# Expression of Nucleostemin Gene in Human Acute Leukemic Cells

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**Abstract: Objective.** To investigate the expression of nucleostemin gene in acute leukemic cells and its link to the pathogenesis of acute leukemia. **Methods.** Specific primers were designed according to the consensus sequence of three NS gene variants. Reverse transcriptase PCR was used to detect the expression level of NS gene in two leukemic cell lines (K562 and HL60), three types (acute myeloblastic leukemia (AML), acute monocytic leukemia ($AM_0L$) and acute lymphoblastic leukemia (ALL)) of primary leukemic cells and bone marrow mononuclear cells (BMMNC) from healthy individuals or patients with benign anemia. β-actin was used as the reference gene. **Results.** NS gene expression were easily detected in K562, HL60, and three groups of leukemic cells, the PCR product intensity ratio of NS gene to β-actin gene in K562, HL60, M1 + M2a, M3, M5a, M5b, and ALL was $0.735 \pm 0.26$, $0.449 \pm 0.19$, $0.687 \pm 0.21$, $0.408 \pm 0.16$, $0.866 \pm 0.27$, $0.448 \pm 0.19$, $0.403 \pm 0.19$, respectively, but the expression of this gene in the BMMNC of healthy individuals or patients with benign anemia were either not detected or too low to be calculated. In myeloid leukemia, the NS gene expression in leukemic cells at early stage of differentiation was significantly higher than the NS gene expression in leukemic cells at later stage of differentiation (K562 > HL60, M1 + M2a > M3, M5a > M5b, $P < 0.01$). **Conclusions.** NS gene is over-expressed in acute leukemic cells, and the expression level in leukemic cells at different differentiation stage is different. The results suggested (1) NS gene expression closely related to the origination and development of leukemia; (2) cancer cells and the stem cells had similarity in some aspects; and (3) NS gene might be a potential target in the treatment of leukemia. [Life Science Journal. 2006;3(2):12-16] (ISSN: 1097-8135).

**Keywords:** nucleostemin; leukemia; stem cell; proliferation; differentiation; cell cycle

**Abbreviations:** ALL: acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; $AM_0L$: acute monocytic leukemia; APL: acute promyelocytic leukemia; ATRA: all-trans retinoic acid; BMMNC: bone marrow mononuclear cells; CML-BC: chronic myelogenous leukemia-blast crisis; HSC: haematopoietic stem cell; IDA: iron-deficiency anemia; MA: megaloblastic anemia; NS: nucleostemin

## 1 Introduction

Nucleostemin gene first cloned by McKay and Tsai in 2002 was found highly expressed in rat embryo stem cells, rat central nervous system stem cells and rat primitive bone marrow cells. NS gene is apparently involved in regulating the proliferating of both stem cells and at least some types of cancer cell[1]. The protein encoded by NS was abundantly expressed while the cells were proliferating in an early multipotential state, but it abruptly and almost entirely disappeared at the start of differentiation. The fact that NS expressed in stem cells and several cancer cell lines, but not in the differentiated cells of adult tissues, suggested its role in maintaining self-renewal of stem cells and cancer cells[1-3]. Leukemia belongs to malignant clonal disease of haematopoietic stem cell (HSC). Leukemia is characterized by the appearance of increased numbers of immature and dedifferentiation leukemic cells in the marrow and blood. NS is involved in the regulatory pathways, but the fundamentals about NS are still unknown. In the present study, we examined the expression of NS gene in leukemia cell line and patients with acute leukemia

by RT-PCR, which would help illuminate the association of NS gene and leukemia.

## 2 Materials and Methods

### 2.1 Cell lines

Two leukemia cell lines, K562 and HL60, were kindly provided by Department of Microbiology and Immunology, School of Medicine, Zhengzhou University and Chinese Academy of Medical Science, respectively. The two cell lines were cultured in RPMI1640 medium (GIBCO-BRI) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃ in 5% $CO_2$ and passaged every 2 – 3 days.

### 2.2 Subjects

The marrow samples of 39 patients (23 female and 16 male; age 15 – 51 years old, median age 33 years old) with acute leukemia and 11 control subjects were obtained from the First Affiliated Hospital of Zhengzhou University. Among them, patients with M1, M2a, M3, M5a, M5b of AML and ALL were 3, 10, 8, 5, 6 and 7 cases, respectively. Eleven control cases included megaloblastic anemia (MA), iron-deficiency anemia (IDA), the secondary anemia and healthy subjects. The diagnosis standards for acute leukemia were based on the WHO classification of malignant haematological diseases[4].

The bone marrow sample was collected from posterior superior iliac crest and mixed thoroughly with EDTA-K2. The cell smear was determined by Wright-Giemsa staining. The blood-EDTA-K2 solution layered over the Ficoll and centrifuged at 2,000 rpm for 20 min. Cells at the interface between the plasma and Ficoll layer were harvested and washed twice with PBS. The mononuclear cells were collected for RNA extraction.

### 2.3 Reagents

Trizol reagent was purchased from Invitrogen (Carlsbad, California, USA). Reverse transcription kit, PCR amplification kit, primers synthesis and sequencing were obtained from Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, China). DNA markers were purchased from MBI (Lansing, Michigan, USA).

### 2.4 RNA preparation and RT-PCR

Total RNA was extracted from K562, HL60 cell lines and cells were separated with Trizol Reagent according to the manufacture protocol and the separated cells were treated with the DNA-free kit to remove residual genomic DNA. The concentration and integrity of the isolated RNA were determined by UV spectrophotometer (0.2 – 0.9 μg/μl) and gel electrophoresis respectively.

A BLAST search in the GenBank database identified the consensus motif (1,833 bp) of three variants (NM014366, NM206825, NM206826) from NS gene. According to the principle of primer designing, we determined specific primers for NS gene: Up-stream primer 5′-AAAGCCATTCGGGTTGGAGT-3 and Down-stream primer 5′-ACCACAGCAGTTTGGCAGCAC-3′. The amplified fragment was 418 bp. β-actin was used as an internal control, and its full lenghth was 315 bp.

Firstly, isolated RNA (5 μg) was reverse transcribed to cDNA in the 20 μl reaction mixture. Secondly, 5 μl cDNA mixture was used for PCR amplification mixture containing 0.2 μM specific primers, 0.04 μM internal control primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 μl 10× buffer and 1 U Taq DNA polymerase in 25 μl reaction solution. The program was accomplished by 5 min at 95 ℃ for initial denaturing, followed by 30 cycles of 95 ℃ for 30 sec, annealing for 30 sec at 56 ℃ and 72 ℃ for 50 sec and a final extension for 5 min at 72 ℃. The amplified product was identified by gel electrophoresis on 1.7% agrose gel. Furthermore, the sequence of NS gene was exactly identical with the access number of GenBank (NM014366, NM206825, NM206826).

### 2.5 Determine density score of amplified product

The amplified bands were scanned by gel analysis system. Ratio = density score of positive band/density score of β-actin. The higher the ratio, the higher the expression level of NS gene.

### 2.6 Statistical analysis

Statistical analysis was performed with SPSS10.0 software. Independent-sample T test was for comparison. $P < 0.05$ was considered statistically significant.

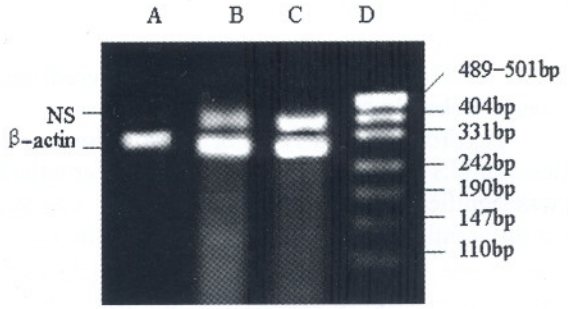


**Figure 1.** Detection of NS expression in HL60 and K562 cell lines
A: health control; B: HL60; C: K562; D: DNA marker

## 3 Results

### 3.1 NS expression in HL60 and K562 cell lines

The expression of NS mRNA in the two cell lines were analyzed by RT-PCR. As shown in Figure 1, there were high levels of NS gene in the two cell lines. Moreover, the expression level of K562 was higher than that of HL60 ($0.735 \pm 0.22$ vs $0.449 \pm 0.15$, $t = 4.623$, $P < 0.01$).

### 3.2 NS expression in patients with acute leukemia

The same results were also observed from patients with acute leukemia (Figure 2). The levels of mRNA in different types of leukemia were presented in Table 1.

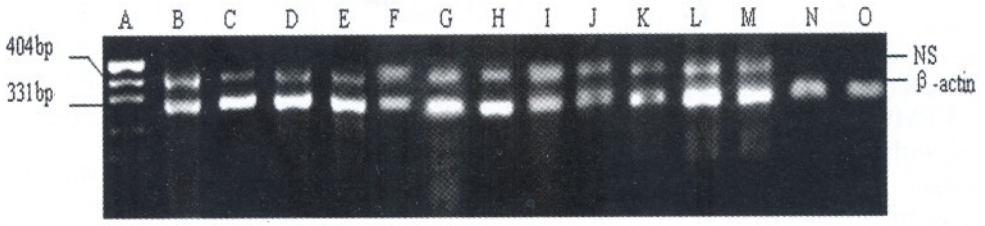


**Figure 2.** Detection of NS expression in acute leukemia cell
A:DNA marker; B,I,J,L: AML-M1,M2a; C,H,K:ALL; D,G:AML-M3; E,F,M: AML-M5; N:MA control; O:health control

**Table 1.** Density score of NS expression in patients with different types of leukemia compared with internal control ($\bar{\chi} \pm SD$)

| Leukemia | M1 + M2a | M3 | M5a | M5b | ALL | Control |
|---|---|---|---|---|---|---|
| Cases | 13 | 8 | 5 | 6 | 7 | 2 |
| Ratio | $0.687 \pm 0.21$ | $0.408 \pm 0.16$ | $0.866 \pm 0.27$ | $0.448 \pm 0.19$ | $0.403 \pm 0.19$ | 0 |

### 3.3 NS expression in healthy subjects and benign anemia subjects

According to the results of RT-PCR, low level or even no NS could be detected in benign anemia and healthy subjects (Figure 3). The reason for low level may be due to the existence of minimal HSC in bone marrow.

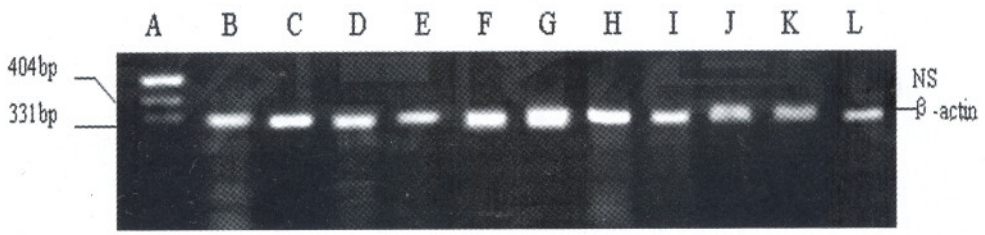


**Figure 3.** Detection of NS expression in healthy subjects and benign anemia subjects
A:DNA marker; B,E,G,I,L:health subjects; C,H,K:MA; D,F:IDA; J:secondary anemia (chronic inflammations)

### 3.4 NS expression in different differentiation stages leukemic cells

According to cell types of acute leukemia, the level of NS gene in the early stage of differentiation was significantly higher than that in the late stage of differentiation (Figure 4). That is, the expression levels of NS in K562, M1 + M2a and M5a were higher than in HL60, M3 and M5b, respectively ($t = 4.623$, 3.054, 4.256; $P < 0.01$).

## 4 Discussion

NS is a newly found p53-binding protein, which exists mainly in the nucleoli of stem cells and various cancer cells, but does not express in committed and terminally differentiated cells[1,5]. The expression level of NS declined obviously during embryo and adult development due to the differentiation of stem cells. *In-vivo* experiments displayed that the NS expression could not even be detected after CNS stem cells were induced to differentiate[2]. In addition, NS expression disappeared before the changes of cell cycle markers during the development of CNS, which indicated the disappearance of NS expression inducing the cell cycle arrest, but not the reverse[1]. Down-regulation of expression of NS gene may result in cell cycle arrest and

cell differentiation[6,7]. Also, NS protein was considered as one of makers of stem cells[8].

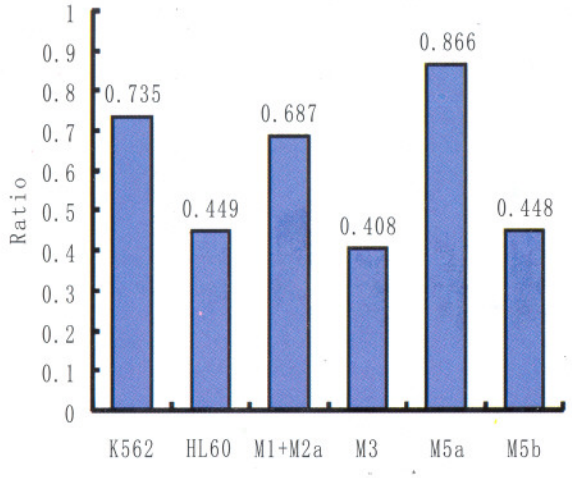


**Figure 4.** Ratio of density score of NS expression in different differentiation stage of leukemia

Acute leukemia is a group of malignant haematopoietic disorders. Researchers have suspected that similar mechanisms might be at work in both cancer cells and stem cells[6,7]. For example, both have the ability not only to indefinitely proliferate, but also to self-renew. It's the same for leukemia cell. Only one difference is that the indefinite proliferation leukemic cells are maintained in certain stage and do not differentiate and mature. As has been recently proposed, leukemia stem cell may locate on initiative stage of haematopoietic cell clone, with self-renewal capacity, and differentiation arrested before cell mature, which are source of the pathogenesis, drug resistance and relapse of leukemia[9]. Evidence shows that many signals pathways that are classically associated with cancer cells may also regulate normal stem cells self-renew[10]. The preliminary studies indicate that protein encoded by NS gene is involved in regulating the proliferation and differentiation of both stem cells and some types of cancer cells.

In the present study, high levels of NS gene expression were found in leukemic cells. This suggested that NS may play an important role in the origination and development of leukemia. We hypothesized that leukemic cells may lead to cell cycle endlessly rather than cellular differentiation due to the existence of a mass of NS protein. Additionally, we collected healthy subjects and benign anemia subjects as control group. From the results of RT-PCR, very low level of NS was detected in only 2 out of 11 cases of control subjects. This could be because the HSC in bone marrow was very little.

It is noteworthy that there were striking differences in NS level of leukemic cells between different stages of differentiation, such as the expression of NS in K562, M1 and M2a, and M5a was higher than that in HL60, M3 and M5b, respectively. According to previous classifications from immunophenotype, morphology and cell enzymology, differentiation stage of K562 is prior to that of HL60. It is close to the differentiation stage of HSC. Because M1, M2a, and M3 belong to myeloblastic cell type and promyelocytic cell type respectively, differentiation stage of the former is earlier than that of the latter. The same is true in M5a and M5b. In conclusion, there is close relationship between NS expression level and proliferation and dedifferentiation of leukemic cell. However, further study is necessary to verify this point.

K562 is a human erythroleukemia cell line derived from a patient with CML-BC. These cells are pluripotent in that they are able to differentiate along the lineage of granulocytic, megakaryocytic, erythroid, monocytic[11,12]. HL60 cell line is derived from human APL. This pluripotent cell line can differentiate into granulocytic and monocytic cell lineages. Environmental conditions such as pH and many chemical inducers can greatly facilitate the differentiation of HL60 cell line into granulocytic and monocytic cell lineages. For example, ATRA has been reported to induce it granulocytic differentiation, and vitamin D can promote it to differentiate into monocytic series[13]. Any way, there are many similarities between leukemic cell and stem cell. This also further confirms the theory of LSC. However, it remains to be determined how inducers act on the expression of NS gene.

In conclusion, NS gene over-expresses in acute leukemia cells, and it closely relates to the origination and development of leukemia. On the one hand, NS plays an important rcle on regulation of the proliferation and differentiation of leukemia cell. Different differentiation stages of leukemic cell showed different expression levels of NS. On the other hand, low level or no expression could be detected in benign anemia subjects and healthy subjects.

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