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Identification and characterization of novel NuMA isoforms

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ABSTRACT

The large nuclear mitotic apparatus (NuMA) has been investigated for over 30 years with functions related to the formation and maintenance of mitotic spindle poles during mitosis. However, the existence and functions of NuMA isoforms generated by alternative splicing remains unclear. In the present work, we show that at least seven NuMA isoforms (categorized into long, middle and short groups) generated by alternative splicing from a common NuMA mRNA precursor were discovered in HeLa cells and these isoforms differ mainly at the carboxyl terminus and the coiled-coil domains. Two "hotspot" exons with molecular mass of 3366-nt and 42-nt tend to be spliced during alternative splicing in long and middle groups. Furthermore, full-length coding sequences of long and middle NuMA obtained by using fusion PCR were constructed into GFP-tagged vector to illustrate their cellular localization. Long NuMA mainly localized in the nucleus with absence from nucleoli during interphase and translocated to the spindle poles in mitosis. Middle NuMA displayed the similar cell cycle-dependent distribution pattern as long NuMA. However, expression of NuMA short isoforms revealed a distinct subcellular localization. Short NuMA were present in the cytosol during the whole cycle, without colocalization with mitotic apparatus. These results have allowed us tentatively to explore a new research direction for NuMA's various functions.

1. Introduction

The large nuclear mitotic apparatus (NuMA) protein has been investigated for over 30 years since it was first identified by Lydersen and Pettijohn for its special distribution on both interphase nucleus and mitotic spindle poles [1,2]. Recent studies have demonstrated important roles of NuMA in completion of mitosis, post-mitotic nuclear reassembly [3,4] and tethering spindle microtubules to the spindle poles [5]. Moreover, abnormal expression of NuMA, including depletion of NuMA by microinjection of NuMA antibodies [6], mutation at amino or carboxyl terminal domains [7], and overexpression of NuMA [8], would result in aberrant spindles. Besides, NuMA participates in spindle positioning, asymmetric cell division [9–12] and some functions in nuclear at interphase, such as functioning as the nuclear scaffold structure [13–16], interacting with splicing factors [17] and promoting homologous recombination repair [18]. However, little information about NuMA isoforms generated by alternative splicing has been reported except for several articles associated with NuMA isoforms by using NuMA antibodies [19–21]. These bands recognized by NuMA antibodies may not truly reflect mature NuMA isoforms for the failure search of the corresponding mRNA sequences in the NCBI Core Nucleotide and UCSC Genome Browser database.

Previous bioinformatics analysis on NuMA mRNA sequence demonstrated that NuMA might have different isoforms which can be categorized into three groups based on the sequence length: long, middle and short (Fig. 1A). It's difficult to obtain the full-length mRNA of long and middle NuMA by traditional PCR for the long length sequence and low abundance, respectively. To overcome this problem, a novel overlap extension PCR, designated as fusion PCR, was developed to integrate an intact DNA sequence by fusing several DNA fragments independent of DNA digestion and ligation [22]. Recent fusion PCR includes two

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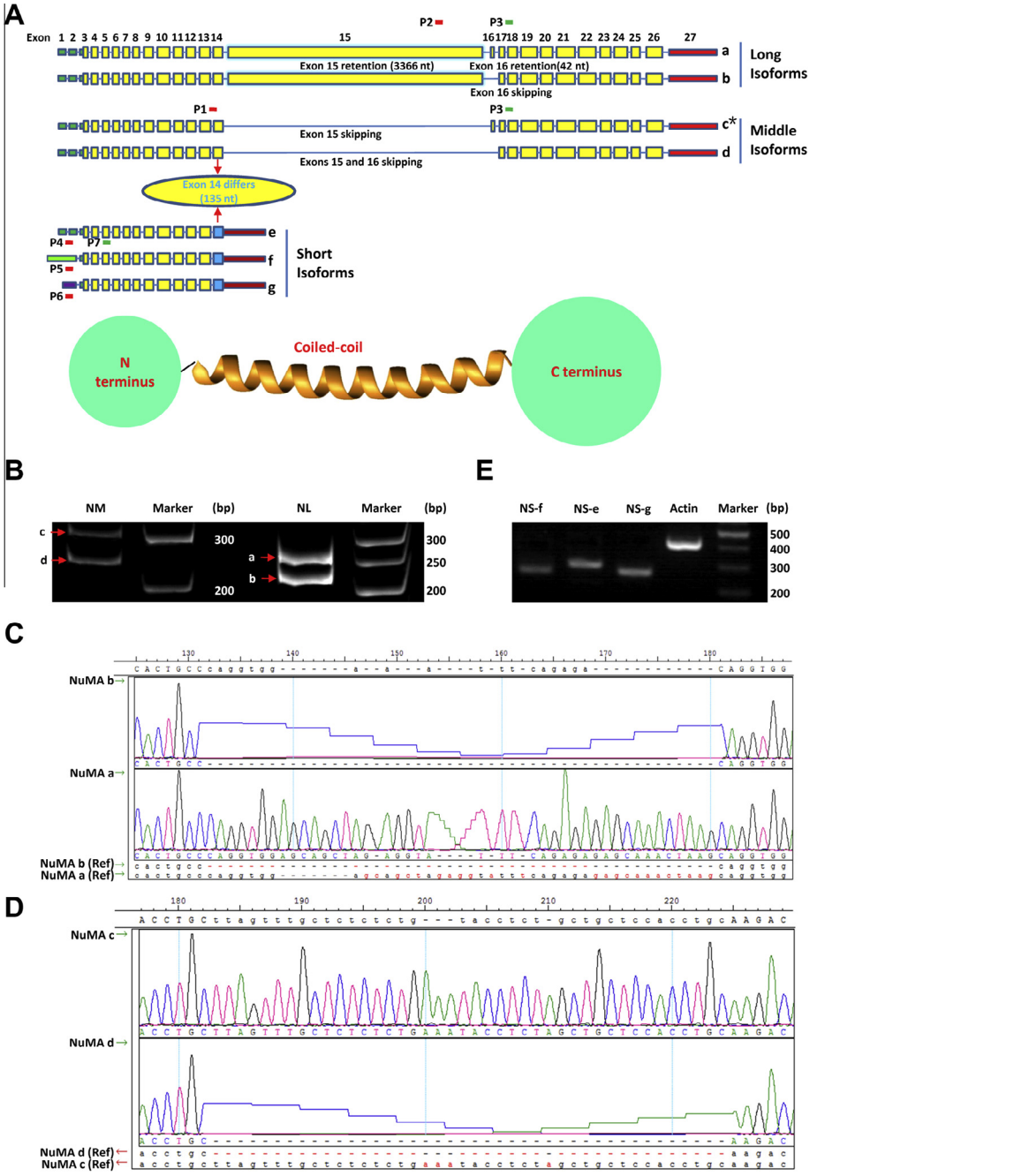

Fig. 1. Three type isoforms of NuMA derived from alternative splicing. (A) Schematic representation of NuMA isoforms. Following the NCBI designation of isoforms (a) and (b), four additional mRNA sequences from the UCSC Genome Browser and one novel splicing product were observed in this study (marked with the asterisk), named as isoforms (c)–(g). The 5′ UTRs are shown as colored bars (deep green, light green, and purple) and the 3′ UTRs are shown as red bars. The exons are indicated by solid rectangles with yellow for internal exons and blue for exons with 135-bp difference in the short NuMA, separated by blue lines indicating the introns. Each pair of PCR primers (P1–P7) is labeled with the cDNA position with red box showing the sense and green box showing the antisense probes. The bottom part describes the three protein domains of NuMA with exon positions lined up with the isoform structures above. (B) Fragments of NuMA isoform in long and middle groups were resolved in Nuclear PAGE Gels. Two bands with molecular mass between 200 and 300 bp were detected in long (a and b) and middle (c and d) types, respectively. (C) Sanger sequencing demonstrated that two products amplified by using P2–P3 primers could be perfectly matched with the reference sequences of long NuMA. (D) Sanger sequencing proved that a novel member in the middle type was discovered with 42-bp exon retention. (E) Three isoforms in the short type were proved to express in HeLa cells in which NuMA-f displayed the lowest expression after normalization with the expression of an internal control (actin). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

steps: (1) target sequences were amplified respectively by specific primers which has a complementary sequence of adjacent segment at its N-terminus. (2) Add the mixture of each fragment in the same reaction system and amplified the fusion fragments by outer primer pairs. Fusion PCR could be used for the in-frame fusion of two functional genes from the same or different species. Furthermore, it could also be used for amplification of long fragments with low copy number. This technology is suitable for amplification of full-length mRNA of long and middle NuMA in this study (see Section 2).

Recent study has described multiple isoforms of NuMA generated by alternative splicing, uncovered the expression pattern of these isoforms in different cell lines, and illustrated the subcellular localization of these isoforms.

2. Materials and methods

2.1. Cells and reagents

All cell lines were cultured with RPMI-1640 medium (HyClone, US) containing 10% fetal bovine serum (Gibco, US) and maintained at 37 °C in 5% CO_2. All reagents used in the present study were of analytical or biochemical grade. 30% acrylamide–bisacrylamide solution, 10% ammonium persulfate, Tris–borate–EDTA (5× TBE) and TEMED were obtained from CWBIO (Beijing, China), A mixture of DNA ladders, trans2K plus marker and 6× loading buffer were purchased from TransGen (Beijing, China). Running buffer was prepared by adding ethidium bromide solution to 1× TBE buffer at a concentration of 0.5 mg/mL. The procedure for extraction of DNA fragments using nuclear PAGE was similar to that described by Kaneta et al. [23].

2.2. Reverse transcription PCR and real-time quantitative PCR

Total RNA was isolated using Trizol (Invitrogen, US). The cDNA was synthesized from 2 μg of total RNA with M-MLV Reverse Transcriptase (Promega, US). The primers for reverse transcription PCR (PCR) and real-time quantitative PCR (qPCR) are listed in Table 1. qPCR was carried out using the TransStart Green qPCR Super Mix kit (TransGen, China) with the cDNA template diluted 1:4 and detected using the iCycler iQTM5 Detection System (BioRad, US). The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3. Fusion PCR

Fusion PCR is a powerful tool to integrate an intact DNA sequence by fusing several DNA fragments independent of DNA digestion and ligation [22]. In this study, this method was applied for obtaining overlength sequence of long NuMA (Fig. S2B) and low abundance sequence of middle NuMA (Fig. S2A). As for long NuMA amplification, the target sequence was split into three fragments for fusion PCR (N_NuMA, 1425 nt; M_NuMA, 3240 nt; C_NuMA, 1846 nt), the homologous sequences between N_NuMA and M_NuMA or M_NuMA and C_NuMA are 89 nt or 54 nt, respectively. In separate PCR reactions, three fragments were amplified using specific primer pair (Table 1). Then, the first fusion PCR was conducted for 13 cycles using equimolar mixture of N_NuMA and M_NuMA without adding any primer. The following step was to amplify and purify the fusion product NM_NuMA, and then carried out the second fusion PCR reaction. Finally, the integrate sequence was amplified by PCR using P8-P9 primer pair. The same operation was conducted to obtain the CDSs of middle NuMA isoforms.

2.4. Expression vectors and transfection

To generate NuMA isoforms fused to the green fluorescent protein (GFP-NL, GFP-NM and GFP-NS), the coding sequences of these isoforms were amplified by polymerase chain reaction (PCR) from fusion PCR-based templates and HeLa cDNA templates and then cloned in frame with GFP in a pEGFP-C1 vector (Clontech). Lipofectamine 2000 (Invitrogen) was used to transfect plasmid DNA HeLa cells.

2.5. Immunofluorescence

Cells grown on coverslips were washed three times in phosphate-buffered saline (PBS) followed by fixation in cold methanol for 10 min. Immunofluorescence analysis was performed as described previously [24]. The primary antibodies included an anti-alpha-tubulin monoclonal antibody (Sigma, US). The secondary antibodies were TRITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, US). The images were acquired using an Olympus BX51 microscope.

2.6. Statistical analysis

The variables were analyzed with the two-tailed student *t*-test using SPSS 13.0 and GraphPad Prism 5.0. The data with the mean ± SD and a P value <0.05 were considered statistically significant.

Table 1
Primers for PCR and qPCR detection.

Target sequence	Primer pair	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Middle NuMA	P1–P3	GAACGAATCCTTCAGGGA	TCAACTGGGCTTGCAGTTCA
Long NuMA	P2–P3	AAGTATGAGGGTGCCAAG	TCAACTGGGCTTGCAGTTCA
Short NuMA_e	P4–P7	AGAGGTACGATTCCGGAG	GCCATGGATTCTGTCAATGA
Short NuMA_f	P5–P7	AAGACCCCCAGAAAGAGT	GCCATGGATTCTGTCAATGA
Short NuMA_g	P6–P7	TTACTTACTGGCCCC	GCCATGGATTCTGTCAATGA
[a]NTOM	P8–P3	TCACCAAGATGACACTCCAC	TCAACTGGGCTTGCAGTTCA
[b]CTOM	P1–P9	GAACGAATCCTTCAGGGA	GGTACTGGCCCTTTAGTGCTT
[c]NTOL	P8–P10	TCACCAAGATGACACTCCAC	CGCTCAGTCTCCAGCATCTCT
[d]MTOL	P11–P12	AGGCCAGCAGGAAGCCA	CTCTCCTCCAGGACCTTGACC
[e]CTOL	P13–P9	GCAGCGGTTCCAGG AAG	GGTACTGGCCCTTTAGTGCTT
GFP-NL	F–R	AGTGAATTCGGAGTGACTGTCTGGCATC (*EcoR* I)	AGTGGATCCCCTTTAGTGCTTTGCCTT (*BamH* I)
GFP-NM	F–R	AGTGAATTCGGAGTGACTGTCTGGCATC (*EcoR* I)	AGTGGATCCCCTTTAGTGCTTTGCCTT (*BamH* I)
GFP-NS	F–R	AATGAATTCGATGACACTCCACGC (*EcoR* I)	ACCGGATCCATTACAGCACACTATTG (*BamH* I)
[f]β-actin	F–R	GAGCTACGAGCTGCCTGA	CCTAG AAGCATTTGCGGTGG
NS_q	F–R	TCCTTTAAGCTGCGGGAG	ACTGGACTCAGCTTTGCACA
[g]GAPDH_q	F–R	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC

[a] NTOM: N terminal of middle NuMA.
[b] CTOM: C terminal of middle NuMA.
[c] NTOL: N terminal of long NuMA.
[d] MTOL: middle part of long NuMA.
[e] CTOL: C terminal of long NuMA; NL: long NuMA; NM: middle NuMA; NS: short NuMA.
[f,g] Housekeeping control.

3. Results

3.1. Three isoform groups of NuMA identified in HeLa

In order to identify the NuMA isoforms, all of the NuMA transcripts on the NCBI Core Nucleotide and UCSC Genome Browser were collected. The NuMA transcripts without mRNA polyadenylation signal adjacent to 3′ UTR or H3K27Ac and H3K4Me3 histone modification next to the 5′ UTR were discarded. Afterwards, a total of 6 NuMA transcripts were further analyzed in this study (Fig. 1A, a, b, and d–g). These transcripts were categorized into long (a and b), middle (d) and short (e–g) groups based on the sequence length and exon usage. As illustrated in Fig. 1A, the protein structure of the long group was characterized by globular head and tail domains that were separated by a coiled-coil domain [2]. The 3366-nt exon coding for the predominant region of the coiled-coil domain for long NuMA was skipped in middle group. Moreover, although the coding sequences of the three short NuMAs, including e, f and g, were identical, different transcription initiation sites might be utilized due to differences in 5′ UTR sequence (Fig. 1A and S1).

Next, these 6 NuMA isoforms were further investigated by PCR amplification using cDNA from HeLa cells and Sanger sequencing. The existence of isoforms a and b was validated by PCR amplification and sequencing using primer pair P2–P3 (Fig. 1A–C). In particular, PCR amplification using P1–P3 primer pair resulted in two products when exploring middle NuMA (Fig. 1A and B). After sequencing, these two fragments were found to differ by retention and skipping of a 42-nt exon (Fig. 1D), which was also skipped by isoform b compared with a. Therefore, we speculated the existence of a novel middle NuMA (designated as isoform c in Fig. 1A, marked with asterisk) which embraced the 42-nt exon. This isoform c was further explored that N- and C-terminal fragments (Fig. S2A and S3A–C) both covering the 42-nt exon were amplified respectively using primer pairs P8–P3/P1–P9 and then sequenced. Except for the 42-nt exon retention, the remaining sequences of N- and C-terminals were identical to that of d. All of these observations indicated that isoform c should be a novel member of NuMA middle group.

As for the short NuMAs, sequencing of the full length coding region demonstrated their identical open reading frames (ORFs) (data not shown). However, due to the differences in 5′ UTR, we were able to discriminate isoforms e, f and g using primers P4 to P7 (Fig. 1A, D and E). Furthermore, the expression of e and g was relatively higher than that of f in HeLa cells (Fig. 1E and S1E), suggesting increased transcription initiation efficiency of Promoter 1 and 3 (Fig. S1A).

3.2. The various expression of NuMA isoforms in different cell lines

After identification of the 3 NuMA isoform groups in HeLa cells, we further investigated the expression pattern of these isoforms in other cell lines.

As mentioned above, the ORFs, as well as the protein products, were identical for the 3 short NuMA isoforms. Therefore, the total mRNA expression of e, f and g was examined in this analysis. qPCR primers targeting a unique 135-nt sequence in the last exon of short NuMAs (blue in Fig. 1A) were designed and applied. As shown in Fig. 2A, expression variation of short NuMA was detected in 12 cancer cell lines derived from tumor tissues and 2 nonneoplastic cell lines. Although the expression was highest in HeLa among the tumor cells, expression of the short NuMA in nonneoplastic cells was at least 2.4-fold higher than that of HeLa, implying the involvement of short NuMA in the inhibition of tumor progression.

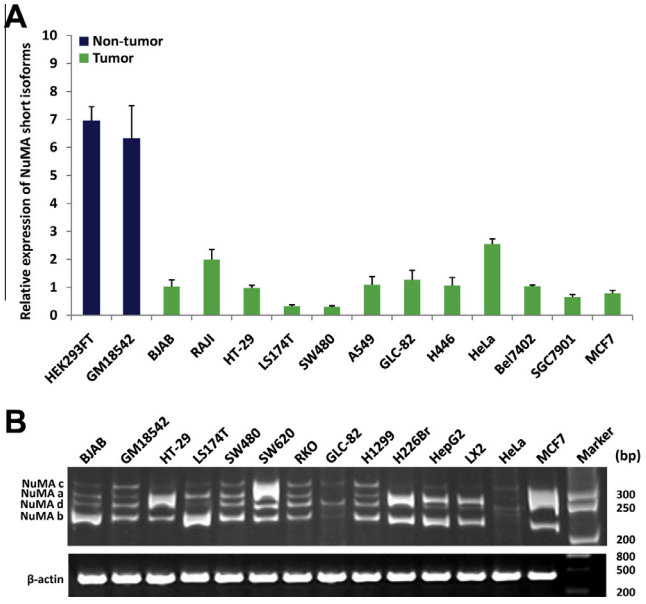

Fig. 2. Expression patterns of NuMA isoforms in different cell lines. (A) The expression of short NuMA was measured in two nonneoplastic cell lines and twelve cancer cell lines by qPCR. Lower expression of short NuMA was detected in cancer cell lines compared with nonneoplastic controls. (B) Expression of each isoform in both long and middle NuMA among different cell lines. The products amplified by using P2–P3 and P1–P3 primers are of 271 bp (a), 227 bp (b), 298 bp (c) and 257 bp (d), respectively. The β-actin was used as an internal control.

The expression of long and middle isoforms was explored using semi-quantitative PCR due to the lack of available specific primers for each isoform. The application of this technique also provided the opportunity to simultaneously compare the expression level of each NuMA isoform among different cell lines, as well as the expression level of different NuMA isoforms within the same cell line. For each of the long and middle isoform, expression variation was observed among 14 different cell lines (Fig. 2B). Furthermore, even for the cancer cell lines derived from the same tissue, such as the HT-29, LS174T, SW480, SW620 and RKO from colon cancer, this kind of expression variation was still evident. In addition, it was also illustrated that the expression level of long isoforms was higher than that of middle ones in 8 cell lines (Fig. 2B).

Taken together, all of these results showed that the expression level for each NuMA isoform group varied in distinct cell lines, suggesting different functions of NuMA isoforms.

3.3. Different localization pattern of short isoforms compared with that of long and middle NuMA

Before studying the cellular localization of NuMA, the full-length ORFs of NuMA isoforms were obtained using fusion-PCR- and conventional-PCR-based approaches (see Section 2). Then recombinant plasmids expressing GFP-tagged NuMA isoforms were constructed and transfected into HeLa cells.

Immunofluorescence analysis showed that GFP-tagged NuMA long isoform (b) was mainly localized in the non-nucleoli region of nucleus during interphase, and translocated to the spindle poles at metaphase (Fig. 3). The distribution pattern of GFP-tagged NuMA middle isoform (c) was similar to that of GFP-tagged isoform b, suggesting that, although the coiled-coil domain was largely deleted, the middle NuMA was involved in microtubule assembly.

In contrast, the GFP-tagged NuMA short isoform was mainly localized at cytoplasm during the whole cell cycle without

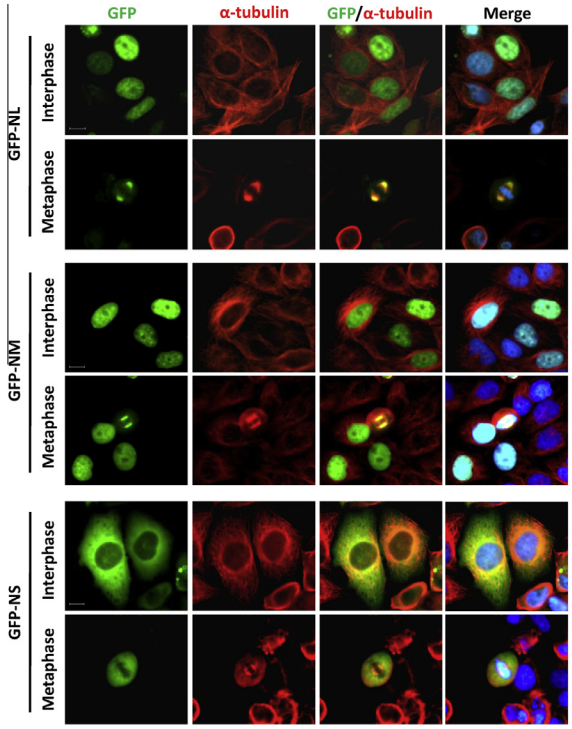

Fig. 3. Subcellular localization of NuMA isoforms from three groups. Immunofluorescence of HeLa cells transfected with GFP-tagged NuMA isoforms (green) using an alpha-tubulin antibody (red) superposed with DAPI staining (blue). During interphase, long and middle NuMAs localized in the non-nucleoli region of nucleus while short NuMA resided in the cytoplasm; at metaphase, long and middle NuMAs translocated to the mitotic apparatus while short NuMA still displayed a uniform distribution in the cytosol. NL: long NuMA; NM: middle NuMA; NS: short NuMA. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

colocalization with mitotic apparatus at metaphase (Fig. 3), implying distinct functions of the short isoforms.

4. Discussion

NuMA is a cell cycle-dependent protein that mainly participated in the processes of mitotic spindle assembly and maintenance, spindle positioning and asymmetric cell division [2,25–28]. During the past 3 decades, the long group was regarded as the only type of NuMA and was extensively investigated. As illustrated in Fig. 1A, the long NuMA was consisted of N-terminus, C-terminus and a coiled-coil domain in the middle. However, in this study, we identified the short NuMA which was only comprised of the globular N-terminus, as well as the middle NuMA with depletion of the 3366 nt exon coding for the large part of coiled-coil domain. It was likely that the function of the 3 categories of NuMA isoforms was different because of their distinct exon utilization. For instance, due to the lacking of C-terminus domain which contained the nuclear localization signal and the microtubule-binding motif, the short NuMA was hardly detected in interphase nuclear and on mitotic apparatus (Fig. 3). Moreover, the increased expression in nonneoplastic cell lines compared with that in cancer cell lines further indicated the potential involvement of short NuMA in suppressing tumor progression (Fig. 2A). The mechanism of inhibition on tumor progression by short NuMA might be mediated through its interaction with actin-like proteins [29], which have already been demonstrated to promote tumor proliferation [30,31]. As another example, although similar cellular localization during the cell cycle was observed for the middle and long NuMA, their interaction with Rae1, a messenger RNA export factor which bound with long NuMA at the coiled-coil domain [2,32], could be different. If this is the case, the skipping of the 3366 nt exon in middle NuMA might account for this difference.

In this study, the different NuMA isoforms were classified into 3 groups based on their CDS length and composition, however, the highly similar exon utilization for each member within the same group did not necessarily mean that they were completely identical. In particular, although the same CDS was utilized for the 3 short NuMA isoforms, differences in their 5′ UTR suggested that the transcription of these short isoforms were under the control of different regulation mechanisms (Fig. S1D). This speculation was further evidenced by the expression differences for the 3 short NuMA in HeLa cells (Fig. 1E). Although the total mRNA expression level for the short isoforms was examined in different cell lines, it was essential to determine the expression level for each short NuMA mRNA and clarify their distinct efficiency in guiding the protein synthesis.

Accordingly, we also postulated that the function of a and b in the long group, as well as that of c and d in the middle group, was not totally identical due to the retention and skipping of the 42-nt exon. However, sequence alignment demonstrated that the effect of the depletion for 42-nt exon showed no impact on the integrity of nuclear localization signal or the microtubule-binding motif at the C-terminus was detected. Therefore, b and c were selected as representatives of the long and middle groups, respectively, and their similarity in cellular localization justified previous prediction on the effect of 42-nt exon depletion. Moreover, among the isoforms a–d, the independence of the alternative splicing events between the 3366-nt exon and the 42-nt exon indicated that two distinct splicing signals might be accounted for the retention and skipping of these two exons.

Due to the similarity among the cDNA and amino acid sequences of different NuMA, it was difficult to distinguish each of the 7 isoforms investigated in this study. However, the isoform-specific analyses were still conducted through the application of a few well-established techniques. On behalf of the long and middle group respectively, the exogenous GFP-tagged b and c isoform of NuMA were applied in the cellular localization analysis instead of the antibody uniquely targeting protein product of each NuMA isoform. Actually, the failure to produce high-specificity and -affinity antibodies recognizing each NuMA compelled us to make such a substitution. Besides, when studying the NuMA isoform expression in different cell lines, semi-quantitative PCR was performed on the same amount of total cDNA to simultaneously determine the expression level of long and middle NuMA isoforms (Fig. 2B) because of difficulties in designing the isoform-specific qPCR primers. Although the application of these techniques only provided incomplete evidence to study NuMA isoforms, important clues, such as differences in function of short and long NuMA, could be inferred from the analyses.

Another technique of note was the fusion PCR applied to obtain the full-length ORF of the long and middle isoforms. Initially, the fusion PCR, also called overlap extension PCR, was first applied for construction of hybrid fusion genes [33]. In this study, the full-length ORF of long or middle isoforms could not be obtained in a single PCR reaction because of their overlength (more than 3 Kb for middle, and about 6 Kb for long). However, we successfully amplified 3 and 2 overlapping fragments for the long and middle isoforms (Fig. S2), and then fusion PCR was applied to obtain the full-length ORF of b and c (Fig. S3). Our work manifested the broad application of fusion PCR in exploring the overlong cDNA of certain genes.

In conclusion, according to the clues provided on the database, we initially identified NuMA isoforms, including c, a newly discovered one, in HeLa cells and then categorized them into long, middle

and short groups. Additionally, the expression level of short NuMA was increased in nonneoplastic cell lines compared with that of cancer cell lines, and the cellular localization of short NuMA was extremely different from that of long NuMA. This preliminary work not only suggested the novel function of NuMA isoforms, but also pointed out new directions for NuMA research.

Conflict of interest

We declare that all authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.104.

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