Original Article

Nucleostemin regulates proliferation and migration of gastric cancer and correlates with its malignancy

Hongxue Wu, Weixing Wang, Shilun Tong, Chong Wu

Department of Gastrointestinal, Renmin Hospital of Wuhan University, Wuhan, Hubei Province, P. R. China

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Abstract: Objective: The aim of this study was to investigate the effects of nucleostemin (NS) knocking down in SGC-7901 gastric cancer cell line and investigates its correlation with the metastasis and TNM stage in gastric cancer (GC) patients. Methods: NS expression was assessed using immunohistochemistry in 421 patients with GC. The correlation between NS expression, clinicopathological features and prognosis was analyzed. NS gene silencing was performed using a specific small interfering RNA (NS-siRNA). The gene expression level of NS was evaluated by PCR. The viability and growth rate of SGC-7901 cells were determined by trypan blue exclusion test. Cell cycle distribution of the cells was analyzed by flow cytometry. Results: High NS expression was correlated with node metastasis, distant metastasis and TNM stage. Kaplan-Meier survival analysis revealed that patients with low NS expression had significantly longer survival than those with high NS expression. Moreover, our results showed that NS knocking down inhibited proliferation and viability of SGC-7901 cells in a time-dependent manner. Cell cycle studies revealed that NS depletion resulted in G1 cell cycle arrest at short times of transfection (24 h) followed with apoptosis at longer times (48 and 72 h), suggest that post-G1 arrest apoptosis is occurred in SGC-7901 cells. Conclusion: Overall, these results point to essential role of NS in SGC-7901 cells, thus, this gene might be considered as a promising target for treatment of GC.

Keywords: Gastric cancer (GC), nucleostemin (NS), SGC-7901, small interfering RNA, apoptosis, target therapy

Introduction

Gastric cancer (GC) is the second leading cause of cancer mortality in the world, and has a particularly high incidence in Asian countries including China [1, 2]. Despite the declining incidence of gastric cancer, there are still over 1 million cases newly diagnosed and 850000 deaths globally each year [3, 4]. The high mortality rate is mainly due to late presentation, since early stage of gastric cancer is either asymptomatic or presents with non-specific symptoms. The survival rate depends on the stage of gastric cancer at the time of diagnosis.

Nucleostemin (NS) is a new protein localized in the nucleolus of most stem cells and tumor cells which regulates their self-renewal and cell cycle progression [5, 6]. The protein coded by NS gene was found in the nucleoli of undifferentiated cells, such as adult and embryonic stem cells, neural stem cells and human bone marrow stem cells but not in differentiated counterpart cells, indicating that NS is silenced during normal cells differentiation [7, 8]. Interestingly, recent reports suggest that NS gene is also abundantly expressed in several human cancer cell lines such as Sw620 (colorectal), Hela (cervical), 5637 (bladder), PC-3 (prostate), and HL-60 (acute myelocytic leukemia) [9-12]. In parallel with significant of this gene in cancer, several knocking down experiments using RNA interference (RNAi) showed that inhibition of NS gene expression markedly inhibited proliferation and cell cycle progression of cancerous cells followed with induction of differentiation and/or apoptosis [11-14]. Recently, a high expression level of NS has been reported in gastric cancer patients [15]. Consistent with this, RNAi-mediated NS knocking down inhibited proliferation and induced differentiation and apoptosis in gastric cancer cell lines [16]. However, importance of
NS in other types of digestive cancers, especially gastric cancers, needs to be addressed.

This study was designed to investigate functional importance and therapeutic potential of NS gene expression and effects of NS knockdown on cell cycle and apoptosis in gastric cancer cell lines. Our result showed that RNA interference (RNAi)-mediated NS silencing induced G1 cell cycle arrest followed with apoptosis in gastric cancer cell lines.

Material and methods

Patients and samples

Based on tissue data availability, 421 cases of GC, between 2009 and 2012, were included in the present study. Formalin-fixed paraffin-embedded tissues were collected from Renmin Hospital of Wuhan University (Wuhan, China). This study was approved by the Ethics Committees of Renmin Hospital of Wuhan University. Informed consent was obtained from all participants and the study was performed in accordance with the Declaration of Helsinki. Follow-up data were obtained from medical records and direct communication with the patients or their relatives. The follow-up period was defined as the time from the date of surgery to the date of patient mortality or the final follow-up in January 2014.

Cell culture and transfection

BGC-823, AGS and SGC-7901 cell lines were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI-1640 in 5% CO2 at 37°C. NS siRNA and scrambled siRNA were purchased from Invitrogen Life Technologies and transfected using Lipofectamine® 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions.

Histopathological evaluation and scoring

Paraffin-embedded serial sections of GC specimens were analyzed for NS protein expression using an anti-NS antibody (Abcam, Cambridge, MA, USA), as described previously [9]. Negative control sections were incubated with pre-immunized rabbit serum (Abcam). Immunostaining was assessed by two independent, blinded pathologists and scored by multiplying the intensity of the staining by the percentage of stained cells. The staining intensity was graded on a scale of 0 to 3. The percentage of stained tumor cells was graded as follows: 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). The final scores ranged from 0 to 12. For any subsequent analysis, scores between 0 and 4 were defined as low expression and those between 5 and 12 were defined as high expression. Inconsistent scores were re-evaluated by two pathologists until a consensus score was established.

PCR

RNA isolation and reverse transcription were performed as previously described [22]. Oligonucleotide primer sequences were as follows: β-actin (264 bp), forward: 5’-GAG ACC TTC AAC ACC CCA GCC-3’; reverse: 5’-AAT GTC AC G CAC GATT TCC C-3’; NS (201 bp), forward: 5’-TCC CCA TCG CCA TCC CC-3’ reverse: 5’-CAC CAT GGC CTC GGC TGG-3’. For all the above genes, amplification was performed under the same cycling conditions (1 minute at 94°C, 50 seconds at 57°C, 1 minute at 72°C), except the number of cycles that were specified for each gene (32 for NS).

Western blot and Immunoprecipitation

SGC-7901 were harvested at specific times after treatment with regents as indicated in each experiment. Cells were mixed with loading buffer and subject to electrophoresis. After electrophoresis, proteins were transferred to polyvinyl difluoride membranes (Pall Filtron) using a semidry blotting apparatus (Pharmacia) and probed with mouse mAbs, followed by incubation with peroxidase-labeled secondary antibodies. Detection was performed by the use of a chemiluminescence system (Amersham) according to the manufacturer’s instructions. Then membrane was stripped with elution buffer and reprobed with antibodies against the non-phosphorylated protein as a measure of loading control. Controls for the immunoprecipitation used the same procedure, except agarose beads contained only mouse IgG.

MTT assay

Cell viability was assessed using an MTT assay. Following transfection, cells were plated in 96-well plates and incubated for 24, 48 and 72 h. A total of 20 μl 5 mg/ml MTT (Sigma-Aldrich) was added to each corresponding test well and
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Table 1. Correlation between NS and clinicopathological variables

<table>
<thead>
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<th>Characteristic</th>
<th>n</th>
<th>low</th>
<th>high</th>
<th>p-value</th>
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<tr>
<td>Total</td>
<td>421</td>
<td>131</td>
<td>290</td>
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<tr>
<td>Age (years)</td>
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<tr>
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<td>65</td>
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<tr>
<td>Moderate</td>
<td>166</td>
<td>54</td>
<td>112</td>
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</tr>
<tr>
<td>Poor</td>
<td>76</td>
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<tr>
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<td>140</td>
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incubated for 4 h at 37°C. The supernatant was then discarded and 200 μl dimethyl sulfoxide was added to each well to dissolve the formazan. Optical density was assessed by measuring the absorbance of each well at 490 nm using a spectrophotometer (SpectraMax Plus384; Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate.

Cell viability assays

An Alamar blue assay was used to measure cell proliferation. This assay is based on the quantitative metabolic conversion of blue, non-fluorescent resazurin to pink, fluorescent resorufin by living cells. After 72 h of incubation, an Alamar blue (Invitrogen) stock solution was aseptically added to the wells to equal to 10% of the total incubation volume. The resazurin reduction in the cultures was determined after a 2-6 h incubation with Alamar blue by measuring the absorbances at 530 nm and 590 nm wavelengths on a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments).

Apoptosis assay

Following maintenance in culture, the cells were harvested and stained with phycoerythrin-conjugated Annexin V according to the manufacturer’s instructions (BD Biosciences). The cells were then analyzed on a FACS Calibur flow cytometer (BD Biosciences). The cells were considered viable if double negative, early apoptotic if positive for Annexin V alone and necrotic or late apoptotic if double positive.

Cell cycle analyses

DNA contents of cells were analyzed using flow cytometry as described previously [12]. Control and transfected cells were harvested and washed twice with PBS (Phosphate Buffer Saline), fixed in 70% ethanol and kept at -20°C until analysis. Then the cells were stained with 20 μg/ml PI containing 20 μg/ml RNase (DNase free) for 2 h. The stained cells were analyzed by flow cytometry (Partec Pas, Germany). The population of G0/G1, S, G2/M and sub-G1 cells was determined using Mulicycle Cell Cycle Software. The results are expressed as percentages of the cells in each phase.

Statistical analysis

Results are expressed as mean ± standard deviation. Data were analyzed using the unpaired two-tailed student’s t test and the log rank test. P values of P<0.05 were considered significant.

Results

Correlation between NS and clinicopathological variables (Table 1)

NS expression was analyzed in 421 GC tissue samples and paired non-cancerous gastric tissue samples. Among the 421 GC tissues, 69.36% (292/372) of cases demonstrated high NS expression, while only 10.22% (129/372) of the matched, non-cancerous gastric tissue samples showed high NS expression (P<0.001). Immunohistochemistry revealed a predominantly nuclear localization of NS (Figure 1) and showed that NS expression was significantly higher in GC tissue than in non-cancerous, normal gastric tissue. In addition, significant differences in NS expression were observed between tumors with node metastasis (P=0.0017), dis-
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Kaplan-Meier survival analysis revealed that the patients with low NS expression had significantly longer survival than those with high NS expression (log-rank, P<0.001; Figure 2A). The correlation between metastatic lymph node NS expression and patient survival was also assessed. Kaplan-Meier analysis revealed that patients with low NS expression in the metastatic lymph nodes had significantly longer overall survival than patients with high metastatic lymph node NS expression (Figure 2B, log-rank P=0.007).

Expression of NS was efficiently inhibited by NS-siRNA in SGC-7901 cells

Based on our preliminary data about high expression level of NS in SGC-7901 cell lines, we examined different RNAi techniques for silencing of this gene in SGC-7901 cells. One of the designed siRNAs, called NS-siRNA, could efficiently inhibit NS expression in SGC-7901 cells (Figure 3). As depicted in Figure 3, NS-siRNA at 200 nM was efficiently delivered into SGC-7901 cells (Figure 3A) and significantly inhibited NS expression in a time-dependent manner (Figure 3B). In fact, no significant reduction in NS expression was observed after 6-12 h NS-siRNA transfection of SGC-7901 cells, whereas NS mRNA level were significantly inhibited between 16 h and 48 h of transfection (Figure 3B and 3C). The inhibition rate of NS expression in comparison with corresponding β2m internal control after 16 h, 24 h and 48 h were about 20%, 23% and 56%, respectively (Figure 3C).

NS siRNA significantly reduces GC cell proliferation and invasion

NS expression was analyzed in three GC cell lines: BGC-823, AGS and SGC-7901 cells. NS
expression was observed to be higher in AGS and SGC-7901 cells than in BGC-823 cells (Figure 4A). Based on this finding, AGS and SGC-7901 cells were used for the subsequent functional analysis. siRNA-induced NS knockdown was confirmed using qPCR and western blot analyses (Figure 4B). NS siRNA was observed to significantly reduce proliferation and invasion in AGS and SGC-7901 GC cells (Figure 4C and 4D).

Knockdown of NS leads to profound morphological and functional changes in SGC-7901 cells

The morphology of SGC-7901 cells after NS-siRNA transfection was shown in Figure 5A. Aggregation of SGC-7901 cells and decreases in cell confluency was typically observed in NS depleted SGC-7901 cells. However, some cell death criteria such as cell shrinking and cell debris were observed after 48-72 h of NS-siRNA transfection. To determine mode of cell death in NS-siRNA transfection cells, we studied apoptosis and necrosis by AO/EtBr double staining of the cells (Figure 5B). The results clearly showed that NS siRNA transfected cells underwent apoptosis after 48 h. The apoptotic criteria, including nuclear fragmentation chromatin condensation, and apoptotic bodies were clearly observed. In these figure, viable cells were equally green whereas early apoptotic cells had bright green blots in their nuclei indicating chromatin condensation and nuclear fragmentation. Late apoptotic cells, however, stained orange and showed condensed and fragmented nuclei. Necrotic cells were uniformly orange. These means that knockdown of NS induces apoptosis in SGC-7901 cells.

Evidence suggests that the cell fate decision is made within G1 phase of cell cycle. Therefore, the cell cycle distribution of NS-siRNA transfected SGC-7901 cells was also studied in this work (Figure 5C). When compared with control cells, NS-siRNA transfected cells showed a significant increase in G0/G1 phase of cell cycle population with concurrent decrease in S and G2M phase after 24 h of transfection. As might be expect, a sub-G1 peak (apoptotic cells) was apparent after longer times of transfection. For example, After 24 h, the G0/G1 cell cycle population of NS-siRNA transfected cells (58%) was higher than control cells (47%). Moreover, the sub-G1 cell population (apoptotic cells) was increased from 21-38% 48-72 h of transfection, respectively. All these indicated that knockdown of NS induces G0/G1 cell cycle arrest in SGC-7901 cells.
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Discussion

Gastric cancer is considered one of the most deadly tumors worldwide. Even with the decline in its incidence, the mortality rate of this disease has remained high, mainly due to its late diagnosis and to the lack of precise prognostic markers. Despite improvements in medical technology, such as the development of new diagnostic imaging methods, gastric cancer remains a silent disease that is frequently diagnosed in advanced stages, which is responsible for its elevated mortality [12]. Additionally, the presence of metastasis in the lymph nodes is a frequent event in this type of neoplasia and is considered an important prognostic marker because it may contribute to the high rates of recurrence and/or gastric cancer mortality [4, 13].

Considering the increasing level of understanding of the molecular basis of tumor biology, several biomarkers have been identified for many types of tumors [14-16]. These biomarkers or molecular markers are molecular entities (DNA, RNA or protein) that can be isolated from biological materials and are useful in the five main areas of cancer study and medicine: cancer screening, diagnosis, tumor classification, prognosis and prediction of a therapeutic response [17]. Despite its importance in translational medicine, some important factors determining the efficiency of a molecular marker assay are the levels of sensitivity and specificity [18], which currently limit their use in clinical practice.

Several reports have suggested that NS is a marker of stem cells that is involved in controlling self-renewal, cell cycle progression and proliferation in both stem cells and cancerous cells. As we took this matter into consideration that NS plays a critical role in cell proliferation, consequently, we examined NS expression and its function in SGC-7901 and AGS cells as a model of GC cell lines. With our knowledge, functional importance of NS in GC has not been studied until now. Our results indicated that NS mRNA was highly expressed in SGC-7901 and AGS cells. This finding is aligned with previous studies based on NS over-expression in several human cancer cell lines [19-24].

Significant correlations were observed between NS expression, metastasis, TNM stage and mortality. Furthermore, these correlations were

Figure 3. Knockdown of NS expression by siRNA in SGC-7901 cell line. A. NS-siRNA delivery into SGC-7901 cells. After 24 h of transfection with fluorescein-labeled NS-siRNA, SGC-7901 cells were harvested and analyzed by fluorescent microscopy. B. RT-PCR study of NS gene expression level in SGC-7901 cells. Following transfection with 200 nM IR- and NS-siRNAs, SGC-7901 cells were collected and mRNA levels of NS were determined by semi quantitative RT-PCR. C. Analyzing of NS mRNA level in SGC-7901 cells. The densitometry analysis of NS mRNA over β2m mRNA data was studied by UVItec software. Each value represents the mean ± SEM of three independent experiments and P<0.05 (*) were considered statistically significant.
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found to be independent of other patient characteristics. These findings indicate that high NS expression may be a useful prognostic marker for GC. NS has been reported to be up-regulated in neuroblastoma, as well as pancreatic, lung, bladder, liver, ovarian and breast cancer [8, 13, 25, 26]. However, the role of NS in GC is yet to be elucidated. In the present study, immunohistochemistry revealed that NS was up-regulated in GC tissues compared with the levels in paired non-cancerous tissues.

In human breast tissue, NS over-expression has been found to contribute to malignant progression through inactivating wild-type p53 and p38 mitogen-activated protein kinase, as well as through decreasing p16 protein expression [27]. NS has also been shown to be a prognostic marker in patients with lung adenocarcinoma [28]. Furthermore, high NS expression has been reported to be correlated with poor prognosis in patients with pancreatic neuroendocrine tumors and medulloblastoma [29, 30]. In the present study, patients with GC with high NS expression were observed to have a worse outcome than those with low NS expression. Furthermore, multivariate analysis suggested that high NS expression was an independent prognostic factor for patients with GC. In vitro experiments in GC cells were also performed and revealed that NS siRNA significantly inhibited GC cell proliferation and invasion. These findings indicate that NS may not only be a prognostic marker, but also a potential therapeutic target.

In our study, role of NS in cell cycle progress and apoptosis of SGC-7901 cells was determined by a NS specific siRNA as a genomic nanoparticle. These oligos led to a significant decrease in the NS mRNA expression. The results showed that NS knocking down inhibited growth of SGC-7901 cells 24 h after transfection. Apoptosis began after 48 h and increased to its highest level after 72 h. Therefore, NS depletion in SGC-7901 cells resulted in growth inhibition at short times and apoptosis at longer times. These results are in

Figure 4. NS siRNA significantly reduces proliferation and invasion in gastric cancer cells. A. NS expression in BGC-823, AGS and SGC-7901 cells detected using western blot analysis. B. siRNA-induced NS silencing confirmed using quantitative polymerase chain reaction and western blot analyses. C. NS siRNA inhibited the proliferation of AGS and SGC-7901 cells. D. NS siRNA inhibited invasion by AGS and SGC-7901 cells. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 for the difference between the two groups.
full agreement with cell cycle results where an accumulation in G1 phase population was observed after 24 h of NS-siRNA transfection. After this time point, however, the cells population at G1 phase decreased and a sub-G1 peak was appeared, suggest that post-G1 arrest apoptosis is exact mode of action of NS-siRNA in SGC-7901 cells. Most literature reports suggest that NS depletion inhibited proliferation and induced cell cycle arrest in cancer cell lines [31-34]. For instance, NS specific siRNA in bladder cancer cells led to G1 cell cycle arrest in prostate PC-3 cells and bladder cancer 5637 cells [27, 32]. However, NS may also induce G2/M cell cycle arrest as the case of bladder cancer SW1710 cells. Apparently, the role of NS in regulation of G1 phase of cell cycle in SGC-7901 cells are in full agreement with most of these literature reports.

Although, several reports point to apoptotic effects of NS depletion in different cancerous cells, induction of apoptosis following G1 cell cycle arrest is a novel finding of this paper. In fact, it has been previously reported that NS depletion induced a rapid apoptosis response in HeLa cells, PC-3 cells, human bladder (5637) cells and HL-60 cells [23, 27, 35]. In our experiments, however, we observed a delayed apoptosis response in SGC-7901 and AGS cells. This may be related to different levels of NS depletion and protein contents of the cells used in distinct experiments.

In conclusion, NS may be a prognostic biomarker for GC and its high expression is associated with poorer prognosis. Further investigations are required to validate the findings of the present study and to elucidate the underlying mechanisms through which NS affects GC.
Disclosure of conflict of interest
None.

Address correspondence to: Weixing Wang, Department of Gastrointestinal, Renmin Hospital of Wuhan University, Wuhan, Hubei Province, P. R. China. E-mail: wangweixingvip1234@163.com

References


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[33] Shin YV, Chu KM. MiRNA as potential biomarkers and therapeutic targets for gastric cancer. World J Gastroenterol 2014; 20: 10432-10439.
