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Biochemical and molecular characterization of novel mutations in GLB1 and NEU1 in patient cells with lysosomal storage disorders

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ABSTRACT

Lysosomes are cytoplasmic compartments that contain many acid hydrolases and play critical roles in the metabolism of a wide range of macromolecules. Deficiencies in lysosomal enzyme activities cause genetic diseases, called lysosomal storage disorders (LSDs). Many mutations have been identified in the genes responsible for LSDs, and the identification of mutations is required for the accurate molecular diagnoses. Here, we analyzed cell lines that were derived from two different LSDs, GM1 gangliosidosis and sialidosis. GM1 gangliosidosis is caused by mutations in the GLB1 gene that encodes β-galactosidase. A lack of β-galactosidase activity leads to the massive accumulation of GM1 ganglioside, which results in neurodegenerative pathology. Mutations in the NEU1 gene that encodes lysosomal sialidase cause sialidosis. Insufficient activity of lysosomal sialidase progressively increases the accumulation of sialylated molecules, and various clinical symptoms, including mental retardation, appear. We sequenced the entire coding regions of GLB1 and NEU1 in GM1 gangliosidosis and sialidosis patient cells, respectively. We found the novel mutations p.E186A in GLB1 and p.R347Q in NEU1, as well as many other mutations that have been previously reported. We also demonstrated that patient cells containing the novel mutations showed the molecular phenotypes of the corresponding disease. Further structural analysis suggested that these novel mutation sites are highly conserved and important for enzyme activity.

1. Introduction

Lysosomes are eukaryotic organelles that function in the degradation of virtually all types of macromolecules. They contain a large number of acid hydrolases, and mutations in the genes that encode these enzymes cause inherited diseases, called lysosomal storage disorders (LSDs) [1]. Functional defects of lysosomal enzymes result in an increase in the number and size of lysosomes and the progressive accumulation of undigested metabolites, which cause cellular dysfunction and disease pathology. More than 50 LSDs have been identified and they can be classified based on the defective enzymes and accumulated macromolecules. Certain lysosomal proteins are functionally and physically associated as multiprotein complexes for efficient digestive reactions. One example of this type of complex consists of three core enzymes, β-galactosidase (β-gal, EC 3.2.1.23), lysosomal sialidase (neuraminidase 1, EC 3.2.1.18) and cathepsin A (EC 3.2.1.18), and mutations in any of these proteins lead to LSD pathology [2].

Mutations in GLB1 gene (HGNG: 4298) that encodes β-gal (GLB1 protein) cause two biochemically and clinically distinct LSDs: GM1 gangliosidosis (OMIM #230500) and Morquio-B disease (MBD, OMIM #256550) [3]. β-gal hydrolyzes a wide range of substrates, and defects in β-gal result in massive accumulations of GM1 ganglioside and keratan sulfate in GM1 gangliosidosis and MBD, respectively. GM1 gangliosidosis is a severe neurodegenerative disease, whereas MBD is not neuropathic but rather is defective in skeletal development. GM1 gangliosidosis is classified into three types based on the age of disease onset (infantile, juvenile and adult types), and the infantile type is the most severe type. β-gal is composed of 677 amino acids, and over 100 mutations in GLB1 gene have been reported in patients with GM1 gangliosidosis and MBD [4]. A structural analysis based on the crystal structure of human β-gal revealed that most mutations found in severe GM1 gangliosidosis patients are located in the protein core. In contrast, many mutations associated with mild GM1 gangliosidosis or MBD are found throughout the protein surface regions [5].

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Sialidosis is a rare genetic disease that originates from mutations in NEU1 gene (HGNC: 7758), and distinct clinical features are known, including mental retardation and skeletal dysplasia [6]. Four sialidase genes (NEU1, 2, 3 and 4) exist in humans, and their protein products differ in the subcellular location and substrate specificity. Human sialidases share high sequence homology, including Asp-box motifs that are critical for sialidase activity. NEU1 encodes lysosomal sialidase that consists of 415 amino acids, and mutations in NEU1 result in the accumulation of sialylated oligosaccharides and glycoproteins. More than 30 mutations in NEU1 genes have been described in sialidosis patients, although correlations between mutation types and clinical phenotypes have not yet been clearly demonstrated. In this paper, we report novel mutations in GLB1 and NEU1 genes that cause GM1 gangliosidosis and sialidosis, respectively. We sequenced GLB1 and NEU1 genes in patient cell lines and established that each cell line showed molecular features of the corresponding disease. We also verified that the novel mutations abolished enzyme activities using *in vitro* assays with overexpressed mutant proteins.

2. Materials and methods

2.1. Cell culture

Human WT (CRL2097) and patient fibroblasts (see Table 1) were purchased from the Coriell Cell Repositories (http://ccr.coriell.org). Fibroblasts were cultured in α-MEM, supplemented with 15% FBS, 1X MEM non-essential amino acids (Gibco), 1 mM sodium pyruvate, 100 mg/ml streptomycin and 100 U/ml penicillin. 293T cells were maintained in DMEM, supplemented with 10% FBS, 100 mg/ml streptomycin and 100 U/ml penicillin.

2.2. cDNA sequencing

RNAs were extracted from fibroblasts using RNiso Plus reagent (Takara) according to the manufacturers' instructions. RNAs were reverse transcribed using Superscript III first-strand synthesis system (Invitrogen). Full-length GLB1 and NEU1 cDNAs were PCR-amplified and sequenced. NEU1 cDNA was further cloned into pCR4-TOPO vector (Invitrogen) for allele-specific sequencing. The oligos that were used for PCR amplification of full-length GLB1 were 5′-CCG CGG CCG CAT GCC GGG GTT CCT GGT TCG CAT-3′ and 5′-GGC TCG AGT ACA TGG TCC AGC CAT GAA TC-3′, and The oligos for full-length NEU1 PCR were 5′-GCT TAA GGG TGA CAT CTG CGC-3′ and 5′-CTG TCT TTC AGG CGT CTC CAG-3′.

2.3. Cloning

To generate GFP fusions of GLB1 and NEU1, WT GLB1 and NEU1 genes were amplified from cDNA of WT fibroblasts and cloned into pEGFP-N3 vector (BD bioscience Clonetech). FLAG tag was inserted between the 3′-end of GLB1/NEU1 and the 5′-end of GFP. Mutant GLB1 and mutant NEU1 constructs were prepared by site-directed mutagenesis of the corresponding WT constructs.

2.4. Enzyme assay

For β-gal assay, 293T cells were transiently transfected with either WT or mutant GLB1-pEGFP plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. Empty pEGFP vector was used as a negative control. Cells were further incubated for 24 h, and the expression of GFP fusion protein was confirmed by the detection of fluorescence signals. Cells were harvested and resuspended in the assay buffer (20 mM citrate pH 4.5, 60 mM NaCl and 1 mM $MgCl_2$). Cells were then lysed by sonication, and cell debris was removed by centrifugation at 4 °C for 10 min. 2 μg of cell lysate was incubated with 2 mM 4-methylumbelliferyl-β-D-galactopyranoside (MUG, Sigma–Aldrich) in 100 μl of assay buffer at 37 °C for 30 min. Reactions were terminated by adding 50 μl of 0.1 M glycine-NaOH, pH 10.5 buffer. Fluorescence signal of the resulting 4-MU was determined with excitation and emission at 365 nm and 450 nm, respectively. Sialidase assay was performed as for β-gal assay with several modifications. Increased amount of cell lysate (20 μg) was incubated with 0.1 mM 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MUNANA, Sigma–Aldrich) for 4 h.

Table 1
Summary of cell line information and sequencing results. Novel mutations are marked in bold. NC: not changed.

Disease	Sequenced gene	Cell line	Mutation				Exon	Reference
			Type	Nucleotide changes		Amino acid change		
				Allele 1	Allele 2			
GM1 gangliosidosis	GLB1	GM00918	Polymorphism	C > T	C > T	P10L	1	[3].
			Missense	A > C	A > C	**E186A**	6	This paper
		GM02439	Polymorphism	C > T	C > T	P10L	1	[3].
			Missense	C > T	C > T	R201C	6	[16].
		GM03589	Polymorphism	C > T	C > T	P10L	1	[3].
			Missense	G > A	NC	C127Y (Het)	3	[8].
			Missense	NC	T > G	W161G (Het)	5	[7].
		GM05335	Missense	G > C	G > C	Q255H	7	[17].
		GM05652	Polymorphism	C > T	C > T	P10L	1	[3].
			Nonsense	C > T	C > T	R351X	10	[7].
			Polymorphism	A > G	A > G	S532G	15	[9].
		GM05653	Polymorphism	C > T	C > T	P10L	1	[3].
			Nonsense	C > T	C > T	R351X	10	[7].
			Polymorphism	A > G	A > G	S532G	15	[9].
		GM10919	Polymorphism	C > T	C > T	P10L	1	[3].
			Missense	C > A	C > A	R148S	4	[9].
			Polymorphism	A > G	A > G	S532G	15	[9].
		GM12369	Polymorphism	C > T	C > T	P10L	1	[3].
			Missense	T > G	T > G	W161G	5	[7].
Sialidosis	NEU1	GM02685	Missense	G > A	G > A	G227R	4	[11].
		GM02921	Missense	NC	T > C	V275A (Het)	5	[18].
			Missense	G > A	NC	**R347Q** (Het)	6	This paper

2.5. X-gal staining

To measure β-gal activity using X-gal as a substrate, cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Fixed cells were incubated in the assay buffer containing 1 mg/ml X-gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide and 2 mM magnesium chloride at 37 °C until cells stained blue.

2.6. Western blotting

30 μg of cell lysate was loaded onto 4–20 % gradient gel (Bio-Rad) and transferred to nitrocellulose membranes. GLB1/NEU1-GFP fusion proteins were detected with anti-GFP antibody (Abcam, ab290, 1:5000), and β-actin was detected with anti-β-actin antibody (Abcam, ab6276, 1:5000).

2.7. Immunocytochemistry

To measure lysosome contents, LysoTracker Red DND-99 (Invitrogen) was added to the culture media at a dilution of 1:20000 for 30 min and removed before detecting fluorescence signals. GM1 accumulation was measured by immunostaining cells with anti-GM1 antibody (Abcam, ab23943, 1:50).

2.8. Structural analysis

Sequence alignment was performed using the ClustalW2 and BoxShade servers. For GLB1 modeling, the crystal structure of human GLB1 (PDB code: 3THC) was used as a template, and the mutant model was generated by PyMOL program. For NEU1 modeling, the template search and homology modeling were performed using the SWISS-MODEL server (http://swissmodel.expasy.org). The crystal structure of human NEU2 (PDB code: 2F13) was selected as a template.

3. Results/discussion

3.1. GLB1 mutations in GM1 gangliosidosis patient cells

We identified six missense, one nonsense and two polymorphic mutations in GLB1 gene in eight GM1 gangliosidosis patient cell lines, and one missense mutation p.E186A was a novel mutation (Table 1). Seven cell lines were derived from the patients diagnosed with infantile type GM1 gangliosidosis, and one (GM02439) was from a juvenile type patient (http://ccr.coriell.org). All of the patient cell lines, except GM03589 line, have homozygous mutations. GM03589 cells contain two heterozygous mutations, both of which were reported to disrupt β-gal activity [7,8]. p.P10L mutation is a common polymorphism that was observed in seven out of eight cell lines. p.S352G polymorphism that is known to decrease β-gal activity was found in three cell lines. Five missense (p.C127Y, p.R148S, p.W161G, p.R201C and p.Q255H) and one nonsense (p.R351X) mutations were previously described. GM00918 cell line has a novel homozygous p.E186A mutation and was investigated for further analysis.

GM00198 patient fibroblast cell line evidently exhibited the molecular phenotypes of GM1 gangliosidosis. A nucleotide change of c.557A > C results in the conversion of glutamic acid to alanine

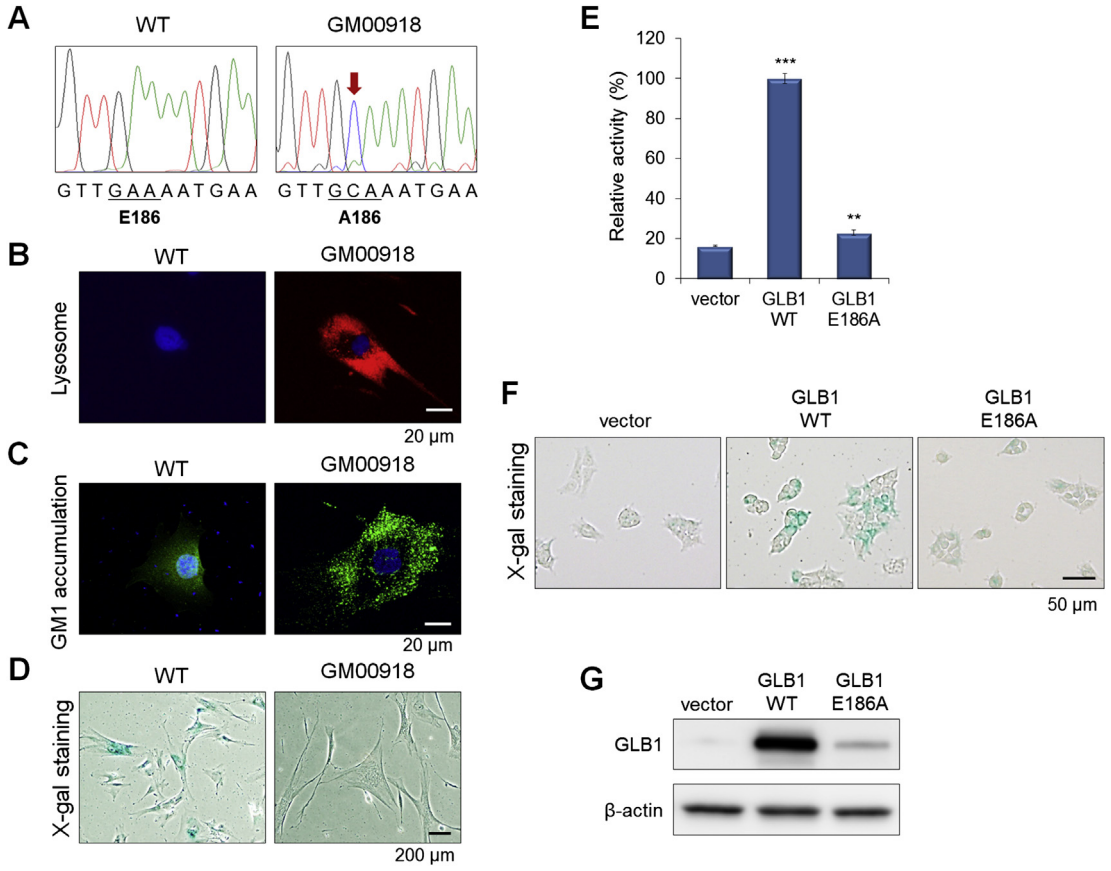

Fig. 1. Characterization of GM1 gangliosidosis patient fibroblasts and E186A mutation. (A) cDNA sequencing of GLB1 gene (B and C) Immunostaining of WT and patient fibroblasts using LysoTracker (B) and anti-GM1 ganglioside antibody (C). (D) X-gal staining of WT and patient fibroblasts. (E) β-gal assay of WT and E186A GLB1 proteins expressed in 293T cells. $^{***}P < 0.001$, $^{**}P < 0.01$, based on the t-test. (F) X-gal staining of 293T cells expressing GLB1 constructs. (G) Western blotting of 293T cells transfected with empty vector, WT or E186A GLB1 constructs. β-actin was used as a loading control.

(Fig. 1A). GM00918 fibroblasts contained markedly elevated levels of lysosomes compared with wild-type fibroblasts (WT, CRL2097), as shown by LysoTracker staining (Fig. 1B). These fibroblasts also showed massive accumulation of GM1 ganglioside, demonstrating that they were derived from a GM1 gangliosidosis patient rather than from a MBD patient (Fig. 1C). β-gal activity was apparently impaired in GM00918 cells, as shown by X-gal staining assay (Fig. 1D). To demonstrate that the loss of β-gal activity was caused by p.E186A mutation in GLB1, we transiently overexpressed GFP fusions of WT and E186A mutant GLB1 proteins in 293T cells. Vector-transfected cells showed approximately 16% β-gal activity that presumably originated from GLB1 in the 293T genome, compared to that of cells transfected with WT GLB1 construct (Fig. 1E). Introducing p.E186A mutation into GLB1 dramatically reduced β-gal activity to 22.9% of WT activity, which was close to the background level (Fig. 1E). Loss of β-gal activity of E186A GLB1 was further validated by X-gal staining (Fig. 1F). The expressions of fusion proteins was examined by Western blotting and GFP signal detection (Fig. 1G and data not shown). E186A mutant was expressed at approximately 25% of WT expression level. We failed to produce E186A GLB1 at a level similar to that of WT, although we tried various transfection conditions. We speculate that p.E186A mutation may decrease protein stability and promote its degradation. Taken together, our data demonstrated that the novel mutation p.E186A in GLB1 significantly reduced enzyme activity and induced molecular symptoms of GM1 gangliosidosis in patient cells.

Structural analyses indicated that the mutations identified in this study were critical for enzyme activity. Eukaryotic GLB1-related proteins are highly conserved among species from fungi to humans (Fig. 2A). They also share lower, but significant degree of homology to prokaryotic proteins, including conserved catalytic residues and overall domain structures [5]. All six missense mutation sites that we identified are well conserved in eukaryotes,

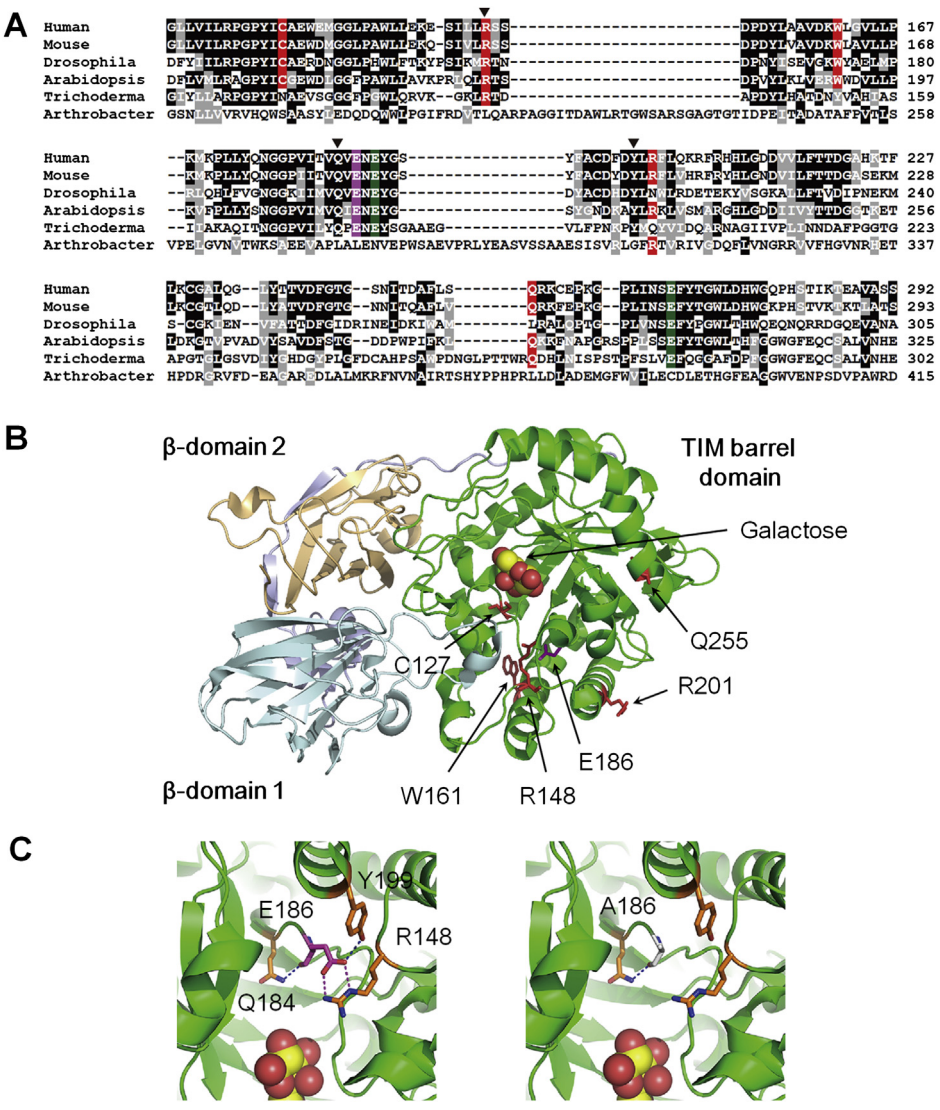

Fig. 2. Structural analysis of GLB1 mutations identified in GM1 gangliosidosis patient cells. (A) Sequence alignment of GLB1-related proteins in eukaryotes (human, mouse, fruit fly, *Arabidopsis thaliana*, *Trichoderma reesei*) and a prokaryote (*Arthrobacter*). Only a select portion of the alignment is presented. Mutation sites are colored red (C127, R148, W161, R201 and Q255) and purple (E186). Two catalytic glutamic acids are colored green. Residues that are predicted to interact with E186 are marked with triangles. (B) The crystal structure of human β-gal. Green, TIM barrel domain; sky blue, β-domain 1; gold, β-domain 2. Mutation sites are represented as sticks with the same color scheme as in (A). Galactose ligand is presented as spheres, and it is bound in the active site. (C) Three-dimensional modeling of E186A mutant. Left, WT has E186 residue shown as purple sticks. Right, the mutated A186 residue is shown in gray. E186-interacting residues are presented as orange sticks. Ion pair interactions are shown as purple dashed lines, and hydrogen bonds are shown as blue dashed lines. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

suggesting that these sites are important for protein function. Two mutation sites, R148 and E186, are more conserved than the other four sites, and E186 residue is very close to the catalytic E188 residue. Three-dimensional analyses based on the crystal structure of human β-gal revealed that all of the mutation sites reside in the TIM barrel domain that encompasses the active site (Fig. 2B) [5]. Four residues, C127, R148, W161 and E186, are located in the proximal ligand-binding site, and C127 is directly involved in ligand binding. Two residues, R201 and Q255, which are less conserved, are relatively distant from the active site, suggesting that they might have smaller impact on activity than the other four sites. The mutation of R201, p.R201C, was indeed identified in patient cells with juvenile type that is milder than infantile type. E186 is located in the protein core and is predicted to interact with three neighboring residues, R148, Q184 and Y199 (Fig. 2C) [5]. All of the residues that interact with E186 are evolutionary conserved, and mutations of these residues have been found in GM1 gangliosidosis patients, suggesting that they are functionally critical residues (Fig. 2A) [9,10]. E186 forms ion pair interactions with R148, and is hydrogen-bonded to Q184 and Y199. Replacement of glutamic acid with the smaller alanine is predicted to abolish ion pair interactions to R148 and hydrogen bonding to Y199, which likely affects the core structure and protein stability.

3.1.1. Analysis of NEU1 gene in sialidosis patient cells

We analyzed cell lines derived from another type of LSD, sialidosis, and identified three missense mutations in NEU1 gene (Table 1). GM02685 line contained a previously described p.G227R mutation [11]. Two heterozygous mutations, p.R347Q and p.V275A, were identified in GM02921, and p.R347Q mutation has not yet been reported. Nucleotide changes of c.1040G > A and c.824T > C that cause arginine to glutamine and valine to alanine amino acid alterations, respectively, were detected as doublets in the sequencing results (Fig. 3A). We further verified that two point mutations reside on different alleles by sequencing multiple clones of NEU1 cDNAs from GM02921 cells. Every NEU1 clone contained only one of the two mutations (data not shown).

We then demonstrated that GM02921 fibroblast cell line showed molecular characteristics of sialidosis. Lysosomal contents were significantly enriched in GM02921 cells compared with WT fibroblasts, as shown by LysoTracker staining (Fig. 3B). Sialidase enzyme activity was abolished in GM02921 cells as previously described (Fig. 3C) [12]. To determine whether p.R347Q mutation alone eliminates sialidase activity, we generated GFP fusion constructs of NEU1 protein that contained either p.R347Q or p.V275A mutation. Residual activities of both mutant NEU1 proteins were less than 5% of WT activity when overexpressed in 293T cells, suggesting that p.R347Q is a novel mutation that eliminates sialidase activity (Fig. 3D). Expression of fusion proteins was analyzed by Western blotting and GFP signal detection (Fig. 3E and data not shown). R347Q mutant and V275A mutant were expressed at 30% and 7.5% of WT level, respectively. We propose that these mutations may disrupt the stability of NEU1 proteins, as we tested a variety of transfection conditions, including higher amounts of plasmid, but were unable to increase their expression levels close to that of WT (data not shown).

We performed structural analyses to further invest the effects of the identified mutations on the structure and function of lysosomal sialidase. Sequence alignment showed that vertebrate sialidases are highly conserved (Fig. 4A). All three mutation sites, G277, V275 and R347, are well conserved, implying that they play important roles in protein function. We also generated a three-dimensional structure model of human NEU1 proteins using homology modeling system (Fig. 4B). Because the human NEU2 protein is the only mammalian sialidase of which structure has been solved to date, we selected its crystal structure as a template [13]. Although NEU2 shares lower

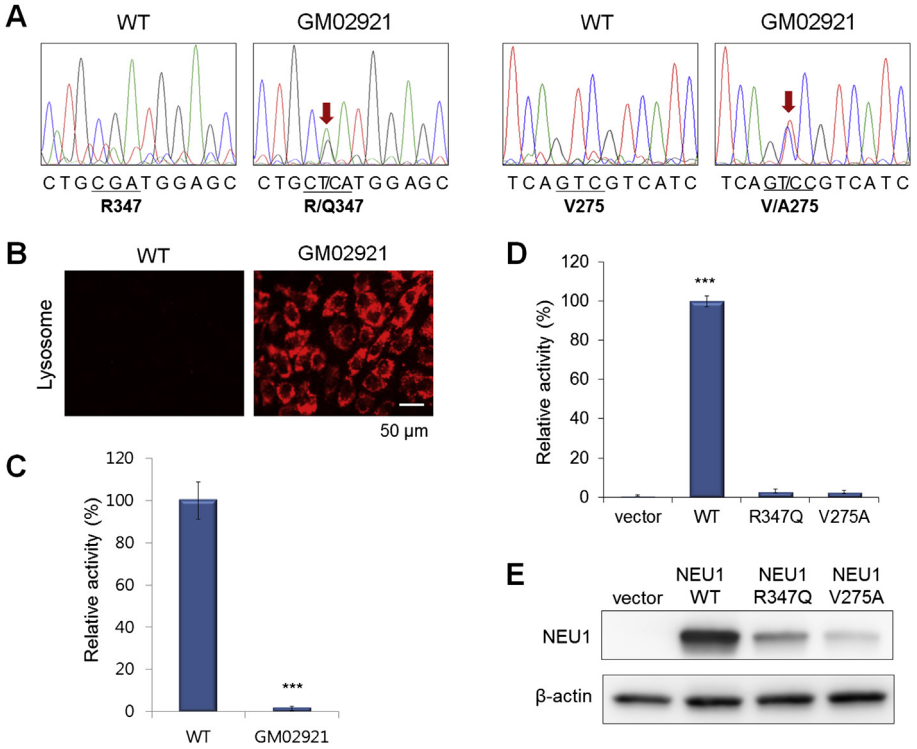

Fig. 3. Characterization of sialidosis patient fibroblasts and R347Q mutants. (A) cDNA sequencing of NEU1 gene in WT and patient cell lines. Doublets indicative of p.R347Q and p.V275A were reproducibly detected. (B) LysoTracker staining of WT and patient fibroblasts (C) Sialidase assay of WT and patient fibroblasts. ***$P < 0.001$, based on the t-test. (D) Sialidase assay using 293T cells overexpressing WT or mutant NEU1 proteins. ***$P < 0.001$, based on the t-test. (E) Western blotting of 293T cells transfected with empty vector, WT, R347Q or V275A NEU1 constructs. β-actin was used as a loading control.

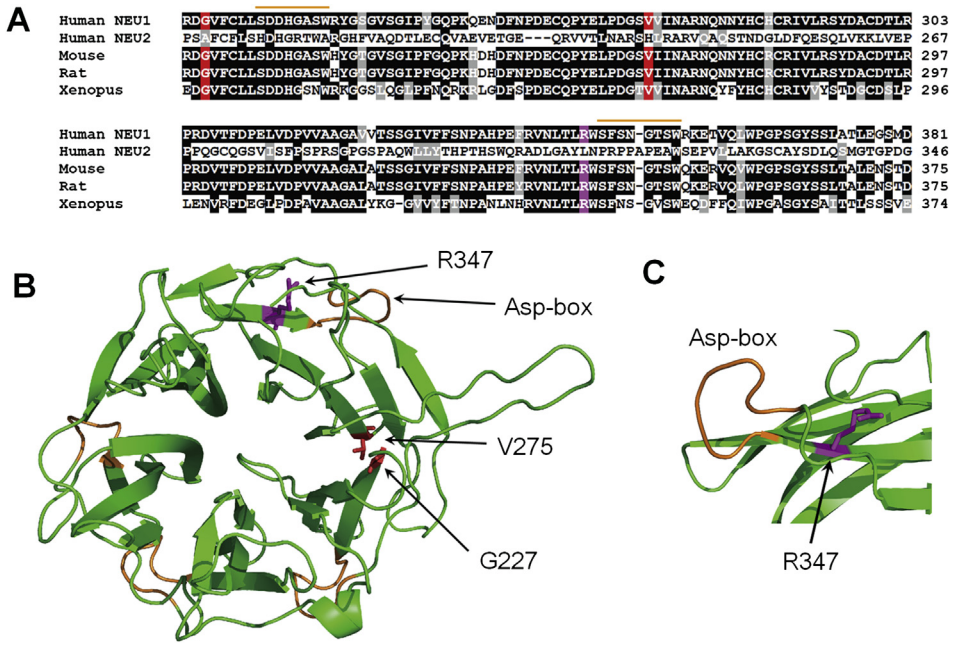

Fig. 4. Structural analysis of mutations in NEU1 protein. (A) Sequence alignment of vertebrate NEU1-related proteins. Alignment is partially presented. Mutation sites are colored red (G277 and V275) and purple (R347). Asp-boxes are indicated with orange lines. (B) Homology modeling of human NEU1 based on the crystal structure of human NEU2. The color scheme is the same as that described in (A). (C) Details of the region adjacent to R347 residue. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

sequence homology to NEU1 than other vertebrate sialidases, human NEU1 and NEU2 still are significantly similar (28% identity), and catalytically critical residues and motifs are conserved in both [14]. Homology modeling predicted that NEU1 forms a canonical sialidase architecture that is composed of many β-strands and has the catalytic site at the central cavity [15]. The three mutation sites are likely not directly involved in catalytic reaction because they are not close to or do not face the central hole. Rather, these sites are anticipated to affect the topology of the loops that contain many enzymatically important residues. G227 and V275 are located at the ends of β-strands, the connecting points to the loops. R347 residue is very close to the Asp-box motif that is highly conserved, and the proper orientation of this motif is required for enzyme activity (Fig. 4C). Mutations of those residues likely change the neighboring loop structures and thus affect enzyme activity.

In this study, we characterized patient cells with GM1 gangliosidosis and sialidosis and demonstrated that these cells exhibited molecular features of the corresponding diseases. We identified six missense, one nonsense and two polymorphisms in GLB1 gene and three missense mutations in NEU1 gene in GM1 gangliosidosis and sialidosis patient cells, respectively. Of these mutations, p.E186A in GLB1 and p.R347Q in NEU1 are novel mutations. Structural analyses predicted that the identified mutations are critical for enzyme activities. Taken together, our study provides information that is required for accurate molecular diagnosis and insight into the effects of the mutations on the enzymatic activity responsible for LSDs.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

J.E.K. and M.S. designed the study, conducted experiments and prepared manuscript. Y.S.S. collected data. M.J.S. helped the literature review and manuscript preparation. Y.S.C. obtained funding and critically revised manuscript.

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