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ORIGINAL ARTICLE

Identification of the rare compound heterozygous variants in the *NEB* gene in a Korean family with intellectual disability, epilepsy and early-childhood-onset generalized muscle weakness

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We examined a Korean family with complex phenotypes characterized by intellectual disability, epilepsy and early-childhood-onset generalized muscle weakness. Since we did not find any abnormality using several conventional genetic tests for detection of chromosomal aberrations, gene copy number variations and mitochondrial gene mutations, we aimed to identify disease-causing genetic alteration(s) in this family. We conducted whole-exome sequencing (WES) in this family. After filtering the WES data, we compared five exome sequences of two affected siblings, one unaffected sibling and the unaffected parents, and we determined the allele frequency of the identified variants in an Asian population. Finally, we selected one candidate variant pair which is unique in the patients and corresponds to an autosomal recessive genetic model. The two affected siblings had the same compound heterozygous variation in the *NEB* gene encoding nebulin, which was composed of two different missense variants: c.2603T>C (p.L868P) in exon 27 and c.21340C>T (p.R7114W) in exon 143. We confirmed these variations by Sanger sequencing. On the basis of the fundamental role of nebulin in the brain and skeletal muscles, we concluded that this compound heterozygous *NEB* variation may be a sound candidate for the disease-causing mutation in this family. Since the patients are characterized by generalized muscle weakness together with neurodevelopmental phenotypes, it is suggested that *NEB* mutations could manifest more diverse phenotypes than those previously described.
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INTRODUCTION

We examined a Korean patient characterized by intellectual disability, epilepsy and early-childhood-onset generalized muscle weakness. Her older brother had a similar clinical phenotype, but her parents and older sister were not affected. Copy number variants have been reported to be common in patients with multiple neurodevelopmental disorders, such as a combination of intellectual disability and epilepsy.[1] Many genes have been reported to be responsible for a variety of congenital myopathies.[2] To identify the causative genetic alterations in the affected siblings, we first performed several genetic tests, including G-band karyotyping for detection of chromosomal aberrations, array comparative genomic hybridization analysis for detection of copy number variation, Southern blot analysis for detection of CGG expansion in the *FMR1*gene which is the causative gene for Fragile X syndrome, and sequencing analysis for detection of mutations in the mitochondrial DNA. However, we did not find any abnormalities with these mutation analyses.

Recently, whole-exome sequencing (WES) with a next-generation sequencer has been shown to be robust in identifying causative gene(s) in inherited disorders.[3] WES in 264 patients with epileptic encephalopathy and in their parents by the Epi4K Consortium and Epilepsy Phenome/Genome Project found many novel gene mutations.[4] Diagnostic exome sequencing analysis in persons with severe intellectual disability found a lot of *de novo* mutations.[5] Therefore, we carried out WES in this family. Here, we report rare compound heterozygous *NEB* variants in Korean patients with intellectual disability, epilepsy and generalized muscle weakness. This is the first report that *NEB* mutations known to cause autosomal recessive nemaline myopathy may also be involved in neurodevelopmental phenotypes. Therefore, we have discussed the possible genotype–phenotype correlations,

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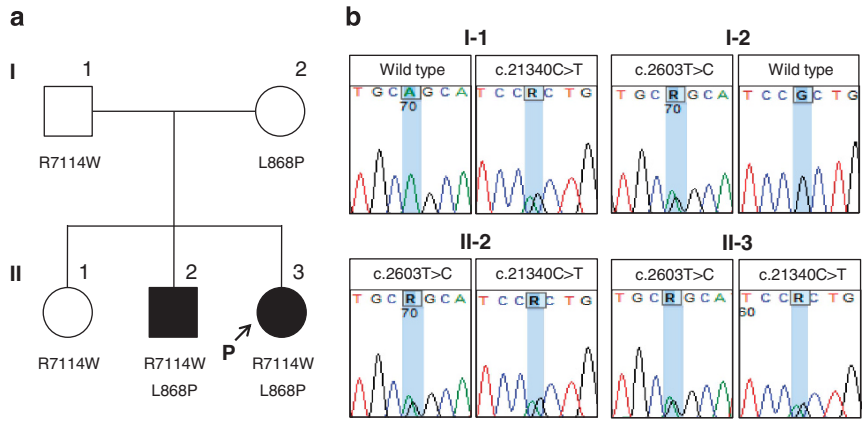

Figure 1 Pedigree of the family in this study and Sanger sequencing results of *NEB* variations. (**a**) Pedigree of the parents (I-1 and I-2), the two affected siblings (II-2 and II-3) and the one unaffected sibling (II-1) who underwent the quintet-based whole-exome sequencing. The letter 'P' indicates a proband of this family. (**b**) Sanger sequencing results of the variations in the *NEB* gene (c.2603T>C and c.21340C>T) in the parents (I-1 and I-2) and in the patients (II-2 and II-3). All chromatograms and sequences are the results of reverse-primer sequencing. The corresponding sequences are shown on top of each chromatogram.

Table 1 Clinical features of the two patients

	Patients	
Functional evaluation	*II-2*	*II-3 (Proband)*
Manual muscle test (extremity grade right/left)	Upper: 2/2	Upper: 3/3
	Lower: 1/1	Lower: 1/1
Full-scale intellectual quotient[a]	<25	<25
Verbal intellectual quotient[a]	<30	<30
Performance intellectual quotient[a]	<30	<30
Social quotient[b]	0.6	16.1
Needle electromyography	Not available	Myopathic findings

[a]Measured by the Korean Wechsler Adult Intelligence Scale.
[b]Measured by the Korean Vineland Social Maturity Scale.

mainly focusing on conjectured nebulin function in neurons as an actin regulator.

MATERIALS AND METHODS

Subjects

Two affected siblings (II-2 and II-3) visited the Department of Physical Medicine and Rehabilitation, Ajou University Hospital, Suwon, Republic of Korea, for their intellectual disability, epilepsy and early-childhood-onset generalized muscle weakness (Figure 1a). To identify the causative genetic alterations in the affected siblings, we first performed several genetic tests, including G-band karyotyping for the detection of chromosomal aberrations, array comparative genomic hybridization analysis for the detection of copy number variation using the Roche NimbleGen CGX-3 135K Whole-Genome Array (Roche NimbleGen, Inc., Madison, WI, USA), Southern blot analysis for the detection of CGG expansion in the *FMR1*gene for Fragile X syndrome, and sequencing analysis for the detection of mutations in mitochondrial DNA. However, we did not find any abnormalities using any of these approaches. We collected peripheral blood samples from the five family members for WES and extracted genomic DNA using standard procedures, where the unaffected parents (I-1 and I-2) and unaffected older sister (II-1) were recruited as controls for the mutation analysis (Figure 1a). This study was approved by the Institutional Review Board Committee of the Ajou University Medical Center.

Whole-exome sequencing with a next-generation sequencer

A pre-enrichment DNA library was constructed according to the Illumina TrueSeq DNA sample preparation guide (Illumina, Inc., San Diego, CA, USA). Exome enrichment was done using Illumina TrueSeq Exome Enrichment probes and streptavidin beads. The enriched exome library was loaded onto flow cells of an Illumina cBot for cluster generation. The flow cells with clusters of the enriched exome libraries were transferred to an Illumina HiSeq2000. High-throughput sequencing was then performed for each captured library to ensure that each sample met the desired average sequencing depth of 40-fold. A full description of the WES methods is included in the Supplementary Methods.

Sanger sequencing

DNA extracted from peripheral blood samples was amplified using PCR with primers to confirm the c.2603T>C in exon 27 (5′-CTACCCTTGGGCATCGTAAC-3′ and 5′-GGCATGTGTGATGTCTTTGC-3′) and the c.21340C>T in exon 143 (5′-GCCCCATCACAGTACCTGAC-3′ and 5′-TGGCCCTCTGGAGTGTTTAC-3′). The PCR product was sequenced on an ABI 3500xL DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

RESULTS

Clinical description

A 15-year-old girl (II-3 in Figure 1a) came to the clinic with complex phenotypes of intellectual disability, epilepsy and generalized muscle weakness of early-childhood onset. She was born by normal vaginal delivery. Her birth weight was 3 500 g. Her development was apparently unremarkable until the age of 12 months. At the age of 13 months, she developed nonfebrile seizure episodes, which were not controlled by various antiseizure medicines. Along with intractable epilepsy, she showed progressive psychomotor regression and eventually became wheelchair-bound at the age of 13 years with no production of meaningful words. She did not show dysmorphic features and had a normal head circumference (53.0 cm, 5th to 25th percentile). She showed generalized muscle weakness, where the manual muscle test revealed grade 3/3 of the upper extremities and grade 1/1 in the lower extremities, respectively (Table 1). She had a 'skin and bones' appearance in all four extremities due to severe muscle atrophy. The patient had severe contracture of multiple joints including bilateral hips, knees, ankles, shoulders, elbows, wrists and interphalangeal joints along with scoliosis (Figure 2). She did not exhibit any signs of spasticity, dystonia or rigidity and her muscle enzymes were within the normal range.

Muscle biopsy is a good method to determine if myopathy is present. However, a muscle biopsy could not be done because the

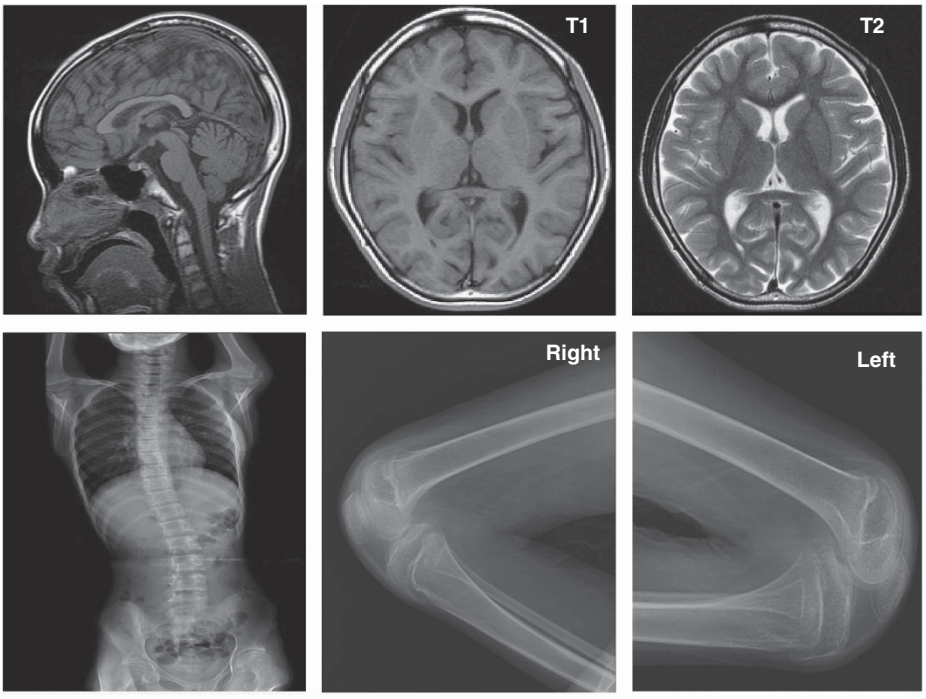

Figure 2 Radiographic findings of the patient (II-3). Magnetic resonance imaging of the brain shows normal findings in the top panel, where sagittal T1-, axial T1- and T2-weighted images are given. The plain X-rays of the spine and knees reveals scoliosis and 130°/150° bilateral flexion contracture of the knees in the bottom panels.

patient refused the procedure. Instead, a needle electromyography test was done in both the tibialis anterior and gastrocnemius muscles of the proband. Although she showed little volitional muscle contraction due to scanty muscle tissue with fibrosis, a few short duration, low amplitude polyphasic motor units were identified as well as a few abnormal spontaneous activities with positive sharp waves and fibrillations, indicating that she has myopathy. While she was able to open and close both eyes with no difficulty, she had considerable difficulty with mastication and swallowing. A videofluoroscopic swallowing study revealed significant delays in both the oral and pharyngeal phase of swallowing, showing fluid aspiration without developing a cough reflex. However, tube feeding has been refused and she has been on oral feeding with pureed diet.

A psychological evaluation using the Korean Wechsler Adult Intelligence Scale and the Korean Vineland Social Maturity Scale showed profound intellectual disability, with a full-scale intellectual quotient of <30, a verbal intellectual quotient of <30, a performance intellectual quotient of <30, and a social quotient of 16.1 (Table 1). She was completely dependent with respect to performance of the activities of living, with a score of zero on the modified Barthel index. Magnetic resonance imaging of the brain showed normal findings (Figure 2). The electroencephalogram study showed generalized epileptiform discharges, with an irregular and discontinuous background rhythm.

Her older brother (II-2 in Figure 1a) had similar clinical phenotypes (Table 1). He showed apparently normal development until the age of 6 months and had become wheelchair-bound by the age of 10 years. Her parents and older sister did not have any myopathic or neurodevelopmental problems. We could not detect any genetic alterations in the affected two siblings by conventional genetic tests.

Whole-exome DNA sequence analysis

We conducted WES analysis of the family members as described above. After data filtering, we compared the five exome sequences from the two affected siblings, the unaffected sibling and their unaffected parents. We excluded non-disease-causing common variants, and extracted exonic and splicing variants that could explain the cause of the disease in the patients according to autosomal dominant or recessive genetic models, or genomic imprinting mutations as described in the Supplementary Methods. As a result, a total of seven compound heterozygous variant pairs of five genes (*PRAMEF2*, *NEB*, *IBSP*, *PRKAG2* and *MLH3*) were identified as candidate variants corresponding to the autosomal recessive genetic model (Supplementary Table S1). Next, we determined the allele frequency of the candidate variants in an Asian population on the basis of the data from the 1000 Genomes Project (http://www.1000genomes.org/), and selected those with an allele frequency of 1% or less. Finally, only one compound heterozygous *NEB* variant pair, which is unique in the patients, was found. The patients had two different *NEB* missense variants, c.2603T>C (p.L868P) in exon 27 and c.21340C>T (p.R7114W) in exon 143, but the unaffected parents and older sister had only one *NEB* variant (either c.2603T>C or c.21340C>T; Table 2). We confirmed these variations in the family members by Sanger sequencing (Figure 1b).

In silico prediction of functional effects for the missense variants

As these two variants have not been reported in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk), we conducted *in silico* prediction of the functional effect of these variations at the amino-acid level using Polymorphism Phenotyping v2 (PolyPhen 2) and Sorting Intolerant from Tolerant predicting methods. The PolyPhen 2 results predicted that both variants are probably damaging, and the Sorting Intolerant from Tolerant results predicted that the p.R7114W variant

Table 2 Characteristics of the identified *NEB* variants in this study

Characteristics	*Variant 1*	*Variant 2*
Genomic position	g.152543967	g.152390806
Exon	27	143
DNA change	c.2603T>C	c.21340C>T
Protein change	p.L868P	p.R7114W
Allele frequency in the Asian population[a]	0.010	0.003
PolyPhen 2 score	0.979	0.999
PolyPhen 2 result	Probably damaging	Probably damaging
SIFT score	0.25	0.02
SIFT result	Tolerant	Intolerant

Abbreviations: PolyPhen 2, Polymorphism Phenotyping v2; SIFT, Sorting Intolerant from Tolerant.
[a]Data from the 1000 Genomes Project (http://www.1000genomes.org/).
GenBank number: NG_009382.2 for genomic DNA, NM_001271208.1 for cDNA and NP_001258137.1 for protein.

is intolerant (Table 2). Furthermore, the amino-acid residues in the variation sites are well conserved in other species (Supplementary Figure S1). These *in silico* prediction results indicate that both of these *NEB* variants may have a deleterious effect on the protein's function (Table 2).

DISCUSSION

We identified the *NEB* variant of alternatively spliced exon 143 in addition to the constitutive exon 27. Clinically the affected subjects are characterized by intellectual disability and epilepsy in addition to congenital myopathy of early-childhood onset. A giant filamentous protein nebulin encoded by *NEB* gene is one of actin regulators like dystrophin, utrophin, α-actinin, β-spectrin and so on.[6,7] Nebulin is expressed abundantly in skeletal muscle as a component of the cytoskeletal matrix.[8] Alternative splicing results in numerous nebulin isoforms differing in their C-terminal regions.[9] Although the isoforms of nebulin are expressed in other organs in addition to skeletal muscle, particularly in the brain,[10] the biological function of nebulin remains unknown in the brain. Mutations in *NEB* cause autosomal recessive congenital myopathies such as nemaline myopathy 2 (NEM2, OMIM #256030), distal myopathy and core-rod myopathy.[11,12] Nemaline myopathy patients having NEB gene mutations present a wide spectrum of morphological features.[13]

The severe generalized muscle weakness concurrent with contracture of multiple joints seen in our patients is compatible with typical clinical findings of autosomal recessive congenital myopathy caused by *NEB* mutations. However, to the best of our knowledge, there have been no reports describing any signs of dysfunction of the CNS such as intellectual disability and/or epilepsy in patients with *NEB* mutations. We concluded that the compound heterozygous *NEB* variation detected herein might be a disease-causing mutation for the following reasons: (1) our patients showed severe early-childhood-onset generalized muscle weakness; (2) nebulin is reported to be expressed in the brain, as well as in the skeletal muscle; (3) nebulin is an actin regulator that seems to have a role in neurons, as well as in skeletal muscle; (4) the two *NEB* variants identified in our patients are very rare in the general population; and (5) the two *NEB* variants are predicted *in silico* to have deleterious effects on the nebulin protein.

The isoforms of nebulin are expressed in other organs in addition to skeletal muscle, particularly in the brain.[10] An immunohistochemical study reported that nebulin is expressed in the cytoplasm of pyramidal neurons and in subcortical endothelial cells in the brain.[10] Therefore, nebulin seems to have a role as an actin regulator in neurons of the human brain as it does in skeletal muscle. Actin is involved in many essential processes in eukaryotic cells, including muscle cells and neurons. Through actin polymerization, actin works as a universal force provider, generating the pressure that biological processes use.[14] Since abnormal morphological changes of neuronal dendrites and spines are reported to be associated with intellectual disability, changes in the cytoskeletal organization of dendrites and spines are likely to affect the structure and function of developing and mature synapses.[15] Therefore, abnormalities of actin-binding proteins could cause dysfunction of the actin cytoskeleton. It seems that synaptic excitability and synaptic plasticity could be altered by a dysfunctional actin cytoskeleton caused by *NEB* mutations. Similar phenomenon is observed in Duchenne muscular dystrophy (OMIM #310200), which is a fatal, recessive, X-linked muscular disease caused by *Dystrophin* gene mutation.[16] Dystrophin is also one of actin regulators like nebulin and is expressed in both muscle fibers and nonmuscular organs including the brain.[17] Deficiency of the brain-specific isoforms of dystrophin protein such as Dp71 and Dp140 is known to be associated with the presence of intellectual disability and seizure in Duchenne muscular dystrophy.[18,19] Therefore, it is not surprising that our patients had intellectual disability and epilepsy, as these are associated with a biallelic mutation of the actin regulator-encoding *NEB* gene.

Our patients had compound heterozygous missense variation in exons 27 and 143. Of the 183 exons in the *NEB* gene, exons 63–66, 82–105, 143–144 and 166–177 are key regions where alternative splicing occurs.[20] Alternative splicing in one of the four regions is likely to be quite elaborate in a developmental stage-specific, organ-specific or muscle type-specific way. Since exons 143–144 are known to be mutually exclusive in alternative splicing, these two exons are never found within the same transcript.[20] A mouse study reported that the transcripts expressing *Neb* exon 127, which corresponds to human *NEB* exon 143, were more prominent in muscles of young mice.[21] Transcripts expressing either exons 143 or 144 are found in both the muscles and brain.[10] They encode a portion of amino acids from the super-repeat 21 in muscles and the linker repeat 1 in the brain, which is close to the carboxy (C)-terminal region. Maintaining physiological Z-disk widths and myofibrillar connectivity is known as one of the functions of nebulin, where the C-terminal region of nebulin interacts with desmin close to the Z-disk.[22,23] This is also possibly relevant for the exon 143 functions of *NEB*. Amino acid sequences encoded by exons 143 or 144 differ in both charge and hydrophobicity and the amino-acid sequence encoded by exon 143 shows complete homology between mouse, rat and human.[21] In the human fetal brain, only exon 144-containing transcripts were detected, whereas exon 143- or 144-containing transcripts were found in adult brains.[10] The human fetal muscle expresses transcripts containing only exon 143, whereas the adult skeletal muscle and heart muscle express transcripts containing exon 143 or 144.[20] This suggests that exon 143 might harbor a regulatory function utilized during muscle maturation. However, there has been no report on mutation involving exons 143 and 144 of *NEB* in human, yet.[11,20,21]

In this context, we concluded that the identified compound heterozygous *NEB* variation may be a sound candidate for the disease-causing mutation in our patients characterized by intellectual disability, epilepsy and early-childhood-onset generalized muscle weakness, suggesting *NEB* mutations could manifest more diverse phenotypes than those previously described. Because the affected family is quite small and there are no strong linkage data in this family, however, more complex non-monogenetic mechanisms involved in the identified *NEB* variations need to be considered. In addition,

functional studies are required to elucidate the possible mechanisms underlying the CNS dysfunction in these rare compound heterozygous *NEB* variants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)