# Comparative effects of *Nucleostemin* silencing in human Molt-4 and Jurkat leukemia T-ALL cells

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## ABSTRACT

Nucleostemin (NS), a stem cell-abundant nucleolar protein, is critical for maintaining the self-renewal and proliferative properties of normal and cancerous stem cells. Recent data suggests that NS signaling is important for proliferation of T-cells and leukemia cells. This study was conducted to verify the role of NS in pathogenesis and treatment of T-cell acute lymphocytic leukemia (T-ALL). Our results revealed that RNA interference-mediated NS silencing primarily affected clonogenicproperty of T-ALL cells by limiting their self-renewal potential *in vitro*.These effects were accompanied with inhibition of proliferation and early apoptosis in Jurkat cells (p53-null) while late apoptosis in Molt-4 (p53 functional) T-ALL cells. Collectively, our results suggest that NS is a critical regulator in self-renewal and apoptosis of differentT-ALL cells. This suggests therapeutic potential of this gene in leukemia.

**Key words:** Acute lymphoblastic leukemia; Apoptosis; Nucleostemin; Gene silencing

## INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive type of leukemia, accounting for 10–15% pediatric and 25% of adult ALL cases [1,2]. The disease is caused by an accumulation of abnormal immature T lymphoblasts as result of multistep genetic alterations in leukemia initiating cells, so-called leukemia stem cells (LSCs) [1,3]. Like other types of leukemia, T-ALL LSCs are characterized by indefinite self-renewal, uncontrolled cell cycle progression, impaired differentiation and loss of sensitivity to apoptosis [2,3]. Although, the treatment outcome in T-ALL has been tremendously improved with the current therapeutic protocols,the average overall survival of the patients with resistance and relapse remains poor[3,4].Due to this relative lack of efficacy, identifying more effective targets and strategies is essential for treat of this type of leukemia.

Signaling circuitscontrolling stem cell self-renewal areabruptly down-regulatedduring differentiation prior to terminal cell divisionbut often reactivated during malignant transformation [5]. To date, the intricate molecular machinery and the signaling mechanisms regulating fate of T-ALL is not clear.In particular, the role of NS developmental signaling pathways in the pathogenesis of T-ALL remains to be delineatedmore [2].NS is reported to be a marker of stem cells that is involved in controlling self-renewal, cell-cycle progression and proliferation in both stem cells and cancerous cells [6]. The expression of thisGTP-binding protein is rapidly decreased during terminal differentiation of stem cells [7,8]. Mechanistically, the effects of NS are mediated via p53, although some p53-independent mechanisms have been reported in cancerous cells [9-11].

Recently, scientists reported that NS gene silencing caused cell-cycle arrest and cell death of leukemia cell lines such as K562 and Molt-4 cells, suggesting that this nucleolar protein might be an attractive molecular target for developing anti-leukemiatherapy [2,12]. We designed this study to better study the importance of NS in self-renewal and apoptosis of T-ALL cells.

## MATERIAL AND METHODS

***Cell line and cell culture***

Molt-4 and Jurkat cell lines were purchased from the Pasteur Institute of Iran and were cultured in RPMI1640Medium with 10%Foetal Bovine Serum (FBS) (Biosera), 100 µg/ml Streptomycin, 100 u/mL Penicillin (Cinagen, Tehran) and was maintained at 37°C in a humidified atmosphere with 5% $CO_2$.

***siRNA design and synthesis***

There double stranded, short interfering RNAs (siRNA) against all NS mRNA variants were designed as previously reported [2]. The sequences for NS-siRNA and IR-siRNA were: 5'-GAACUAAAACAGCAGCAG AdTdT-3' and 5′-UGA CGA UCA GAA UGC GAC UdTdT-3′, respectively.

***Transfection of cells with NS-siRNA***

For transfection, $2\times10^5$ cells/well were cultured in 100µL of RPMI1640 medium supplemented with 10% serum within 24 well plate (SpL Life sciences, South Korea). In brief, different concentrations of siRNA solutions were mixed separately with HiPerFect transfection reagent (Qiagen, USA) in 100µL serum free medium RPMI1640 for each well. After 15 minutes incubation at room temperature, the resulting mixture was moved to the cell containing well in the plate. After 6 hours, 400 µl of culture medium containing12% serum and antibiotic were added to each well.

***Real Time quantitative PCR (q-PCR)***

Total RNA was extracted from cells with the RNX plus kit (Cinagen, Tehran). Equal volume of total RNA (1 µg) of each sample was treated with DNaseI enzyme andOligodt primer (Fermentase, EU). The RNA converted to cDNA using the reveres transcription reaction by using the RT enzyme (Fermentase, EU). RT-qPCR was performed using SYBR Greenby Rotor Gene 6000 machine (Applied Biosystems)under following cycle conditions: 95°C for 10 minutes, followed by 40 cycles at 57°C for 30 seconds and 72°Cfor 30 seconds. Results were normalized against ß-actin expression. Each QPCR was performed on at least three different experimental samples and each reaction was performed in triplicate. The forward primer and reverse primer of *NS* were 5`-AAAGCCATTCGGGTTGGAGT -3` and 5`-ACCACAGCAGTTTGGCAGCAC -3`, respectively. Human *β2Microglobulin ($\beta_2m$)* gene was used as a control for adjusting the relative amounts of total RNA between the samples. $\beta_2m$ forward and reverse primers were: 5'-CTACTCTCTCTTTCTGGCCTG-3' and 5'-GACAAGTCTGAATGCTCCAC-3', respectively.

***Growth, viability and Self-renewal assays***

Trypan blue exclusion test were used to study the cell growth and viability. For this purpose, $2\times10^5$ cells were seeded in each well of 24 well plates. After 12, 24, 48, 72 h, the number of viable and non-viable cells for each well was counted using Trypan blue exclusion test and hemocytometer; finally, we drew growth and viability curves for each group [2]. For measuring self-renewal capacity of the cells, Colony formation assay was performed in semisolid culture medium containing 0.9 % methylcellulose and 10 % FBS. In brief, 400 cells were placed into 1.2% methylcellulose, 30% FBS, 1% bovine serum albumin (BSA), 10 µM 2-mercaptoethanol and 2 mMof L-glutamine. Colonies consisting of >50 cells were counted using an inverted microscope at 12-14 days, then harvested and replating in methylcellulose [13].

***Morphological evaluation of the apoptosis***

Apoptosis was first detected by Fluorescent microscopy. In this way control and transfected cells were washed in cold PBS and gently mixed with a mixture of Acridine orange (AO, 1 µg/ml) and ethidium bromide (EtBr,1µg/ml) solution (1 : 1, v/v). The suspension was put on a microscopic slide and viewed under a fluorescent microscopy (Nikon E-1000, Japan).

***Statistical analysis***

Statistical analysis was performed with Microsoft Excel 2010 and SPSS14. Independent-sample t-test was used for comparison. $P < 0.05$

was considered statistically significant. All experiment was replicated at least in triplicate.

## RESULTS

### *NS-siRNA efficiently silenced NS in T-ALL cells*

Theq-PCR results confirmed high expression levels of NS in Molt-4 and Jurkatcellsas compared with IR-siRNA that was used as a control for nonspecific effects of siRNA transfection (Figure 1). When we used NS-siRNA, a significant decrease at the level of NS mRNA was observed at 12 h post-transfection followed with moreinhibitory effects (more than 50%) at 24 h and 48 h (Figure 1). For instance, the NS gene expression was reduced by about52% in Molt-4 cells and 60%in Jurkat cells at 48 h post-transfection (Figure 1).

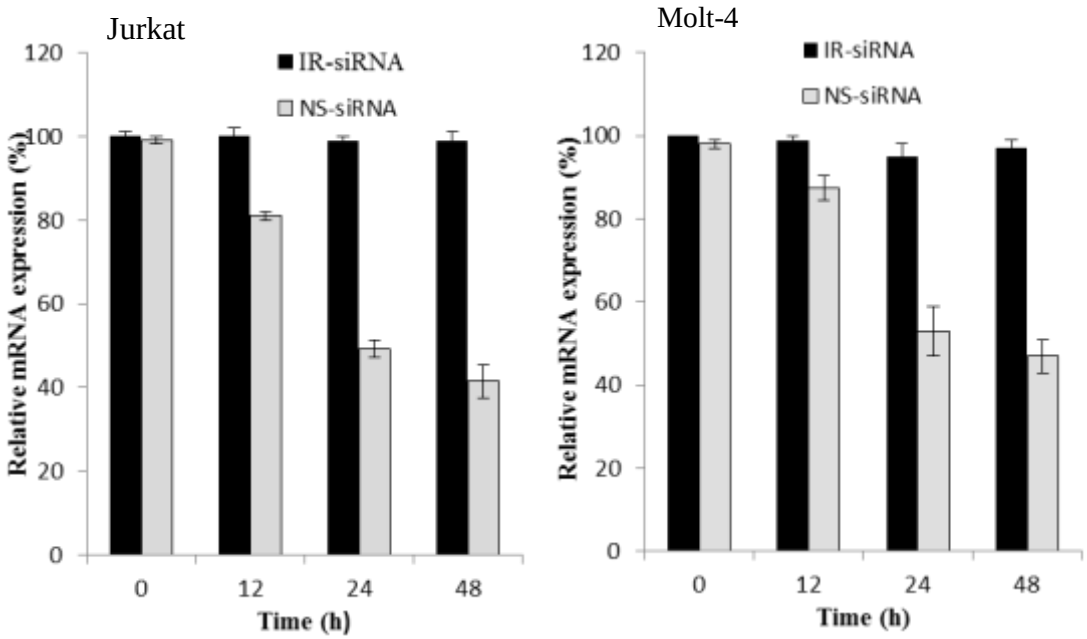


**Figure 1.** Efficacy of NS-siRNA in NS depletion in Molt-4 and Jurkat cells. Following transfection of both Molt-4 and Jurkat cells with IR- and NS-siRNAs, the mRNA was extracted, and the gene expression level was determined by q-PCR. In comparison with IR-siRNA transfected cells,the gene expression level of NS was statistically significant($P$<0.05) in all indicated times after NS-siRNA transfection. In all experiments$\beta_2m$was used as a control for gene expression level. The results were presented as % of control gene ± SD.

### *The growth, viability and self-renewal capacity of T-ALL cells were inhibited by NS-siRNA*

To study biological consequence of NS depletion, the growth and the viability of Molt-4 and Jurkatcellswere evaluatedin a time-dependent manner. AsshowninFig. 2A, NS-siRNA reduced proliferation of bothMolt-4and Jurkat cells by 26.3±3.7 % and 29.0±2.5 % respectively at 24 hours post-transfection, respectively. The growth inhibitory effects were more prominent at 48hours and 72 hours post-transfection(Figurure 2A); by 40.0±5 and 50.5 ±2.5% in Molt-4 and 44.5±3.0 and 49.3±2.7% in Jurkat cells, respectively. Theviabilityof control and NS-siRNAtransfected cellswas also studiedby Trypan blue exclusion test (Fig. 2B). In comparison with IR-siRNA, no significant decrease in viability of Molt-4 cells was observed at 24 h of NS-siRNAtransfectionwhile the viability was significantly reduced (24.5±2.5% %) in Jurkat cells at this condition. The viability of both cell typeswas significant decreased at 48 hours (by 22.4±5% for Molt-4 and 35.2±2.5% % for Jurkat) and 72 hours (by 46.75±2.5% for Molt-4 and

41%±4.3% for Jurkat) after transfection with NS-siRNA(Figure 2B).

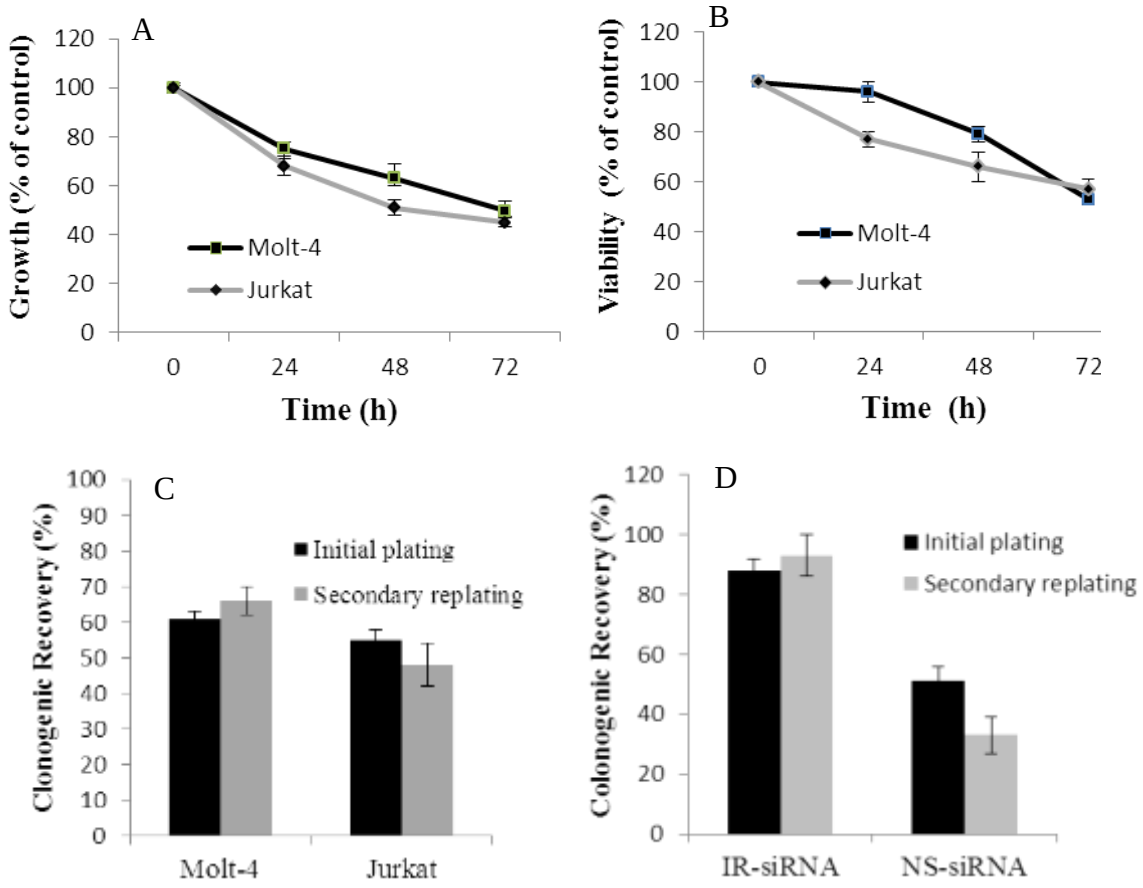


**Figure 2.** Effects of NS-siRNA in growth, viability and self-renewal of Molt-4and Jurkat cells. The growth inhibition (A) and viability (B) ofboth cells were determined bytrypan blue exclusion test at 24 h, 48 h and 72 h after transfection with NS-siRNA, as mentioned in materials and methods.The results were presented as % of control( IR-siRNA transfected) ± SD. Self-renewal capacity was determined by clonogenic recovery of the cells(C and D) following transfection with NS-siRNA.C: After transfection of cells with NS-siRNAand plated in duplicate in methylcellulose, the colonies werecounted at 12–14 days (initial plating). D: The primary colonies were also recloned (secondary replating) to exactly verify self-renewal potential of the cells.

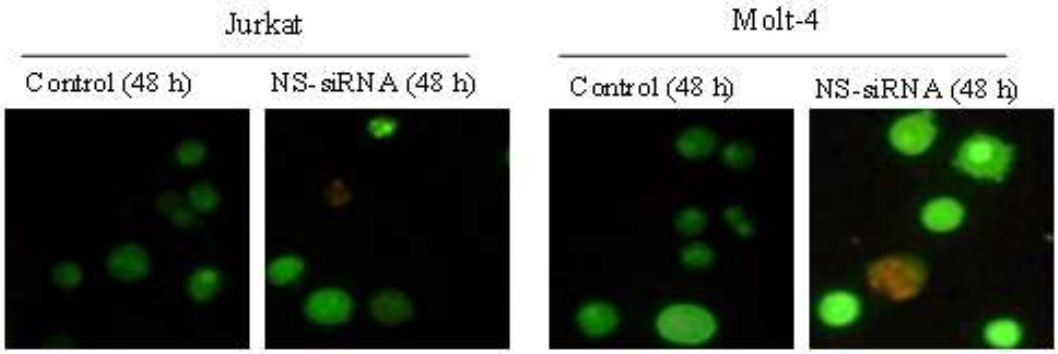


**Figure 3.** Effects of NS depletion on apoptosis induction of Molt-4 and Jurkat cells. NS-siRNA transfected cells at 48 h after transfection. The cells were stained with AO/EtBr and occurrence of apoptosis was observed by fluorescent microscopy (40×).In these figures, viable cells were equally green and early apoptotic cells had bright green blots in their nuclei. Late apoptotic cells, however, stained orange and showed condense and fragmented nuclei. Chromatin condensation (short arrows) and apoptotic bodies (long arrow) are clearly observed.

***Apoptosis is induced following NS silencing in T-ALL cells***

To know whether apoptosis is ultimate fate of T-ALL-depleted cells, we studied condensation a fragmentation of nuclear DNA by AO/EtBr staining. In this test, viable cells were equally green and early apoptotic cells had bright green blots in their nuclei. Late apoptotic cells, however, stained in orange color and showed condense with fragmented nuclei. Based on results presented in Fig. 3 an early apoptosis (48hours after NS-siRNA transfection), was observed in p53− Jurkat cells. In contrast, no detectable apoptotic cellswere observed inp53+ Molt-4 cells at 48 h of NS depletion (Figure 3). However, at longer times of NS depletions (72 hours and 96 hours) a substantial increase in apoptosis was observed in both cell types (data not shown).

## DISCUSSION

The problems with current therapeutic protocols in T-ALL treatment are drug resistance and relapsingof the disease. Indeed, a fraction of LSCs with high self-renewal capacity may remain after current therapies treatment &that may cause relapse and therapeutic failure [4]. To get an exact view of the therapeutic potential of NS in leukemia, we selected two different models of leukemia Molt-4 and Jurkat cells based on p53 status. By using molecular targeting of one of the most important genes, NS, the fate of the cells was studied.*NS*is expressed in many cancer cell lines such as gastric cancer, lung cancer, leukemia (K562 and HL-60), prostate (PC-3), bladder (5637), Crivical (Hela) and mammary tumors (MCF-7) and preferentially exist in many stem cell- populations [14-19]. Therefore,*NS* could be a potent target therapy in most cancers due to its inhibitory effect on the proliferation rate of cells. [6]. In this regard, our data not only confirms these previous reports on importance of this gene in leukemia but also further elucidates the downstream events modulated by NS in T-ALL cells.

Firstly, we showed the effects of RNA interference (RNAi) in reducing *NS* gene expression in a different human T-ALL cell lines. Our results also showed that 24 hoursafter NS silencing, a significant growth inhibition is observed in both Molt-4 and Jurkat cells followed with more inhibitory effects at longer intervals (48 hours and 72 hours). Similar results obtained on different leukemia cells where rate of cell proliferation decreased 24 hours after NS inhibition [2,20]. In addition, NS depletion reduced proliferation of many different cancerous cell lines such as PC-3, 5637 and Hela cells [11,16,18]. This may be due to major regulatory role of NS in promoting cell-cycle and self-renewal of stem and cancer cells [6].Therefore, we also examined the *in vitro* effects of NS depletion on clonogenic growth. Upon serial replating (without further silencing of NS), secondary colony formation was significantly inhibited by NS-siRNA, suggesting critical roles of NS in controlling self-renewal of T-ALL cells.

These findings suggest that *NS* gene silencing by NS-siRNA induced reduction in the rate of cell proliferationand represents a major regulatory role for *NS* in promoting cell cycle and self-renewal of stem and cancer cells.

Apoptosis induction is a typical event that is observed following NS depletion. It is noticeable that Jurkatcells were more sensitive at shorter times of post-transfection (48 hours) than Molt-4 cells. Molt-4 cells were also susceptible to apoptosis, albeit with different kinetic; apoptosis and concurrent decrease in viability occurred only after 48 hours of NS depletion (Figure 3). Indeed, Molt-4 cells with functional p53 protein were somewhat resistant to apoptosis than Jurkat cells (which has a defective p53 pathway) and a delayed apoptosis after 48 hours and 72 hours of NS-transfection was observed in Molt-4 cells. Both early and late apoptosis induction after NS depletion havealso been reported in several studies. For example, in PC-3 prostate and HL-60 cells, NS depletion resulted in growth inhibition and rapid apoptotic response [11,20]. In K562 leukemia cells, however, a delayed apoptotic response has been observed [12].

The severe effects of NS silencing on the viability and apoptosis of p53-null Jurkatcells (Figures 2 and 3) is ratherunexpected, because it has been previously demonstrated that apoptosis induction after NS depletion is mediated through p53. A possible explanation may be the level of NS

knocking-down in both cells; based on Figure 1, NS-siRNA more potently depleted NS in Jurkat cells than Molt-4 cells. Another possible explanation for this apparent discrepancy may be the differences in phenotype and differentiation status of both cells of NS knocking-down in both types of cells. Also, it may be concluded that NS effects are mediated via p53-independent pathways, as previously reported [2, 10, 12].

## CONCLUSION

In conclusion, *NS*is expressed at high levels in T-ALL cells. Following NS-depletion, the Molt-4 and the Jurkat cells exhibited inhibition of self-renewal and proliferation andshowed apoptotic response. These results highlight the importance of this stem cell-related gene as a therapeutic target for leukemia.

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## REFERENCES

1. Cox CV, Martin HM, Kearns PR, Virgo P, Evely RS, et al. Characterization of a progenitor cell population in childhood T-cell acute lymphoblastic leukemia. Blood2007; 109(2):674-82.
2. Rahmati M, Moosavi MA, Zarghami N. Nucleostemin knocking-down causes cell cycle arrest and apoptosis in human T-cell acute lymphoblastic leukemia MOLT-4 cells via p53 and p21Waf1/Cip1 up-regulation. Hematology 2014;19(8):455-62.
3. Gilliland DG1, Jordan CT, Felix CA. The molecular basis of leukemia. Hematology Am SocHematolEduc Program 2004;80-97.
4. Tosello V, Ferrando AA.The NOTCH signaling pathway: role in the pathogenesis of T-cell acute lymphoblastic leukemia and implication for therapy.TherAdvHematol 2013;4(3):199-210.
5. Moosavi MA, Yazdanparast R. Distinct MAPK signaling pathways, p21 up-regulation and caspase-mediated p21 cleavage establishes the fate of U937 cells exposed to 3- hydrogenkwadaphnin: differentiation versus apoptosis.ToxicolApplPharmacol 2008;230(1):86-96.
6. Tsai RY, McKay RD. A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Dev 2002; 16 (1): 2991–3003.
7. Tsai RY, McKay RD. A multistep, GTP-driven mechanism controlling the dynamic coupling of nuclesostemin. J Cell Biol 2005; 168(2):179-84.
8. Ma H and Pederson T. Nucleostemin: a multiplex regulator of cell-cycle progression. Trends in Cell Biology 2008; 18(12): 575-9.
9. Ma H, Pederson T. Depletion of the nucleolar protein nucleostemin cause G1 cell cycle arrest via the p53 pathway. MolBiol Cell 2007; 18(7):2630-5.
10. Jafarnejad SM, Mowla SJ,Matin MM. Knocking-down the expression of nucleostemin significantly decreases rate of proliferation of rat bone marrow stromal stem cells in an apparently p53-independent manner. Cell Prolif 2008; 41(1):28-35.
11. Liu R, Zhang Z, Xu Y. Down regulation of nucleostemin causes G1 cell cycle arrest via a p53-independent pathway in prostate cancer PC-3 cells. UrolInt 2010; 85(2):221-7.
12. Seyed-Gogani N, Rahmati M, Zarghami N, Asvadi-Kermani I, Hoseinpour-Feyzi MA, Moosavi MA. Nucleostemin depletion induces post-G1 arrest apoptosis in chronic myelogenous leukemia K562 Cells. Adv Pharm Bull 2014; 4(1):55-60.
13. Tsai RY.Turning a new page on nucleostemin and self-renewal. J Cell Sci 2014;127(18):3885-91.
14. Liu SJ, Cai ZW, Liu YJ, Dong MY, Sun LQ, Hu GF, Wei YY, Lao WD. Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies. World J Gastroenterol 2004;10(9):1246-9.
15. Gao HX, Gao XF, Wang GQ, Wang ES, Huang W, Huang P. In vitro study of Nucleostemin gene as potential therapeutic target for human lung

carcinoma. Biomed Environ Sci2012; 25(1):91-7.
16. Nikpour P, Mowla SJ, Jafarnejad SM, Fischer U, Schulz WA. Differential effects of Nucleostemin suppression on cell cycle arrest and apoptosis in the bladder cancer cell lines 5637 and SW1710. Cell Prolif 2009;42(6):762-9.
17. De Nevi E, Marco-Salazar P, Fondevila D, Blasco E, Pérez L, Pumarola M. Immunohistochemical study of doublecortin and nucleostemin in canine brain. Eur J Histochem 2013; 20;57(1):e9.
18. Liu SJ, Zhang ZH, Zhang DQ, Sui XM, Liu YJ, Cai ZW, Yuan XY, Sun LQ, Hu GF, Liu RL.Gene profiling after knocking-down expression of nucleostemin in Hela cells using oligonucleotide DNA microarray. J ExpClin Cancer Res 2006;25(4):575-83.
19. Lin T, Meng L, Li Y, Tsai RY. Tumor-initiating function of nucleostemin-enriched mammary tumor cells. Cancer Res 2010;70(22):9444-52.
20. You Y, Li X, Zheng J, Wu Y, He Y, Du W, Zou P, Zhang M.Transcript level of nucleostemin in newly diagnosed acute myeloid leukemiapatients.Leuk Res 2013; 37 (12) :1636-41.