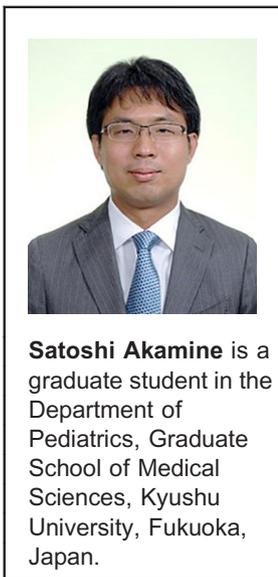


Early-onset epileptic encephalopathy and severe developmental delay in an association with de novo double mutations in *NFI* and *MAGEL2*

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SUMMARY

Advance in the exome-wide sequencing analysis contributes to identifying hundreds of genes that are associated with early-onset epileptic encephalopathy and neurodevelopmental disorders. On the basis of massive sequencing data, functional interactions among different genes are suggested to explain the common molecular pathway underlying the pathogenic process of these disorders. However, the relevance of such interactions with the phenotypic severity or variety in an affected individual remains elusive. In this report, we present a 45-year-old woman with neurofibromatosis type I (NFI), infantile-onset epileptic encephalopathy, and severe developmental delay. Whole-exome sequencing identified de novo pathogenic mutations in *NFI* and the Schaaf-Yang syndrome-associated gene, *MAGEL2*. Literature-curated interaction data predicted that *NFI* and *MAGEL2* proteins were closely connected in this network via their common interacting proteins. Direct conversion of fibroblasts into neurons in vitro showed that neuronal cells from 9 patients with *NFI* expressed significantly lower levels of *MAGEL2* (54%, $p = 0.0047$) than those from healthy individuals. These data provide the first evidence that pathogenic mutations of *NFI* deregulate the expression of other neurodevelopmental disease-associated genes. De novo mutations in multiple genes may lead to severe developmental phenotypes through their cumulative effects or synergistic interactions.

KEY WORDS: Early-onset epileptic encephalopathy, Whole-exome sequencing, De novo mutation, Neurofibromatosis type I, *MAGEL2*, Direct conversion, Functional interaction.

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Neurofibromatosis type 1 (NF1) is a neurocutaneous syndrome with autosomal dominant patterns of inheritance.¹ Affected individuals show characteristic skin lesions of café-au-lait spots, multiple tumors, and variable degree of mental disability with or without autistic features. The *NF1* gene is located at chromosome 17q11.2, encoding 2,839 amino acid protein neurofibromin.² *NF1* mutations cause the hyperactive extracellular signal-regulated kinase (ERK) pathway, thereby leading to accelerated cell growth and tumor formation.³ Hyperactive signals in synaptic ERK pathways are associated with both epilepsy and mental development in childhood.⁴ Previous studies demonstrated that the prevalence (0.76%) of infantile spasm (IS) or early-onset epileptic encephalopathy (EOEE) in NF1 has been reported to be higher than those (0.02–0.05%) in general populations.⁵ Neuroimaging studies also revealed that cortical and subcortical lesions were occasionally found in NF1 patients.⁶ However, specific brain lesions or genetic backgrounds have not been disclosed for NF1 in the majority of cases with IS/EOEE. We experienced a case of a 45-year-old female with NF1, EOEE, and severe developmental delay. Atypical phenotypes in the present case were studied on the basis of genetic as well as biological backgrounds.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board at Kyushu University (#461-00). Written informed consent was obtained from the parents. Experiments were conducted under a stringent compliance to the institutional guideline and our experimental protocol (23-53). Case report and experimental procedures for whole-exome sequencing (WES), in vitro conversion of neurons, quantitative polymerase chain reactions (PCR), and bioinformatics are presented in Appendix S1 and Figs. S1, S2).

RESULTS

De novo mutations in *NF1* and *MAGEL2*

De novo mutations in *NF1* (NM001042492.2: c.4835 + 1G>T) and *MAGEL2* (NM019066.2: c.219C>G, c.224delC) were validated with Sanger sequencing (Figs. 1A,B). The former mutation occurred at the splicing junction of exon 36 in *NF1*, disrupting the functional expression of neurofibromin. The latter was mapped to the coding region of *MAGEL2* and was considered to produce a truncated form of *MAGEL2*. We further investigated whether the mutated allele was located on the paternal allele.⁷ Methylation-sensitive digestion with *SmaI* followed by PCR amplification and direct sequencing of the flanking region revealed that the mutated allele remained intact after the *SmaI* digestion (Fig. 1B). We thus confirmed that the de novo *MAGEL2* mutation occurred at the maternal allele, which was fully methylated in lymphocytes. Microarray-

based comparative genome hybridization (CMA ver. 8.1 at Baylor MGL; data not shown) excluded that this case carried pathogenic copy number variants (CNVs).

Functional interaction between *NF1* and *MAGEL2*

The de novo *MAGEL2* mutation in this case was unlikely a primary cause of the patient's developmental phenotypes. We considered the double mutations of *NF1* and *MAGEL2* as an extremely rare genetic event⁸ (Table 1). We alternatively interpreted this event to be possibly relevant with the unusual NF1 phenotype of this case. To explore whether *NF1* and *MAGEL2* might work as genetic modifiers for each other, we used STRING, a protein-protein interaction database (<http://string-db.org/>). This open database predicted that *NF1* and *MAGEL2* were directly or indirectly connected via common binding proteins in a functional network consisting of 35 proteins (nodes) and 84 interactions (edges) (Fig. S2A). Given the number of edges expected to be 48, these 35 proteins were considered to have 1.8-fold enriched protein-protein interactions ($p = 2.25 \times 10^{-6}$). Thus, this network was suggested to have functional enrichment in certain molecular pathways. Indeed, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses showed that signaling molecules associated with “chromatin binding (GO:0003682),” “RNA polymerase (GO:0003899 and KEGG:3020),” and “RAS signaling pathways (KEGG:4014)” were enriched in this network (Figs. S2B,C).

Altered expression of *MAGEL2* in NF1 patient-derived neurons

To validate the possible functional interplays between *NF1* and *MAGEL2*, we examined whether pathogenic mutations in *NF1* might influence neuronal expression of *MAGEL2*. We determined the transcriptional activation of *NF1* and *MAGEL2* using the direct conversion system of fibroblast into neuron in vitro. Quantitative analyses on mRNA expression showed that *MAGEL2* expressions in fibroblasts did not differ between the patients and controls. The neuronal conversion induced robust increase in the *MAGEL2* expression (Fig. 1C). Notably, the neuronal expression of *MAGEL2* was decreased to 54% of that in healthy controls ($n = 9$ for each group, $p = 0.0047$, Student's t test; Fig. 1C). The neuronal expression of *MAGEL2* in the present case was 41% of that in healthy controls ($p = 0.0189$, Student's t test; data not shown). Neuronal conversion also induced higher expressions of imprinted genes (*UBE3A* and *CYFIP1*) at the chromosome 15p11.2 region than those in fibroblasts (Fig. 1D). However, induced neurons from NF1 patients and healthy controls expressed these genes at comparable levels. These data confirmed the epistatic regulation of *MAGEL2* expression by *NF1* and illustrated the specific effects of the *NF1* mutations on transcriptional activation of *MAGEL2* during the neuronal conversion.

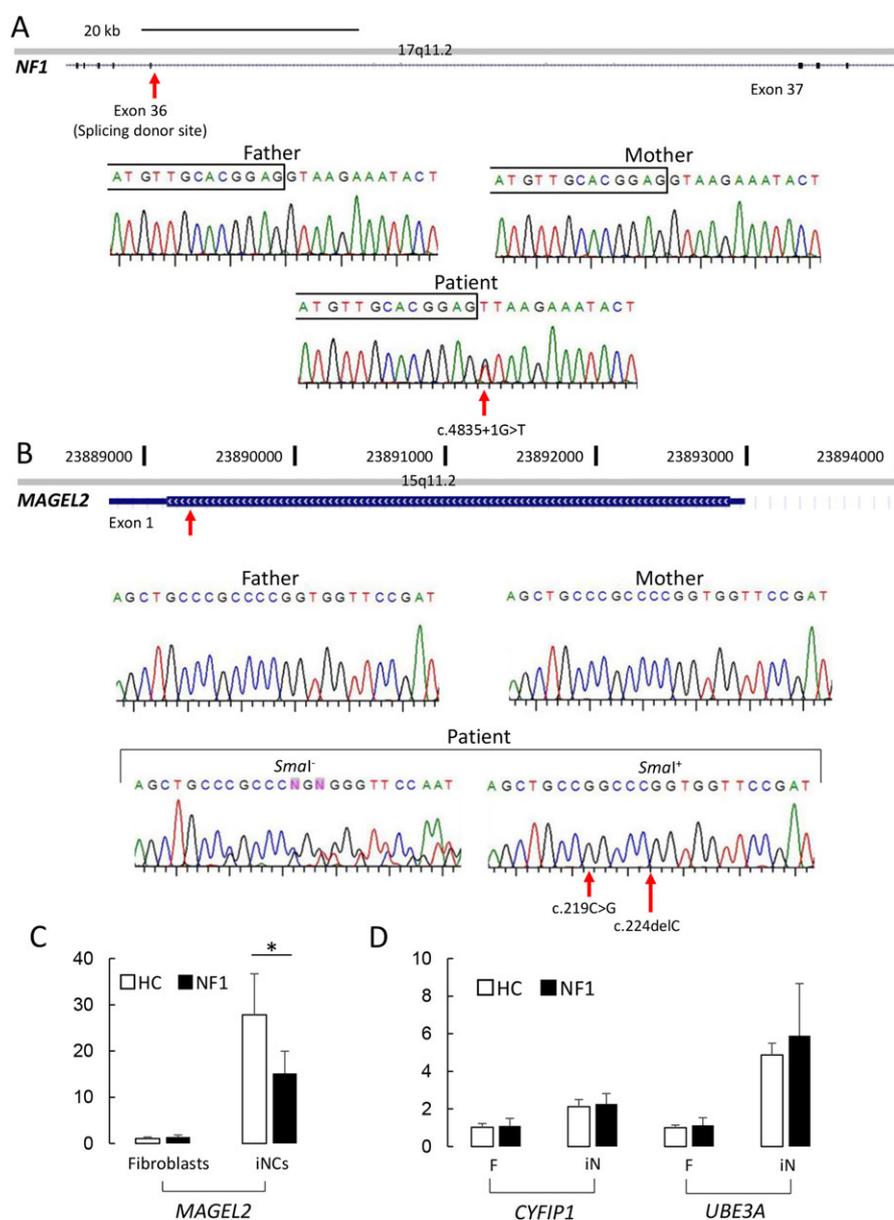


Figure 1.

De novo mutations identified in the present case and functional interaction between *NF1* and *MAGEL2*. **(A)** De novo mutation at the splicing junction of exon 36 in *NF1*. The sequence chromatograms of father, mother, and the patient are shown. Red arrow indicates that this mutation occurred at chr17:29592358 (NM_001042492.2:c.4835 + 1G>T). Boxed letters above the sequencing data denote exonic sequences. **(B)** *MAGEL2* mutation in the present case. Aligned data illustrate that the mutation occurred de novo in this patient at chr15:23892666, 23892671 (NM_019066.4:c.219C>G, c.224delC). Sequencing results before (*Sma*⁻) and after the *Sma* digestion (*Sma*⁺) indicate that this mutation occurred at the methylated (or maternally inherited) allele. **(C)** *MAGEL2* mRNA expression before and after neuronal induction. White and black bar plots represent the relative expression levels in indicated cells from healthy controls (n = 9) and *NF1* patients (n = 9), respectively. **(D)** *CYFIP1* and *UBE3A* expressions in fibroblasts and induced neurons. The gene expression profiles were quantitated *in vitro* using the cells from healthy controls and *NF1* patients (n = 9 for each group). Values in C and D are shown as mean ± SD of each group. Asterisk indicates the p value of less than 0.05 (Student's t test).

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To test whether *NF1* mutations might affect other genes associated with EOEE, we randomly selected 6 EOEE-associated genes (*CDKL5*, *CHD2*, *ARX*, *KCNT1*, *SCN1A*, and *TRIM8*)^{9–13} and assessed their expression profiles in both

fibroblasts and induced neurons (Fig. S3). As expected, *in vitro* conversion of fibroblasts into neurons resulted in 2- to 5-fold higher expression of these six genes than those in fibroblasts. When we compared their expression levels in

Table 1. Clinical features of the present case in comparison with those of neurofibromatosis type I and Schaaf-Yang syndrome

	Present case	Neurofibromatosis type I	Schaaf-Yang syndrome
Mutated gene	<i>NF1</i> , <i>MAGEL2</i>	<i>NF1</i>	<i>MAGEL2</i>
(Band Locus)		(17q11.2)	(15q11.2)
Phacomatosis and associated lesion			
Café-au-lait spots	+	+	–
Neurofibroma	+	+	–
Lisch nodules of iris	–	+	–
Dysmorphism			
Bitemporal narrowing of facial appearance	–	–	+
Almond-shaped palpebral fissures	–	–	+
Small hands	+	–	+
Joint contractures	–	–	+
Tumorigenesis and skeletal problem			
Brain tumor			
Abnormal MRI signal	+	+	–
Bone fracture	–	+	–
Neurological sign			
Feeding problem	–	–	+
Hypotonia	+	+	+
Developmental delay	+	+	+
Autism spectrum disorder	+	+	+
Seizure	+	+	–

neurons, only *KCNT1* was expressed at a significantly lower level (69%) in *NF1* neurons than that in healthy controls ($p = 0.011$, Student's *t* test; Fig. S3). All the other genes were expressed in neurons from *NF1* patients at similar levels to those in controls. These data suggested that *NF1* mutation attenuated the expression of a subset of EOEE-associated genes.

DISCUSSION

We presented a Japanese woman who had dysmorphic appearance, severe intellectual disability, and intractable epilepsy with a history of infantile-onset seizures. This is the first case of *NF1* carrying another *de novo* mutation in a gene that is known to cause different neurodevelopmental disorders. Previous studies demonstrated that *NF1* patients are susceptible to the onset of epileptic encephalopathy, such as IS and EOEE.¹⁴

The WES and subsequent analyses led us to the following two discussions: First, the severe phenotypes of this patient resulted solely from the *NF1* mutation regardless of the *MAGEL2* mutation. Second, the double mutations in *NF1* and *MAGEL2* exerted cumulative or synergistic effects to produce more severe phenotypes than those expected for

individuals with a single gene mutation in either *NF1* or *MAGEL2*.

The first perspective might be valid taking the allele-specific expression of *MAGEL2* into account. Indeed, truncating *MAGEL2* mutations proved to be critical for dysmorphic appearance and hypothalamic dysfunctions exclusively when the mutations occurred in the paternal alleles.⁷ Despite these facts, experimental studies have shown that *MAGEL2* was widely expressed in embryonic as well as in the adult brains.¹⁵ Moreover, allele-specific expression of imprinted genes varies over time and by region in neuronal subpopulations.¹⁵ Recent studies have shown that *MAGEL2* and *NF1* were expressed from the maternal allele in embryonic tissues under certain conditions, suggesting that Prader-Willi syndrome-associated genes in the chromosomal region at 15q11–q13 have epigenetic flexibility.^{16,17} With our findings of lower expression of *MAGEL2* in neurons from *NF1* patients, we considered that the *MAGEL2* mutation on the maternal allele caused only negligible effects on neuronal phenotype, whereas it could reach the pathogenic level when the additional mutation in *NF1* coincided. Therefore, it cannot be safely concluded that the *MAGEL2* mutation in the maternal allele was irrelevant to the neurodevelopmental phenotypes in this case.

MAGEL2 protein belongs to a family of melanoma-associated antigen (MAGE) domain-containing molecules, which has been characterized as highly expressed genes/proteins in various types of tumors.¹⁸ Among MAGE family proteins, NRAGE was shown to interact with p75 neurotrophin receptor and to promote nerve growth factor (NGF)-dependent apoptosis, suggesting that NRAGE could be a component of intracellular signaling pathways.¹⁹ Similarly, *NF1* is involved in the NGF-dependent survival of embryonic sensory and sympathetic neurons.²⁰ These data supported our hypothesis that MAGE family proteins and *NF1* may cooperatively regulate the maturation of neurons under certain molecular pathways, as the network in this study illustrated.

The interaction database and subsequent bioinformatics analysis revealed that the *NF1*- and *MAGEL2*-containing network was significantly enriched in the proteins associated with particular cellular functions, such as RAS signaling. Although physiological functions of *MAGEL2* in neurons remain unknown, this result raised a new possibility that *MAGEL2* may regulate the RAS signals cooperatively with *NF1* in the developing brain. Thus, the hypomorphic mutations in the two genes may cause more profound effects on neuronal dysfunctions than those resulting from the mutation in either gene.

According to the protein interaction database, *NF1* and *MAGEL2* were unlikely to constitute a protein complex. Rather, our experimental data supported evidence that *NF1* acts as an epistatic regulator of *MAGEL2* expression. In line with these data, we conceptualized that the *de novo*

mutation of *MAGEL2* exaggerated neuronal dysfunctions owing to the *NF1* mutation and hyperactive RAS-ERK signaling. Thus, the severe neurological phenotype of our case can be regarded as an extended model of digenic inheritance or the transheterozygote at two loci.^{21,22} To determine their additive or synergistic effects on RAS signaling and neurological deficits, more studies using genetically engineered mice will be necessary.

Missing pieces are left for our future study to investigate whether and how recessive mutations inherited from the patient's parents could modify the phenotypes of other cases with atypical phenotypes of NF1. Accumulation of genome-wide analysis data for NF1 patients with IS and EOEE will clarify these issues.

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Dismorphic appearance of the present case.

Figure S2. Bioinformatics analyses on interaction between *NF1* and *MAGEL2*.

Figure S3. Expression of epileptic encephalopathy-associated genes in fibroblasts and induced neurons.

Appendix S1. Materials and methods.