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Research Article

Modulation of SMN nuclear foci and cytoplasmic localization by its C-terminus

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Abstract. The survival of motor neuron (*SMN1*) gene product, SMN, is detected both in the cytoplasm and in nuclear gems and cajal bodies. We show here that SMN exon 6 is essential both for formation of its nuclear foci and for its cytoplasmic localization. However, exon 7 inhibits the formation of SMN nuclear foci but promotes SMN cytoplasmic localization. More interestingly, we find that a random C-terminal tag of five or more amino acids downstream of exon 6 is sufficient to inhibit the occurrence of multiple nuclear foci and to promote cytoplasmic localization of SMNΔ7, the primary product of the *SMN2* gene. Moreover, SMNΔ7 proteins that bear spinal muscular atrophy mutations in exon 6 either showed defects in nuclear foci formation or enhanced cytoplasmic localization. We conclude that exon 6 and exon 7 synergistically regulate SMN distribution that may require specific exon 6 motifs but is independent of specific sequences in exon 7.

Key words. Spinal muscular atrophy; survival motor neuron; SMN; tudor domain; Y-G motif.

Spinal muscular atrophy (SMA) is the most common hereditary disease causing infant mortality. The disease is characterized by the progressive degeneration of spinal motor neurons, resulting in paralysis and muscular atrophy. SMA is classically subdivided into three types (type I–III) based on the clinical severity and the onset age, type I, the Werding-Hoffman syndrome, being the most severe form [1]. There are two survival of motor neuron (*SMN*) genes: *SMN1* and *SMN2*. The *SMN1* gene produces mostly full-length SMN protein while the *SMN2* gene generates mostly exon 7-skipping protein (SMNΔ7) and a slight amount of full-length protein. Loss of function of the *SMN1* gene leads to SMA. The *SMN2* gene, on the other hand, is present in the majority of SMA patients but is insufficient to compensate for the *SMN1* gene defects. The functions of SMN are not yet fully understood. Through multiple interacting proteins, SMN has been implicated in assembly of small nuclear RNPs [2–4], pre-mRNA splicing [5], transcription [6–8] and apoptosis [9–11]. However, why depletion or lack of SMN protein targets only motor neurons needs clarification.

SMN is ubiquitously expressed and localized in both the cytoplasm and the nucleus, where it concentrates in gems and/or cajal bodies [12, 13]. SMN has been structurally and functionally conserved during evolution. The SMN proteins from species such as yeast, *Caenorhabditis elegans* and *Drosophila*, retain a number of properties identified in the human SMN, including RNA-binding activity and self-association [14–16]. The Y-G box of SMN exon 6 is the most conserved region. Several missense mutations have been identified in this region from SMA patients, suggesting that exon 6 is critical for SMN function [17]. However, one intriguing and puzzling fact is that exon 7, a region that is considered essential for SMN protein, is poorly conserved. Recently, Zhang and his col-

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leagues showed that exon 7 of human SMN protein is responsible for SMN cytoplasmic localization. They further mapped exon 7 and found that a five amino-acid (4–8) sequence Gln-Asn-Gln-Lys-Glu (QNQKE) in exon 7 is the cytoplasmic localization signal [18]. In contrast, our recent work shows that *Drosophila* SMN, exon 7 of which is diversified from human SMN and does not contain QNQKE, is mainly localized in the cytoplasm [11, 19], suggesting that mechanisms other than a simple sequence signal may play roles in the localization of SMN protein. To investigate these mechanisms, we generated a series of constructs with point mutations or with peptide replacements. We observed that exon 6 is not only essential for the formation of SMN nuclear foci but for SMN cytoplasmic localization as well. While exon 7 also modulates both of the cellular events, there is no requirement for specific sequences.

Materials and methods

Plasmids

Full-length, deletion mutant and missense mutant SMN or SMN7 cDNAs were cloned at the C terminus of green fluorescent protein (GFP) between the *Bgl*II and *Sal*I sites of pEGFP-C1 (Clontech). Natural SMNΔ7 (SMNΔ7n) and tagged SMNΔ7 cDNAs were generated by PCR with DNA sequences corresponding to tag peptides in the reverse primers. All plasmids were verified by sequencing analysis.

Cell culture and protein localization examination

HeLa cells were cultured at 37 °C in DMEM with 10% FBS on glass coverslips and transfected with plasmids expressing GFP fusion proteins by the standard calcium phosphate method. Twenty-four hours post transfection, cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed three times with PBS, permeablilized in 0.1% Triton X-100 in PBS for 3 min, and then incubated with 1 μg/μl of Hoechst for 15 min for nuclear staining. Cells were then washed twice with PBS and mounted on glass slides for examination of protein localization under an immunofluorescence microscope.

Results

Exon 7 inhibits nuclear aggregation and promotes cytoplasmic localization in a manner without sequence specificity

A recent report by Zhang et al. [18] suggested that a five-amino-acid sequence QNQKE in exon 7 of SMN protein serves as a signal for SMN cytoplasmic localization. Consistent with their results, we showed that the exon 7-skipping SMNΔ7 (exon 7 deleted and without exon 8 sequences) is mostly localized in bright foci in the nucleus, but full-length SMN forms cytoplasmic granules in response to stress [16, 19]. However, these data cannot explain why the *Drosophila* SMN that lacks the putative QNQKE signal is localized in the cytoplasm. To investigate the discrepancy, we first compared the amino acid sequences of exon 7 in species from human to yeast (fig. 1A), and found that exon 7 is very diversified and no consensus QNQKE signal exists in most species. We next replaced exon 7 in GFP-hSMN (human) with a 6×His tag peptide. We found that the fusion protein (SMN exons 1–6 + 6×His) was mainly localized in the cytoplasm with a few bright foci in the nucleus in HeLa cells (fig. 1B), similar to the localization pattern observed in wild-type SMN, indicating that no specific amino acid sequence in exon 7 is required to inhibit SMN nuclear localization.

To further test this notion, we analyzed the exon 7 sequence. We found that exon 7 from all species is more hydrophilic (data not shown). Therefore, we randomly selected five amino acids, Glu (negative charged), Gln (neutral), Lys (positively charged), Ile and Val (hydrophilic), based on their charges, hydrophobicity and

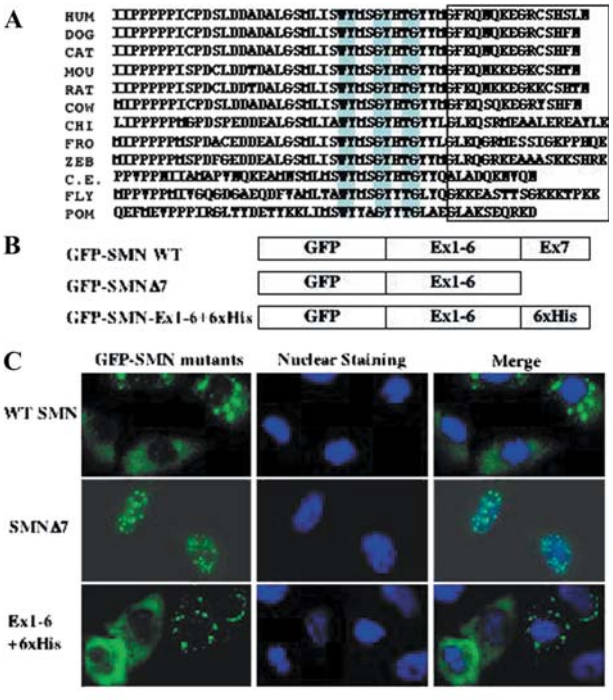

Figure 1. Exon 7 modulates SMN distribution in a non-sequence-specific manner. Ex, exon(s); HUM, human; MOU, mouse; CHI, chicken; FRO, Frog; ZEB, zebrafish; C.E., *Caenorhabditis elegans*; POM, *Schizo-saccharomyces pombe*. (*A*) Alignment of SMN C termini from different species. The conserved Y-G motif in exon 6 is highlighted. The diversified exon 7 sequences are boxed. SMNs from *Drosophila* and several other species do not contain QNQKE, the putative cytoplasmic localization signal [18]. (*B*) GFP-fusion constructs. (*C*) Plasmids were transfected into HeLa cells. Cells were stained with Hoechst (blue) 24 h after transfection, and examined under a fluorescence microscope.

polarity to test the effects of peptide properties on SMN localization. We generated constructs with six identical residues to replace exon 7 for each of these amino acids. Constructs were transfected into HeLa cells and examined under fluorescence microscopy. We observed that all fusion proteins with 6×Glu, 6×Gln, 6×Lys, 6×Ile or 6×Val at the C terminus of SMN exons 1–6 were localized into the cytoplasm, indicating irrelevance of the hydrophilic property to localization function of exon 7 (fig. 2A). We next investigated the effects of the length of C terminal tags on SMN localization. We generated constructs with 5×His, 4×His, 3×His, 2×His and 1×His added to the C terminus of SMN exons 1–6. We observed that five histidines are sufficient to promote the cytoplasmic localization of SMN. The length of a tag is correlated with the percentage of SMN cytoplamic distribution: the longer the tag, the more SMN localized into the cytoplasm (fig. 2B). Consistent with these results, we found that the natural SMNΔ7 (SMNΔ7n), the main product of the *SMN2* gene that is composed of exons 1-6 together with four amino acids Glu-Met-Leu-Ala (EMLA) derived from exon 8 at the C terminus, showed a similar nuclear distribution pattern to GFP-SMNΔ7 (only exons 1–6), but with a much stronger cytoplasmic presence in about 20–30% of transfected cells as seen for SMN exons1–6 + (2 or 3)×His (fig. 2B, C, D).

SMN exon 6 is essential for both SMN nuclear polymerization and cytoplasmic localization

The facts that the GFP-SMN exons 1–6 fusion protein is strongly distributed in the nuclear foci, and that exon 7 inhibits nuclear foci formation in a non-sequence-specific manner raise a possibility that signals in other regions of SMN coordinate or counteract exon 7 effects. To study this further, we generated a series of constructs that express GFP-SMN mutant fusion proteins (fig. 3A). Immunofluorescence assays indicated that SMN exons 3–6 (fig. 3B) exhibited identical nuclear distribution pattern to exons 1–6 (fig. 1C), while with exons 3–5 localization was similar to that of GFP alone, although 30% of transfected cells formed cytoplasmic granules. SMN exon 3–4, 6 (without exon 5) formed multiple but smaller nuclear foci, and SMN exon 3,5–6 (without exon 4) only formed a few small nuclear foci. Furthermore, exons 4–6 without exon 3 were unable to form nuclear foci as seen in exons 3–6 (fig. 3B). These results suggest that both exons 3 (tudor domain) and 6 are essential for nuclear foci formation. While exon 4 may also be important, exon 5 is not essential but may facilitate nuclear foci formation. On the other hand, similar to GFP, GFP-SMNΔ6 was diffusely distributed throughout the cell with a higher concentration in the nucleus but without formation of nuclear foci (fig. 3C), while all

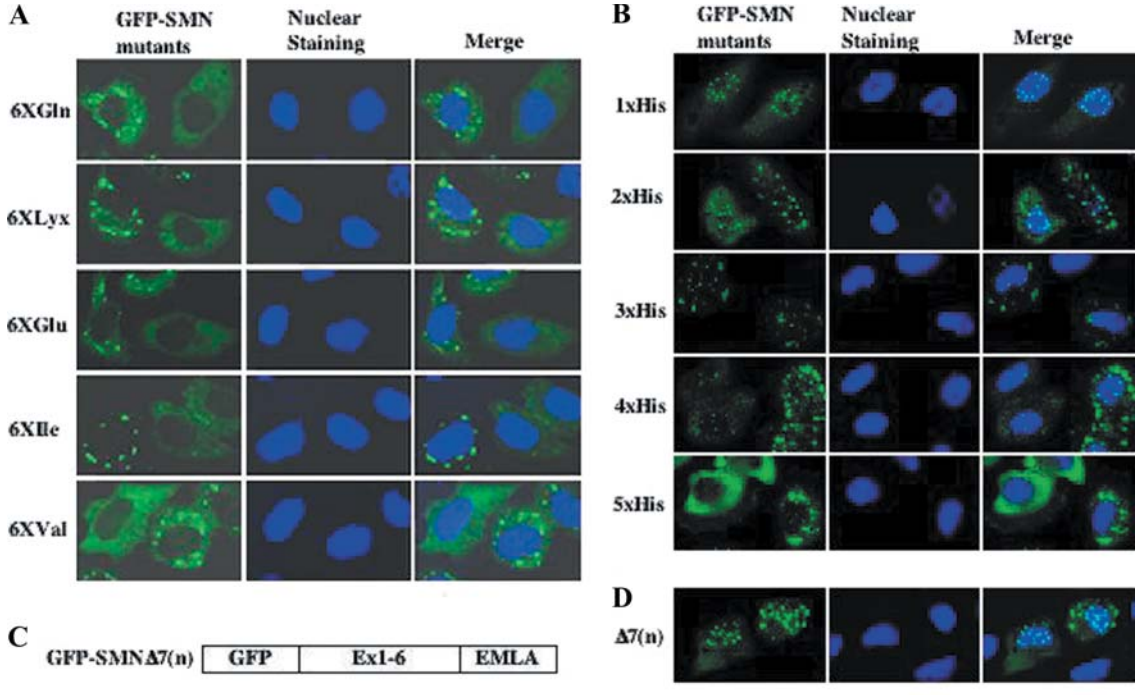

Figure 2. Replacements of exon 7 with non-specific amino acids and with different lengths. (*A*) Six amino acids representing distinct properties (charge or hydrophobicity) were fused to GFP-SMN Ex1–6 to replace exon 7 of SMN. Plasmids were transfected into HeLa cells and examined under a fluorescence microscope. Nuclei were counterstained with Hoechst (blue). (*B*) Distribution of SMNs with exon 7 replaced by one to five histidines. (*C*) Construct of natural SMNΔ7 [SMNΔ7(n)] that includes exons 1–6 and four amino acids from exon 8. (*D*) Distribution of SMN Δ7(n) in HeLa cells.

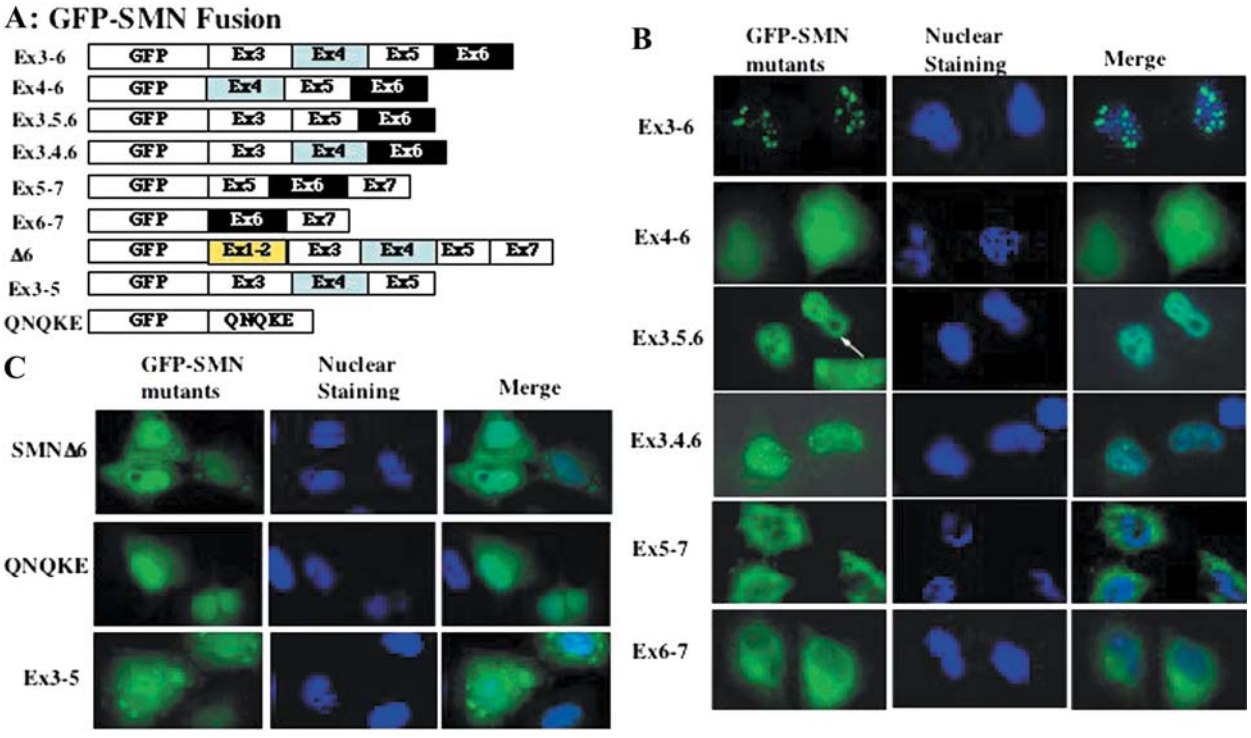

Figure 3. Effects of exon 6 and the tudor domain on the formation of SMN nuclear foci and cytoplasmic localization. (*A*) GFP-fusion constructs to Examinate the impact of different exons on SMN localization were transfected into HeLa cells and examined under a fluorescence microscope. (*B*) Exons 3 and 6 play key roles in nuclear foci formation. (*C*) Exon 6 is critical for SMN cytoplasmic localization but QNQKE has no effects on GFP localization. Nuclei were counter-stained with Hoechst (blue).

other deletion mutants containing exons 6–7 showed predominant localization in the cytoplasm (fig. 3C and unpublished data). These results suggest that exon 6 is essential not only for formation of nuclear foci but may also coordinate with exon 7 for cytoplasmic localization. To further test this hypothesis, we fused exon 6–7 to the C terminus of GFP and found exons 6–7 predominantly recruited GFP into the cytoplasm (fig. 3A, C). We also fused the 26 amino acids of the exon 6 C terminus (including the Y-G motif) and exon 7 to GFP. This fusion protein was mainly retained in the nucleus, similar to the GFP control (data not shown), suggesting that full exon 6 may be required for the cytoplasmic targeting. In addition, when we fused the peptide QNQKE that was considered as a SMN cytoplasmic signal to the C terminus of GFP, no obvious distribution difference between GFP-QNQKE and GFP was observed (fig. 3C).

SMNΔ7 with mutations from SMA patients displays defects in nuclear polymerization or promotes cytoplasmic localization

Except for G279V(C) and A2G, all other SMN missense mutations derived from SMA patients reside in the tudor domain and exon 6. Even G279V(C) is adjacent to exon 6 (Y-G motif). To test whether the mutations in the tudor domain and exon 6 affect the formation of SMN nuclear foci or SMN localization, we generated constructs of GFP-SMNΔ7 bearing mutations E134K (tudor domain), S262I, Y272C, T274I or G275S (exon 6). Examining the GFP signal in transfected HeLa cells, we observed that SMNΔ7 mutants E134K and Y272C exhibited a defective nuclear localization pattern without the formation of nuclear foci. Mutant S262I displayed a similar distribution pattern to that of wild-type SMN exons 1–6, but with significantly smaller granules, suggesting that the ability to self-polymerize was underminded. Interestingly, T274I and G275S, two mutations adjacent in the Y-G motif in exon 6, were predominantly localized in the cytoplasm, implying that both of these mutations gain the function for cytoplasmic targeting. However, SMNΔ7 T274I failed to form nuclear foci, while SMNΔ7 G275S retained this ability (fig. 4). Considering the facts that E134K and Y272C cause the most severe form of SMA (type I), and that mutations S262I, G275S and T274I are identified in type II or III SMA patients [17], the ability to form SMN nuclear foci (or gems) might contribute to pathogenesis of SMA. Furthermore, we also examined the ability of full-length SMN with mutations Y272C and T274I to form gems. Though the number of gems in Y272C and T274I was smaller than that of wild-type SMN, the difference was not as dramatic as for constructs without exon 7 (SMNΔ7) (data not shown). Full-length mutants

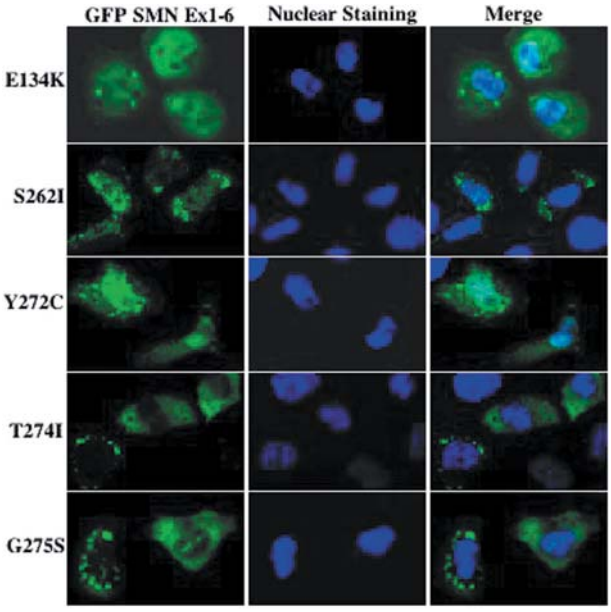

Figure 4. SMNΔ7 with mutations identified from SMA patients displays defects in the formation of SMN nuclear foci and/or promotes cytoplasmic localization. GFP-SMNΔ7 mutants were transfected into HeLa cells and examined under a fluorescence microscope. The nucleus was counterstained with Hoechst (blue).

may themselves not form gems but bind to endogenous SMN that localizes to gems.

Discussion

SMN appears to have multiple functions. The nuclear SMN has been suggested to be involved in mRNA splicing [5], rRNA and/or tRNA processing and perhaps in transcription [16, 20]. In the cytoplasm, SMN functions as a specificity factor essential for the efficient assembly of Sm proteins on U snRNAs [4]. More recently, it has been shown that SMN is required for localization of β-actin mRNA in neuronal growth cones [21]. SMN also plays an important role in apoptosis [10, 11]. Furthermore, our recently published data suggest that SMN is localized in stress granules (SGs) and that SMN triggers SG formation when over-expressed [19]. Since regional cellular localization of SMN protein, either in the nucleus, cytoplasm, axons or in SGs, may be correlated with its specific functions, examination of SMN distribution, in particular that of mutant SMNs, may shed light on what cellular processes, such as splicing of neuronal-specific genes, Sm assembly or protection/transportation of neuronal- specific mRNAs in axons and/or in SGs, are defective in motor neurons in SMA patients. To investigate how SMN localization is modulated and to pinpoint which sequences regulate its distribution, we generated deletion/mutation constructs. We observed that exon 6 is not only critical for the formation of SMN nuclear foci but is also essential for SMN cytoplasmic localization (fig. 3). Exon 7, on the other hand, modulates both of these cellular events (figs. 2, 3). In addition, we showed that mutations in the tudor domain and exon 6 in SMNΔ7 constructs redistributed the proteins mostly into the cytoplasm or compromised formation of nuclear foci (fig. 4), although less dramatic effects have been observed in full-length mutant SMNs. We deduce that formation of SMN foci may play a role in the pathogenesis of SMA.

One intriguing observation in this report is that replacement of exon 7 with a random peptide of five to six or more amino acids displays a similar distribution pattern as that of the full-length SMN (fig. 2), and perhaps partially restores the functions of SMNΔ7, suggesting that the specific sequence of exon 7 may not be critical. This notion is supported by further analysis of exon 7 sequences, which show poor conservation among species (fig. 1). Since the *SMN2* gene is still intact in most SMA patients, experiments could be designed to screen compounds/drugs that modulate translation machineries to read through the normal stop codon (UAG) in *SMN2Δ7* mRNA. A new isoform of SMNΔ7 protein with a peptide of 12 amino acids at the C terminus of SMNΔ7 could be synthesized from exon 8 of the *SMN2* mRNA and redistributed to regional compartments where the full-length SMN functions. This strategy, employing compounds/drugs such as gentamycin, has proved to be effective for patients with cystic fibrosis [22] and seems promising for treatment of Hurler's disease and Duchenne muscular dystrophy [23, 24]. It may also benefit SMA patients.

Finally, the results presented here indicate that, due to their different C termini, differences in protein distribution were observed among SMNΔ7, a construct including SMN exon 1 to exon 6, SMNΔ7n, the primary product of the *SMN*2 gene that contains a four amino-acid peptide from exon 8, and constructs with extra polylinker sequences at the C terminus of SMNΔ7 (figs. 1–3) [19]. Therefore, caution should be taken when data generated from these different protein isoforms are interpreted.

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