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Genotype-phenotype correlation of gangliosidosis mutations using in silico tools and homology modeling

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ABSTRACT

Gangliosidoses, including GM1-gangliosidosis and GM2-gangliosidosis (Tay-Sachs disease and Sandhoff disease), are lysosomal disorders resulting from enzyme deficiencies and accumulation of gangliosides. Phenotypes of gangliosidoses range from infantile, late-infantile, juvenile, and to the adult form. The genotype-phenotype correlation is essential for prognosis and clinical care planning for patients with a gangliosidosis condition. Previously, we have developed a method to establish the genotype-phenotype correlation of another lysosomal disease, mucopolysaccharidosis type I, with in silico tools. This same method was applied to analyze the genotype and phenotype of 38 patients diagnosed with a gangliosidosis disease in the United States. Out of 40 mutations identified, 3 were novel, including p.Tyr192His and p.Phe556Ser of the *GLB1* gene and p.Gly461Val of the *HEXA* gene. Furthermore, the mutant protein structure of all missense mutations was constructed by homology modeling. A systemic structural analysis of these models revealed the specific mechanisms of how each mutation may lead to the disease. In summary, the method developed in this study holds promise as a tool that can be broadly applicable to other lysosomal diseases and monogenic diseases.

1. Introduction

The gangliosidoses are inherited metabolic disorders resulting from the accumulation of gangliosides in the central nervous system, which leads to severe and progressive neurological impairment [1]. They are categorized into GM1-gangliosidosis and GM2-gangliosidosis, and both diseases are autosomal recessive. GM1-gangliosidosis (MIM #230500) is due to mutations in the *GLB1* gene, leading to deficiency of lysosomal enzyme β-galactosidase and subsequent accumulation of GM1-gangliosides [1]. Notably, the mutations in the GLB1 gene can also lead to another lysosomal disease, mucopolysaccharidosis type IV B (MPS IVB, MIM # 253010), or Morquio syndrome type B. MPS IVB is mainly a skeletal disease due to the accumulation of keratan sulfate [2]. GM2-gangliosidoses, including Tay-Sachs disease (MIM #272800) and Sandhoff disease (MIM #268800), are due to mutations in the *HEXA* and *HEXB* genes encoding the α and β subunits, respectively, of lysosomal enzyme β-hexosaminidase A, resulting in accumulation of GM2 gangliosides [1].

Phenotypes of GM1- and GM2-gangliosidoses can be generally classified as infantile, late-infantile, juvenile, and adult. Patients with the infantile form exhibit symptoms during infancy, presenting with progressive neurological impairment and death in early childhood [3,4]. A late-infantile form has also been reported, in which patients exhibit symptoms between one and three years of age and may live into later childhood [4,5]. The onset of symptoms in the juvenile form is usually between three to five years of age, manifested as ataxia, dysarthria, hypotonia, and dysphagia [6,7]. The lifespan of the juvenile form ranges from late childhood to early adulthood [1]. In contrast, the adult (or late-onset) form exhibit symptoms in early or mid-adulthood. The symptoms include limb-girdle weakness, ataxia, neuromuscular weakness, and eventual loss of ability to ambulate independently. The symptoms include limb-girdle weakness, ataxia, neuromuscular weakness, and eventual loss of ability to ambulate independently [8–10]. In addition, difficulties with speech may develop. Patients may also develop psychiatric changes [4,6,7]. The lifespan of the adult form varies greatly [7]. There are no effective therapies for GM1- and GM2-gangliosidoses, with palliative measures being the current standard of care. There are many continuing efforts, however, to develop therapeutic protocols, which include establishment of novel animal models [11], enzyme replacement therapy [12], substrate reduction therapy [13], bone marrow transplantation [14], and gene therapy [15] in animal models.

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At this time, understanding the genotype-phenotype correlation of gangliosidoses is critical in understanding the patient's prognosis and planning for different stages of palliative care. As the era of newborn screening emerges, the relationship between the genotype and phenotype will play a greater role in planning critical care, especially in light of increasing efforts towards developing effective treatments for patients with gangliosidoses. Newborn screening is being performed in an increasing number of lysosomal diseases. Newborn screening of gangliosidoses is not currently done but is anticipated to become routine as future therapies become available. One method of newborn screening in lysosomal disease is with assays of the defected enzyme. Another potential method is metabolomics profiling with reverse phase liquid chromatography (RPLC) [16]. However, it is still difficult to reliably predict phenotypes of the disease, such as late-infantile or juvenile versus adult phenotypes. Methods to identify the phenotypes of diseases are urgently lacking. Identification of the phenotypes is crucial because phenotypes with a worsened prognosis, such as infantile gangliosidoses, require more urgent and intensive interventions. Furthermore, the ability to predict phenotypes from genotypes would allow enrichment of disease subtypes in clinical trials, advancing the development of treatments.

In a previous study, we demonstrated a single amino acid mutation prediction (SAAMP) algorithm to predict whether a missense mutation is pathogenic or benign for MPS I disease [17]. This method integrates the prediction outcome of multiple bioinformatics tools and achieves a high sensitivity (94%) and specificity (80%). More recently, a SAAMP 2.0 algorithm, which has better sensitivity and specificity, has been developed. When assessed in a total of 13 lysosomal diseases, it yielded a further improved sensitivity (95%) and specificity (90%), which outperformed the mainstream bioinformatics tools evaluated [18]. In the study reported herein, the in silico method was application was expanded to analyze the genotype-phenotype correlation of patients with gangliosidoses. Additionally, 3D structural analysis was also conducted to elucidate the mechanisms of how each mutation can lead to the disease.

2. Methods and materials

2.1. Patients

This study was conducted under a clinical trial, The Natural History of Gangliosidoses (NCT00668187), of the Lysosomal Disease Network (U54NS0657698) which is part the National Institutes of Health (NIH) Rare Diseases Clinical Research Network (RDCRN). Patients participating in this study were enrolled in the natural history study. The study was conducted at the University of Minnesota with Institutional Review Board (IRB) approval and IRB-approved consent of the patients or patients' parents/legal guardians. The diagnosis of each patient was confirmed through genetic sequencing and biochemically by enzyme assays. The clinical course, and thus the phenotype, of each patient was documented through retrospective chart review and prospective clinical care.

2.2. Predicting functional impacts of missense mutations

A total of 7 bioinformatics tools were used as previously described [17]. These tools include: Sorting Intolerant From Tolerant (SIFT, http://sift.jcvi.org/) [19], Polymorphism Phenotyping (PolyPhen, http://genetics.bwh.harvard.edu/pph2/) [20], I-Mutant (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) [21], PROtein Variation Effect ANalyzer (PROVEAN, http://provean.jcvi.org/index.php) [22], Protein ANalysis THrough Evolutionary Relationships (PANTHER, http://www.pantherdb.org/) [23], Single Nucleotide Polymorphism Database & Gene Ontology (SNPs&GO, http://snps.biofold.org/snps-and-go/snps-and-go.html) [24], Predictor of Human Deleterious Single Nucleotide Polymorphism (PHD-SNP, http://snps.biofold.org/phd-snp/phd-snp.html) [25].

Each bioinformatics tool predicts whether a single mutation is pathogenic or benign with an inherent index indicating the confidence of the prediction. SIFT focuses on predicting the effect of a single nucleotide polymorphism (SNP) through sequence preservation over the evolutionary time. PolyPhen utilizes a sequence and structure-based method to predict the possible impact of SNP. I-Mutant is a support vector machine (SVM) based predictor of protein stability changes introduced by a SNP. PROVEAN is a sequence-based predictor that estimates whether a SNP affects the protein function. SNPs&GO is a SVM based web server that combines protein structural/functional parameters and sequence analysis derived information. PHD-SNP is a SVM web server based on evolutionary information. PANTHER is a protein family and subfamily database that predicts the frequency of occurrence of an amino acid at a particular position in homologous sequences.

2.3. Homology modeling and 3D structural analysis

Iterative Threading ASSEmbly Refinement (I-TASSER, http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to build the three-dimensional structure model with replica-exchanged Monte Carlo simulations [26]. The Swiss-PDB viewer was used to analyze the models generated by homology modeling. Additionally, Project Have yOur Protein Explained (HOPE; http://www.cmbi.ru.nl/hope/home) was used for analyzing the structural impacts of these mutations as previously described [17].

3. Results

3.1. Patients description

A total of 38 patients with infantile, late-infantile or juvenile gangliosidoses were enrolled and analyzed for correlations between genotypes and phenotypes. Patients with the infantile gangliosidoses are described in our previous paper [3]. Patients with the late-infantile or juvenile gangliosidoses are described in this paper (summarized in Table 1). Across both studies, there are patients with infantile GM1-gangliosidosis (n = 8), late-infantile GM1-gangliosidosis (n = 4), juvenile GM1-gangliosidosis (n = 6), infantile Tay-Sachs disease (n = 9), late-infantile Tay-Sachs disease (n = 1), juvenile Tay-Sachs disease (n = 3), infantile Sandhoff disease (n = 6), and juvenile Sandhoff disease (n = 1). Patients originated from different regions in the United States. Genotype-phenotype association was evaluated using a method previously reported [17,18], based on clinical and biochemical results of subjects enrolled in this study, as well as literature reports. There was one pair of siblings, and these patients had late-infantile GM1 (genotype p.Arg201Cys/p.Arg201Cys). A total of 40 mutations were identified, including 30 missense/nonsense mutations, 4 splicing mutations, and 6 insertions/deletions. A total of 3 novel mutations were identified in this study, which were the p.Tyr192His and p.Phe556Ser in the *GLB1* gene, and p.Gly461Val in the *HEXA* gene.

3.2. In silico prediction

As previously described, a total of seven bioinformatics tools were applied to predict the severity of the missense or nonsense mutations. As shown in our previous studies, the SAAMP 1.0 and SAAMP 2.0 algorithm can predict whether a mutation is pathogenic or benign by integrating the prediction outcomes of the aforementioned tools. The prediction outcomes of each individual tool and the SAAMP algorithm are shown in Table 2. All these mutations were predicted to be 'pathogenic' by the SAAMP algorithm, confirming the accuracy and reliability of this method.

Table 1
Demographic information of late-infantile and juvenile GM1 and GM2 gangliosidoses patients. Abbreviations: F, female; M, male; mo, months old of age; MRI, magnetic resonance imaging; N/A, not available; yo, years old of age.

Diagnosis	Gender	Race	Genotype	Age at clinical finding	Initial clinical finding leading to diagnosis	Age at diagnosis
Late-infantile GM1	M	Caucasian	p.Arg201Cys/p.Lys578Arg	1 yo	Abnormal gait noted around 1 year of age. Work -up at 2 years of age for muscular dystrophy	22 mo
Late-infantile GM1	M	French Canadian, Irish, Polish	c.75 + 2dupT/p.Phe556Ser	1 yo	Difficulties with speech. Never learned to speak words	21 mo
Late-infantile GM1	M	Caucasian	p.Arg201Cys/p.Arg201Cys	2 yo	Strabismus at 2 years of age. MRI at 2 years old showed demyelination and MRI at 4 years of age showed little to no change in myelination pattern.	5 yo
Late-infantile GM1	M	Caucasian	p.Arg201Cys/p.Arg201Cys	2 yo	Speech and language delays. (Has older sibling diagnosed with late-infantile GM1)	19 mo
Late-infantile GM1	F	N/A	p.Leu228Pro/p.Asn669Lysfs*53	19 mo	Not walking	18 mo
Juvenile GM1	F	Korean, German, Irish	p.Leu155Arg/unknown	4 yo	Falling down frequently and falling off chair	5.5 yo
Juvenile GM1	F	Irish, German, Korean	p.Arg148Ser/p.Tyr192His	2.5 yo	Falling. Other symptoms noted after initial symptoms: dyspraxia, speech delays with poor articulation and limited vocabulary, dysmyelination on brain MRI, general developmental delays, inattention, hyperopia and astigmatism	10 yo 5 mo
Juvenile GM1	F	Caucasian	p.Arg201His/p.Arg351Ter	4 yo	Developmental delays exhibited as loss of words at age 4 years	12 yo
Juvenile GM1	M	Caucasian	p.Arg201His/p.Ala301Val	2 yo	Developmental delays in speech	8 yo
Juvenile GM1	F	N/A	p.Thr82Met/p.Gly123Arg	N/A	N/A	N/A
Late-infantile Tay-Sachs	F	Irish, Norwegian, German	c.570G > T/c.1273_1277dupATATC	17 mo	Difficulty ambulating	14 mo
Juvenile Tay-Sachs	F	Mexican, Italian, German, Native American and Dutch	c.1274_1277dupTATC/p.Gly461Val	N/A	N/A	6 yo
Juvenile Tay-Sachs	F	African, French Canadian	p.Arg178His/p.Arg499Cys	N/A	N/A	3 yo
Juvenile Tay-Sachs	F	N/A	c.77G > A (p.Trp26Stop)/p.Arg499His	N/A	N/A	8 yo

3.3. Establishment of 3D models

To analyze the 3D structural change introduced by the missense or nonsense mutations, structural analysis was performed by comparing the native and mutant protein structures. First, the native structure of *GLB1* [27], *HEXA*, *HEXB* proteins were extracted from the Protein Data Bank (PDB, http://www.rcsb.org/). The mutant models were constructed by I-TASSER for further structural analysis. An illustration of detailed structural changes is shown in Fig. 1. The total energy of these models was calculated and shown in Table 3. Out of all 23 models, 11 had significantly higher energy than the native models, indicating that the stability of these mutants was severely affected. The remaining 12 mutant models had similar total energy to the native models. Therefore, these mutations may affect the protein by other mechanisms than affecting stability, which may include impairing the active site or binding abilities with other proteins or substrates.

3.4. Systemic structural analysis

To gain further insights into the impact of the missense or nonsense mutations, a systemic method was designed, which analyzes individual mutation in the following five features: amino acid properties (charge, size, and hydrophobic status), contacts, conservation, domain, and structure.

3.4.1. Amino acid properties

The size, charge, and hydrophobic status of the amino acids were evaluated in missense mutations.

3.4.1.1. Size change. In ten mutations, the mutant residue was larger than the wild-type residue. If the mutation in the wild-type residue was buried in the core of the protein, the larger mutant residue was sometimes too large to fit. This applied to the following mutations: six mutations of *GLB1* (p.Arg68Trp, p.Thr82Met, p.Gly123Arg, p.Leu155Arg, p.Ala301Val, p.Lys578Arg), three mutations of *HEXA* (p.Leu127Arg, p.Arg170Trp, p.Gly461Val) and one mutation of *HEXB* (p.Gly301Arg).

In contrast, seven mutations resulted in smaller residues compared to the wild-type residues. If the residue was buried in the core, this mutation led to an empty space within the protein. This characteristic was found in five mutations of *GLB1* (p.Arg148Cys, p.Tyr192His, p.Leu228Pro, p.Tyr270Asp, p.Phe556Ser), one mutation of *HEXA* (p.Arg178His), and one mutation of *HEXB* (p.Val493Gly).

For residues on the surface of proteins that were smaller or larger than the wild-type counterpart, four mutations were predicted to lead to loss of external interactions with other molecules or other residues of the protein. In the study populations, this type of external interaction defect was found with one mutation of *GLB1* with a larger mutant residue (p.His281Tyr) and one mutation of *GLB1* (p.Arg201Cys) with a smaller mutant residue, and two mutations of *HEXA* with smaller mutant residues (p.Arg499His and p.Arg499Cys).

3.4.1.2. Charge change. A positively or negatively charged wild-type residue was replaced with a neutral mutant residue in five mutations. Examples of these mutations are three mutations of *GLB1* located in the core of the protein (p.Asp441Asn, p.Arg68Trp, p.Arg148Cys) and two mutations of *HEXA* (p.Arg170Trp, p.Arg178His). If a neutral mutant residue on the surface replaced a charged wild-type residue, a loss of normal interactions with other molecules was predicted. This applied to one mutation of *GLB1* (p.Arg201Cys) and two mutations of *HEXA* (p.Arg499His and p.Arg499Cys).

A neutral wild-type residue was replaced by mutant residues with positive or negative charges in six mutations. When the mutation was in the core of the protein, the mutation introduced a charge in a buried residue, causing problems in protein folding. This situation applied to one mutation of *HEXA* (p.Leu127Arg), one mutation of *HEXB*

Table 2
Phenotype prediction by in silico tools. The prediction outcomes of each individual tool are listed in the table. 'D' stands for 'disease' prediction, while 'N' stands for 'neutral' prediction. The 'P' stands for 'pathogenic' prediction by the SAAMP algorithm. The cut-off value of the SAAMP algorithm is set as 0.5. I, II and III represent infantile, late-infantile, and juvenile GM1 or GM2 gangliosidosis, respectively.

Mutation	Phenotype	Bioinformatic Tool								
		I-Mutant	PANTHER	SNP&GO	PROVEAN	PolyPhen	SIFT	PHD-SNP	SAAMP 2.0	Pathogenic index
GLB1										
p.Arg68Trp	I	D	D	D	D	D	D	D	P	1
p.Thr82Met	III	N	D	D	D	D	N	D	P	0.75
p.Gly123Arg	III	D	D	D	D	D	D	D	P	1
p.Arg148Cys	I	D	D	D	D	D	D	D	P	1
p.Leu155Arg	III	D	D	D	D	D	D	D	P	1
p.Tyr192His	III	D	D	D	D	D	D	D	P	1
p.Arg201Cys	II	D	D	D	D	D	D	D	P	1
p.Leu228Pro	II or III	D	D	D	D	D	D	D	P	1
p.Tyr270Asp	I	D	D	D	D	D	D	D	P	1
p.His281Tyr	I	N	D	D	D	D	D	D	P	1
p.Ala301Val	III	D	D	N	D	D	D	D	P	1
p.Asn318Asp	I	D	D	D	D	D	D	D	P	1
p.Asp441Asn	I	D	D	D	D	D	D	D	P	1
p.Phe556Ser	II	D	N	N	D	D	D	D	P	1
p.Lys578Arg	I, II	D	D	D	D	D	D	D	P	1
HEXA										
p.Leu127Arg	I	D	D	D	D	D	D	D	P	1
p.Arg170Trp	I	D	D	D	D	D	D	D	P	1
p.Arg178His	III	D	D	D	D	D	D	D	P	1
p.Gly461Val	III	D	D	D	D	D	D	D	P	1
p.Arg499Cys	III	D	D	D	D	D	D	D	P	1
p.Arg499His	III	D	D	D	D	D	D	D	P	1
HEXB										
p.Gly301Arg	I	D	D	D	D	D	D	D	P	1
p.Val493Gly	I	D	D	D	D	D	D	D	P	1

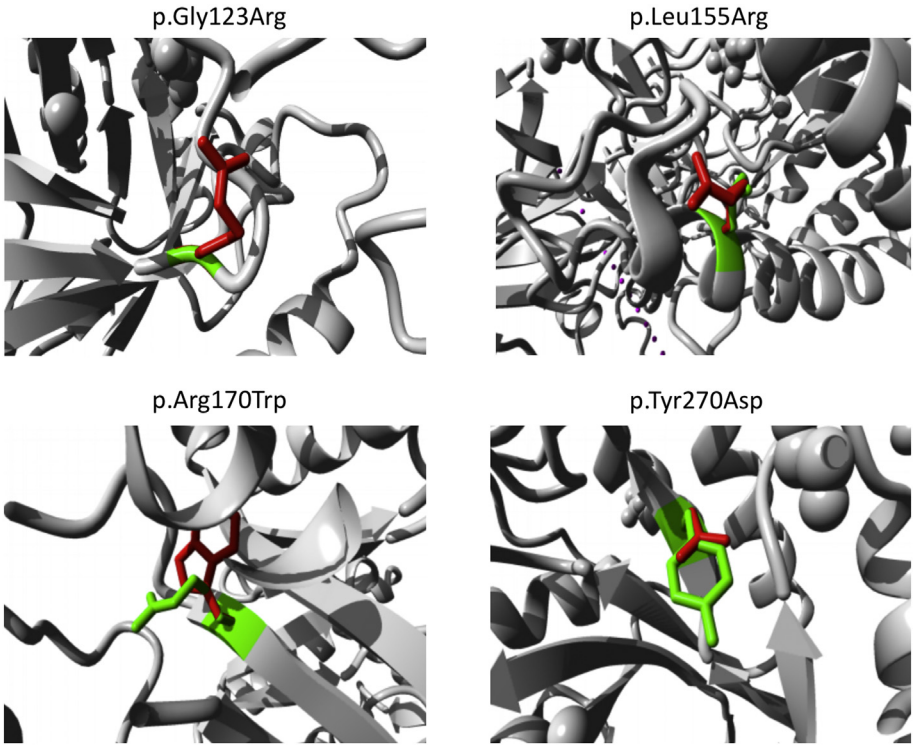

Fig. 1. Close-up view of superimposed structure of native and mutant residues. The main protein core is shown in gray color while the wild-type and mutant residues are shown in red and green color, respectively. The following mutants were shown: p.Arg170Trp, p.Gly123Arg, p.Leu155Arg and p.Tyr270Asp. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(p.Gly301Arg), and four mutations of *GLB1* (p.Leu155Arg, p.Gly123Arg, p.Tyr270Asp, p.Asn318Asp).

3.4.1.3. Hydrophobic status change. A wild-type residue which was more hydrophobic than the mutant residue was detected in ten mutations. This mutation caused a loss of hydrophobic interactions in the core of the protein, thereby disturbing correct protein folding. This situation applied to eight mutations of *GLB1* (p.Arg68Trp, p.Thr82Met, p.Leu127Arg, p.Arg148Cys, p.Leu155Arg, p.Tyr192His, p.Tyr270Asp, p.Phe556Ser), one mutation of *HEXA* (p.Arg170Trp), and one mutation of *HEXB* (p.Val493Gly).

Table 3
Total energy of native and mutant protein structure models.

Gene	AA change	Total energy after minimization (KJ/mol)
GLB1	Native	−57,984
	p.Arg68Trp	−57,813
	p.Thr82Met	−33,553
	p.Gly123Arg	−33,956
	p.Arg148Cys	−58,109
	p.Leu155Arg	−58,777
	p.Tyr192His	−33,389
	p.Arg201Cys	−33,221
	p.Leu228Pro	−32,189
	p.Tyr270Asp	−57,033
	p.His281Tyr	−33,559
	p.Ala301Val	−58,629
	p.Asn318Asp	−33,438
	p.Asp441Asn	−33,753
	p.Phe556Ser	−33,435
	p.Lys578Arg	−33,351
HEXA	Native	−28,732
	p.Leu127Arg	−28,999
	p.Arg170Trp	−28,413
	p.Arg178His	−30,469
	p.Gly461Val	−29,532
	p.Arg499Cys	−28,442
	p.Arg499His	−28,424
HEXB	Native	−59,199
	p.Gly301Arg	−56,720
	p.Val493Gly	−29,294

3.4.2. Contacts

Wild-type residues may form salt bridge and/or hydrogen bond with other residues (summarized in Table 4). The difference in charge between the wild-type and mutant residues is highly likely to disturb the ionic interaction (salt bridge). This applied to four mutations of *GLB1* (p.Arg68Trp, p.Arg148Cys, p.Arg201Cys, p.Asp441Asn) and four mutations of *HEXA* (p.Arg170Trp, p.Arg178His, p.Arg499His, p.Arg499Cys). Moreover, the size difference between wild-type and mutant residues may place the new residue in an incorrect position to make the original hydrogen bond. This applied to eight mutations of *GLB1* (p.Arg68Trp, p.Thr82Met, p.Arg148Cys, p.Tyr192His, p.Arg201Cys, p.Tyr270Asp, p.His281Tyr, p.Lys578Arg) and one mutation of *HEXA* (p.Arg170Trp). Additionally, the difference in hydrophobicity between the wild-type and mutant residues would affect hydrogen bond formation. Such was the case with six mutations of *GLB1* (p.Thr82Met, p.Arg148Cys, p.Tyr192His, p.Arg201Cys, p.Tyr270Asp, p.His281Tyr), and one mutation of *HEXA* (p.Arg170Trp).

As for p.Arg178His of *HEXA*, the wild-type residue has interactions with a ligand, (3aR,5R,6S,7R,7aR)-5-(hydroxymethyl)-2-methyl-5,6,7,7a-tetrahydro-3aH-pyrano[3,2-d][1,3]thiazole-6,7-diol. The different properties between the wild-type and mutant residues can result in loss of interactions with the ligand and impair the protein function. In both the PDB-file and in the Protein Interfaces Surfaces and Assemblies (PISA), this residue was found to be involved in a multimer contact. This confirmed that the residue contacting other proteins. Moreover, this mutation introduces a smaller residue at this position, which may be too small to make multimer contacts. As for p.Tyr270Asp of *GLB1,* the wild-type residue has interactions with a ligand annotated as (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)piperidine-3,4,5-triol. The difference in properties between the wild-type and mutant residues can easily cause loss of interactions with the ligand, thereby disturbing the protein function. As for p.Thr82Met of *GLB1* and p.Gly461Val of *HEXA,* the mutated residues are not in direct contact with a ligand. However, the mutation could affect the local stability, thereby affecting the ligand-contacts made by one of the neighboring residues.

3.4.3. Conservation

In general, mutations to highly conserved residues damage the protein.

All mutations analyzed were located near a highly conserved region. For mutations p.Arg68, p.Gly123, p.Tyr270 of *GLB1*, and p.Arg499 of *HEXA*, the region was 100% conserved. As for p.Arg201Cys, p.His281Tyr, and p.Phe556Ser of *GLB1*, and p.Arg170Trp of *HEXA*, neither the mutant residues nor another residue type with similar properties were observed at this position in other homologous sequences. Based on conservation scores, these mutations are highly likely damaging to the protein. As for p.Asp441Asn, p.Leu155Arg, p.Tyr192His, and p.Asn318Asp of *GLB1* and p.Leu127Arg, p.Arg178His, and p.Gly461Val of *HEXA*, the mutant residues were not among the other residue types observed at this position in other homologous proteins. However, residues that have some properties in common with these mutated residues were observed. This indicates that, in some rare cases, these mutations might not be damaging to the protein. As for p.Leu228Pro, p.Arg148Cys, p.Thr82Met, p.Ala301Val, and p.Lys578Arg of *GLB1*, the mutant residues were among the residues at this position observed in other sequences. This indicates that homologous proteins exist with the same residue type as the mutant at this position, and these mutations may not be damaging to the protein.

3.4.4. Domain

All the mutations described in the previous paragraph were located in a certain domain that is crucial for the enzymatic activity. Therefore, these mutations can disturb the normal functions of these domains. Moreover, all these mutations were in contact with another domain that is also important for the activity. The interactions between these domains could be disturbed by these mutations, which likely affected the function of the protein.

3.4.5. Structure

The mutations with large structural impacts are discussed in detail as follows. For mutation p.Leu228Pro of *GLB1*, the wild-type residue is

Table 4
Interactions between the wildtype amino acid residues with other residues. The wildtype amino acids are involved in hydrogen bond and salt bridge with other residues, and the mutations lead to the loss of such interactions.

Gene	Mutation	Hydrogen bond	Salt bridge
GLB1	p.Asp441Asn	Arg457, Lys493, Met480, Lys493	Arg457, Arg482, Lys493, Arg590
GLB1	p.Asp68Trp	Asp67	Asp67, Asp342, Asp568
GLB1	p.Arg148Cys	Glu186	Glu186, Glu188 and Asp221
GLB1	p.Arg201Cys	Asp198	Asp 196, Asp198
GLB1	p.Tyr270Asp	Glu268	
GLB1	p.His281Tyr	Asp275	
GLB1	p.Lys578Arg	Tyr444	Asp441, Glu478, Glu620
GLB1	p.Thr82Met	Ile55	
GLB1	p.Tyr192His	Leu147	
HEXA	p.Arg170Trp	Phe167, Lys197	Glu114
HEXA	p.Arg178His		Asp175, Asp207, Asp208, Glu323, Glu462
HEXA	p.Arg499His, p.Arg499Cys		Glu482, Glu498, Glu506

located in an α-helix. This mutant proline disrupts an α-helix since it is not located at one of the first three positions of that helix. Similarly, p.Leu155Arg of *GLB1* was located in an α-helix, and the mutation converts the wild-type residue into a residue that does not conform to an α-helix. Therefore, this mutation would disturb the helix and thereby affect the structure of the protein. As for p.Thr82Met, p.Tyr192His, p.Tyr270Asp, and p.Phe556Ser of *GLB1*, the wild-type residue was predicted to be located in its preferred secondary structure, a β-strand. The mutant residue prefers to be in another secondary structure, therefore the local conformation may be slightly destabilized.

As for p.Gly123Arg of *GLB1,* p.Gly461Val of *HEXA*, and p.Gly301Arg of *HEXB*, the wild-type residue is a glycine, the most flexible of all residues. This flexibility might be necessary for the protein function, and mutation of this glycine can disturb this function. In addition, only glycine is flexible enough for unusual torsion angles. As a result, these mutations can force the local backbone into an incorrect conformation and thereby disturb the local structure. As for p.Val493Gly of *HEXB*, the mutant residue glycine is very flexible and may disturb the required rigidity of the protein at this position.

3.5. Disease subtype deduction for GM2-gangliosidoses

The SAAMP 2.0 algorithm is robust in predicting whether a mutation is pathogenic or benign, however, it is not sensitive enough to predict disease subtypes. Therefore, based on previous literature, the association between genotype and disease subtypes of GM2-gangliosidosis (infantile, juvenile or adult) was deducted in Tables 5 and 6. Three novel mutations were evaluated in this study: p.Gly461Val, p.Tyr192His, and p.Phe556Ser. The mutation is p.Gly461Val was found in a patient diagnosed with juvenile Tay-Sachs disease. The mutation was found in trans with c.1274_1277dupTATC (p.Tyr427Ilefs) in *HEXA*, which leads to a frameshift mutation and is predicted to lead to a complete loss of enzyme activity. The mutation p.Gly461Val is likely to be associated with the juvenile phenotype. The mutation p.Tyr192His was found in trans with p.Arg148Ser in a patient diagnosed with juvenile GM1-gangliosidosis. The mutation p.Arg148Ser was associated with the infantile phenotype in a previous paper, therefore p.Tyr192His is expected to be associated with the juvenile phenotype [17]. The mutation, p.Phe556Ser was found in a patient diagnosed with late-infantile GM1-gangliosidosis and was in trans with c.75 + 2dupT, a known mutation associated with the infantile phenotype. Therefore, p.Phe556Ser is likely to be associated with the late-infantile phenotype of GM1-gangliosidosis.

Table 6
Deduction of phenotype severity through mutations in the *HEXB* gene through investigations of previous literature.

Mutation	Phenotype	References	Mutation	Phenotype	References
p.Trp57Cys	Juvenile	[64]	p.Cys360Arg	Unknown	[66]
p.Ser62Leu	Infantile	[65]	p.Pro417Leu	Adult	[4]
p.Ala97Pro	Unknown	[66]	p.Tyr456Ser	Adult	[71]
p.Cys137Tyr	Juvenile	[4]	p.Asp459Ala	Juvenile	[72]
p.Thr150Leu	Infantile	[4]	p.Gly483Ser	Infantile	[73]
p.Thr150Pro	Infantile	[47]	p.Gly484Glu	Infantile	[47]
p.Ile207Val	Unknown	[67]	p.Val493Gly	Infantile	[27]
p.Thr209Ile	Infantile	[47]	p.Asp494Gly	Juvenile or Adult	[74]
p.His212Asn	Infantile	[47]	p.Trp503Arg	Unknown	[66]
p.His235Tyr	Unknown	[68]	p.Pro504Ser	Adult	[4]
p.Ser255Arg	Unknown	[69]	p.Arg505Gln	Adult	[4]
p.Tyr266Asp	Infantile	[41]	p.Leu513Pro	Infantile	[75]
p.Gly282Glu	Infantile	[41]	p.Arg533His	Adult	[76]
p.Arg284Gln	Juvenile	[70]	p.Arg533Cys	Infantile	[47]
p.Thr295Arg	Unknown	[66]	p.Cys534Tyr	Infantile	[47]
p.Cys309Phe	Infantile	[47]	p.Arg539Cys	Unknown	[41]
p.Gly353Arg	Infantile	[4]	p.Ala543Thr	Unknown	[77]

4. Discussion

GM1- and GM2-gangliosidoses have variable phenotypes (infantile, late-infantile, juvenile, and adult), like other lysosomal diseases. The wide variations in phenotype result in considerable differences in the urgency of initiating treatment. Residual enzyme activity provides a rough estimate on the phenotype. However, with currently available laboratory methods, there is no clear correlation between clinical presentation of patients with gangliosidoses and the percentage of the enzyme activity reported in leukocytes or fibroblasts [2,41]. Therefore,

Table 5
Deduction of phenotype severity through mutations in the *HEXA* gene through investigations of previous literature.

Mutation	Phenotype	References	Mutation	Phenotype	References	Mutation	Phenotype	References
p.Met1Thr	Infantile	[28]	p.Ser210Phe	Infantile	[48]	p.Arg393Pro	Infantile	[34]
p.Met1Leu	Infantile	[29]	p.Phe211Ser	Infantile	[32]	p.tRP420cYS	Infantile	[40]
p.Met1Val	Infantile	[30]	p.Ser226Phe	Unknown	[35]	p.Phe434Leu	Juvenile or Adult	[41]
p.Pro25Ser	Adult	[28]	p.Ala246Thr	Infantile	[45]	P.Leu451Val	Unknown	[49]
p.Tyr37Asn	Juvenile	[31]	p.Gly250Ser	Unknown	[44]	p.Gly454Ser	Infantile	[32]
p.Leu39Arg	Infantile	[32]	p.Gly250Glu	Juvenile	[44]	p.Gly454Asp	Infantile	[57]
p.Cys58Tyr	Juvenile or Adult	[33]	p.Gly250Val	Unknown	[49]	p.Gly455Arg	Infantile	[50]
p.Glu114Lys	Infantile	[34]	p.Arg252His	Juvenile or Adult	[50]	p.Cys458Tyr	Infantile	[58]
p.Leu127Arg	Infantile	[27]	p.Arg252Leu	Infantile	[51]	p.Met459Val	Unknown	[29]
p.Leu127Phe	Infantile	[35]	p.Asp258His	Infantile	[52]	p.Gly461Val	Juvenile	This study
p.Arg166Gly	Juvenile	[36]	p.Thr259Ala	Unknown	[53]	p.Glu462Val	Infantile	[34]
p.Arg170Gln	Infantile	[37]	p.Pro260Ser	Unknown	[41]	p.Asp465Asn	Infantile	[59]
p.Arg170Trp	Infantile	[27]	p.Trp266Gly	Infantile	[41]	p.Trp474Cys	Juvenile	[60]
p.Arg178His	Juvenile	[38]	p.Gly269Ser	Adult	[54]	p.Gly478Arg	Infantile	[44]
p.Arg178Leu	Infantile	[39]	p.Gly269Asp	Infantile	[35]	p.Ala479Thr	Unknown	[29]
p.Arg178Cys	Infantile	[40]	p.Ser279Pro	Juvenile	[55]	p.Glu482Lys	Infantile	[39]
p.His179Arg	Unknown	[41]	p.Asn295Ser	Infantile	[51]	p.Leu484Pro	Infantile	[58]
p.His179Tyr	Infantile	[41]	p.Met301Arg	Infantile	[32]	p.Trp485Arg	Infantile	[61]
p.Tyr180His	Adult	[42]	p.Asp314Val	Unknown	[35]	p.Tyr497Cys	Unknown	[62]
p.Val192Leu	Infantile	[43]	p.Asp322Asn	Infantile	[34]	p.Arg499His	Juvenile	[32]
p.Asn196Ser	Unknown	[44]	p.Asp322Val	Unknown	[53]	p.Arg499Cys	Juvenile	[30]
p.Lys197Thr	Juvenile or Adult	[32]	p.Asp322Tyr	Infantile	[34]	p.Arg504His	Juvenile	[42]
p.Val200Met	Adult	[43]	p.Ile335Phe	Unknown	[56]	p.Arg504Leu	Infantile	[47]
p.Trp203Gly	Infantile	[45]	p.Gln336His	Unknown	[41]	p.Arg504Cys	Infantile	[32]
p.His204Arg	Infantile	[32]	p.Gln374Arg	Infantile	[45]	p.Phe521Leu	Juvenile or Adult	[63]
p.Asp207Glu	Infantile	[46]	p.Ile388Met	Unknown	[44]			
p.Asp208Val	Infantile	[47]	p.Val391Met	Unknown	[54]			

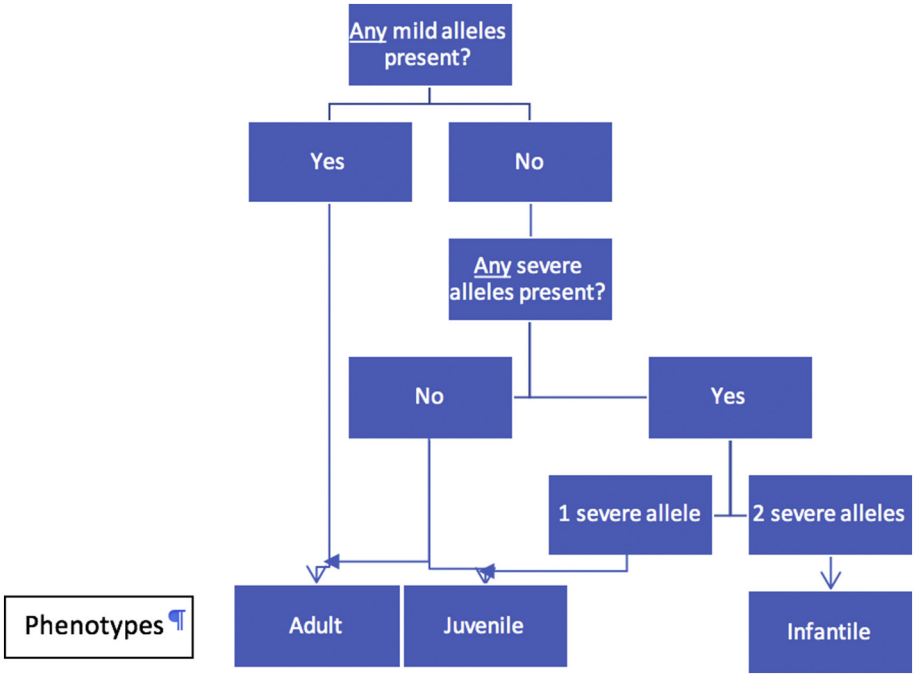

Fig. 2. Predicting disease subtypes based on mutations in autosomal recessive diseases. A general protocol for predicting disease subtypes of autosomal recessive diseases was proposed.

the residual activity is not a good indicator of the severity of the disease. Diagnosis is based on clinical evaluation and laboratory testing of respective enzyme activity; however, the average time from onset of symptoms to diagnosis is often greater than 5 years for the adult onset phenotype. In the infantile phenotype, onset of symptoms to diagnosis usually occurs well after the disease has caused severe neurological impairment and severe disability. [78,79].

As the era of newborn screening expands and more treatments become available for lysosomal diseases, improving understanding of genotype-phenotype correlation will become increasingly important. For variants of unknown significance (VUS), our SAAMP algorithm provides a powerful method to predict whether a missense mutation is 'pathogenic' or 'benign'. This SAAMP algorithm provides unprecedented sensitivity and specificity when compared to the other individual bioinformatics tools tested [17,18].

To determine the phenotype of a patient, mutations on both alleles need be considered. Predicting disease phenotypes based on genotypes is a major barrier in initiating appropriate treatment. In this study, phenotypes and mutations on both alleles of patients from other reports were analyzed and the phenotype of each mutation was deduced manually. We improved upon our previous work in MPS I [17] and GM1-gangliosidosis [18]. Establishment of such a database and making it readily accessible to clinicians and researchers will be remarkably beneficial. To predict the disease subtypes based on each mutation, four general assumptions are recommended for autosomal recessive diseases (Fig. 2). 1) The phenotype is infantile only if both alleles are severe. 2) The phenotype is adult if any of the alleles are mild. 3) The phenotype is juvenile if the alleles are intermediate and severe. 4) Juvenile or adult phenotypes can occur if both alleles are intermediate [17]. Notably, there may be heterogeneity in phenotype even among siblings with the same mutation due to genetic background. This phenomenon makes phenotype prediction more complicated.

Based on the phenotypes of our patients and previously reported patients, we propose the effects of mutations on the allele functionality (Tables 5, 6) in GM2-gangliosidosis. Clinicians can identify select the mutation in their patients from this table, determine its impact on the allele, then determine the predicted phenotype using Fig. 2. If a genotype present in our patients was also identified in previous reports, we cross-referenced the phenotype (p.Thr82Met, p.Arg148Cys, p.Arg148Ser, p.Arg201His, and p.Ala301Val) [80–82].

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