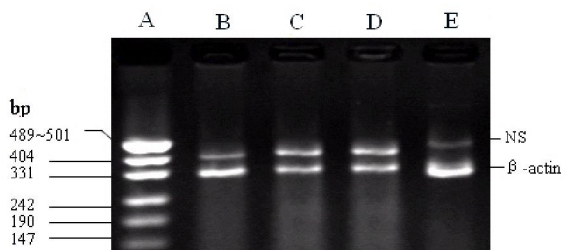


Figure 1. RT-PCR result of human leukemia HL-60 cells treated 48 hours with NS-shRNA (A, DNA Mark; B, R1, treated by NS-shRNA-1; C, C1, blank control; D, C2, negative control; E, R2, treated by NS-shRNA-2).

3.1.2 NS protein change

Cells were harvested for Western blotting after transfected for 48 hours, the expression of NS protein was significantly lower than the blank and negative control group. The figure of Western blotting has been shown as below (figure 2). Then the gel was analyzed by gray-scale scanning. The gray-scale result of the negative control group was supposed 1.000, then the result of the other two groups were detected respectively, and the results were shown in the table below (table 1). Finally, the inhibition rate was calculated by the formula: $(1.000 - 0.479) \times 100\% = 52.1\%$

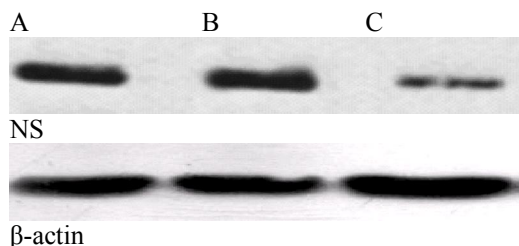


Figure 2. Detecting of NS protein by Western blotting in HL-60 cells transfected for 48 hours (A, blank control; B, negative control; C, transfected group).

Table 1. Inhibition rate of NS protein in HL-60 cells transfected for 48 hours

Group	Gray-scale result	Inhibition rate of NS protein (%)
Blank control	1.053	\
Negative control	1.000	0
Transfected group	0.479	52.1

3.2 Morphology changes observed in culture

Some cells in transfected group were disintegrated or broken 24 hours after incubated with NS-shRNA-2, vesiculose projections could also be seen, the nuclei disappeared, and the fragments spread around radially (Figure 3).

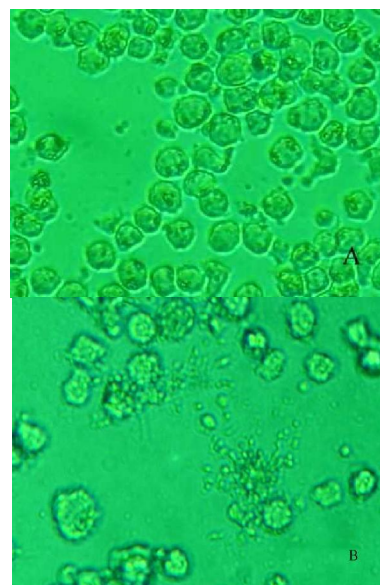


Figure 3. The cataplasma bodies of HL-60 cells dispersed radially after transfected with NS-shRNA-2 for 24 hours (1 000×) (A, HL-60 cells in control group; B, HL-60 cells transfected for 72 hours).

3.3 Morphology changes detected by Wright-Giemsa staining

24 hours after transfection, nuclear fragmentations and "apoptosis" appeared in a few cells, and the

amount of nuclear fragmentations were larger. By the 48th hour, there were more apoptotic cells and more “apoptosis”, and the amount was larger with time going on. The apoptotic cells showed some features as follows: cells shrunk and became smaller, the nuclei were broken, cell debris with nuclear fragmentations that varying in shape and size could be seen easily, apoptosis appeared (Figure 4).

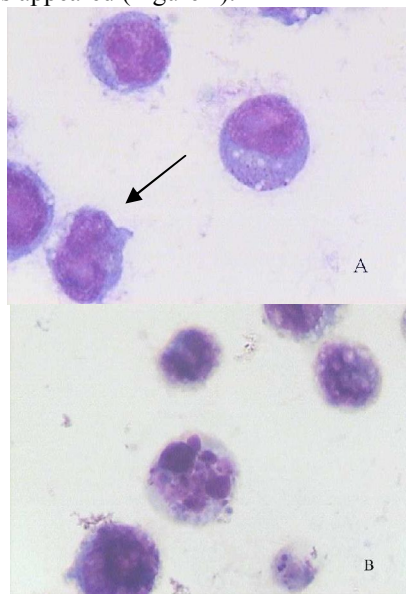


Figure 4. The apoptosis characters in HL-60 cells after transfected with NS-shRNA-2 (1000×, Giemsa staining, the arrows indicate the apoptosis characters) (A, HL-60 cells in the control group; B, HL-60 cells transfected for 72 hours).

3.4 Detection of apoptosis by flow cytometry (FCM)

Cells were collected for FCM after transfected with NS-shRNA-2 for 24 hours, and FCM Annexin V FITC was used to detect the apoptosis rate (reported as $\bar{x} \pm SD$ according to three repeated researches) of HL-60 cells. The apoptosis of HL-60 cells was induced by down-regulation of NS protein, which was caused by the transfection of NS-shRNA, and the apoptosis rate increased from (3.12±0.38)% to (25.32±3.06)%, $P < 0.05$, the difference was statistically significant between the two groups (Figure 5, Table 2).

3.5 Detection of apoptosis by TUNEL technology

Cells both in the transfected group and the control group were harvested and detected by TUNEL technology 48 hours later, and the positive cell number and positive rate were both calculated. The apoptosis inducing effect of NS-shRNA-2 was significant, the difference was statistically significant between the two groups (Figure 6, Table 3).

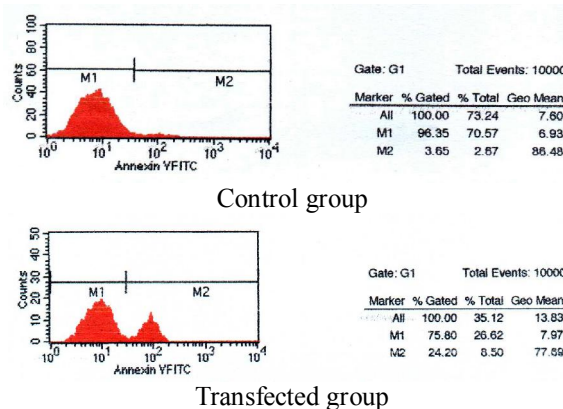


Figure 5. The apoptosis rate of HL-60 cells transfected with NS-shRNA-2 for 24 hours (%).

Table 2. The apoptosis rate of HL-60 cells transfected with NS-shRNA-2 for 24 hours (%)

Group	Apoptotic cell numbers	Non-apoptotic cell numbers	Apoptosis ($\bar{x} \pm SD$) %
Control group	312	9688	(3.12±0.38)
Transfected group	2532	7468	(25.32±3.06) *

* $P < 0.01$, compared with control group, examined by four table χ^2 test.

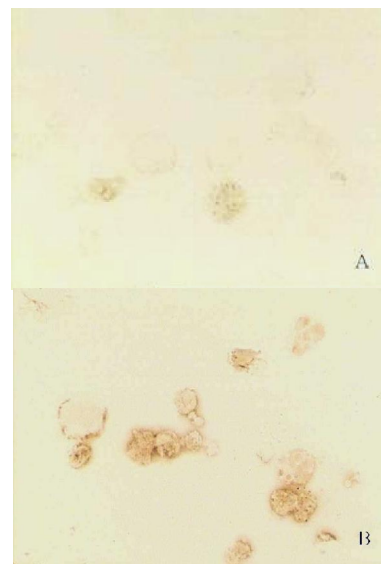


Figure 6. HL-60 cells apoptosis induced by NS-shRNA-2 with TUNEL method (400×). A: Control group; B: Transfected group.

Table 3. Detection results of cell apoptosis induced by NS-shRNA-2 with TUNEL method

Group	Total cell numbers	Positive cell numbers	Apoptosis rate (%)
Control group	300	10	(3.30±1.52)
Transfected group	300	82	(27.30±3.21) *

* $P < 0.01$, compared with control group, examined by t test.

4. Discussion

The products of Nucleostemin gene are p53-binding protein with molecule weight of 61 KD. NS protein is probably a specific regulatory factor responsible for stem cells and cancer cells acrossing G₂/M checkpoint, so NS may play an important role in cell proliferation and differentiation, and keep cells in non-differentiated state^[1,8,9]. Our previous studies had discovered that continuous overexpression of NS gene and NS protein existed in leukemia HL-60 cells, which has close relationships with the occurrence and development of acute leukemia^[3-7]. RNAi (RNA interference) is a process of post-transcriptional gene silencing mediated by small double-stranded RNA (dsRNA), which can inhibit the expression of target gene with its specificity, efficiency and rapidity^[10-12]. We designed and constructed two NS specific shRNA, and successfully transfected them into HL-60 cells, respectively. The RT-PCR showed that NS gene was proved to be down-regulated and NS-shRNS-2 was more effective. Probably this is because the "position effect" which means different NS-shRNA targets different site of NS gene. But this effect is still unclear up to now. Some of the cells treated with NS-shRNA shrunk or broke into pieces, the nuclei were disappeared, karyorrhexis and "apoptosis bodies" appeared, and the amount of "apoptosis bodies" was larger as time went on, observed in culture or by Wright-Giemsa staining. Apoptosis rate increased according to FCM and Tunel methods, showing the existence of apoptosis, which suggested that cell apoptosis could be induced by the blocking of NS gene and NS protein in HL-60 leukemia cells. Tsai and Liu had experimented on solid tumor cells, and discovered that while inhibiting the expression of NS gene and NS protein, the reproductive activity would be weaken in the meanwhile, and part of the cells quit their cell cycles^[1,2], and in our previous studies, we treated HL-60 cells with NS-shRNA, and the results were similar, so in such a case, if the weaken of cell reproductive activity is related with the increase of apoptosis, is a question deserves deep consideration. Inducing apoptosis in tumor cells is one of the strategy to treat malignant tumor^[13-14]. A number of studies have proved that high expression of NS gene is a common phenomenon in malignant tumor, if the apoptosis of tumor cells could be induced to weak cell reproductive activity by blocking NS gene, NS can be established as a candidate gene for the theoretical therapy principle of malignant tumor. But the mechanism of apoptosis caused by NS, and the signal transduction pathway in the process are unclear, and need to be discussed by further experiment.

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