The Short Isoform of Nuclear Mitotic Apparatus Protein 1 Functions as a Putative Tumor Suppressor

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Abstract

Background: Nuclear mitotic apparatus protein 1 (NuMA1) had been reported to produce three groups of isoforms categorized as long, middle, and short groups, of which short NuMA displayed distinct localization patterns compared to long and middle isoforms. However, the function of short NuMA was not clear in the progress of cancer formation. This study aimed to unveil the role of short NuMA in cancer pathogenesis.

Methods: The expression levels of short isoforms were explored in paired gastric carcinoma (GC) samples and different cell lines. Furthermore, the short isoform behaved as a putative tumor suppressor based on cell proliferation and cell colony formation assays. Pull-down assay and whole-genome gene expression analysis were carried out to search candidate interaction partners of short NuMA.

Results: The expression of short NuMA was highly expressed in S and G2 phases of the cell cycle; compared with nontumor tissues, short NuMA downregulated in nine GCs (GC1 [0.131, P = 5 × 10⁻⁴]; GC2 [0.316, P = 3 × 10⁻⁵]; GC3 [0.111, P = 6 × 10⁻⁴]; GC4 [0.456, P = 0.011]; GC5 [0.474, P = 0.001]; GC6 [0.311, P = 0.004]; GC7 [0.28, P = 3 × 10⁻⁴]; GC8 [0.298, P = 0.007]; and GC9 [0.344, P = 0.002]). Besides, high expression of short NuMA significantly inhibits cell growth (2.43 × 10⁴ vs. 2.97 × 10⁴, P = 0.0029) and cell clone information in vitro (70 vs. 2, P = 1.67 × 10⁻⁴). Short NuMA could bind with alpha-actinin-4 (ACTN4), a putative tumor promoting gene. Overexpression of short NuMA could tremendously decrease the expression of MYB proto-oncogene like 2 (MYBL2) of about 92-fold, which played an important role in the cell cycles.

Conclusions: Short isoform of NuMA might be functioned as a putative role of tumor suppressor. Further studies should be made to illuminate the relationship between ACTN4, MYBL2, and tumor progression.

Key words: Cell Cycle; Nuclear Mitotic Apparatus Protein 1; Short Isoform; Tumor Suppressor

Introduction

Nuclear mitotic apparatus protein 1 (NuMA1) encodes a large protein that forms a structural component of the nuclear matrix.¹⁻³ The encoded protein interacts with microtubules and plays a role in the formation and organization of the mitotic spindle during cell division.⁴⁻⁵ It has been reported that ectopic expression of NuMA, including interference of NuMA by microinjection of its antibodies,⁶ mutations at the spherical domains at the amino or carboxyl terminal domains,⁷ and overexpression of NuMA,⁸ would bring on aberrant spindles, such as multipolar spindles. Three groups of isoforms (long, middle, and short) generated by alternative splicing of NuMA1 were reported by analyzing expressed short tags (ESTs) produced by NuMA1.⁹ According to our previous reports, the localization pattern of proteins expressed by the short isoform was different from the other two isoforms. Proteins expressed by the

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long and middle isoforms of NuMA were located in nuclear during interphase and spindles during metaphase. Interestingly, products expressed by the short isoform were confined in the cytoplasm. Our previous result also showed that the short isoform was downregulated in many cancer cell lines determined by real-time quantitative polymerase chain reaction (PCR). However, little is known about its function of the short isoform in the pathogenesis of cancer. In our analysis, the short isoform was also significantly underexpressed in paired gastric carcinomas (GCs). Cell proliferation assay displayed that proteins of short isoform could significantly inhibit the proliferation of HeLa cells. Cell colony formation assay showed that overexpression of short NuMA could tremendously prohibit the formation of cell colonies. It is indicated that short isoform of NuMA1 could be functioned as tumor suppressor. Pull-down assay showed that short products of NuMA1 could bind with actin-like proteins, such as alpha-actinin-4 (ACTN4), a tumor-promoting protein. Whole-genome gene expression analysis found that the MYB proto-oncogene like 2 (MYBL2) was decreased about 91 times in short NuMA overexpressed cells. MYBL2 could activate the cell division cycle 2, cyclin D1, and insulin-like growth factor-binding protein 5 genes. However, further analysis was needed to unveil the exact mechanism for short NuMA to suppress the expression of MYBL2.

**Methods**

**Materials**

The cell lines were cultured with RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and maintained in 5% CO₂ at 37°C. The DNA ladders and Trans2K Plus Marker were purchased from TransGen Biotech (Beijing, China). Running buffer was prepared by adding ethidium bromide solution to 1 × Tris/boric acid/EDTA buffer at a concentration of 0.5 mg/ml. AS2037 (an autoimmune antibody for recognizing NuMA) and AS2057 (an autoimmune antibody for recognizing NuMA) were selected in gentamicin (G418; Life Technologies, USA) at a concentration of 800 μg/ml. After 2 weeks of selection, cell lines determined by real-time quantitative polymerase chain reaction (PCR). The first-strand cDNA was produced from 2 μg total RNA with M-MLV Reverse Transcriptase (Promega, USA). Primers for reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qPCR) were listed in Table 2. The qPCR was performed using the SYBR Premix Ex Taq II (Tli RNaseH Plus) (TAKARA, Japan) with the cDNA template and detected using the ABI 7500 Real-time PCR System (Life Technologies, USA). The relative expression of the target genes was calculated using the 2^(-ΔΔCt) method.

**Expression vectors and transfection**

The coding sequence of NuMA1 short isoforms was amplified using FastPfu DNA Polymerase (Transgen Biotech, Beijing, China) with cDNAs from SGC7901 cells. The amplicon was cloned in frame with green fluorescent protein (GFP) or glutathione S-transferase (GST) in the pEGFP-C1 or pGEX-4T-2 vectors, respectively. Lipofectamine 2000 (Invitrogen, USA) was used to transfect plasmid DNA into HeLa cells. Twenty-four hours after transfection of pEGFP-C1-NS, stable transfectants were selected in gentamicin (G418; Life Technologies, USA) at a concentration of 800 μg/ml. After 2 weeks of selection, clones of resistant cells were isolated. Integration

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Table 1: Detailed information about nine gastric carcinoma samples

| No. | Gender | Age (years) | TNM | Stage | DM | Operation date | Death date | FFD | ST (days) | SS |
|-----|--------|-------------|-----|-------|----|----------------|------------|-----|-----------|----|
| 1   | Male   | 48          | T3N0M0 | 2     | NA | October 16, 2003 | August 15, 2009 | October 8, 2008 | 2137 | Dead |
| 2   | Male   | 58          | T3N2M0 | 3b    | NA | July 22, 2003 | November 30, 2005 | August 1, 2005 | 862  | Dead |
| 3   | Male   | 68          | T3N3M0 | 4     | NA | August 21, 2003 | July 3, 2006 | May 9, 2004 | 1047 | Dead |
| 4   | Male   | 62          | T4N1M0 | 4     | Pancreas tail | October 14, 2003 | September 22, 2008 | September 15, 2008 | 1803 | Dead |
| 5   | Male   | 62          | T3N1M0 | 3a    | NA | September 17, 2003 | November 21, 2005 | September 9, 2005 | 796  | Dead |
| 6   | Male   | 74          | T4N1M0 | 4     | Diaphragm invasion | September 9, 2003 | February 1, 2005 | September 21, 2004 | 511  | Dead |
| 7   | Male   | 65          | T2N1M0 | 2     | NA | December 8, 2003 | December 31, 2006 | December 23, 2005 | 1119 | Dead |
| 8   | Male   | 65          | T3N2M0 | 3b    | NA | September 28, 2003 | December 30, 2004 | August 11, 2004 | 368  | Dead |
| 9   | Male   | 64          | T3N1M0 | 3a    | NA | August 5, 2003 | July 31, 2008 | June 15, 2008 | 1822 | Dead |

TNM: Tumor/lymph node/metastasis; DM: The distal metastasis; FFD: Final follow-up date; ST: Survival time; SS: Survival state; NA: Not available.
of transfected plasmid DNA was confirmed by PCR and Western blot analyses.

**Immunofluorescence assay**

The procedures for immunofluorescence assay were adapted from our previous report.[9,10] Cells grown on coverslips were washed three times with phosphate-buffered saline (PBS) followed by fixation in cold methanol for 10 min. The primary antibodies included AS2033 (a centriole-specific autoimmune antibody) and AS2057 (an autoimmune antibody for recognizing NuMA). The secondary antibodies were TRITC-conjugated donkey anti-human IgG (Jackson ImmunoResearch Laboratories, USA). The images were acquired using an Olympus Confocal FV100 Microscope.

**Soft agar colony formation assay**

The procedures for colony formation assay were adapted from previous reports.[11,12] 1 × 10^4 transfected cells were resuspended in 1 ml Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum and 0.3% low-melting agar (GE Healthcare, USA) and plated on 2-cm dishes containing a solidified bottom layer made of 0.6% agar in DMEM. Then, the agar plates were placed in the incubator. Twenty days later, colonies with a diameter 100 μm were counted using a low-power (×10) magnification microscope.

**Pull-down assay**

The soluble GST-tagged short NuMA1 was extracted and then washed in a Glutathione Sepharose column with 20 ml PBS buffer to remove the alcohol residue. The bacterial lysates passed the column at a flow rate of 1 ml/min and were washed with 30 ml PBS buffer containing 1% Triton. A 1 ml elution buffer was added containing reduced glutathione, and the mixture was incubated for 10 min.

The cells (mitotic and interphase cells) were collected and washed with PBS three times. NTEN300 and protease inhibitors were added on ice for 30 min, and then the cells were centrifuged at 13,400 ×g for 20 min to extract whole proteins. Washing with Glutathione Sepharose beads was performed with TEN100 buffer three times, followed by incubating with 25 μg purified GST-tagged short NuMA1 with 25 μl beads for 4 h at 4°C. The beads were washed with 200 μl TEN100 buffer and centrifuged at 160 ×g for 5 min; this was repeated three times. The cell lysates were incubated with the beads at 4°C for 24 h. The beads were washed with TEN100 buffer three times, followed by the addition of 25 μl 2× sodium dodecyl sulfate (SDS) loading buffer. The mixture was boiled for 5 min and centrifuged at 13,400 ×g for 20 min. The clear supernatants were electrophoresed by SDS-PAGE and stained with Coomassie brilliant blue. The bands with differential staining were sliced for identification by matrix-assisted laser desorption ionization time of flight mass spectrometer (MALDI-TOF-MS).

**DNA microarray**

Total RNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA), in accordance with the manufacturer’s instructions. The integrity of the RNA was checked by electrophoresis (Agilent 2100 Bioanalyzer). The procedure for microarray analysis was based on the standard Agilent Technologies protocol. DNase treatment of the RNA was done during the purification procedure using an RNase-Free DNase Kit (Qiagen). Twenty micrograms of total RNA was reverse transcribed using an oligo dT12-18 primer and aminoallyl-dUTP. The cDNA was then reacted with N-hydroxysuccinimide esters of Cy3 or Cy5 (GE Healthcare, Buckinghamshire, UK), according to the manufacturer’s instructions. Dye molecules were separated from the labeled products using a QIAquick PCR Purification Kit (Qiagen). Cy3-labeled cDNA from the control sample was mixed with the same amount of Cy5-labeled cDNA from the test sample. The mixture was then applied to the microarray (Whole G4112A, covering 41,000 unique genes and transcripts; Agilent Human Genome), and hybridization was performed for 17 h at 60°C, according to the manufacturer’s instructions. After hybridization, the slides were washed and scanned using a confocal laser scanner (Agilent G2565BA). The fluorescence intensities on the scanned images were quantified, corrected for background fluorescence, and normalized using global normalization methods, based on the assumption that the median value of the fluorescence intensities of both samples should be the same.

**Statistical analysis**

Data were statistically analyzed using SPSS statistical software version 20.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation (SD) for quantitative variables or as percentages for qualitative variables. A P < 0.05 was considered statistically significant.

**Results**

Proteins encoded by short isoforms were localized in the cytoplasm during cell cycle

As for the short NuMAs, according to the UCSC genome browser, there existed three types of short isoforms transcribed...
from alternative promoters with identical open reading frames (ORFs) [Figure 1a]. To study the localization pattern of proteins encoded by short isoforms, the ORF of short isoforms was cloned in frame with GFP in pEGFP-C1 and transfected into HeLa cells. Our previous immunofluorescence analysis[9] showed that GFP-fused long and middle isoforms of NuMA were mainly localized in the nucleus during interphase and the spindle poles at metaphase. Due to lack of coiled-coil and C-terminus domains, the GFP-tagged short isoform of NuMA was mainly localized at the cytoplasmic region during the whole cell cycle [Figure 1b]. Besides, the expression of short NuMA was highly expressed in S and G2 phases of the cell cycle determined by real-time quantitative polymerase chain reaction (qRT-PCR) [Figure 1c].

**Short nuclear mitotic apparatus protein suppressed cell growth**

The expression levels of short isoforms were assessed by qRT-PCR in matched tumor/nontumor tissues from nine GCs; compared with nontumor tissues, short NuMA displayed significantly lower expression in paired tumor tissues [Figure 2a]. Moreover, to investigate the function of short isoforms, GFP-tagged short NuMA1 (GFP-NS) was transfected into HeLa cells to assess the influence of its overexpression on the cell growth, with GFP alone as the control. As shown in the cell growth curve in Figure 2b, the overexpression of short isoform incurred a significant decrease in the proliferation rate at the 4th day (GFP-NS vs. GFP-Blank = 2.43 × 10^5 vs. 2.97 × 10^5, \( P = 0.0029 \)). Cell colony formation experiment displayed that short NuMA possessed the ability to greatly decrease the formation of colonies compared with cells transfected with GFP alone [2 vs. 70, \( P = 1.67 \times 10^{-45} \); Figure 2c].

**Short isoforms could bind with alpha-actinin-4**

The ORF of short NuMA1 was fused in frame with GST in pGEX-4T-2 system. The GST-tagged protein was immobilized on GTH (glutathione)-coated resins. Proteins bound with product of short NuMA were electrophoresed by SDS-PAGE and stained by Coomassie brilliant blue [Figure 3]. The differentially stained bands (band 1, 2, and 3) were sliced.
and detected by MALDI-TOF-MS [Figure 3 and Table 3]. Interestingly, except NuMA1 itself, actin-like proteins such as ACTN1, ACTN4, and actin cytoplasmic 2 were found to bind with short isoform of NuMA. Considering that ACTN4 played important roles in promoting tumor cell proliferation, the direct interaction relationship awaits further investigation.

**DISCUSSION**

NuMA is a cell cycle-associated protein that involved in mitotic spindle assembly and maintenance, spindle positioning, and asymmetric cell division. To our knowledge, the long isoform of NuMA was regarded as the only type of NuMA and was extensively investigated during the last three decades. However, based on mRNA and EST sequences, the short isoform of NuMA was identified and confirmed in different tissues. Compared with the long isoform proteins, the putatively encoded protein from short NuMA1 mRNA only contained the globular N-terminus of the long isoform protein. Since the C-terminus of NuMA containing the nuclear localization signals (NLSs), we deduced that products encoded by the short isoform would mainly exist in the cytoplasm region, instead of the nuclear region or spindle poles. As the immunofluorescence assay revealed, the long and middle isoforms existed in the nuclear during interphase of the cell cycle and spindle poles during the metaphase. However, the proteins of the short isoform mainly localized in the cytoplasmic region during the whole cell cycles. According to our previous results, the expression of short isoform was significantly downregulated in many cancerous cell lines. The expression levels of short isoforms were further checked in nine pairs of matched GC tissues in this study. As expected, short isoform was greatly decreased in the gastric cancer tissues. Therefore, we inferred that proteins of short isoforms could play a role as a putative tumor suppressor during the pathogenesis. Cell growth curve analysis showed that Short NuMA possessed the ability to inhibit the proliferation of HeLa cells. Cell colony formation assay displayed that the short isoform could greatly restrain the formation of cell colonies.

Next, a pull-down assay was performed to capture proteins possessing the ability to bind short NuMA. Interestingly, only actin-like proteins were pulled down, such as ACTN1, ACTN4, and actin cytoplasmic 2 (ACTG1). Actin-like proteins had been reported to be related with the pathogenesis of tumors. ACTN4 had already been demonstrated to

| Item | Accession | Peptides | AAs | MW (×10^3) | Description                           |
|------|-----------|----------|-----|-------------|---------------------------------------|
| NC   | P02769    | 14       | 607 | 69.2        | Serum albumin                         |
|      | P00761    | 5        | 231 | 24.4        | Trypsin                               |
| 1    | IPI00006196.3 | 19       | 2101 | 236.0       | Isoform 2 of nuclear mitotic apparatus protein 1 |
| 2    | IPI00013808.1 | 29       | 911  | 104.8       | Alpha-actinin-4                       |
| 3    | IPI00909239.1 | 17       | 887  | 102.6       | Actinin, alpha 1 isoform c            |
|      | IPI00021440.1 | 25       | 375  | 41.8        | Actin, cytoplasmic 2                  |

NC: Negative control; MW: Molecular weight; AAs: Amino acids; MALDI-TOF-MS: Matrix-assisted laser desorption ionization time of flight mass spectrometer.
Figure 3: SDS-PAGE for the pulled-down proteins by short NuMA1. HeLa cells from interphase (lane 6–9) and mitosis (lane 2–5) were captured and lysed independently. Lane 1, Bovine serum albumin (5 μg); lane 2, GST beads + lysate (mock); lane 3, GST-CDCA4 + GST beads + lysate; lane 4, GST-Blank (GB) + GST beads + lysate (negative control); lane 5, GST-NS (GNS) + GST beads + lysate; lane 6, GST-NS (GNS) + GST beads + lysate (negative control); lane 7, GST-Blank (GB) + GST beads + lysate; lane 8, GST-CDCA4 + GST beads + lysate; lane 9, GST beads + lysate (mock); lane 10, PageRuler prestained protein ladder (180,000, 130,000, 95,000, 72,000, 55,000, 43,000, 34,000, and 26,000). Arrows represented bands for mass spectrometry. NuMA1: Nuclear mitotic apparatus protein 1; GST: Glutathione S-transferase.

Table 4: Top ten of differentially expressed genes affected by short NuMA1

| Ratio (GFP_B/GFP_NS) | Downregulated genes | Ratio (GFP_B/GFP_NS) | Upregulated genes |
|----------------------|---------------------|---------------------|------------------|
| 96.21947             | ASB9                | 0.09259974          | LOC651536        |
| 91.93533             | MYBL2               | 0.08897984          | SLC2A3           |
| 9.407033             | EPB4I               | 0.07938801          | SCGB3A2          |
| 8.985102             | EPB4IL3             | 0.07224974          | PTGIS1           |
| 8.939297             | HS.127715           | 0.0711004           | CIDECA           |
| 8.878217             | FAM89A              | 0.06929374          | KCNJ16           |
| 8.713161             | ATXN2L              | 0.04026269          | C9orf70          |
| 8.349680             | RGN EF              | 0.03015087          | LOC643912        |
| 8.151778             | AES                  | 0.01847595          | ZNF467           |
| 8.116800             | KIAA00895L          | 0.00970121          | HS.385760        |

GFP_B: GFP blank; GFP_NS: GFP-tagged short NuMA1; NuMA1: Nuclear mitotic apparatus protein 1; GFP: Green fluorescent protein.

Figure 3: SDS-PAGE for the pulled-down proteins by short NuMA1. HeLa cells from interphase (lane 6–9) and mitosis (lane 2–5) were captured and lysed independently. Lane 1, Bovine serum albumin (5 μg); lane 2, GST beads + lysate (mock); lane 3, GST-CDCA4 + GST beads + lysate; lane 4, GST-Blank (GB) + GST beads + lysate; lane 5, GST-NS (GNS) + GST beads + lysate; lane 6, GST-NS (GNS) + GST beads + lysate; lane 7, GST-Blank (GB) + GST beads + lysate; lane 8, GST-CDCA4 + GST beads + lysate; lane 9, GST beads + lysate (mock); lane 10, PageRuler prestained protein ladder (180,000, 130,000, 95,000, 72,000, 55,000, 43,000, 34,000, and 26,000). Arrows represented bands for mass spectrometry. NuMA1: Nuclear mitotic apparatus protein 1; GST: Glutathione S-transferase.

Promote tumor cell proliferation.

ACTN4 harbors a functional LXXLL receptor interaction motif, interacts with nuclear receptors in vitro, and potently activates transcription mediated by nuclear receptors. Therefore, a new hypothesis was put forward regarding the functional mechanism of short NuMA during cancer progression. The overexpression of short NuMA competitively binds with ACTN4 and inhibits ACTN4’s nuclear localization which decreases the expression of genes controlling cell cycle progression. We also analyzed the differentially expressed genes affected by short NuMA by gene expression chips. Totally, 1158 differentially expressed genes (389 genes upregulated and 769 genes downregulated) were selected, of which MYBL2 was downregulated about 91 times in short NuMA overexpressed cells [Table 4]. The protein encoded by MYBL2 is a member of the MYB family of transcription factor genes and is a nuclear protein involved in cell cycle progression. MYBL2 could be phosphorylated by cyclin A/cyclin-dependent kinase 2 during the S phase of the cell cycle. It has been shown to activate the cell division cycle 2, cyclin D1, and insulin-like growth factor-binding protein 5 genes. However, it is not clear about the exact mechanism for short NuMA to suppress the expression of MYBL2.

In conclusion, based on our previous results, the structure of NuMA1 short isoform was distinct from the long and middle isoforms, for it only contained the N-terminal globular domain without the NLS. Short isoform of NuMA might be functioned as a putative role of tumor suppressor. This preliminary work suggested a novel function of NuMA short isoform and also pointed out new frontier for NuMA research.

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Conflicts of interest

There are no conflicts of interest.

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