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Novel Sequence Variants in the *NPC1* Gene in Egyptian Patients with Niemann-Pick Type C

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Abstract

BACKGROUND: Niemann-Pick disease type C (NPC) is a rare, autosomal recessive, progressive neuro-visceraldisease caused by biallelic mutations in either *NPC1*gene (95% of cases) or *NPC2* gene.

AIM: This caseseries study aimed at the molecular analysis of certain hot spots of *NPC1* genein NPC Egyptian patients.

METHODS: The study included 15 unrelated NPC patients and selected parents,as well as20 healthy controls of matched sex and age. Clinical investigations were performed according to well established clinical criteria. Assessment of the chitotriosidase level, as an initial screening tool for NPC, was done in all cases. Polymerase chain reaction amplification of *NPC1* exons (17–25) encountering the hotspot residues (855–1098 and1038–1253) was carried out followed by direct sequencingfor mutational analysis.

RESULTS: All includedpatients with mainly neurovisceral involvement were characterized. The onset of the disease varied from early-infantile (58.3%) to late-infantile (26.7%) and juvenile-onset (6.7%). Ahigh chitotriosidase level wasobservedin all patients. Molecular analysis of *NPC1* (exons 17–25) confirmed 15 mutant alleles out of 30 studied ones. They included two novel homozygous missense variants (p.Ser1169Arg and p.Ser1197Phe) and previously reportedfour mutations (p.Arg958*, p.Gly910Ser, p.Ala927Glyfs*38, and andp.Cys1011*).

CONCLUSION: The two studied amino acid residues (855–1098 and 1038–1253) could beconsidered aspotential hotspot regions in *NPC1* Egyptian patients.

Introduction

Niemann-Pick disease type C (NPC, OMIM# 257220) is a progressive pan-ethnic autosomal recessive lysosomal lipid storage disorder, with an estimated incidence of 1 in 100,000 live birthsworldwide [1]. It is characterized by impaired intracellular lipid trafficking leadingto the aggregation of unesterified cholesterol and sphingolipids in different body organs such as liver, spleen, and brain [2]. The disease has an extremely heterogeneous clinical presentation, includes visceral abnormalities, neurological, and psychiatric manifestations that appear alone or in combination with various ages of onset [3]. Splenomegaly with or without hepatomegalyand prolonged neonatal jaundice are common visceral manifestations. The classic neurological symptoms mainly include “cerebellar ataxia, dysarthria, dysphagia, cataplexy, and progressive dementia” in addition to vertical supranuclear gaze palsy (VSGP) which represents a major disease characteristic feature [4].

NPC is caused mainly by a biallelic mutation in either of *NPC1 (*95% of cases) or *NPC2* (4% of cases) gene [5]. *NPC1* is located on chromosome 18q11–12, contains 25 exons, and encodes a 1278 amino acid glycoprotein with 13 transmembrane domains [6]. Up till now, more than 400 various *NPC1* mutations have been described in NPC patients, including insertions, deletions, duplications, nonsense, and missense mutations [7]. Although these mutations are scattered throughout the gene, the cysteine-rich luminal loop (amino acid residues: 857–1015) was reported as a hot spot regionencountering around one-third of the revealed mutations. While, in other literature, the putative sterol sensing domain was reported as a second hot spot region, including around 20% of revealed mutations [8]. In addition, the amino acid residues(1038–1253) in the c-terminal carboxyl end of protein were also previously mentionedas a hot spotfor pathogenic variants. Some

NPC1 mutations showed recurrent distribution globally or among different populations. p.Ils1061Thr is the most common reported mutation and widely distributed in Western Europe. p.Pro1007Ala is reported as the second most recurrent mutationin different populations, especially in Germany. p.Arg1186His is widely distributed inthe Czech, while Gly992Trp is individualized to Nova Scotian patients [9].

Disease-specific drugs such as miglustat can slow down neurological disease progression [10]. New therapeutic approaches such as gene therapy showed promising results *in vitro* and *in vivo* and it is supposed to be applied in the clinical stage in the near future [11]. In our study, we investigated certain clinical, biochemical, and molecular features in 15 NPC Egyptian patientsto contribute a better disease characterization in Egypt. We aimed at the characterizationof certain *NPC1* hotspot regions (residues: 855–1098 and 1038–1253), especially they also encounter the most common *NPC1* mutations worldwide, hopefully, to contribute in disease diagnosis confirmation and proper genetic counseling among NPC Egyptian patients.

Patients and Methods

The current caseseries study was conducted on 15 NPC Egyptian patients with an age range between 6 months and 10 years(mean ± SD, 3.2 ± 3 years)andsome selected parents as well as 20 healthy controls with matched age and sex during the period from September 2017 to November 2019.

The patients were referred to the clinical genetics and biochemical genetics departments at the National Research Center (NRC), Cairo, Egypt. All cases were diagnosed as NPC according to their clinical presentation and biochemical studies. They were also subjected to history taking include: Age of patients, sex, age of disease onset, and consanguinity. Blood samples were obtained from patients after having written informed approval from their parents or their legal guardians. The study protocol was approved by the Medical Research Ethics Committee at NRC.

Clinical examinations

All included patients were clinically diagnosed, according to Millat *et al*. [12]. Abdominal ultrasonography, magnetic resonance imaging (MRI) of the brain, and electroencephalography were performed for all patients.

Biochemical studies

Peripheral leukocytic β-glucocerebrosidase and peripheral leukocytic acid sphingomyelinase enzymes were measured to exclude similar metabolic diseases such as Gaucher and Niemann-Pick Type A and Type B diseases [13], [14]. Evaluation of chitotriosidase biomarker as a recommended screening tool for NPC was done [15].

Molecular studies

DNA extraction

DNA was extracted from peripheral blood lymphocytes from 15 NPC patients, selected parents, and 20 healthy controls by salting out technique [16]. The DNA concentration was measured at a 260/280 nm absorbance ratio by NanoDrop 2000c Spectrophotometer (Thermo Fisher).

Polymerase chain reaction (PCR) amplification of NPC1 exons (17–25)

PCR was performed in a total volume of 30 µl containing 100–200 ng genomic DNA, 30 pmol of each primer,230 µM of dATP, dCTP, dTTP, and dGTP, 2.5µM Mgcl2, 1 X Taq buffer and 2.5 U Taq polymerase. PCR was performed for nineexons of *NPC1* gene. The primers sequencewas previously described byTarugi *et al*. [17], Table S1. Amplification of the nine exons was performed with an initial denaturation step at 95°C for 5 min followed by 35cycles (each comprising 94°Cfor 40 s, 65°Cfor 40 s, and 72°Cfor 40 s) and a final extension step at 72°Cfor 10 min. The reaction was carried out in BioRad thermal cycler. Each PCR product was verifiedby electrophoresis on a 1.5%(w/v) agarose gel and visualization by UV light.

Purification of the PCR product

PCR products were purified using EXO/SAP PCR Purification Kit(Thermo Fisher Scientific Baltics, UAB, Lithuania);2 µl EXO/SAP cocktail (4 U Exo I and 0.4 U SAP) were added to 6 µl PCR product. The mix was incubated at 37°Cfor 60 min, then at 80°Cfor 20 min.

Direct DNA sequencing of NPC exons (17–25)

PCR amplified fragments were sequenced in both directions using primers and a BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Warrington, UK), samples were run on an ABI PRISM Genetic Analyzer 3500 (Applied Biosystems). Sequences were compared using Basic Local Alignment Search Tool [18].

All variants were described according to nomenclature recommendation by the human genome variations society [19]. Sequence analysis of *NPC1*was

analyzed regarding the reference sequence of(GenBank accession no. *NPC1*, NM_000271.4).

In silico analysis

All annotated variants were examined in several databases, including ClinVar [20], HGMD [7], and gnomAD databases [21].

Functional prediction analysis, including PolyPhen2 [22], SIFT [23], and Mutation Taster [24], was used to evaluate the functional effect of novel non-synonymous amino acid substitutions. *NPC1*mutation tolerance was further analyzed usingthe MetaDome webserver [25].

Prediction of protein structural effect after novel nonsynonymous variations was performed by Project HOPEto predict structural variation between native protein and the variant model [26]. Protein sequence and structure were retrieved from UniProt "Universal Protein Resource" [27]. The UniProt ID number of the aligned human sequences of *NPC1*: "UniProt: O15118."

Results

Clinical data

This study included 15 Egyptian patients belongingto15 unrelated families. Consanguinity was certain in 12 families (80%). They were9 males (60%)and6 females (40%) and their age ranged from 6 months to 10 years(mean ± SD, 3.2 ± 3 years). The onset of the disease varies from early infantile to juvenile form. Accordingly, our patients were classified into early infantile (<2 years) in 7 patients (46.7%), late infantile (2–5 years) in 4 patients (26.7%), and juvenile (5–16 years) in 1 patient (6.7%). Hepatosplenomegaly was the most common disease systemic feature in (60%) of cases, while splenomegaly restricted to 33.3% of them. Neurological manifestations were obvious in 12 patients (80%), classified as early infantile-onset (58.3%), late infantile-onset (33.3%), and juvenile-onset (8.3%). Dysphagia, cataplexy, hypotonia, delayed developmental milestones, speech retardation, loss of previously acquired skills, ataxia, epilepsy, and vertical supranuclear gaze palsy VSGP were themain observable neurological manifestations. Brain MRI was performed in all patients, around (46.7%) of casesshowed cerebral and cortical atrophy, whilemild cortical atrophic changeswere observed in (33.3%), on the other hand around (20%)showed normal MRI. Detailed clinical data are illustrated in Table 1.

Biochemical studies

Assessment of peripheral leukocytic β-glucocerebrosidase and peripheral leukocytic acid sphingomyelinase enzymes showed normal values in all patients, while a remarkable elevation in chitotriosidase activitywas observed in all patients(Table S2).

Molecular studies

Molecular analysis of 9 exons of *NPC1* (exons 17–25) confirmed sixdifferent mutations. Two homozygous novel missense variants in the amino acid residues (1038–1253) were detected and they have already been submitted to ClinVar under accessions SCV001197997 and SCV001197998. The novel mutations were p.Ser1169Arg in the cytoplasmic loop L and p.Ser1197Phe in the 12th TM domain (Figure 1). Molecular analysis of parents showed heterozygous status for the novel variants, and the 20 healthy individuals showed wild type sequences. Four previously reported mutations were detected in the cysteine-rich luminal loop classified as, one nonsense homozygous mutationp. Arg958*, one heterozygous missense mutation p.Gly910Ser, additionally, two homozygous frameshift mutations p.Ala927Glyfs*38, p.Cys1011* (Figure S1). Positions of novel revealed variants can be visualized in *NPC1* protein topology produced by Protter software (Figure S2) [28].

In silico results

All revealed variants were not identified in the overall populations as referenced inthe gnomADdatabase expect p.Gly910Serand p.Arg958*which showed minor allele frequency (0.000007953)and (0.000003982), respectively. Both mutations were also reported as pathogenic variants in the ClinVar database. The novel sequence variations were evaluated through the alignment of amino acid sequences from eight different organisms and alterations are located within conserved residues suggesting an effect on proteins functions or structures (Figure S3). Pathogenicity of novel missense variations was confirmed using different web-based tools (PolyPhen2, SIFT, and Mutation Taster). According to the guidelines by the American College of Medical Genetics and Genomics, both of p.Ser1169Arg and p.Ser1197phe were found to be likely pathogenic variants. MetaDome web server showed that two novel variants are located in regions intolerant for missense variation. According to project HOPE, novel missense variantsp. Ser1169Arg and p.Ser1197Phe were predicted to make harmful effects on proteins binding and folding processes causing subsequently protein malfunction (Figure 2). *In silico* analysis, results are all summarized in (Table 2).

Table 1: Clinical phenotypes and molecular characterization of *NPC1* (exon 17–25) in NPC patients

Patient no	Sex	Age at neurological symptoms	Age at diagnosis	Early infantile	Late infantile	Juvenile form	Clinical manifestations	MRI results	Consanguinity	Nucleotide change/(hz-ht) effect on protein (Exon)	Protein domain	Geographical distribution
1	M	1.5 year	3 years	Early Infantile			Hepatosplenomegaly, delayed developmental and motor skills and hypotonia	Cerebral atrophy Cortical atrophy	No			Baniswif
2	M	1.5 year	2 years	Early Infantile			Splenomegaly, hypotonia, and delay walking	Mild cortical atrophic changes	Yes			Menia
3	F	6 years	8 years			Juvenile Form	Delayed developmental motor milestones, speech delay, epilepsy and hypotonia	Cerebral atrophy Cortical atrophy	Yes	c.3590C>T(hz) p.Ser1197Phe (exon 23)	12th TM domain	Baniswif
4	F	22 months	2 years	Early Infantile			Hepatosplenomegaly, hypotonia Delayed developmental milestone and psychomotor retardation	Mild cortical atrophic changes	Yes	c. 3507C>G (hz) p.Ser1169Arg (exon 23)	Cytoplasmic loop L	Menofia
5	F		6 months				Hepatosplenomegaly		No	c.2728G>A (ht) p.Gly910Ser (exon 18)	Cysteine-rich luminal loop	Menofia
6	M	8 months	1.5 year	Early Infantile			Hepatosplenomegaly, hypotonia and delayed motor skills	Mild cortical atrophic changes	Yes			Giza
7	F	4.5 years	9 years		Late infantile		Splenomegaly, loss of speech progression, unsteady gait, hypotonia, and VSGP	Cerebral atrophy Cortical atrophy	Yes	c.3590C>T (hz) p.Ser1197Phe (exon 23)	12th TM domain	Beniswif
8	M	26 months	3 and 10/12 years		Late infantile		Hepatosplenomegaly, loss of speech progression, unsteady gait, and hypotonia	Cerebral atrophy Cortical atrophy	Yes			Giza
9	M	6 months	2 years	Early Infantile			Splenomegaly, hypotonia and delayed developmental milestone	Mild cortical atrophic changes	Yes	c.2872C>T(hz) p.Arg958*(exon 19)	Cysteine-rich luminal loop	Fayoum
10	M	2 years and 2 months	3 years		Late Infantile		Hepatosplenomegaly, dysphagia Cataplexy, delayed developmental milestone, psychomotor and speech retardation, and ataxia	Cerebral atrophy Cortical atrophy	Yes	c.3032-3038delins11bp(hz) p.Cys1011* (exon 20)		Giza
11	F	11 months	3 years	Early Infantile			Hepatosplenomegaly, hypotonia Delayed developmental milestone	Cerebral atrophy Cortical atrophy	Yes	c.2779Gdup(hz) p.Arg927Gfs*38 (exon 18)	Cysteine-rich luminal loop	Tanta
12	M		11 months				Hepatosplenomegaly		Yes			Giza
13	M	4 years	10 years		Late Infantile		Splenomegaly, VSGP, hypotonia Dystonia and psychomotor and speech retardation	Cerebral atrophy Cortical atrophy	Yes	c.3590C>T (hz) p.Ser1197Phe (exon 23)	12th TM domain	Beniswif
14	M		One and 1/12 years				Hepatosplenomegaly		No			Cairo
15	F	6 months	1 year	Early infantile			Splenomegaly and hypotonia	Mild cortical atrophic changes	Yes			Fayoum

M: Male; F: Female; ht: Heterozygous; hz: Homozygous.

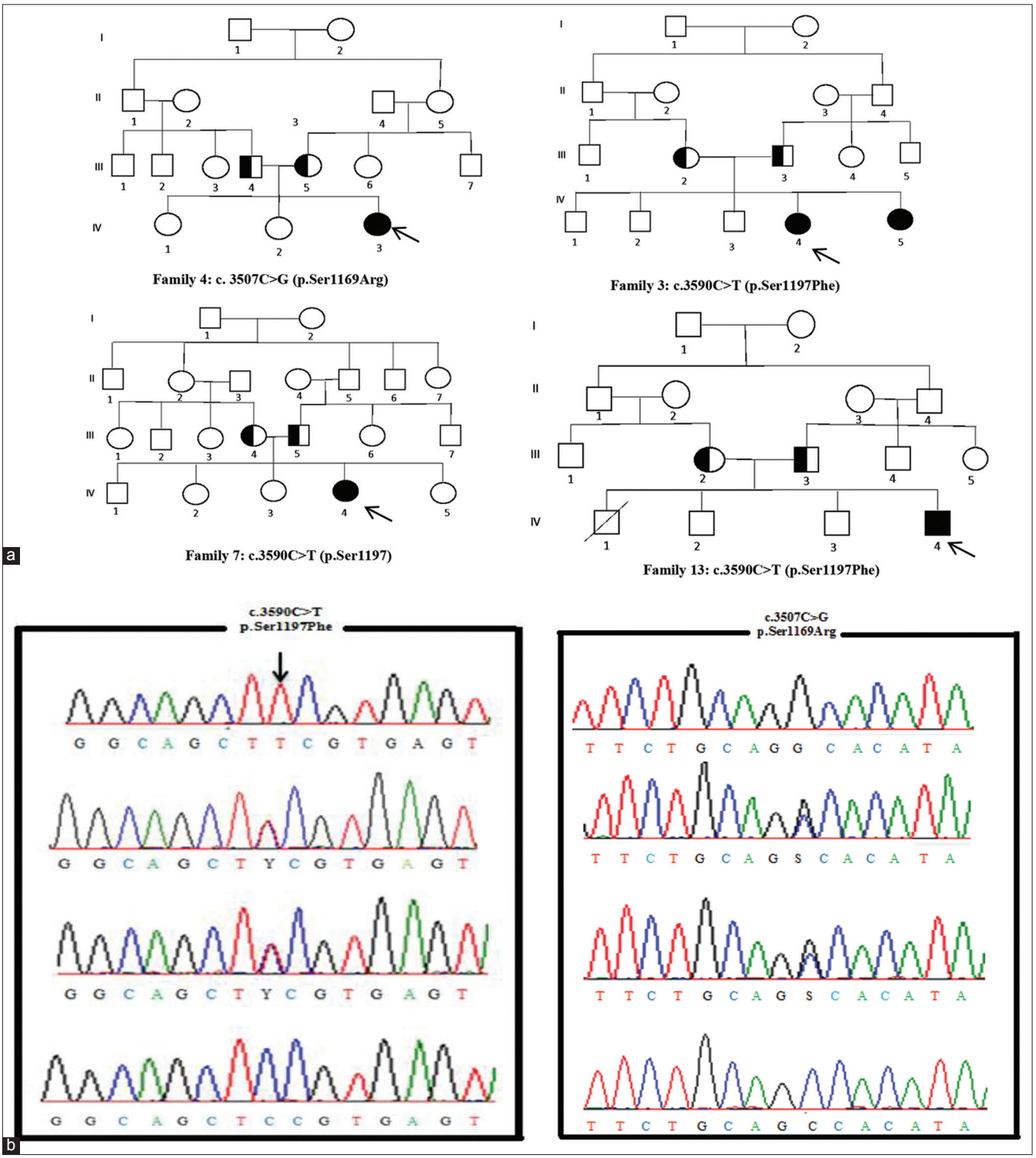

Figure 1: Pedigrees and chromatograms of four families 4, 3, 7, 13. (a) Squares indicate males; circles indicate females; the black symbols indicate affected individuals; the half-black symbols indicate carriers, arrows indicate the molecularly studied probands. (b) Novel variants of NPC1 in 4 probands. The upper chromatogram in each frame represents the variant sequence, the lower one depicts the reference sequence, and the two middle sequences represent the parent's heterozygous sequences

Discussion

NPCis an autosomal recessive lysosomal storage diseasemainly caused by homozygous or compounds heterozygous mutations in *NPC1 (*95% of cases) [1]. Early disease diagnosis is very crucial for proper initiation of treatment and inhibition of disease progression [3]. In the current study, 15 Egyptian patients clinically and biochemically diagnosed as NPC. As previously mentioned, NPC exhibits broad clinical heterogeneity and wide presentation [29], which is illustrated herein. Ourpatients showed a remarkable phenotypic variability and their age ranged from

Table 2: *In silico* analysis of novel revealed variants

cDNA variant (NM_000271.4)	Mutation effect on protein (NP_ 000262.1)	GnomAD	PolyPhen-2	SIFT	Mutation taster	MetaDome tolerance	ClinVar	HOPE
c.3590C>T	p.Ser1197Phe	0	Probably damaging	Deleterious	Pathogenic	Slightly intolerant	No entry	Protein malfunction
c. 3507C>G	p.Ser1169Arg	0	Probably damaging	Deleterious	Pathogenic	Intolerant	No entry	Protein malfunction

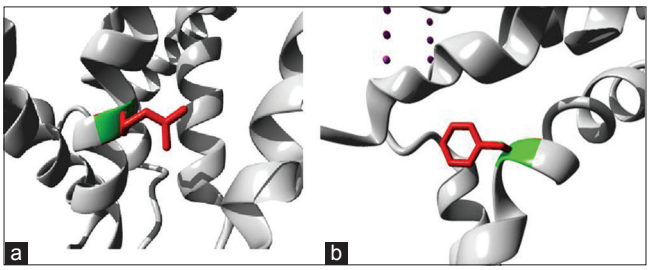

Figure 2: Structural variation between native protein and the variant models according to hope project, (a) zoom in variantp. Ser1169Arg, the protein is colored gray, the side chains of the wild type (serine) and the mutant (Arginine) residue are colored green and red, respectively, (b) zoom in variant p.Ser1197Phe, the protein is colored gray; the side chains of the wild type (serine) and the mutant (phenylalanine) residue are colored green and red, respectively

6 months to 10 years old. Around 80% ofcases were diagnosed after the presence of neurologicalmanifestations, while the diagnosis was only established according to systemic signs in other cases (20%), systemic manifestations such as hepatosplenomegaly or splenomegaly were obvious in the majority of cases (93.3%) this is consistent with previous literature where systemic manifestations areusually present preceding to neurological onsetwith the exception of few percentages of patients(approximately,15%) [30]. The early infantile-onset was the most common form in our study;it represents(46.5%) of cases followed by the late infantile-onset (26.7%) while the juvenile onset was only present in (6.7%) of cases. This agrees with the previous studies that reported the early infantile form as the most frequent disease form in Southern Europe (>20% of cases) and the Middle East [30]. However, a previous Egyptian study reported the neonatal form as a predominating form, followed by an early infantile onset of disease [31]. Remarkably, although severe neonatal cholestatic jaundice is more common in classic NPC, this feature was absent in the current study. Herein, the majority of pediatric patients' manifestations (≤4 years) such as splenomegaly, hepatosplenomegaly, hypotonia, delayed neuro-psychomotor, ataxia, and cataplexy are in agreement with the common previously reported NPC signs in pediatric patients [1]. Clinical manifestations observed in included patients (>4 years) such as cognitive decline, epilepsy, and VSGP were more commonly described in younger NPC patients [30]. Denoting the biochemical analysis, high chitotriosidase levels were detected in all included patients, which is a useful initial screening tool for disease diagnosis. Although other previous studies showed an elevated level of chitotriosidase in NPC patients, other assessments are required for proper diagnosis, as it is not a specific NPC diagnostic marker [32]. Consequently, disease diagnosis must be finally confirmed by molecular analysis, which isalso considered as the only major tool for prenatal diagnosis, family studies, and carrier detection. Up till now, more than 400 disease-causing *NPC1* mutationshave been reported [33]. Although they are widely scattered over the geneother studies considered some regions such as residues(855–1098) in the cysteine-rich luminal loop and residues(1038–1253) which shares 34.7% with PTC1, as hot spot regions for NPC disease mutations[34]. In this study, molecular analysis of nine exons (17–25) of *NPC1* encountering the previously suggested hot spot residues(855–1098) and (1038–1253) revealed six different mutations (15 mutant alleles) in eightunrelated patients. Regarding the high consanguinity rate in our population, homozygous mutations were predominated. Patients showed different geographical distributions, they originatefrom different governorates all over Egypt. Around 23% (7/30) of the 30 studied alleles showed variations in the cysteine-rich luminal loop (residues 855–1098), while 26.7% (8/30) of them showed alterations at the amino acid residues(1038–1253). Distribution of mutations in the two studied amino acid residues (855–1098 and 1038–1253)was consistent with the previously described [34], [35]; moreover, a recent Egyptian study showed that around 37% of detected mutant alleles were in the currently studied hotspot regions [31], confirming that they can also be considered as hot spot regions in NPC Egyptian patients. Although the missense mutations p.Ile1061Thr, p.Arg1186His, p.Pro1007Ala, and Gly992Trp were previously reported as the most common NPC mutations [9], none of them were detected in the present study;however, p.Ile1061Thr was reported in the heterozygous state coupled with p.Ser627Arg in an Egyptian patient with the late infantile onset of disease [31]. Among severely affected patients, we identified one nonsense homozygous mutation, p.Arg958* inpatient9 led to protein truncation in exon 19 within the cysteine-rich luminal loop between TM8 and 9. The patient showed early infantile onset of disease, splenomegaly was the main systemic feature, while neurological manifestations such as hypotonia and delayed developmental milestone started to appear at the age of 6 months, the same mutation was also reported in a previous Egyptian study in two unrelated patients with neonatal and infantile onset of disease [31]. However, it was observed in the compound heterozygous state in a case with adult-onset of disease in a study applied in Western Europe and the USA [36]. This illustrates the severity of the mutation in the disease progression, as all the reported cases carryingthe homozygous p.Arg958* mutation was of early disease onset. However, the case

previously mentioned reported by Bauer *et al*., [36], the neurological manifestations appeared in the adult stage due to the less severe missense mutation p.Asp874Val. Two frameshift mutations (c.3032_3038delins10bp p.Cys1011*) and (c.2779dupG p.Ala927Glyfs*38) were separately observed in the homozygous form in patients 10 and 11, respectively, both mutations led to losing of essential domains of *NPC1* protein and subsequent protein function impairment. Patient 10 showed a late infantile onset of disease and was suffering from hepatosplenomegaly in the early stage of life, while after 2 years of age, progressive neurological manifestations began to appear such as, dysphagia, cataplexy, delayed developmental milestone, psychomotor, speech retardation, and ataxia. Patient 11 was presented with hepatosplenomegaly, hypotonia, and delayed developmental milestone by the age of 2 years, with an early infantile onset of disease. The latter two frameshift mutations were not reported before in any populations rather than Egyptians. Two previously reported that Egyptian patients were described with the early infantile onset of disease [31]. Among cases with a late infantile-onset, we identified the novel missense variation p.Ser1169Arg in patient 4, her parents were both heterozygous carriers and showed normal phenotypes. The patient was suffering from hepatosplenomegaly, hypotonia, delayed developmental milestones, and psychomotor retardation that appeared after 2 years of age. The mutation p.Ser1169Arg is located in the cytoplasmic loop L between the 11thand 12th transmembrane domains. Functional effect prediction programs confirmed the possible pathogenesis of this novel variant. Moreover, it is found to be located within conserved regions when multiple alignment analysis was applied in eightdifferent species. Within the same region, p.Cys1168Tyr mutation was previously detected in a patient with the late infantile onset of disease, where immunoblotting of the mutated *NPC1* protein showed a diminished amount of the protein [35]. This confirms the essential role of this region within the cytoplasmic loop L for the protein activity process. On the other hand, these predictions were confirmed by project HOPE, which illustrated the difference in size, charge, and hydrophobicity value between the wild residue serine and mutant arginine. The exchange of the buried neutral serine residue with larger positively charged arginine could abolish the core structure of the domain and affect protein binding. In addition, loss of hydrophobic interaction between the protein core and membrane lipids may be developed due to the less hydrophobic mutant residue arginine, which affects protein function. Another novel missense variant p.Ser1197Phe was observed in a homozygous state in three different patients (3, 7, and 13). Although, they were not relatives; the three patients were from the same village. Sequencing of parents showed the heterozygous status of the variant. Patients 7 and 13had a late-infantile onset of disease and showed nearly similar manifestations. Splenomegaly was the main systemic feature, while neurological manifestations began after 4 years of age and included loss of speech progression, unsteady gait, and hypotonia in addition to VSGP. Concerning patient3, no systemic signs could be observed; she showed the juvenile form of the disease. Although three latter patients share the same variantp. Ser1197Phe, patient 3 showed different disease onset and clinical manifestations which highlight the extreme phenotypic heterogeneity of the disease. Despite considerable advances in biochemical andgenetic understanding of the disease, the cause of extensivephenotypic variability in NPC remains unclear [29]. The detected variant Ser1197phe is located within the12th transmembrane domain in a highly conserved region was confirmed by multiple alignment analysis. Functional effect prediction programs such asSIFT, PolyPhen2, andMutationTasterpredicted splice site alteration and possible pathogenicity after this novel variant. According to project HOPE, phenylalanine residue is larger and more hydrophobic than serine residue which may affect the protein contact with the lipid-membrane. In addition, in wild protein, the buried serine residue forms a hydrogen bond with glutamine at position 707, while in the mutant phenylalanine residue; loss of hydrogen bonds in the protein core is acquired dueto the difference in size and hydrophobicity of phenylalanine, leading to disruption of the protein correct folding.

Finally, mutation p.Gly910Ser was observed in the heterozygous state in patient 5 (6 months of age) who only suffered from hepatosplenomegaly while neurological manifestations havenot yet manifested on clinical evaluation, the same mutation was also reported in the heterozygous state coupled with p.Cys516Tyr mutation in a newborn infant with acute liver failure to parents of mixed Armenian and Turkish descent [37]. It was also observed in a patient with a juvenile form of the disease in the heterozygous state with p.Pro474Leu missense mutation in a previous Italian study [17]. Here, we could not detect the other heterozygous variant suggesting that patient may have another variant either in a more proximal portion of the *NPC1*or in *NPC2*.

Conclusion

Our study as one of the limited molecular studies on *NPC1* gene of NPC Egyptian patients was able to investigate certain clinical, biochemical, and molecular characteristics in patients. It provides valuable information to enhance the recognition of some variations associated with NPC disease in Egyptians. The novel *NPC1* variants p.Ser1169Arg and p.Ser1197Phe were mostly correlated with the late-infantile onset of the disease. Distribution of detected mutations revealed

that the two studied amino acid residues (855–1098 and 1038–1253) could be considered as potentialhot spot regions in *NPC1* Egyptian patients. Although NPC was variably distributed all over Egypt, a recurrent mutation within the same village illustrates the harmful impact of consanguineous marriage which would extend further from the unit of the family. Finally, the use of *in silico* bioinformatics tools for analysis of the pathogenicity of novel variants isa helpful, time-saving, and relatively accurate test. However, functional analysis of protein would be of great importance, especially in studies examining the disease pathogenesis and pathway.

Ethical Statement

This research was approved by the scientific ethics committee at the NRC.

Informed Consent

The patients' parents or their legal guardiansand controlswere consulted and agreed to participate in the study. Written informed consent was obtained from them.

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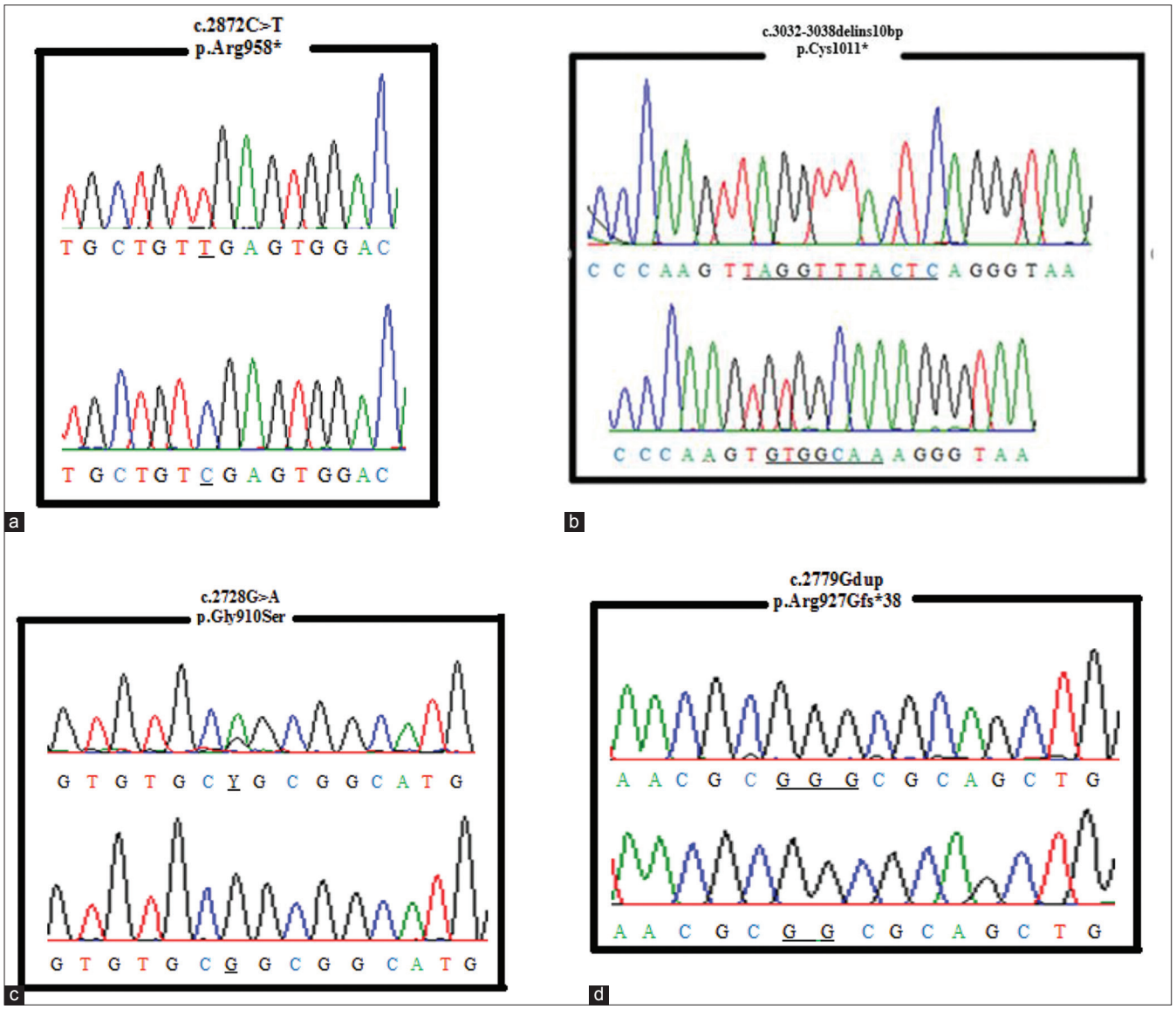

Figure S1: The revealed reported mutations of NPC1 (a) represents the homozygous nonsense mutation p.Arg958 detected in patient 9; (b) shows the homozygous frameshift mutation p.Cys1011* detected in patient 10; (c) shows the heterozygous missense mutation p.Gly910Ser revealed in patient 5; (d) represents the homozygous frameshift mutation p.Arg927Gfs*38 observed in patient 11. The upper chromatogram in each frame represents the variant sequence; the lower one shows the reference sequence*

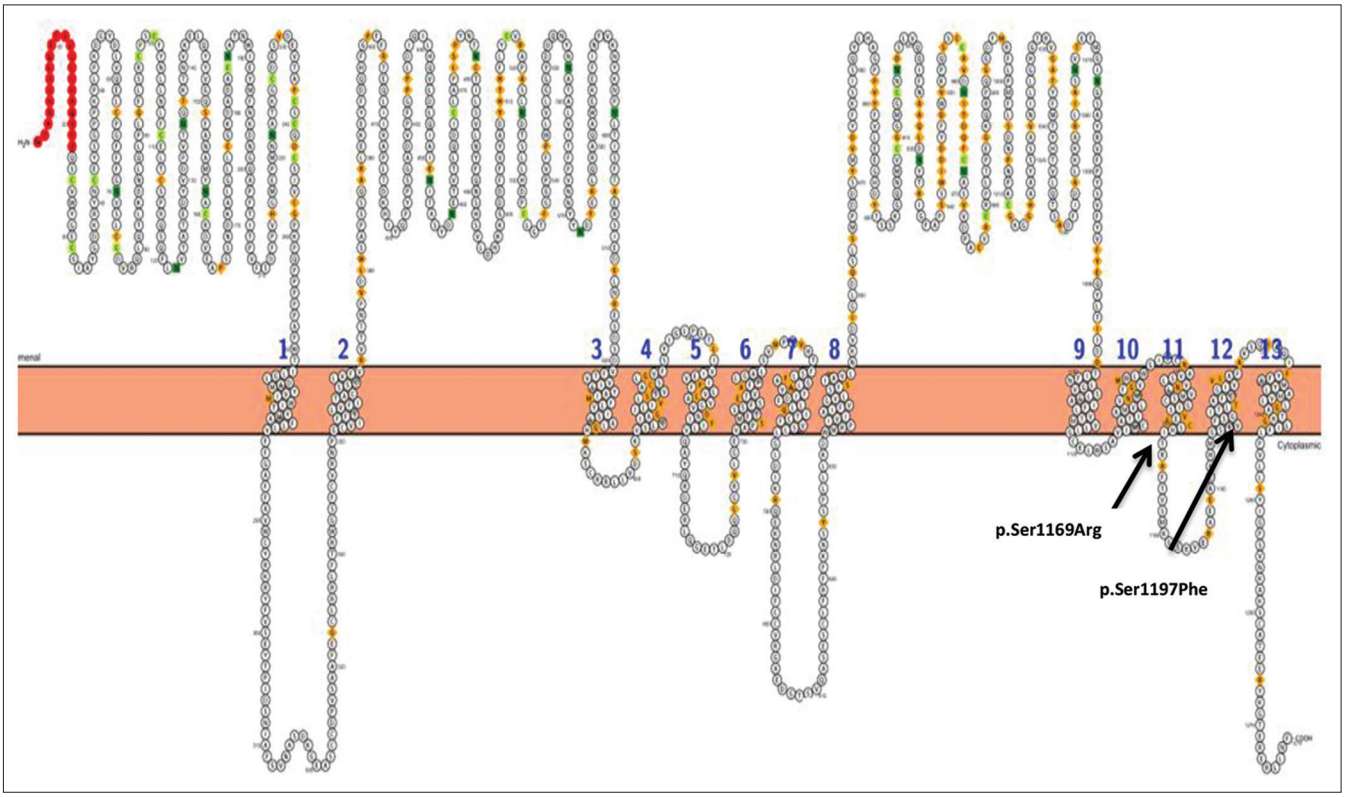

Figure S2: NPC1 protein topology and distribuition of the two novel missense variants (p.Ser1169Arg) and (p.Ser1197Phe). The 13 transmembrane domains of NPC1 are represented in the middle; cytosolic domains are shown at the bottom part, and luminal domains are shown at the upper part of the figure. Novel sequence alterations are indicated at their positions

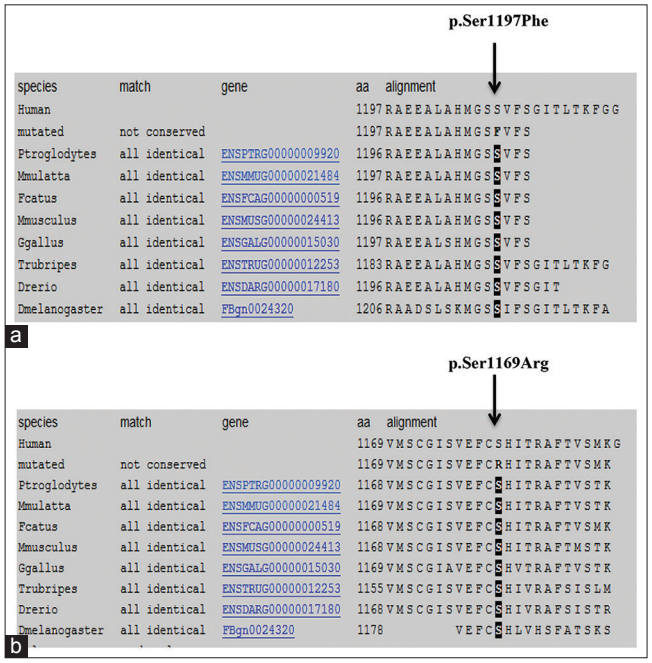

Figure S3: Cross-species conservation of the NPC1 protein sequence in relation to the two novel variants, (a) p.Ser1197Phe and (b) p.Ser1169Arg

Table S1: Primer sequences used to amplify *NPC1* exons (17–25)

Exon No.	Sequence	Fragment size
17F	F: 5_-CCTGTACTCCCTATTAGCCTGTCAT-3_	322
17R	R: 5_-ACTTGCTTGAAACACCTACGTGCATG-3	
18F	F:5_-TGCTTAGTTACTATCAGAGTGTTCAC-3_	291
18R	R:5_-CCTCCTCCGCTGCTTCTGAAGTA-3_	
19F	F:5_-CTGTGGAGCAGGTCAGTAACCCT-3_	245
19R	R:5_-GTATAAACTGAGGCACGATGCAAATG-3_	
20F	F: 5_-CTTCTAACAGTCCTCCCTGCA-3_	247
20R	R: 5_-CTGTCTTAGCCCAGTCCTCTC-3_	
21F	F: 5_-TGCTTAGCCTCAAGTGCTCAGAT-3_	337
21R	R: 5_-ACCCAGTGTAGGCCCTTTGCTG-3_	
22F	F: 5_-GCGAGCTTTAATGAGGCCTCC-3_	295
22R	R: 5_-GACAAACTGAGACTGTATGAGGA-3_	
23F	F: 5_-CAGGGTGCCCTGGGTAATTAGCA-3_	292
23R	R: 5_-GATCCAGACTCTTCAGTCACTGAG-3_	
24F	F: 5_-TTCAATTACAGGTTGGTAAAAGTGGTT-3_	297
24R	R: 5_-CTTGAAAAGAATGCCTCAGGATAGA-3_	
25F	F: 5_-TTCCAAAGTGGGATTACAGGCGTG-3_	221
25R	R: 5_-GACCGACCCTTAGACACAGTTCAG-3_	

Table S2: Biochemical analysis of 15 studied NPC patients

Patient no.	Chitotriosidase μmol/l/h Reference level (4–80 μmol/L/h)	Sphingomyelinase nmol/g.prot/h Reference level (6–47 μl/g.prot/h/g.prot/h)	β-glucocerebrosidase μmol/g.prot/h Reference level (1–5 μmol/g/h)
1	101.3	13.4	1
2	88	23.4	1.5
3	107	11	1.2
4	282	30	4.7
5	195	25	3.2
6	171	7.3	2.6
7	159	8.7	2.5
8	242	7	3.5
9	380	14	2.5
10	195	25	3.2
11	615	17	1.3
12	329	7.3	2.8
13	188	1.7	1.8
14	215	13.7	5
15	692	9	1.5

no: Number.