Original Article

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

Wang-Sen Qin[1], Jin Wu[2], Yang Chen[3], Fa-Cai Cui[1], Fu-Ming Zhang[1], Guan-Ting Lyu[4], Hong-Mei Zhang[2]

[1]Department of Clinical Laboratory, Henan Provincial People's Hospital, Zhengzhou, Henan 450003, China
[2]Department of Clinical Oncology, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032, China
[3]Central Laboratory, Haikou People's Hospital, Haikou, Hainan 570208, China
[4]Department of Blood Transfusion, Tangdu Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032, China

Wang-Sen Qin and Jin Wu contributed equally to this work.

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, $P = 5 \times 10^{-4}$]; GC2 [0.316, $P = 3 \times 10^{-5}$]; GC3 [0.111, $P = 6 \times 10^{-4}$]; GC4 [0.456, $P = 0.011$]; GC5 [0.474, $P = 0.001$]; GC6 [0.311, $P = 0.004$]; GC7 [0.28, $P = 3 \times 10^{-5}$]; GC8 [0.298, $P = 0.007$]; and GC9 [0.344, $P = 0.002$]). Besides, high expression of short NuMA significantly inhibits cell growth (2.43×10^5 vs. 2.97×10^5, $P = 0.0029$) and cell clone information *in vitro* (70 vs. 2, $P = 1.67 \times 10^{-45}$). 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.[4-6] It had been reported that ectopic expression of NuMA, including interference of NuMA by microinjection of its antibodies,[7] mutations at the spherical domains at the amino or carboxyl terminal domains,[5] 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

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.211535

Address for correspondence: Dr. Hong-Mei Zhang, Department of Clinical Oncology, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710032, China
E-Mail: zhm@fmmu.edu.cn

Received: 26-03-2017 **Edited by:** Li-Min Chen
How to cite this article: Qin WS, Wu J, Chen Y, Cui FC, Zhang FM, Lyu GT, Zhang HM. The Short Isoform of Nuclear Mitotic Apparatus Protein 1 Functions as a Putative Tumor Suppressor. Chin Med J 2017;130:1824-30.

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).[9] 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% CO_2 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. AS2033 (a centriole-specific autoimmune antibody) and AS2057 (an autoimmune antibody for recognizing NuMA) were maintained in our laboratory. Detailed GC samples were described in Table 1.

Synchronization of cell cycle

The rapidly growing adherent HeLa cells were shaken at a specific time interval. The collected cells were dispersed in the medium and were at the M phase. The cell culture was continued at the M phase for another 1–10 h until they were at the G1 phase. The cultured cells reach an exponential growth phase with medium containing 2 mmol/L thymine ribonucleoside (TdR) after 16 h. The cells were washed with Hanks buffer 2 or 3 times, and fresh medium was added to culture the cells for another 9 h. Medium containing 2 mmol/L TdR was added to the culture for another 16 h, and the cells in the medium were washed with Hanks buffer another 2 or 3 times. These cells were at the G1/S phase. If the culture was continued for another 2–7 h, they would reach the S phase.

Reverse transcription polymerase chain reaction and real-time quantitative polymerase chain reaction

Total RNA was isolated using TRIZOL reagent (Invitrogen, USA). 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 $2^{-\Delta\Delta Ct}$ 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

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	September 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.

Table 2: Primers for RT-PCR and qRT-PCR

Primer	Sense primers (5'→3')	Anti-sense primers (5'→3')
GFP-NS	AATGAATTCGATGACACTCCACGC	ACCGGATCCATTACAGCACACTATTG
GST-NS	AGTGGATCCAAGATGACACTCCACGCCA	ACCGTCGACCGATTACAGCACACTATTGAACC
NS_q	TCCTTTAAGCTGCGGGAG	ACTGGACTCAGCTTTGCACA
GAPDH_q	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC

GFP-NS: GFP-tagged short NuMA1; GST-NS: GST-tagged short NuMA1; NS_q: Quantitative primers for NuMA1 short isoform; GAPDH_q: Quantitative primers for GAPDH; RT-PCR: Reverse transcription polymerase chain reaction; qRT-PCR: Real-time quantitative polymerase chain reaction; NuMA1: Nuclear mitotic apparatus protein 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

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] 1×10^3 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

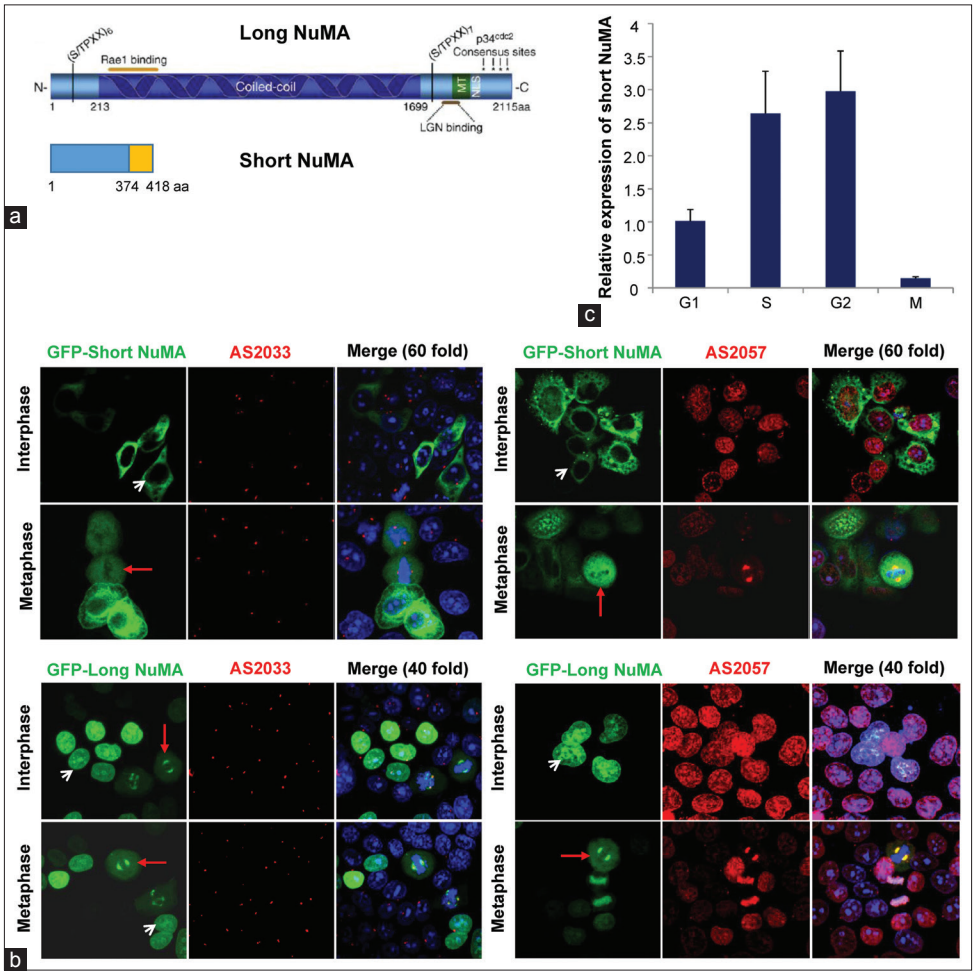

Figure 1: Expression of short NuMA1 in cell cycles and subcellular localization. (a) Structures for long and short isoforms of NuMA1. (b) white arrow heads represent interphase cells; red arrows represent metaphase cells. AS2033 and AS2057 autoimmuno-antibodies could specifically recognize the antigen of centrosome and NuMA, respectively. S2057 (an autoimmune antibody for recognizing NuMA). The secondary antibodies for immunofluorescence assay were TRITC-conjugated donkey anti-human IgG (Jackson ImmunoResearch Laboratories, USA). The magnification folds for short NuMA and long NuMA were 60 and 40, respectively. Subcellualr localization of short NuMA1 at interphase and metaphase. AS2033 (red) represented the centrosome localization and AS2057 (red) represented the NuMA localization. (c) Expression of short isoform detected by quantitative PCR in different cell cycles. NuMA1: Nuclear mitotic apparatus protein 1; PCR: Polymerase chain reaction.

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.[13-17] 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.[13] However, based on mRNA and EST sequences, the short isoform of NuMA was identified and confirmed in different tissues.[9] 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 *NuMA1* 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.[9] 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.[18] ACTN4 had already been demonstrated to

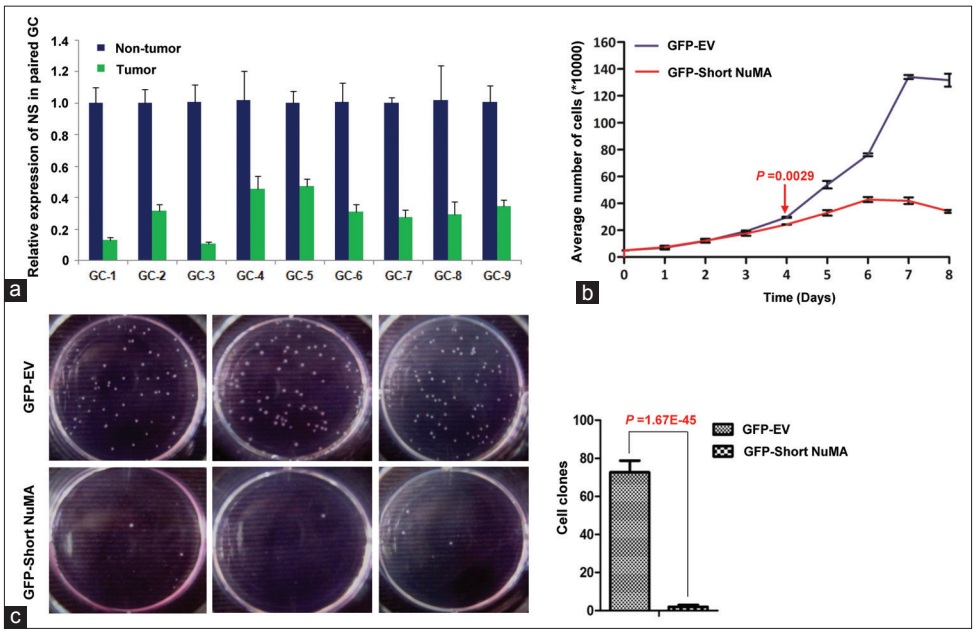

Figure 2: Short NuMA1 functioned as tumor suppressor. (a) Expression of short isoform highly decreased in gastric cancerous tissues. (b) Overexpression of short isoform inhibited the cell proliferation. (c) Overexpression of short isoform decreased the formation of cell colonies. NuMA1: Nuclear mitotic apparatus protein 1.

Table 3: Proteins bound with short isoform identified by MALDI-TOF-MS

Item	Accession	Peptides	AAs	MW (×10³)	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
	IPI00909239.1	17	887	102.6	Actinin, alpha 1 isoform c
3	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.

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	*EPB41*	0.07938801	*SCGB3A2*
8.985102	*EPB41L3*	0.07224974	*PTGS1*
8.939297	*HS.127715*	0.07111004	*CIDEC*
8.878217	*FAM89A*	0.06923974	*KCNJ16*
8.713161	*ATXN2L*	0.04026269	*C9ORF70*
8.349680	*RGNEF*	0.03015087	*LOC643912*
8.151778	*AES*	0.01847595	*ZNF467*
8.116800	*KIAA0895L*	0.00970121	*HS.385760*

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

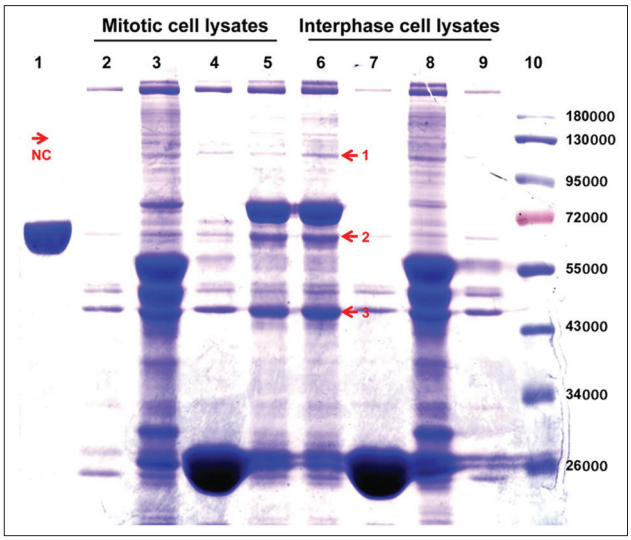

Figure 3: SDS-PAGE for the pulled-down proteins by short NuMA1. HeLa cells from interphase (lane 6–9) and mitosis (lane 2–5) were captured and lysed independently. Lane 1, Bovine serum albumin (5 μg); lane 2, GST beads + lysate (mock); lane 3, GST-CDCA4 + GST beads + lysate; lane 4, GST-Blank (GB) + GST beads + lysate (negative control); lane 5, GST-NS (GNS) + GST beads + lysate; lane 6, GST-NS (GNS) + GST beads + lysate; lane 7, GST-Blank (GB) + GST beads + lysate (negative control); 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.[19-23] ACTN4 harbors a functional LXXLL receptor interaction motif, interacts with nuclear receptors *in vitro*, and potently activates transcription mediated by nuclear receptors.[23] 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.[24] MYBL2 could be phosphorylated by cyclin A/cyclin-dependent kinase 2 during the S phase of the cell cycle.[25-28] It has been shown to activate the cell division cycle 2, cyclin D1, and insulin-like growth factor-binding protein 5 genes.[29] 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.

Financial support and sponsorship

This work was supported by grants from the National Natural Science Foundation (No. 81101490 and No. 81572699).

Conflicts of interest

There are no conflicts of interest.

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