Identification of novel mutations among Iranian NPC1 patients: a bioinformatics approach to predict pathogenic mutations

Rezvan Abtahi1, Parvaneh Karimzadeh2, Omid Aryani3, Diba Akbarzadeh4, Shadab Salehpour5, Alireza Rezayi6, Seyed Hassan Tonekaboni2, Reza Zolfaghari Emameh7 and Massoud Houshmand1,8*

Abstract

Background: Niemann-Pick disease type C (NPC) is a rare lysosomal neurovisceral storage disease caused by mutations in the NPC1 (95%) or NPC2 (5%) genes. The products of NPC1 and NPC2 genes play considerable roles in glycolipid and cholesterol trafficking, which could consequently lead to NPC disease with variable phenotypes displaying a broad spectrum of symptoms.

Materials: In the present study 35 Iranian NPC unrelated patients were enrolled. These patients were first analysed by the Filipin Staining test of cholesterol deposits in cells for NPC diagnostics. Genomic DNA was extracted from the samples of peripheral blood leukocytes in EDTA following the manufacturer’s protocol. All exon–intron boundaries and coding exons of the NPC1 gene were amplified by polymerase chain reaction (PCR) using appropriate sets of primers. Thereafter, the products of PCR were sequenced and analysed using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The variants were reviewed by some databases including the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). Moreover, all the variants were manually classified in terms of the American College of Medical Genetics and Genomics (ACMG) guideline.

Results: The sequence analysis revealed 20 different variations, 10 of which are new, including one nonsense mutation (c.406C>T); three small deletions, (c.3126delC, c.2920_2923delCCTG, and c.2037delG); and six likely pathogenic missense mutations, (c.542C>A, c.1970G>A, c.1993C>G, c.2821T>C, c.2872C>G, and c.3632T>A). Finally, the pathogenicity of these new variants was determined using the ACMG guidelines.

Conclusion: The present study aimed to facilitate the prenatal diagnosis of NPC patients in the future. In this regard, we identified 10 novel mutations, and verified that the majority of them occurred in six NPC1 exons (5, 8, 9, 13, 19, and 21), that should be considered with a high priority for Iranian patients’ cost-effective evaluation.

Keywords: Niemann-Pick C, Molecular Study, New Mutation

Introduction

Niemann-Pick type C (NPC) disease, is a rare genetic and neurodegenerative disorder induced by intracellular accumulation of free cholesterol and gangliosides into lysosomes or late endosome systems [1]. The NPC was estimated to affect at least one person per 100,000 individuals [2, 3]. Of note, the patients affected by this disease are clinically heterogeneous with a broad spectrum of phenotypes and age of onset is variable. The onset and strictness of illness are determined by the degree of functional disruption in cholesterol trafficking [4]. In the majority of cases, the most common symptoms...
are neurological and psychiatric symptoms and arise between 4 and 16 years of age [5]. However, the clinical spectrum is ranged from a neonatal fatal disorder to an adult-onset chronic disease. Correspondingly, the neurological symptoms are manifested as mental deterioration, dystonia, dysarthria, dysphagia, ataxia, psychomotor retardation, cataplexy, and various types of seizures usually combined with vertical supranuclear gaze palsy [6, 7]. The neurodegenerative symptoms are often preceded by some visceral complications such as cholestatic jaundice and hepatosplenomegaly [8]. In the NPC patients, the Filipin staining of the cultured fibroblasts has been used extensively as a diagnostic test [9]. The Filipin staining reveals the abnormal intracellular accumulation of cholesterol in fibroblast cells by showing a strong fluorescence in perinuclear vesicles. [10]. Besides, there are several immunologically and ultra-structurally similarities in the brains of patients suffering from Alzheimer's disease and NPC such as the existence of neurofibrillary tangles, endosomal and lysosomal abnormalities [11]. Moreover, the foamy histiocytes (Niemann–Pick cells) could be identified in the bone marrow [8].

NPC is a heterogeneous disorder with two genetic complementation groups [12]. Accordingly, in approximately 95% of NPC patients, mutations are present in the \( \text{NPC}1 \) gene (MIM 607623) and the remaining patients have mutations in the \( \text{NPC}2 \) gene (MIM 601015) [13]. The \( \text{NPC}1 \) gene has 25 exons spanning more than 47 kb in length and is located on chromosome 18q11 [14]. It encodes a mRNA with roughly 4.9-kb that gives origin to a polypeptide with 1,278 amino acids. The \( \text{NPC}1 \) protein includes 13 transmembrane domains, six small cytoplasmic loops, three large and four small luminal loops, and one cytoplasmic tail [15, 16]. A cysteine-rich domain (residues 855–1,098) was identified in the third luminal loop [15]. All these functional domains are affected by mutations, which are spread through the \( \text{NPC}1 \) gene. Of note most mutations are located on the cysteine-rich domain, including a hot spot region from residues 927 to 958 [17]. Moreover, the region between the amino acid positions 1,038 and 1,253 is known as another hot spot region. This region was shown to have 35% similarity with the Patched 1 (PTC1) protein, namely between residues 974 and 1,180 [18].

The majority of the variations in the \( \text{NPC}1 \) gene are missense mutations, small deletions, and insertions [19, 20]. The most important cause of neuronal apoptosis in NPC was recognized to be the accumulation of intracellular free cholesterol in large amounts in the late endosomes or lysosomes, which are caused by a genetic deficit in cholesterol trafficking [21, 22]. However, identification of molecular defects in this disease can be considered as an important confirming diagnostic procedure, allowing a precise and fast prenatal diagnosis. In this study, the analysis of the \( \text{NPC}1 \) gene was performed in 35 Iranian patients with NPC, which as a result, led to the detection of 10 new \( \text{NPC}1 \) mutations. The present study aimed to provide additional information on the genotype of NPC disease among the Iranian patients.

**Patients and materials**

**Patients**

We studied 35 Iranian unrelated patients diagnosed as NPC using Filipin staining from 2014 to 2018. Documented consent was obtained from patients as approved for the entire study protocol by the NIGEB ethics committee (IR. NIGEB.EC.1397.8.23. B). Filipin staining of skin fibroblasts was performed in the Centogene GmbH Rostock, Germany (Clinical characteristics and genotype of the NPC patients were summarized in Table 1).

**Blood sampling and DNA extraction**

Blood samples were obtained from the Special Medical Center (SMC) and Taban Medical Laboratory, Tehran, IRAN.

Genomic DNA was extracted from the peripheral blood leukocyte samples in EDTA, using the QIA amp kit (QIA amp® DNA Micro Kit #56304, QIAGEN, Hilden, Germany) according with the manufacturer’s protocol.

**Polymerase chain reaction (PCR) amplification and sequencing analysis**

All 25 coding exons in 24 amplicons and the flanking regions of the \( \text{NPC}1 \) gene were amplified by PCR using the primers listed in Table 2. The PCR mixture contained 2 ng DNA template, 20 pmol each primer, 2.5 μL 10 X PCR buffer, and 5 U AmpliTaq in a total volume of 25 μL. The PCR cycle conditions were as following: an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60–63°C for 1 min, and elongation at 72°C for 1 min, with a final incubation for 10 min at 72°C. The PCR amplification products were analysed by 1.5% agarose gel electrophoresis. The PCR products were sequenced using Big Dye Terminator sequencing chemistry (ABI) and the ABI3100 automatic DNA sequence.

The Sequence data were analysed using freely available software (Finch TV) and compared to the query sequence (NM_000271). The variants were reviewed by databases such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) to determine whether they had been reported previously as pathogenic.

Moreover, all the variants were manually reviewed based on the American College of Medical Genetics and Genomics (ACMG) guideline. Besides, an aggregated
Table 1 Clinical and molecular data encountered in 35 NPC patients from Iran

| Patient No | Gender | Age | Age of onset | Clinical phenotype | Filipin test | Dysphagia | Hepatomegaly | VSGP | Splenomegaly | Ataxia | Dementia | DNA change | Protein change | Genotype | Report Status | Reference |
|------------|--------|-----|--------------|--------------------|-------------|----------|--------------|------|--------------|--------|----------|------------|----------------|----------|--------------|-----------|
| 1          | M      | 10  | 6            | J                  | +           | +        | +            | Yes  | Yes         | Yes    | Yes      | c.2776G>A    | p.Ala926Thr     | R         | homo        | [23]      |
| 2          | F      | 8   | 3            | L-I                | +           | +        | +            | Yes  | Yes         | Yes    | Yes      | c.2920-2923delctg | p.Phe974profTyr8 | Not.R    | homo        | new       |
| 3          | M      | 13  | 10           | J                  | +           | +        | No           | No   | Yes         | Yes    | Yes      | c.1970G>A    | p.Gly657Asp    | Not.R    | homo        | new       |
| 4          | M      | 6   | 2            | E-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.1990G>A    | p.Val664Met   | R         | hetero      | [18]      |
| 5          | F      | 12  | 5            | L-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.506A>T      | p.Asn169Ile    | R         | homo        | [24]      |
| 6          | M      | 15  | 10           | J                  | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.1990G>A    | p.Val664Met   | R         | hetero      | [18]      |
| 7          | M      | 5.5 | 1 m*         | E-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.3632 T>A    | p.Val1211Glu  | Not.R    | hetero      | new       |
| 8          | M      | 23  | 11           | J                  | +           | +        | Yes          | Yes  | Yes         | No     | No       | c.287 T>C    | p.Asn918Tyr   | R         | hetero      | new       |
| 9          | F      | 7   | 3            | E-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.406C>T      | p.Gln136Ter   | Not.R    | homo        | new       |
| 10         | F      | 12  | 5            | L-I                | +           | +        | Yes          | Yes  | Yes         | No     | No       | c.287 T>C    | p.Asn918Tyr   | R         | hetero      | new       |
| 11         | M      | 3   | 1 m          | E-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.551G>A      | p.Cys184Tyr   | R         | homo        | [23]      |
| 12         | F      | 20  | 9            | J                  | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.281 T>C    | p.Asn918Tyr   | R         | hetero      | new       |
| 13         | F      | 11  | 4            | L-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.3632 T>A    | p.Val1211Glu  | Not.R    | hetero      | new       |
| 14         | M      | 6   | 6 m          | E-I                | +           | +        | Yes          | Yes  | Yes         | Mild   | Mild     | c.1993C>G    | p.Leu665Val   | R         | hetero      | new       |
| 15         | F      | 22  | 12           | J                  | +           | +        | Yes          | Yes  | Yes         | Mild   | Mild     | c.287 T>C    | p.Asn918Tyr   | R         | hetero      | new       |
| 16         | M      | 4.5 | 2 m          | E-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.3100G>A    | p.Gly1034Arg  | R         | homo        | [3]       |
| 17         | M      | 9   | 1            | E-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.1192C>T    | p.His391Tyr   | R         | homo        | [26]      |
| 18         | M      | 17  | 5            | L-I                | +           | +        | Yes          | Yes  | Yes         | Yes    | Yes      | c.3632 T>A    | p.Val1211Glu  | Not.R    | hetero      | new       |
| 19         | F      | 25  | 13           | J                  | +           | +        | No           | Yes  | Yes         | Mild   | Mild     | c.287 T>C    | p.Asn918Tyr   | Not.R    | hetero      | new       |
| 20         | F      | 21  | 13           | J                  | +           | +        | Yes          | Yes  | Yes         | Mild   | Mild     | c.287 T>C    | p.Val1211Glu  | Not.R    | hetero      | new       |
| 21         | F      | 11  | 7            | J                  | +           | +        | Yes          | Yes  | Yes         | Severe | Severe   | c.1990G>A    | p.Val664Met   | R         | hetero      | [18]      |
| 22         | M      | 2   | 2 m          | E-I                | +           | +        | Yes          | Yes  | Yes         | No     | No       | c.1970G>A    | p.Gly657Asp   | Not.R    | homo        | new       |
| 23         | F      | 16  | 9            | J                  | +           | +        | Yes          | Yes  | Yes         | Severe | Severe   | c.1970G>A    | p.Gly657Asp   | Not.R    | homo        | new       |
| Patient No | Gender | Age (y) | Age of onset (m) | Clinical phenotype | Dysphagia | Hepatomegaly | VSGP | Sphincteromégalie | Dystonia | Ataxia | Dementia | DNA change | Protein change | Reference |
|------------|--------|---------|-----------------|--------------------|-----------|-------------|------|-------------------|----------|--------|----------|------------|----------------|----------|
| 24         | F      | 6       | 3.5             | L-I                | +         | +           | Yes  | Yes               | Yes      | Yes    | Yes      | Severe     | p.His1042Gl-nfsTer14 | Homo     |
| 25         | M      | 2.5     | 7               | E-I                | +         | +           | Yes  | Yes               | Yes      | Yes    | Yes      | Severe     | p.Ser357Leu | Homo     |
| 26         | M      | 3       | 3               | E-I                | +         | +           | Yes  | Yes               | Yes      | Yes    | Yes      | Severe     | p.Gly1034Arg | Homo     |
| 27         | M      | 17      | 13              | L-I                | +         | +           | Yes  | Yes               | Yes      | Yes    | Yes      | Severe     | p.Gly1034Arg | Homo     |
| 28         | F      | 2       | 1.5             | E-I                | +         | +           | Yes  | Yes               | No       | No     | Yes      | Mild       | p.Leu472Pro | Homo     |
| 29         | M      | 7.5     | 15              | L-I                | +         | +           | Yes  | Yes               | No       | No     | Yes      | Severe     | p.Leu472Pro | Homo     |
| 30         | F      | 27      | 10              | J                  | +         | +           | Yes  | Yes               | No       | No     | Yes      | Mild       | p.Leu472Pro | Homo     |
| 31         | M      | 8       | 2.5             | L-I                | +         | +           | Yes  | Yes               | No       | No     | Yes      | Severe     | p.Leu472Pro | Homo     |
| 32         | M      | 19      | 18              | J                  | +         | +           | Yes  | Yes               | No       | No     | Yes      | Severe     | p.Leu472Pro | Homo     |
| 33         | M      | 12      | 10              | L-I                | +         | +           | Yes  | Yes               | No       | No     | Yes      | Severe     | p.Leu472Pro | Homo     |
| 34         | F      | 8.5     | 4               | L-I                | +         | +           | Yes  | Yes               | No       | No     | Yes      | Severe     | p.Leu472Pro | Homo     |

M: Male, F: Female, m: month, J: Juvenile, E-I: Early-Infantile, L-I: Late-Infantile, VSGP: Vertical supranuclear gaze palsy
R: Reported, Not R: Not Reported
knowledge-based tool, VarSome (https://varsome.com/), was employed to evaluate variants comprehensively.

**In silico analysis**

Novel missense variations were analysed using three computational methods, including PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and PANTHER (http://pantherdb.org/about.jsp) to predict the functional impact of novel amino acid changes.

PolyPhen predicts the possible impact of an amino acid substitution on the structure and function of a human protein. The other two in silico approaches were based on evolutionary conservation. Multiple sequence alignments (MSA) in NPC1 from different species were performed using BoxShade server (version 3.21) to verify the conservation degree.

**Results**

Sequence analysis of extracted DNA obtained from the blood samples of 35 patients led to the identification of 20 different variants, 10 of which were previously reported: p.(Asn169Ile), p.(Cys184Tyr), p.(Ala926Thr), p.(Val664Met), p.(Leu472Pro), p.(Asn478Thr), p.(Tyr394His), p.(Gly1034Arg), p.(Ser357Leu), and p.(His398Tyr). These variants were found in the homozygous state in 16 patients (Table 3).

The remaining 10 variations identified include six missense mutations, namely p.(Gly657Asp), p.(Ser941Pro), p.(Arg958Gly), p.(Val1211Glu), p.(Leu665Val), and p.(Ala181Asp), one nonsense mutation p.(Gln136*), and three small deletions, (c.3126delC, c.2920_2923delCCTG, and c.2037delG). None of them have been previously reported. Only five of these 10 variants were found in homozygosity, including the nonsense mutation p.(Gln136*), the missense mutation p.(Gly657Asp), and the three small deletions, (c.3126delC, c.2920_2923delCCTG, and c.2037delG). It is important to notice that in the patients analysed no mutations were observed in any of the intronic flanking regions.

The new variants’ pathogenicity, including six likely pathogenic and four pathogenic variants, was determined in terms of the ACMG guidelines.

The predicted functional effects of novel missense variants were determined using the pre-computed values of the SIFT and PANTHER for the tolerated/deleterious
effects and Polyphen2 for the benign/damaging effects (Table 4). Stop codon and deletions in the \( \text{NPC1} \) gene were not considered.

MSA of the \( \text{NPC1} \) proteins obtained from human (O15118), chimpanzee (H2QEC5), mouse (O35604), chicken (F1NQT4), and zebrafish (F1QNG7) was performed using Clustal Omega and BoxShade server (version 3.21) (Fig. 1).

Observational studies of several national cohorts have categorized patients by age at the onset of neurological manifestations. By considering findings of these studies, the patients were categorized into early-infantile (<2 years old), late-infantile (2–6 years old), juvenile (6–15 years old), and adult (≥15 years old)-onset forms [25]. In this study, the juvenile-onset, early-, and late-infantile onset disease cases were by this order the most frequent disease forms, respectively.

In all patients included in this study, the manifestations usually started in the first decade. The mean age at the time of onset was 5.5 years old (1 m-13y). The disease was diagnosed 2–27 years post the initial clinical presentation. It is noteworthy that variable ages of onset and different age-dependent manifestations, make \( \text{NPC} \) a complex, complicated, and underdiagnosed disease.

Further, dysphagia and splenomegaly were observed in all patients, while hepatomegaly was detected in most patients. Additionally, the neurological features, including vertical supranuclear gaze palsy, cerebellar ataxia, and dementia were highly variable.

**Discussion**

The clinical features, age at the clinical symptoms onset, and the rate of neurological symptoms’ progression are highly variable in the \( \text{NPC} \) disease. In the present study, at the younger age of onset, more severe disease phenotypes were usually observed. Moreover, more than 395 pathogenic variations have been identified for \( \text{NPC} \) (HGMD Professional). Most of these variations are associated with missense mutations (71%) [16]. A small number of the prevalent variations have been described, including p.(Ile1061Thr) and p.(Pro1007Ala) in the patients from western European descent [29], p.(Arg518Gln) from Japan, and p.(Pro474Leu) from Italy. However, none of these variations was detected in our study [30]. In this work, the c.2821 T > C, p. (Ser941Pro) and c.2872C > G, p. (Arg958Gly) variations, both in exon 19, were the two most common mutations found among \( \text{NPC} \) patients analysed. Additionally, the majority of the variants were found in exons 5, 8, 9, 13, 19, and 21 (80%) and few mutations have been identified in other \( \text{NPC} \) exons.

Ten of the twenty variants reported are new variations (not previously reported). Among these, the nonsense mutation and the three small deletions were found highly deleterious.

The new p.(Gln136*) mutation gives origin to a premature termination codon that results in the loss of protein expression and function. Furthermore, the three small deletions were identified in the patients p2, p24, and p32 in the homozygous state, and their presence result in frameshifts.

The remaining six new variants were nsSNP (non-synonymous single nucleotide polymorphisms), which were analysed using in silico methods. SIFT and PolyPhen web tools predicted 100% of nsSNPs as "deleterious" and "probable damaging" to protein's structure and function. By its turn, PANTHER predicted 5 of those 6 nsSNPs as deleterious (83.3%) but one of them (Ala181Asp) was classified as tolerated (Table 4). The variant p. (Gly657Asp) was identified in homozygosity, and the heterozygous state was identified in the \( \text{NPC1} \) gene of their parents.

The cysteine-rich loop, which is known as a functionally significant protein–protein interaction site,
Table 4  novel variants of NPC1 gene were detected in 35 Iranian patients and in silico analysis of missense variants (Stop codon and deletions in NPC1 gene were not considered)

| NO | Exon No | DNA change | Protein change | Patient No | State | SIFT | Polyphen | PANTHER |
|----|---------|------------|----------------|------------|-------|------|---------|---------|
|    |         |            |                |            |       | PROVEAN Prediction (cutoff = -2.5) | Score Prediction (cutoff = 0.5) | subPSEC Prediction (cutoff = -3) |
| 1  | 4       | c.406C>T   | (p.Gln136Ter)  | 9          | homo  | -    | -       | -       | -       |
| 2  | 5       | c.542C>A   | (p.Ala181Asp)  | 12         | hetero| -4.642| 0.818   | 2.9001  | Tolerated |
| 3  | 13      | c.1970G>A  | (p.Gly657Asp)  | 3,22,23    | homo  | -7   | 1       | -5.63539| Deleterious |
| 4  | 13      | c.1993C>G  | (p.Leu665Val)  | 15         | hetero| -2.667| 0.966   | 3.19574 | Deleterious |
| 5  | 13      | c.2037delG | (p.Leu680CysfsTer3) | 32       | homo  | -    | -       | -       | -       |
| 6  | 19      | c.2821T>C  | (p.Ser941Pro)  | 4,7,8,10,12,13,15,18 | hetero| -4.66 | 1       | -5.14243| Deleterious |
| 7  | 19      | c.2872C>G  | (p.Arg958Gly)  | 7,8,10,13,19 | hetero| -6.191| 0.899   | 4.0903  | Deleterious |
| 8  | 20      | c.2920_2923delCCTG | (p.pro974profsTer8) | 2        | homo  | -    | -       | -       | -       |
| 9  | 21      | c.3126delC | (p.His1042GlnfsTer14) | 24       | homo  | -    | -       | -       | -       |
| 10 | 24      | c.3632T>A  | (p.Val1211Glu) | 6,18,19,21 | hetero| -4.576| 1       | -4.67659| Deleterious |

*PROVEAN* Protein Variation Effect Analyser, *PSEC* position-specific evolutionary conservation

*PANTHER* Protein Analysis Through Evolutionary Relationship, *SIFT* sorting Intolerant From Tolerant
has a ring-finger motif and contains nearly one-third of the NPC1 variations [31, 32]. In Fig. 2 is presented the location of the detected novel variants on the NPC1 protein structure. It is noteworthy that two of the 10 detected novel variants, including p. (Ser941Pro) and p. (Arg958Gly), were located in the cysteine-rich loop (residues 927–958 a hot spot region of the gene). These variations were found in a heterozygous state in four studied patients, including p7, p8, p13, and p10.

According to findings of our study and the ACMG guideline, some mutations, including p.(Ala181Asp), p.(Gly657Asp), p.(Leu665Val), p.(Ser941Pro), p.(Arg958Gly), and p.(Val1211Glu)are likely pathogenic; these variations according to the ACMG guideline: a) are absent in any frequency database, such as the gnomAD and 1000 Genomes Project, that indicate this mutation is rare (PM2); b) are located in a well-established functional domain (PM1); c) are missense variants in a gene in which benign missense variations are rare so a common mechanism of the disease is considered as the missense variants (PP2), and d) have computational proof supporting a deleterious result on the gene (PP3). Furthermore, p.(Ser941Pro) and p.(Arg958Gly) variants are new missense changes at an amino acid residue, where the other pathogenic missense changes have been seen before (PM5). Based on this evidence, these mutations are classified as likely pathogenic.

In some variations, including p.(Gln136*), c.2037delG, c.2920_2923delCCTG, and c.3126delC: a) the null variants (including frameshift, nonsense, initiation codon, and splice sites) exist in a gene, where LOF (Loss Of Function) is a known mechanism of disease (PVS1); b) the deleterious effect on the gene is supported by computational evidence (PP3); c) they are absent in all frequency database files such as the gnomAD and 1000 Genomes Project, meaning that these mutations are rare (PM2).

Based on this evidence, these mutations can be classified as pathogenic. In the current study, the aggregated knowledge-based tool, VarSome, was used to review the variants comprehensively.

In this study, 24 out of 35 patients were found to be homozygous, and the remaining patients (11 patients) were either in the heterozygous or in the compounded heterozygous states along with the second mutation.

Seven patients were homozygous, and 11 patients were compound heterozygous for the novel mutations. Furthermore, p. Gly657Asp was detected in three patients (p3-p22-p23), and c.2037delG, c.2920_2923delCCTG, c.3126delC, and c.406C>T p.(Gln136*) were detected in the patients. p2, p9, p24, and p32, respectively. In the
presence of the all 3 deletions and of the nonsense mutation, severe symptoms were observed.

The new mutations, p.Ala181Asp, p.Ser941Pro, p.Arg958Gly, p.Leu665Val, and p.Val1211Glu were observed in heterozygosity. In all these cases, the manifestations of the disease were early/late infantile and juvenile (p4-p7-p8-p10- p12-p15-p18-p19).

Based on the findings of the present study, no straightforward genotype–phenotype correlations can be established due to the type of new mutations, except in case of the deletions, which presence is always accompanied with severe manifestations of the disease.

The high number of homozygous patients in the present study, could be explained by the high prevalence of consanguineous marriages in Iran and despite patients are not relative they may have common ancestors.

Furthermore, in the present study, some common polymorphisms were found in some of the studied patients, that includes p. (His215Arg) (p6, p12, p21), p. (Ile642Met) (p32), and p. (Ile858Val) (p21). This result is compatible with the previous studies performed in Portugal and Germany [33, 34].

Conclusions
In conclusion, the mutation screening of 35 Iranian patients with NPC was described, resulting in 10 novel pathogenic and likely pathogenic NPC1 gene variants. The Niemann-Pick is a neurological disorder with a broad spectrum of clinical features. An alternative tool used to confirm the diagnosis of NPC was mutation screening. Finally, the detection of mutations will facilitate carrier screening of family members and prenatal diagnosis.

Abbreviations
NPC: Niemann-Pick C; PCR: Polymerase chain reaction; NCBI: National Center for Biotechnology Information; NIGEB: National Institute of Genetic Engineering and Biotechnology; PROVEAN: Protein Variation Effect Analyser; PSEC: Position-specific evolutionary conservation; PANTHER: Protein Analysis Through Evolutionary Relationship; SIFT: Sorting Intolerant From Tolerant; HMG-R: Hydroxyl methyl glutaryl coenzyme A reductase; SMC: Special Medical Center; nsSNP: Non-synonymous single nucleotide polymorphisms.
Acknowledgements
The authors thank all the patients who were involved in this study and consent to use their samples and information in the manuscript by signing the consent form.

Authors' contributions
RA: study concept and design, Acquisition of data, Analysis and interpretation of data drafting of the manuscript. OA, SS, AR, SHT, and PK all contributed to sample and data collection. DA: analysis and interpretation of data. RZE: advisor of the study and critical revision of the manuscript for important intellectual content. MR: corresponding authors; supervisor of the study; organized, and designed the study, and approved the manuscript. The manuscript was approved by all the authors.

Funding
No funding was obtained for this study.

Availability of data and materials
The variants in this article are available in National Center for Biotechnology Information (NCBI) and the datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The written consents were obtained and documented through the following ethics approval and consent to participate:

1. Department of Medical Genetics, National Institute for Genetic Engineering and Biotechnology (NIGEB) ethics committee (IR. NIGEB.EC.1397.8.23. B).

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Author details
1. Department of Medical Genetics, National Institute for Genetic Engineering and Biotechnology (NIGEB), 14965/161, Tehran, Iran.
2. Pediatric Neurology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
3. Special Medical Center, Tehran, Iran.
4. Student’s Research Committee, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
5. Department of Pediatric Endocrinology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
6. Department of Pediatrics and Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), 14965/161, Tehran, Iran.
7. Department of Medical Laboratory Science, Knowledge University, Erbil, Kurdistan Region, Iraq.

Received: 4 May 2021 Accepted: 29 December 2021
Published online: 27 January 2022

References
1. Sitarska D, Lugowska A. Laboratory diagnosis of the Niemann-Pick type C disease: an inherited neurodegenerative disorder of cholesterol metabolism. Metab Brain Dis. 2019;34(5):1253–60.
2. Sobrido M-I, Bauer P, De Koning T, Klopopstock T, Nadjar Y, Patterson MC, et al. Recommendations for patient screening in ultra-rare inherited metabolic diseases: what have we learned from Niemann-Pick type C? Orphanet J Rare Dis. 2019;14(1):20.
3. Yang CC, Su YN, Chou PC, Fietz MJ, Yu CL, Hwu WL, et al. Six novel NPC1 mutations in Chinese patients with Niemann-Pick type C disease. J Neurochem Psychiatry. 2005;76(4):592–5.
4. Rego T, Farrand S, Goh AM, Eratne D, Kelso W, Mangelsdorf S, et al. Psychiatric and cognitive symptoms associated with Niemann-Pick C disease: neurobiology and management. CNS Drugs. 2019;33(2):125–42.
5. Mengel E, Pineda M, Hendriksz CJ, Walterfang M, Torres JV, Kolb SA. Differences in Niemann-Pick disease Type C symptomatology observed in patients of different ages. Mol Genet Metab. 2017;120(3):180–9.
6. Nevsimalova S, Malinova V. Cataplexy and sleep disorders in Niemann-Pick type C disease. Curr Neurol Neurosci Rep. 2015;15(1):522.
7. Macias-Vidal J, Rodriquez-Pascual L, Sánchez-Ollé G, Lluch M, Vilageliu L, Grínberg D, Coll MJ. Spanish NPC Working Group. Molecular analysis of 30 Niemann-Pick type C patients from Spain. Clinical genetics. 2011;80(1):39–49.
8. Bosch M, Fajardo A, Alcalá-Vida R, Fernández-Vidal A, Tebar F, Enrich C, et al. Hepatic primary and secondary cholesterol deposition and damage in Niemann-Pick disease. Am J Pathol. 2016;186(3):517–23.
9. Mukherjee S, Maxfield FR. Lipid and cholesterol trafficking in NPC. Biochim Biophys Acta. 2004;1685(1–3):28–37.
10. Hammerschmidt T, Ribas OgOs, Saraiva-Pereira ML, Bonatto MP, Kessler RG, Souza FTS, et al. Molecular and biochemical biomarkers for diagnosis and therapy monitization of Niemann-Pick type C patients. Int J Dev Neurosci. 2018;66:18–23.
11. Kågedal K, Kim WS, Appelquist H, Chan S, Cheng D, Agholme L, et al. Increased expression of the lysosomal cholesterol transporter NPC1 in Alzheimer’s disease. Biochim Biophys Acta. 2010;1801(8):831–8.
12. Millat G, Chikhi K, Naarckeien S, Slaet DE, Fensom AH, Higaki K, et al. Niemann-Pick disease type C: spectrum of H1 mutations and geno- type/phenotype correlations in the NPC2 group. Am J of Hum Genet. 2001;69(5):1013–21.
13. Cvitanović-Šojat L, Malenica M, mONIKA K, Žigman T, Kužnik K, Bielean A. Niemann-Pick disease type C: mutations of NPC1 gene and the course of disease. Paediatr Croatica. 2014;58(1):255–60.
14. Polese-Bonatto M, Bock H, Farias ACS, Mergener R, Matte MC, Gil MS, et al. Niemann-pick disease type C: Mutation spectrum and novel sequence variations in the human npc1 gene. Mol Neurobiol. 2019;56(6):9426–35.
15. Davies JP, Ioannou YA. Topological analysis of Niemann-Pick C1 protein reveals that the membrane orientation of the putative sterol-sensing domain is identical to those of 3-hydroxy-3-methylglutaryl-CoA reductase and sterol regulatory element binding protein cleavage-activating protein. J Biol Chem. 2000;275(32):24367–74.
16. Ioannou YA. The structure and function of the Niemann-Pick C1 protein. Mol Genet Metab. 2000;71(1–2):175–81.
17. Vanier M, Millat G. Niemann-Pick disease type C. Clin Genet. 2003;64(4):269–81.
18. Park WD, O’Brien JF, Lundquist PA, Vockley CW, Karnes PS, et al. Identification of 58 novel mutations in Niemann-Pick disease type C. Correlation with biochemical phenotype and importance of PTC1-like domains in NPC1. Hum Mutat. 2003;22(4):313–25.
19. Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NS, et al. Human gene mutation database (HGMD®): 2003 update. Hum Mutat. 2003;21(6):577–81.
20. Patterson M. Niemann-Pick Disease Type C. 2000 Jan 26 [updated 2020 Dec 10]. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Grripp KW, Mirzaa GM, Amemiya A, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle, 1993–2022. PMID: 20301473.
21. Frolova O, Zielinski SE, Crowley JR, Dudley-Rucker N, Schaffer JE, Ory DS. NPC1 and NPC2 regulate cellular cholesterol homeostasis through generation of low-density lipoprotein cholesterol derived oxysterols. J Biol Chem. 2003;278(29):25517–25.
22. Koh CHV, Cheung NS. Cellular mechanism of UL1666A-mediated apotosis in cultured murine cortical neurons: bridging Niemann-Pick disease type C and Alzheimer’s disease. Cell Signal. 2006;18(11):1844–53.
23. Fernandez-Valero E, Ballart A, Iturriaga C, Lluch M, Macías J, Vanier M, et al. Recommendations for patient screening in ultra-rare inherited metabolic disorders by cholestantriol screening. Mol Genet Metab. 2003;80(1):39–49.
24. Reunert J, Fobker M, Kannenberg F, Du Chesne I, Plate M, Wellhausen J, et al. Rapid Diagnosis of 83 Patients with Niemann Pick Type C Disease. Identification of 25 new mutations in 40 unrelated Spanish Niemann-Pick type C patients with Niemann-Pick Type C and Alzheimer’s disease. Cell Signal. 2006;18(11):1844–53.
25. Fernandez-Valero E, Ballart A, Iturriaga C, Lluch M, Macías J, Vanier M, et al. Recommendations for patient screening in ultra-rare inherited metabolic disorders by cholestantriol screening. Mol Genet Metab. 2003;80(1):39–49.
26. Reunert J, Fobker M, Kannenberg F, Du Chesne I, Plate M, Wellhausen J, et al. Rapid Diagnosis of 83 Patients with Niemann Pick Type C Disease and Related Cholesterol Transport Disorders by Cholestantriol Screening. EBioMedicine. 2016;4:170–7.
26. Tonekaboni SH. Approach to NPC Disease. Iranian J Child Neurol. 2015;9(4):7.

27. Zhan X, Lin N, Zhang HW, Gao XL, Qiu WJ, Han LS, Ye J, Gu XF. Blood 7-ketocholesterol level, clinical features and gene mutation analysis of 18 children with Niemann-Pick disease type C. Zhonghua Er Ke Za Zhi. 2016;54(6):419–23.

28. Asl SN, Vakili R, Ghaemi N, Eshraghi P. The Report of Three Rare Cases of the Niemann-Pick Disease in Birjand, South Khorasan, Eastern Iran. Iranian journal of child neurology. 2017;11(3):53.

29. Millat G, Marçais C, Rafi MA, Yamamoto T, Morris JA, Pentchev PG, et al. Niemann-Pick C1 disease: the I1061T substitution is a frequent mutant allele in patients of Western European descent and correlates with a classic juvenile phenotype. The American Journal of Human Genetics. 1999;65(5):1321–9.

30. Garver WS, Jelinek D, Meaney FJ, Flynn J, Pettit KM, Shepherd G, et al. The National Niemann-Pick Type C1 Disease Database: correlation of lipid profiles, mutations, and biochemical phenotypes. J Lipid Res. 2010;51(2):406–15.

31. Greer W, Dobson M, Girouard G, Byers D, Riddell D, Neumann P. Mutations in NPC1 highlight a conserved NPC1-specific cysteine-rich domain. The American Journal of Human Genetics. 1999;65(5):1252–60.

32. Watari H, Blanchette-Mackie EJ, Dwyer NK, Watari M, Burd CG, Panel S, et al. Determinants of NPC1 expression and action: key promoter regions, posttranscriptional control, and the importance of a “cysteine-rich” loop. Exp Cell Res. 2000;259(1):247–56.

33. Bauer P, Knoblich R, Bauer C, Finckh U, Hufn A, Kropp J, et al. NPC1: complete genomic sequence, mutation analysis, and characterization of haplotypes. Hum Mutat. 2002;19(1):30–8.

34. Ribeiro I, Marcão A, Amatoa O, Miranda MS, Vanier MT, Millat G. Niemann-Pick type C disease: NPC1 mutations associated with severe and mild cellular cholesterol trafficking alterations. Hum Genet. 2001;109(1):24–32.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.