主論文の要旨

HnRNP L and hnRNP LL antagonistically modulate PTB-mediated splicing suppression of *CHRNA1* pre-mRNA

（HnRNP L と hnRNP LL は PTB による *CHRNA1* pre-mRNA のスプライシング抑制を拮抗的に制御する）

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Background

Congenital myasthenic syndromes (CMSs) are a family of inherited neuromuscular disorders characterized typically by muscle weakness and easy fatigability. These are caused by defects in genes coding for presynaptic, synaptic, and postsynaptic proteins at the neuromuscular junction (NMJ). We investigated a 53-year-old man having severe myasthenic symptoms involving all voluntary muscles since birth, a decremental electromyographic response, and no circulating anti-AChR antibodies. We explored to identify the underlying cause and molecular mechanism governing the disease in the patient.

Results

An intercostal muscle biopsy of the patient was obtained at age 41. Electrophysiological studies and endplate ultrastructure investigation by electron microscopy revealed that the safety margin of neuromuscular transmission in the patient was compromised by endplate acetylcholine receptor (AChR) deficiency (Fig. 1). Most of the CMSs are caused by postsynaptic defects due to recessive mutations in genes coding for postsynaptic proteins at NMJ. After observing postsynaptic AChR deffficiency in the patient's endplate, we presumed the presence of pathogenic mutation in AChR subunit genes. Direct sequencing of *CHRNA1*, *CHRNB1*, *CHRND*, and *CHRNE* encoding the AChR α, β, δ, and ε subunits, respectively, revealed two heterozygous mutations in *CHRNA1*. The first mutation was a G-to-A substitution at nucleotide position 1261 (αG421R), and the second mutation was a G-to-A substitution at the 23rd nucleotide of the αP3A exon (αP3A23'G>A) (Fig. 1). Family analysis revealed that the mutations were heteroallelic and recessive. Exon P3A is alternatively spliced in humans and great apes, and the transcript including exon P3A, P3A(+) yields a nonfunctional α subunit. Allele-specific RT-PCR of biopsied muscles revealed that αP3A23'G>A mutation markedly enhanced incorporation of exon P3A into mature mRNA and prevented expression of the functional P3A(-) transcript, lacking exon P3A (Fig.1). On the other hand αG421R generated the P3A(-) transcript to similar levels as that of normal controls (Fig. 1). Therefore we examined the effects of αP3A23'G>A on pre-mRNA splicing of *CHRNA1*.

Inorder to dissect the splicing *cis*-element, we constructed a minigene harboring exons 2 to 4 of *CHRNA1* in the pRBG4 mammalian expression vector (Fig. 2). We engineered patient's mutation (αP3A23'G>A) and confirmed that the minigene indeed recapitulated the effect of the identified mutation on splicing of the pre-mRNA containing exon P3A in the transfected cultured cells. To examine whether the identified mutation disrupts an ESS (exonic splicing silencer) or generates an ESE (exonic splicing enhancer), we introduced five artificial mutations between nucleotide positions 22 and 24. All mutants enhanced incorporation of exon P3A (Fig. 2), indicating that G at position 23 as well as its flanking nucleotides constitute an ESS and αP3A23'G>A mutation disrupts it. We also inserted exon P3A and its flanking introns between the two proprietary constitutive exons of the modified exon-trapping vector pSPL3, and confirmed that αP3A23'G>A can recapitulate the aberrant splicing in a heterologous context (Fig. 2).

Having identified the critical *cis*-element of splicing, we next sought for a responsible *trans*-factor that regulates inclusion or skipping of exon P3A. Exploiting the RNA affinity purification assay, mass spectrometry and immunobloting, we identified that an RNA binding protein, hnRNP L normally binds to exon P3A and that the mutation gains a *de novo* binding affinity for its paralogue, hnRNP LL (Fig. 3). Interestingly, a binding motif of hnRNP L is not abolished by the mutation. Indeed, depletion of hnRNP LL from nuclear extract restores binding of hnRNP L to the mutant exon P3A (Fig. 3). Binding of hnRNPs L and LL are thus competitive for the mutant exon P3A, and hnRNP LL displaces hnRNP L from the mutant site.

siRNA-mediated knockdown of hnRNPs L and LL revealed that hnRNP L silences and hnRNP LL enhances inclusion of exon P3A (Fig. 4). We artificially tethered hnRNPs L and LL to the site of mutation by exploiting bacteriophage MS2 coat protein as fusion tag and a reporter minigene replacing the native target with MS2 binding site. We found that hnRNP L silences and hnRNP LL enhances the inclusion of exon P3A by binding to the identified *cis*-element, but not in other sites (Fig. 4). Lack of binding of hnRNP L and gain of binding of hnRNP LL thus account for exclusive inclusion of exon P3A in the patient muscle.

Molecular dissections of hnRNPs L and LL revealed that a unique proline-rich region (PRR) dictates the splicing suppressing activity of hnRNP L, and lack of PRR in hnRNP LL account for its splicing enhancing activity (Fig. 4). Further analysis exploiting co-

imunoprecipitation experiments disclosed that PRR of hnRNP L is responsible for its binding activity to a known splicing repressor, polypyrimidine tract-binding protein (PTB) (Fig. 5). Therefore, hnRNP LL, lacking PRR, incapacitates to interact with PTB. Functional analysis by siRNA mediated down regulation of hnRNP L or/and PTB disclosed that skipping of exon P3A is co-operatively regulated by both factors (Fig. 5).

Further mechanistic analysis using *in vitro* splicing experiments revealed that binding of hnRNP L to the target sequence in exon P3A stabilizes association of PTB to the upstream PPT, which precludes binding of $U2AF^{65}$ to the PPT and binding of U1 snRNP to the downstream 5' splice site, causing defect in exon P3A definition to produce exon P3A-skipped mRNA (Fig. 6 and 7). In the disease-linked mutant *CHRNA1* pre-mRNA, the binding of hnRNP LL to the mutated sequence in exon P3A excludes the competitor hnRNP L, allowing association of $U2AF^{65}$ and U1 snRNP to the upstream PPT and downstream 5’ splice site, respectively, that leads to proper exon P3A definition to produce the exon P3A-included mRNA (Fig. 6 and 7).

Discussion

Exonic and intronic mutations that affect *cis*-acting splicing elements have been reported in many diseases. However, a splicing mutation on a nonfunctional exon has not been studied well as such mutations were often regarded as a rare polymorphism. The present study underscores the importance of including nonfunctional exons in mutation analyses. In course of investigation, we have characterized three important splicing regulators, PTB, hnRNPs L and LL, analyzed their preferred binding motifs, scrutinized their functional consequences, and dissected the structural domains responsible for regulation on splicing of *CHRNA1*. We demonstrate here a novel mechanism of PTB-mediated inhibition of exon definition, in which hnRNP L facilitates binding of PTB to the upstream PPT that suppresses subsequent association of $U2AF^{65}$ and U1 snRNP in exon-defined E complex. Another example of PTB-hnRNP association has been reported with *PKM* encoding the pyruvate-kinase-M, where PTB and hnRNPs A1/A2 cooperate in excluding exon 9 to increase lactate production in cancer cells (Clower et al, 2010). Together, hnRNP proteins appear to be functional partners of PTB, when binds to the upstream PPT, for inhibiting E complex formation, leading to the subsequent splicing suppression. Our analysis of aberrant splicing in a congenital myasthenic syndrome uncovers a novel switching mechanism of PTB-mediated splicing suppression by hnRNP L and hnRNP LL.