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• GASTRIC CANCER •

Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies

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Abstract

AIM: To examine the role of nucleostemin in the growth regulation of gastric cancer, liver cancer and other cancers.

METHODS: RT-PCR was used to clone the fragment of nucleostemin cDNA from HEK 293 cells. Eighteen kinds of malignant tumor tissues including gastric adenocarcinoma and liver cancer tissues, 3 kinds of benign tumor tissues, 3 kinds of benign hyperplastic tissues and normal tissues were employed to examine nucleostemin gene expression by RT-PCR, Slot blot, Northern blot and *in situ* hybridization.

RESULTS: We successfully cloned a 570 bp fragment of nucleostemin-cDNA from HEK-293 cells. All detected malignant tumor tissues, benign tumor tissues, and benign hyperplastic tissues had high levels of nucleostemin expression. Nucleostemin was also expressed in human placenta tissue at a high level. In terminally differentiated normal human adult kidney and mammary gland tissues, no nucleostemin expression could be detected.

CONCLUSION: Nucleostemin can help regulate the proliferation of both cancer cells and stem cells. It might play an important role in the growth regulation of gastric cancer, liver cancer and other cancers.

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INTRODUCTION

Perhaps the most important and useful property of stem cells is self-renewal. Through this property, striking parallels can be found between stem cells and cancer cells: tumor may originate from the transformation of normal stem cells. Similar signaling pathways may regulate self-renewal in stem cells, and cancer cells[1]. McKay and Tsai found a novel gene nucleostemin (NS) in rat embryo stem cells, the rat central nervous system (CNS) stem cells and rat primitive bone marrow cells. NS gene was apparently involved in regulating the proliferation of both stem cells and at least some types of cancer cells[2]. 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. Our recent data also showed that NS expressed in human gastric cancer (SGC-7901) cells, human hepatocarcinoma (HepG2) cells, human cervical cancer (Hela) cells, human osteosarcoma (OS-732) cells, human mammary (MMK-7) cells and human embryo kidney (HEK-293) cells[3]. Coordinated control of self-renewal and commitment to differentiation is key to maintaining the homeostasis of the stem cell compartment[4,5], when deregulated it may contribute to cancer pathogenesis[6,7]. The identification of stem-cell-specific proteins and the elucidation of novel regulatory pathways that ensure the integration of these processes, are therefore of fundamental importance[8,9]. It seems that NS is involved in the regulatory pathways, but the fundamentals about NS are still unknown. For example, why does NS express in stem cells and cancer cells but not in differentiated cells? What are the tumor types that misexpress NS and the underlying mechanism? And what is the timing of NS reactivation in cancer cells.

Gastric cancer is common in China, and its early diagnosis and treatment are difficult[10,11]. To investigate the mechanism of carcinogenesis of gastric cancer would facilitate its diagnosis and treatment. Therefore, NS may take part in the growth regulation of tumor tissues and hyperplastic tissues. So we employed a number of malignant tumor tissues, benign tumor tissues, benign hyperplastic tissues and normal tissues to examine the expression of NS gene by RT-PCR, Slot blot, Northern blot and *in situ* hybridization, which would help illuminate the mechanism of self-renewal of cancer cells.

MATERIALS AND METHODS

Materials

We collected tens of tumor tissue samples including gastric adenocarcinoma, liver cancer, bladder carcinoma, pancreatic cancer and esophagus squamous carcinoma samples. We also collected several kinds of normal tissue samples such as normal kidney tissues and muscle tissues. Human embryo kidney (HEK-293) cells were cultured in DMEM containing 100 mL/L FCS at 37 ℃ in 50 mL/L CO_2[12,13]. The cells were kept in logarithmic growth phase by trypsin digestion and reinoculation every 2-4 d[14,15]. DMEM cell culture medium was purchased from Gibco Corporation; fetal calf serum (FCS) and trypsin from Hyclone Corporation; restriction enzymes and T4 DNA ligase from Promega Corporation. PCR primers were synthesized by Shanghai Shenyou Company and DNA sequencing was also performed by this company.

Cloning of NS cDNA fragment

Total RNA in HEK 293 cells was isolated using total RNA

isolation kit (Promega Corporation). A 2 μg total RNA was used in reverse transcription (RT) reaction. Up-stream primer, 5' -ggatccatgaaaaggcctaagttaaagaaagc (*Bam*HI site underlined); Down-stream primer, 5' -aagcttgctctccaaattctcctttggta (*Hin*dIII site underlined). PCR protocol (94 ℃ 30 s→50 ℃ 30 s→72 ℃ 30 s) was run for 30 cycles and PCR product was about 570 bp. PCR product was separated by agrose gel electrophoresis, recovered with gel extraction kit (Omega Corporation), and ligated with pGEM-T vector. The ligate was transformed into competent *E.coli* JM 109 cells. The correct transformant was identified by restriction enzyme analysis and DNA sequencing.

Isolation of total RNA in tissue samples and RT-PCR

About 100 mg tissues of each sample was used to isolate total RNA using total RNA isolation kit (Promega Corporation). RT protocol and PCR protocol including PCR primers are the same as the above. GAPDH was used as a loading control, its up-stream primer, 5' -ggtggacctgacctgccgtctaga, and its down-stream primer, 5' -ttactccttggaggccatgtggg. PCR protocol (94 ℃ 30 s→55 ℃ 30 s→72 ℃ 20 s) was run for 25 cycles.

Analysis of slot blot

Twenty μg total RNA of each sample was added into slots, and transferred onto nylon membrane. Slot blot analysis was carried out using ^{32}P-labeled NS fragments and human 18S RNA as probes in the hybridization.

Analysis of Northern blot

Equal volume of total RNA of each sample was used in denaturing agarose gel electrophoresis, and after that RNA was transferred onto nylon membrane. Northern blot analysis was made using ^{32}P labeled NS fragments and human 18S RNA as probes in the hybridization.

Preparation of cRNA probes

Nucleostemin cDNA fragment was inserted into pGEM-T vector, and large quantities of plasmid was harvested with Plasmid Extraction Kit (Promega Corporation). After linearization by *Nco*I restriction enzyme, SP6 promoter was used to drive the transcription of NS-cRNA probes *in vitro* and in this system Biotin labeled rUTPs was introduced into NS-cRNA probes. In the same way, after linearization by Not I restriction enzyme, T7 promoter was used to drive the transcription of sense NS-cRNA probes *in vitro*, which acted as the negative control.

Detection of in situ hybridization histochemistry

The expression of NS mRNA was detected *in situ* by Biotin SP-HRP method[16,17]. Pretreatment of paraffin-embedded specimens referred to the previously used methods[13]. In brief, before *in situ* hybridization, sections were deparaffinized in xylene (7.5 min, twice) and rehydrated in graded ethanol. Sections were then rinsed in 2 changes of diethylpyrocarbonate-treated distilled water and rinsed in 10 mmol/L citrate buffer, pH 6.0. Sections were placed in glass racks (20 slides/rack) and submerged in approximately 250 mL citrate buffer in covered glass tubes. Sections were then microwaved in a 900-watt microwave (Panasonic Matsushita Electric, Danville, MA) 3 times for 5 min each time at full power, topped up with additional citrate buffer to compensate for evaporation. Slides were allowed to cool slightly, then were rinsed in 2 changes of diethylpyrocarbonate-treated distilled water, dehydrated in graded ethanol, and dried. Hybridization of the labeled probes to the tissue sections was carried out using a previously described method[18]. Sense NS-cRNA probes and SP-HRP solution were used as negative controls. And the tissue sections were counterstained by hematoxylin.

RESULTS

Gain of NS-cDNA fragment

We successfully cloned a 570 nt fragment by RT-PCR in HEK-293 cells. Then the fragment was ligased with pGEM-T vector and the recombinant vector was named pGEM-T-NS. pGEM-T-NS was digested by BamH I and HindIII enzymes, yielding a 3.0 kb fragment of vector and a 570 bp fragment of NS cDNA (Figure 1). The result of sequencing indicated our sequence of NS cDNA was completely identical to the sequence in Genebank. Sequencing revealed that no base mutation occurred in the sequence of our cloned NS-cDNA fragment.

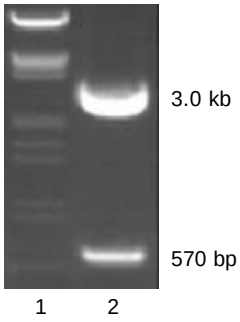

Figure 1 Molecular cloning of nucleostemin cDNA fragment. 1.DNA marker: λDNA, *Eco*RI+ *Hin*d III. 2. pGEM-T-NS, *Bam*HI+*Hin*d III.

NS expression detected by RT-PCR

According to the results of RT-PCR, all above malignant tumor tissues, benign tumor tissues, and benign hyperplastic tissues had high levels of NS expression, so did the normal human placenta tissues. And a lower level of NS expression in normal human adult muscle tissues could also be detected. However, no NS expression could be detected in normal human adult kidney tissues and mammary gland tissues. Partial results of RT-PCR are shown in Figure 2.

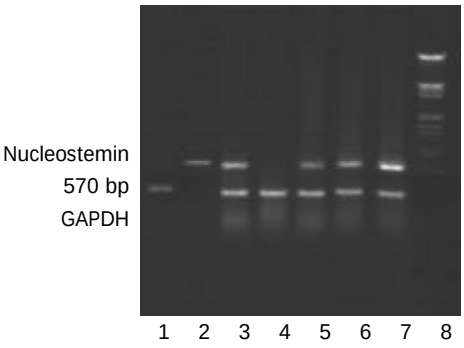

Figure 2 Detection of NS expression in various tumor tissues and normal human adult kidney tissues using RT-PCR. 1. GAPDH cDNA-fragment sample. 2. NS-cDNA fragment sample. 3. Renal carcinoma tissue. 4. Normal human adult kidney tissue. 5. Bladder carcinoma tissue. 6. Liver cancer tissue. 7. Gastric adenocarcinoma tissue. 8. DNA marker: λDNA, *Eco*R I+*Hin*d III.

NS expression by slot blot

Slot blot analysis indicated that NS expressed in all above malignant tumor tissues, benign tumor tissues, and benign hyperplastic tissues, which was in agreement with the results of RT-PCR. However, no NS expression could be detected in normal human adult kidney tissues, mammary gland tissues and muscle tissues. Partial results of Slot blot analysis are shown in Figure 3.

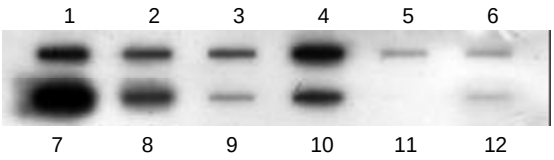

Figure 3 Detection of NS expression in various tumor tissues and normal human adult kidney tissues by Slot blot. 1. Liver cancer tissue. 2. Bladder carcinoma tissue. 3. Pancreatic cancer tissue. 4. Cardia adenocarcinoma tissue. 5. Gastric adenocarcinoma tissue. 6. Esophagus squamous carcinoma tissue. 7. Denatured NS-cDNA sample. 8. Ovary serous cystadenocarcinoma. 9. Ampulle carcinoma tissue.10. Renal carcinoma tissue. 11. Normal human adult kidney tissue. 12. Benign hyperplasia tissue of prostate.

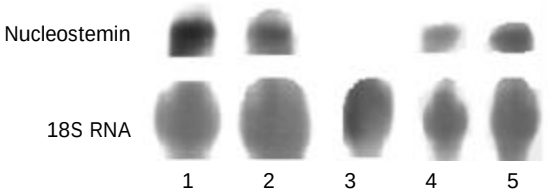

Figure 4 Detection of NS expression in various tumor tissues and normal human adult kidney tissues by Northern blot. 1. Gastric adenocarcinoma tissue. 2. Renal carcinoma tissue. 3. Normal human adult kidney tissue. 4. Liver cancer tissue. 5. Bladder carcinoma tissue.

Detection of NS expression by Northern blot

The results of Slot blot were further confirmed by Northern blot analysis. NS misexpressed in all these malignant tumor tissues, benign tumor tissues, and benign hyperplastic tissues. But the results of Northern blot analysis showed NS did not express in normal human adult kidney tissues, mammary gland tissues and muscle tissues. Partial results of Northern blot analysis are shown in Figure 4.

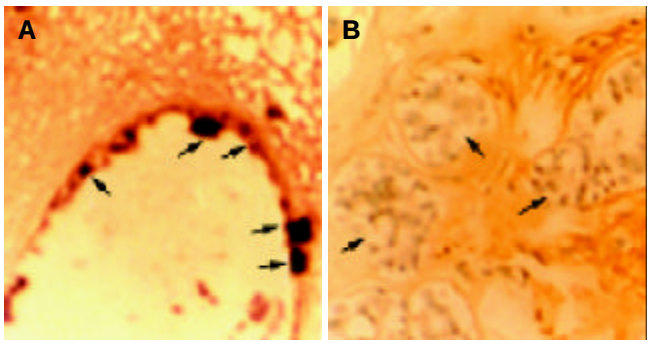

Figure 5 *In situ* hybridization detection of nucleostemin mRNA in invasive ductal breast carcinoma and normal mammary gland tissue by SP-HRP method. Counterstained with hematoxylin. A: Ductal carcinoma cells showed strong staining *in situ* (arrows). B: Normal mammary glandular epithelial cells showed no positive staining, only with azury nuclear counterstaining (arrows).

NS expression in tumor tissues confirmed by in situ hybridization

The Biotin SP-HRP (streptavidin-biotin-peroxidase-DAB) system reacted with 0.05% DAB (3-3' , 4-4' -diaminobenzidine tetrahydrochloride) containing 0.1 mL/L H_2O_2, producing brown positive staining. And counterstain was performed by hematoxylin, producing azury nuclear staining. The results of *in situ* hybridization conformed to those of Slot blot analysis and Northern blot analysis. Positive staining was located in cytoplasm of cancer cells, but no positive staining in cytoplasm of normal adult tissue cells such as normal kidney cells and mammary gland cells. The results of *in situ* hybridization detection of nucleostemin mRNA in invasive ductal breast carcinoma and normal mammary gland tissue are indicated in Figure 5**.** Ductal carcinoma cells showed strong staining in cytoplasm **(**Figure 5A), while normal mammary glandular epithelial cells showed no positive staining, only with azury nuclear counterstaining (Figure 5B).

DISCUSSION

Nucleostemin (NS) is a newly found p53-binding protein, which exists mainly in the nucleoli of stem cells and some various cancer cells, but does not express in committed and terminally differentiated cells[2]. The expression level of NS declined obviously during embryo and adult development due to the differentiation of stem cells. For example, it was expressed preferentially in neuroepithelial precursors during embryogenesis and diminished during the central nervous system (CNS) differentiation. The expression of NS was also found in adult bone marrow hematopoietic stem cells, however could not be found in committed B-lymphocytes and granulocytes. *In vivo* experiments displayed that the NS expression could not even be detected after CNS stem cells were induced to differentiate. Recently, it was confirmed that NS expression also existed in ES cells and mesenchymal stem cells of mice[19]. *In vivo*, NS expression disappeared before the changes of cell cycle markers during the development of CNS, which indicated the disappearance of NS expression induced the cell cycle arrest, but not the reverse. Moreover, non-cycling cells increased by silencing NS expression with small interfering RNA (siRNA) in CNS stem cells and U2OS cancer cells[2].

We successfully cloned a 570 bp fragment of NS-cDNA from HEK-293 cells. The full length of NS-cDNA was 1 650 bp and the cloned fragment was located in the 5' -terminal of the full length. The fragment had high specificity and non-homology with other genes by BLAST analysis, so it was equal to probes in the screening of gene expression. From the results of RT-PCR, Northern blot and Slot blot analysis, we found that all these malignant tumor tissues, benign tumor tissues, and benign hyperplastic tissues had high levels of NS expression, which indicated that NS played an important role in the self-renewal of these cancer cells and hyperplastic cellls. At the same time, NS expressed in human placental tissues also at a high level, possibly due to the existence of a mass of placental stem cells[20-23]. A small quantity of NS expression could be found in normal human muscle tissues by RT-PCR, however no expression could be detected by Slot blot and Northern blot analysis. The reason might be that a few of myoblasts with the characteristic of stem cells existed in muscle tissues[24,25], so only by the sensitive RT-PCR technique could a very small volume of NS expression be detected (figures not provided). In terminally differentiated normal human adult kidney tissues and mammary gland tissues, no NS expression could be detected as no cells possess the characteristics of stem cells or cancer cells in these tissues. The analysis of *in situ* hybridization indicated that NS gene expressed in cancer cells, but not in normal adult tissue cells, such as normal kidney cells and mammary gland cells. These results were in agreement with McKay' s[2].

All these results displayed that NS might play an important role in the proliferation regulation of cancer cells and stem cells. A working hypothesis of NS deduced from all information involved displayed that NS accumulated predominantly in the nucleolus and NS localized into nucleoplasm after binding with GTP[26]. In the nucleoplasm, NS and p53 existed in a protein complex, and subsequently the growth-suppressive function of p53 was inhibited. During cell differentiation, when NS expression was downregulated, p53 was released from the protein complex, which allowed the stabilization or activation of p53 and induction of target genes critical for cell cycle exit

and differentiation, such as $p21^{CIP}$. Members of the E2F family of transcription factors play a crucial role in cell proliferation control by regulating the expression of genes important for DNA synthesis and cell cycle progression[27]. A functional E2F factor is composed of a heterodimer between an E2F polypeptide (E2F1- E2F6) and a DP polypeptide(DP1 and DP2). The transcriptional activities of E2F factors are regulated through association with the Rb(retinoblastoma) tumor suppressor protein and the other pocket proteins, p107 and p130. Binding of a pocket protein inhibits the transcriptional activation capacity of E2F factors and, in at least some cases, can convert E2F to repressors of transcription. The activity of Rb is regulated during the cell cycle by cdks(cyclic dependent kinase), primarily by cdk4 and cdk6, in association with D-type cyclin and cyclin E in association with cdk2. Phosphorylation of Rb by these cdks results in the dissociation of Rb from E2F and the depression/activation of E2F-regulated genes. The E2F-DP transcription complex determined the start of S phase and induced the expression of some genes which were necessary to the progress of S phase, such as Cyclin E, Cyclin A, proliferating cell nuclear antigen (PCNA) and dihydrofolate reductase (DHFR). The product of Cip1, $p21^{CIP1}$, blocked the process of phosphorylation of Rb by cdks and Cyclin complex, so E2F-DP in non-phosphorylation state still bound with RB and could not be freed. Only freed E2F-DP could gain its activity to promote the expression of these genes, and as a result, DNA synthesis was carried out and cells would pass through S phase.

Our results were in agreement with the working hypothesis mentioned above. There were high levels of NS expression in cancer cells, hyperplastic tissue cells and stem cells, so NS inhibited the p53-induced cell differentiation or apoptosis and these cells could easily pass through cell cycles. NS helped regulate the proliferation of both cancer cells and stem cells, although the precise mechanism was not yet clear. The focus on NS has potential therapeutic implications. For example, the growth of gastric cancer may be suppressed by silencing the NS expression with some molecular techniques, which would greatly improve the validity of its treatment.

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