Expanding the genetic spectrum of Mucolipidosis in Egyptian patients: Recurrent and novel *GNPTAB* and *GNPTG* genes variants

Original Article

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ABSTRACT

Mucolipidosis term describes several clinical conditions that combine features of both mucopolysaccharidoses and sphingolipidoses. Mucolipidosis typeII (MLII) and MLIII are two forms of a rare autosomal recessive lysosomal storage disorder. Both result from deficiency of the N- acetylglucosamine (GlcNAc)-1-phospho- transferase enzyme. This enzyme is a hexameric complex; 2α, 2β and 2γ encoded by two genes; *GNPTAB* & *GNPTG* gene. In this study, we report the underlying genetics of 4 different ML Egyptian patients who were recruited according to their clinical presentations, and β-Hexosaminindase -A enzyme activity assay. Whole exome sequencing for the four patients was carried out revealing 4 different mutations with all patients homozygous for their corresponding mutations. Two frameshift mutations were found in *GNPTAB* gene; c.2693dupA in exons 13 & c.3503_3504delTC in exon 19. The other two mutations were mapped to the *GNPTG* gene; a novel nonsense mutation c.658A>T in exon 4, and the splice site mutation c.233+1G>A. both mutations were associated with mild phenotype.

Key Words: Genetics, *GNPTAB* gene, *GNPTG* gene, Molecular, Mucolipidosis.

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INTRODUCTION

Mucolipidosis as a term describe several clinical conditions that combine features of both mucopolysaccharidoses and sphingolipidoses. Mucolipidosis typeII (MLII; formerly known as I-cell disease) and MLIII (formerly known as pseudo-Hurler dystrophy) were found to be linked by the same molecular mechanisms. They represent two forms of a rare autosomal recessive lysosomal storage disorder with a worldwide estimated incidence of 2.5 and 10 cases per 1,000,000 live births. (**Leroy and Demars, 1967; Bargal *et al.*, 2006; Matos *et al.*, 2020**)

Both result from deficiency of the N- acetylglucosamine (GlcNAc)- 1-phospho- transferase enzyme. This enzyme is responsible for generation of mannose-6-phosphate (M6P) targeting signals. More than 70 lysosomal enzymes require those M6P targeting signals. Only M6P-modified enzymes can bind to M6P receptors to mediate their transport from the trans-Golgi network to lysosomes via the endosomal compartment (**Velho *et al.*, 2019; Sachs *et al.*, 2020**).

Thus, in ML II & ML III, newly synthesized lysosomal enzymes are mis-targeted to lysosomes and hyper secreted in the extracellular environment causing a 10–20-fold higher plasma activity of almost all lysosomal hydrolases than in normal controls. Subsequently, these enzymes become deficient intracellularly and various non-degradable macromolecules accumulate in lysosomes. Enlarged lysosomes are then dysfunctional, which strongly impairs cellular function and homeostasis (**Velho *et al.*, 2019; Matos *et al.*, 2020**).

According to the age of onset, progression rate and severity of symptoms patients of mucolipidosis can be classified into ML type II and ML type III. ML II (MIM #252500) is the most severe form of the disease and is typically evident at birth. It is usually progressive and causes death during the first decade of life mostly due to respiratory insufficiency. ML II patients have coarse facial features and hypertrophic gingiva. Main clinical manifestations include psychomotor retardation, developmental delay, and severe skeletal abnormalities; like craniosynostosis, osteopenia, neonatal hyperparathyroidism, rickets, thoracic deformity, kyphosis, deformed long tubular bones, hip dysplasia, clubfeet, dysostosis multiplex, and joint contractures. In most severe cases of ML II, bone dysplasia with shortened and curved bones might be evident prenatally. Organomegaly, and cardio-respiratory involvement are

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common in ML II children, of which cardiac hypertrophy or thickening and insufficiency of the mitral valve typically occur (**Velho *et al*., 2019; Pasumarthi *et al*., 2020**).

ML type III represents the more attenuated form of the disease with later onset and slower disease course, which gives a longer life expectancy to adulthood. Despite being a milder form, it has common skeletal manifestations that allow its diagnosis in childhood. Joint stiffness, carpal tunnel syndrome, and pain in hips, shoulders, hands, and/or ankles, waddling gait, as well as spinal deformities, are the most common observations in ML III patients. Yet they are not unique for ML III, thus, MLIII can be misinterpreted as a rheumatologic disorder like juvenile arthritis or progressive pseudorheumatoid arthropathy of childhood. Other signs like craniofacial dysmorphism, organomegaly, and cardiorespiratory problems are often absent or less prominent than in MLII. Patients with intermediate phenotypes were reported to share features of both ML II and ML III. They have the same physical growth characteristics as ML II and express the speech and motor phenotypes of ML IIIα/β (**Tüysüz *et al*., 2018; Pasumarthi *et al*., 2020**).

The GlcNAc- 1-phosphotransferase enzyme (EC 2.7.8.17) is a hexameric complex; 2α, 2β and 2γ. The membrane- bound α- and β- subunits are synthesized as a common α/β-precursor and encoded by *GNPTAB* gene, and the soluble γ subunit is encoded by *GNPTG* gene (**Tiede *et al*., 2005; Velho *et al*., 2019**).

The six subunits are assembled in the endoplasmic reticulum (ER) as an inactive complex, which is then transferred to the Golgi apparatus to be activated. Activation occurs by site- 1 protease (S1P) enzyme that proteolytic cleavage of the α/β-precursor into active α-and β- subunits. The γ-subunit then binds directly to the α-subunit enhancing the hexameric activity for M6P modification of certain enzymes. The common membrane-bound α/ β-subunit precursor is encoded by the *GNPTAB* gene, and the soluble, regulatory γ-subunit is encoded by the *GNPTG* gene. (**De Pace *et al*., 2015; Velho *et al*., 2015; Sachs *et al*., 2020**).

ML II occurs due to variations in *GNPTAB* gene only in contrast to ML III that is caused by pathogenic variations either in *GNPTAB* gene (MLIII alpha/beta, MIM # 252600) or *GNPTG* gene (MLIII gamma, MIM# 252605). Mutations in *GNPTAB* gene that abolish the GlcNAc- 1-phosphotransferase activity, like frameshifts and nonsense mutations, are associated with the severe ML II. ML III on the other hand was found to result from mutations in both *GNPATB* & *GNPTG* genes. From this molecular fact, ML III is further classified into two subgroups; ML III alpha/beta (MIM #252600) and MLIII gamma (MIM #252605), respectively. Both subtypes have different levels of the residual GlcNAc- 1-phosphotransferase activity and variable clinical presentations. In general, *GNPTG* mutations are associated with a milder phenotype and better prognosis compared with *GNPTAB* mutations (**Soares *et al*., 2020; Ludwig *et al*., 2021**). 63A

GlcNAc- 1- phosphotransferase deficiency is associated with 5- to 20-fold elevation of several plasma lysosomal enzymes, suggesting ML II or ML III. Subtype determination and diagnosis confirmation is done by *GNPTAB* and *GNPTG* gene sequencing for genotype characterization (**Velho *et al*., 2019; Pasumarthi *et al*., 2020**).

GNPTAB and *GNPTG* gene mutational pattern in Egypt is still not well defined. This study aims to add to our understanding of the genetic pattern of ML II & ML III among Egyptian patients.

PATIENTS AND METHODS:

Ethical aspects

The study design and results were approved by Medical Research Ethics Committee of the NRC. Written informed consents to participate in this study were obtained from legal guardians of the patients prior to medical examination and genetic testing.

Patients

The study included four affected children from 4 unrelated families, 7 available family members, and one amniotic fluid sample. The parents and other siblings were included when available. Three females & one male patient, were referred from the Clinical Genetics Clinics and Biochemical Genetics Department at the National Research Centre (NRC). Their age ranged from 2 to 11 years. The patients were complaining from skeletal deformities with variable phenotypic presentations, associated with biochemical increase in the extracellular activity of β-Hexosaminidase-A enzyme above reference range in accordance with specific electrophoretic separation pattern of urinary glycosaminoglycans (GAGs). Amniotic Fluid sample from family 2 was also included.

Methods

Clinical and radiological evaluations were performed for all the patients. The biochemical analysis measuring β-Hexosaminidase-A enzyme activity in plasma, as a representative of Mannose-6-phosphate dependent enzymes, was performed according to the protocol proposed by Kolter and Sandhoff, 2005.

Blood samples of 3-5 ml of peripheral blood from patients and all available family members were collected. Genomic

DNA was extracted from leukocytes using PAXgene Blood DNA extraction kit (PreAnalytiX, USA) according to the manufacturer's protocol. DNA concentration and purity were determined by UV-Spectrometric method using NanoDrop 2000 (Thermo Scientific, USA). The dsDNA was quantified using DeNovix®, a flourescence-based method.

Whole-exome sequencing was performed on a HiSeq platform (Illumina, USA). Targeted Sanger sequencing for PCR products of patients for validation and segregation in other available members of each family for their related gene was performed using ABI 3500 Genetic Analyzer 8-Capillary (Applied Biosystems, USA). Specific primers designed and conditions are available upon request.

For the prenatal sample, genomic DNA was extracted from 20cc amniotic fluid sample using a GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, USA) according to the manufacturer's protocol, and then, Sanger sequencing was used to detect the specific mutation site of the proband.

Alamut Genova 1.4 (http://www.interactive-biosoftware.com/alamut-genova/) was used for in silico analysis and interpretation of the detected variants.

RESULTS:

1- Clinical results

The study included four families with 3 affected females and a male patient. All clinical data are summarized in (Table 1). For patients' 1 & 2 the skeletal symptoms' onset was early before the first year of life. Their milestones were delayed. Skeletal survey of the patients showed the characteristic dysostosis of MPS including coarse facies and short stature. Muscle hypotonia was also presented. The first sign and symptom for patients' 3 & 4 was delayed till the age of 3 years. Their facies weren't coarse and showed less severe and slowly progressive symptoms compared to the first 2 patients.

The detailed clinical data of patient 1 are not available as the patient died soon after referral. Blood samples for both biochemical and molecular analysis were obtained during the patient's first visit to our clinics.

Since birth patient 2 was dyspneic and congenital heart disease was confirmed with Echo. The patient suffered from craniocynostoses and hearing loss. At the age of 26 months, she developed generalized tonic convulsions. Short stature was also evident on examination (-3.6 SD) (Fig. 1). The family had an elder male sibling that died at the age of 4 months and suffered from skeletal deformities.

Patient 3 symptoms were much milder, limited to camptodactyly; which was corrected surgically at the age of 3 years old, progressive joint stiffness, knock knees, abnormal gait, backache, average mentalities, and delayed milestones. Yet, the patient's facies were not dysmorphic and his anthropometric measures showed short stature (- 1.98 SD), other measures were within normal (Fig. 2).

The main complaint of patient 4 was the abnormal gait and corneal clouding. Her milestones were 6 months delayed and the disease progression was slow. The patient's facies were not coarse but her stature tends to be short (-3.5 Std). Joint stiffness along with knock knees restricted her range of motion.

2- Biochemical Results

Using 2D electrophoretic separation, urinary GAGs were high for age in all patients compared with reference groups and showed the same specific pattern different from that of MPS as reported before in Essawi *et al*., 2021.

The β-Hexosaminidase-A enzyme activity was ranged from 341 to 1067 nmol/l/h (Table 1). All the results were above the normal range of *GNPTAB* enzyme activity (n: 50-200 nmol/l/h.).

Table 1: Clinical data of the four studied patients.

Patient	Consang-Uinity	Short Stature	Milestones	Coarse Facies	Cardio-pulmonary Complications	Enzyme Activity (nmol/l/h)
1	+	+	Delayed	+	NA	1067
2	+	+	Delayed	+	+	563
3	+	+	Delayed	-	-	365
4	+	+	Delayed	-	-	341

NA: Not available due to patients' death.

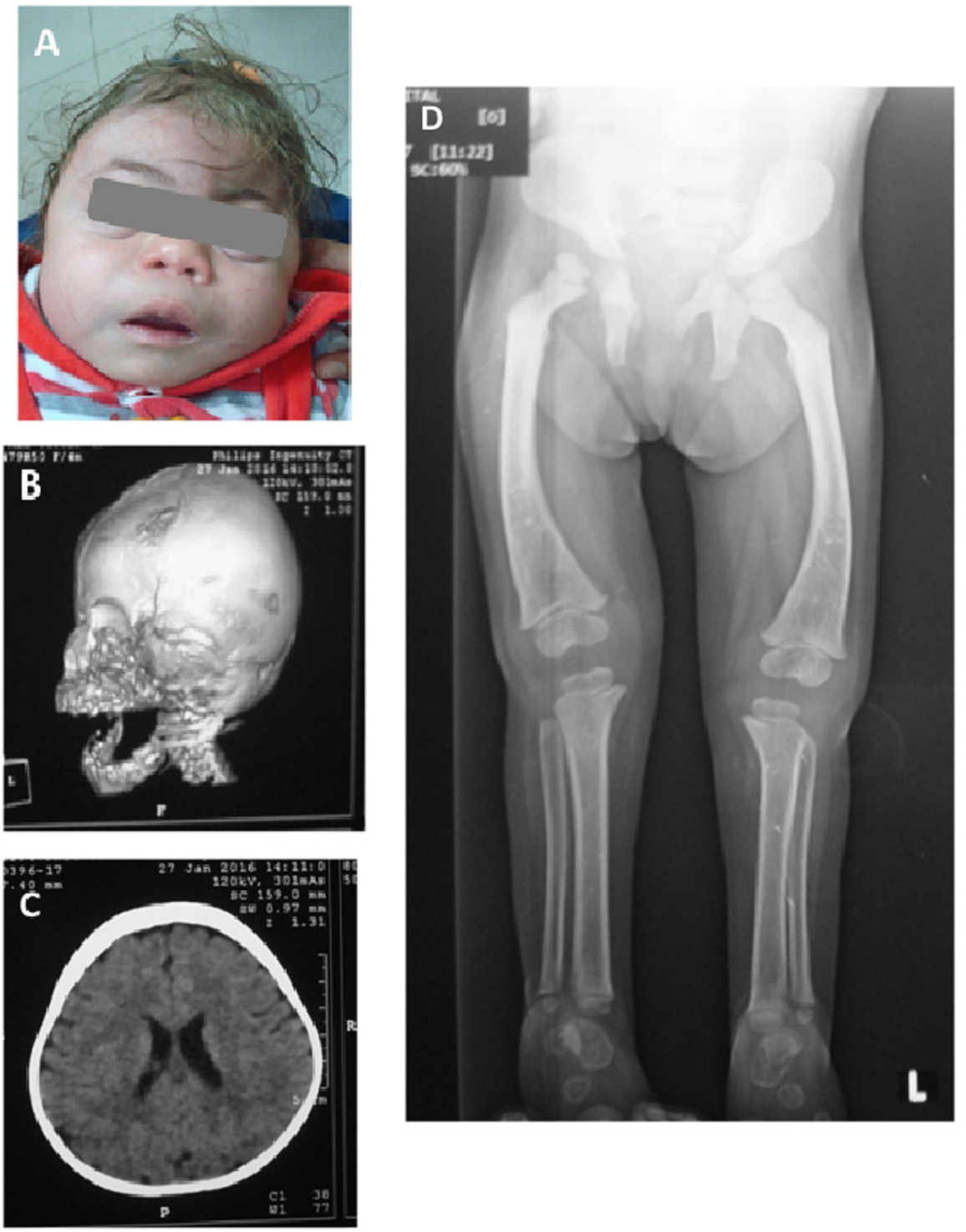

Fig. 1: Patient 2 at the age of 26 months; (A): Note the coarse face. (B): 3D skull CT showing coronal and metopic craniosynostosis. (C): Brain CT showing mildly dilated lateral ventricles. (D): pelvis and lower limbs showing dysostosis multiplex.

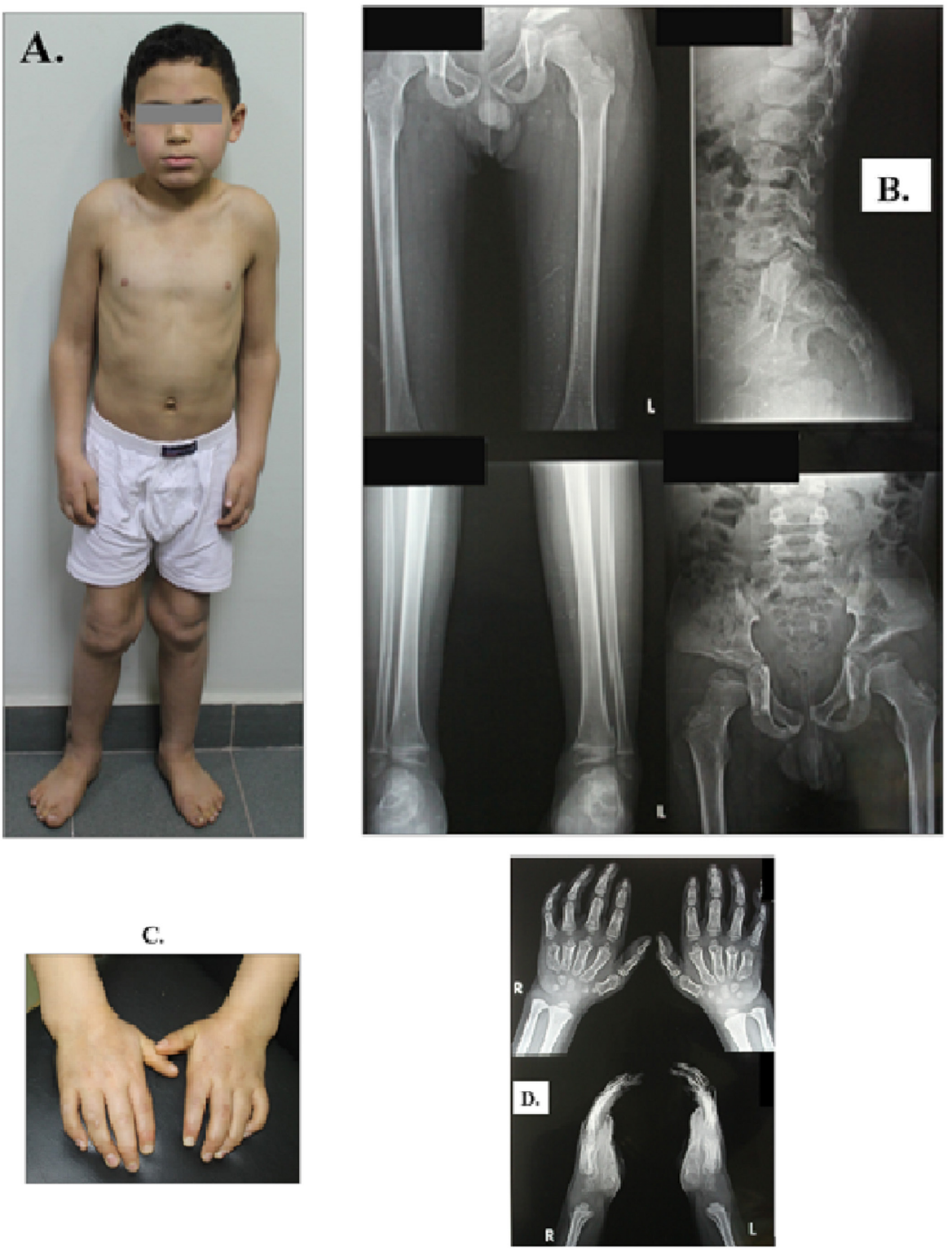

Fig. 2: Patient no. 3 at the age of 11 years and 4 months; A. Full body view showing short neck and broad metaphyses more evident at the knee joints. B. X-rays long bones of limbs showing broad irregular metaphyses and hypoplastic epiphyses, and spine X-ray showing abnormal shape and irregular end plates. C. Photograph of both hands showing tapering fingers with stiffness, clawing and camptodactyly of distal phalanges. D. Postero-anterior and lateral view X-rays of both hands showing camptodactyly and distal tapering of phalanges.

3- Molecular Results

Two patients had pathogenic mutations in *GNPTAB* gene, both were frame-shift disease causing mutations. Patient 1 has homozygous mutation c.2693dupA (p.Y899VfsX21) in exon 13, and homozygous mutation (c.3503_3504delTC) in exon 19 has detected in patient 2 (Fig. 3 & 4). The amniotic fluid sample analyzed for family 2 revealed the absence of previously reported mutation of *GNPTAB* gene of the affected sister.

The other two patients had 2 novel homozygous mutations in *GNPTG* gene; one is a splice site mutation and the other is nonsense mutation. Intron 4 mutation c.233+1G>A (IVS4+1 G>A) was detected in patient 3 and in heterozygous pattern in his mother (Fig. 5). Alamut Genova 1.4 software® analysis for c.233+1G>A (IVS4+1G>A) mutation of *GNPTG* gene showed changes in the splice factors. Original splice site has 2 binding sites; one for SRp55 & the other for SF2 / ASF (IgM- BRCA1). The new splice has 3 binding sites; one for SRp40, second for SF2 / ASF (IgM- BRCA1) & third for for SF2 / ASF. This suggests the creation of a new possible site and would alter the splicing of the mutated Gene. In silico analysis tools has confirmed that c.233+1G>A mutation is pathogenic and disease causing one (Fig. 6).

The last patient had a nonsense novel mutation in *GNPTG* gene; c.658A>T (p.k220*). This mutation changed Lysine into a stop codon terminating the gene's transcript to 220 amino acids instead of 305 amino acids (Fig. 7).

Both the splice site and the nonsense mutations have registered to the ClinVar database with accession numbers VCV000552294 and VCV000917517 respectively.

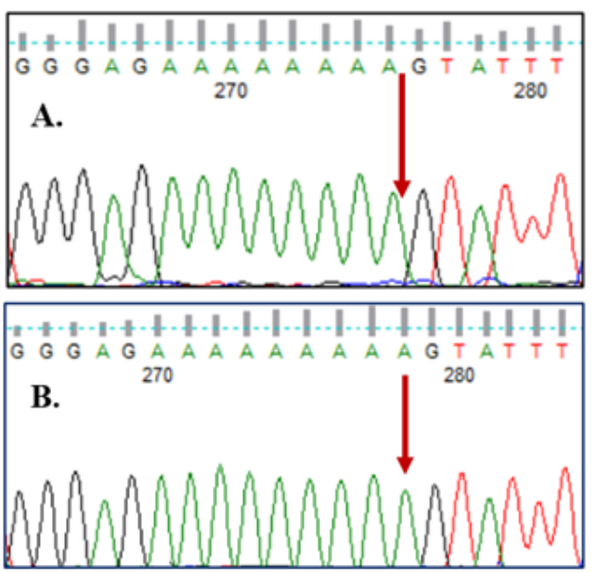

Fig. 3: Sequence chromatogram of exon 13 of *GNPTAB* gene in patient 1. A. normal control with wild type sequence, B. Mutation c.2693dupA (p.Y899VfsX21) in homozygous pattern found in patient 1. Site of mutation is denoted by the red arrows.

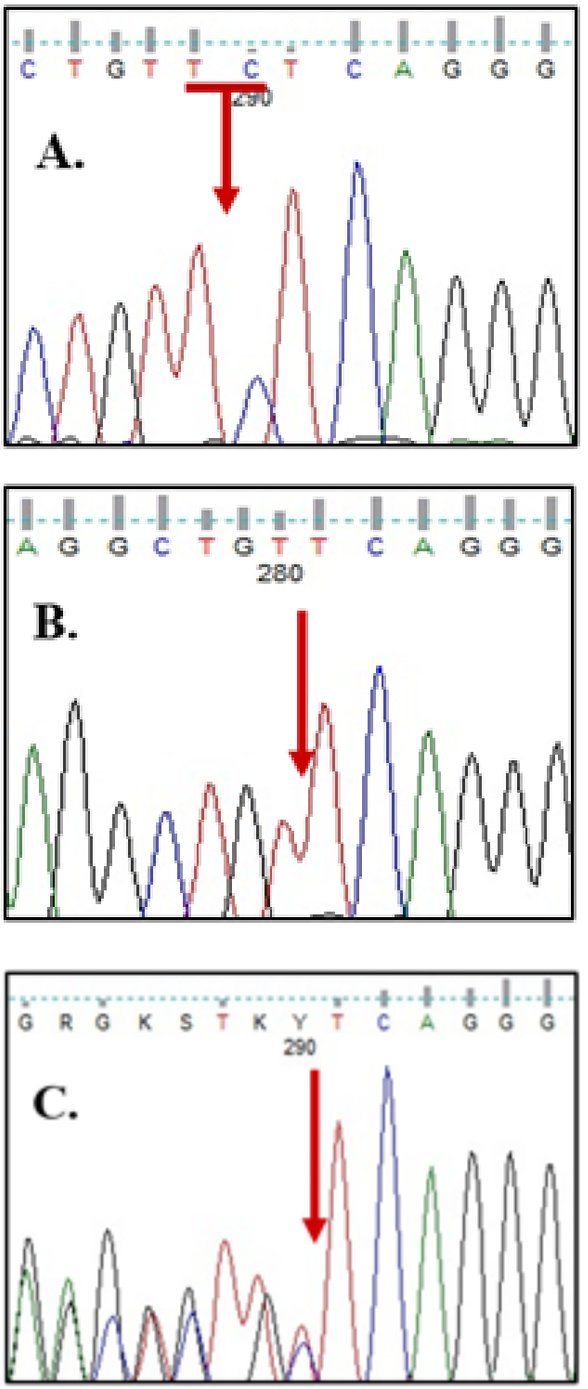

Fig. 4: Sequence chromatogram of exon 19 of *GNPTAB* gene in patient 2 .A. normal control with wild type sequence, B. Mutation c.3503_3504delTC (p.L1168QfsX5) in homozygous pattern found in patient 2. C: Heterozygous pattern for c.3503_3504delTC (p.L1168QfsX5) mutation found in both parents. Site of mutation is denoted by the red arrows.

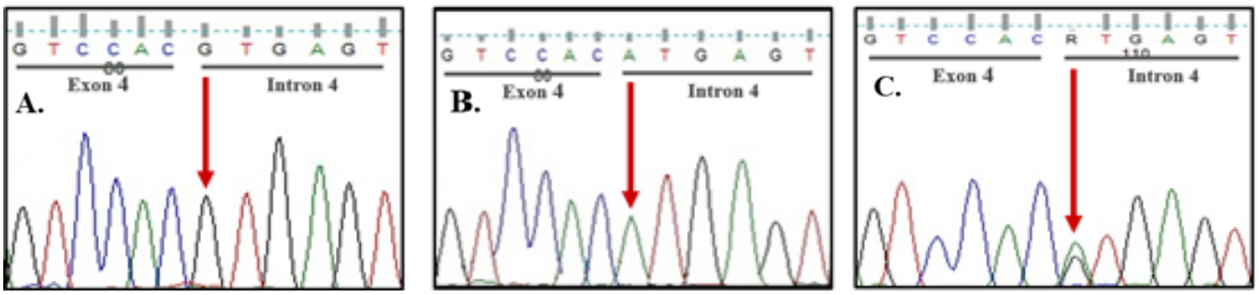

Fig. 5: Sequence chromatogram of exon 4 of *GNPTG* gene in patient 3. A. normal control with wild type sequence, B. Mutation c.233+1 G>A (IVS4+1 G>A) in homozygous pattern found in patients 3, C: Heterozygous pattern for c.233+1 G>A (IVS4+1 G>A) mutation found in the parents and brother. Site of mutation is denoted by the red arrows.

Fig. 6: Alamut® splice site changes prediction for c.233+1 G>A (IVS4+1 G>A) mutation of *GNPTG* gene. Original splice site has 2 binding sites; yellow for SRp55 & violet for SF2 / ASF (IgM- BRCA1). The new splice has 3 binding sites; green for SRp40, violet for SF2 / ASF (IgM- BRCA1) & pink for for SF2 / ASF.

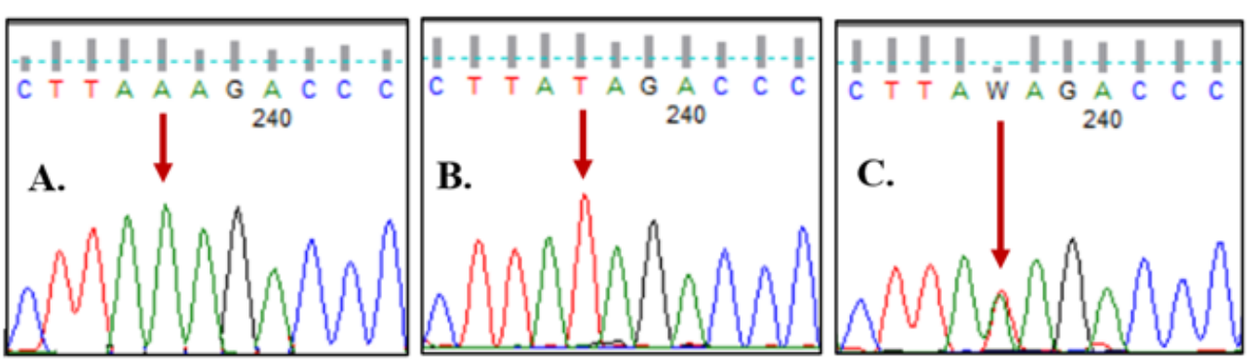

Fig. 7: Sequence chromatogram of exon 4 of *GNPTG* gene in patient 4. A. normal control with wild type sequence, B. Mutation c.658A>T (p.k220*) in homozygous pattern found in patient 4, C: Heterozygous pattern for c.658A>T (p.k220*) mutation found in both parents. Site of mutation is denoted by the red arrows.

DISCUSSION

Mucolipidosis type II and type III (ML II & ML III) are two autosomal recessive lysosomal storage disorders with disturbed metabolism of many mucopolysaccharides or glycosaminoglycans giving rise to clinical features like those encountered in patients with mucopolysaccharidosis (MPS). ML II is usually recognizable at birth by failure to thrive and developmental delay. Dysostosis multiplex, coarse facies, short stature, hypertrophic gingiva, retarded psychomotor development and cardiac valve involvement are the most common clinical features.Both result from impaired trafficking of lysosomal hydrolases to lysosomes due to total or near total deficiency of GlcNAc-phosphotransferase enzyme. ML III represents the milder, slowly progressive form of the disorder with less severe features allowing prolonged survival (**Pohl *et al*., 2010; Yang *et al*., 2013; Kasapkara *et al*., 2017**).

GlcNAc-phosphotransferase is a hexameric complex six subunits, 2α, 2β and 2γ with the *GNPTAB* gene encoding the α /β subunits while the γ subunit is encoded by the *GNPTG* gene. According to the human genome mutation database (http://www.hgmd.cf.ac.uk/ac/index.php. HGMD professional 2.2021) more than 311 different *GNPTAB* mutations have been reported. Of which 105 mutations responsible for ML II, 43 associated with ML III α / β, and 18 causes stuttering. While for *GNPTG* gene, 69 mutations were reported with 26 causing ML III γ and 15 causing stuttering. (**Qian *et al*., 2013; Schweizer *et al*., 2013; Ludwig *et al*., 2017**).

The four patients were recruited according to their clinical presentations, and β-Hexosaminindase -A enzyme activity assay. Whole exome sequencing for the four patients revealed two mutations in *GNPTAB* gene in exons 13 & 19; and 2 different mutations in *GNPTG* gene in patients with mild phenotype. All patients were homozygous for their corresponding mutations.

In patient 1, a duplication mutation c.2693dupA (p.Y899VfsX21) in exon 13 resulted in a several changes in the *GNPTAB* gene open reading frame; first, Tyrosine, hydrophobic aromatic amino acid, at position 899 was changed to Valine, a much smaller aliphatic hydrophobic amino acid. Second, the open reading frame changes entirely for 20 amino acids and finally, a stop codon is generated 21 amino acids downstream this mutation leading to a premature truncation of translation. This mutation was reported in a heterozygous pattern with a nonsense mutation in exon 10. The patient showed near total loss of phosphotransferase activity with craniofacial and skeletal deformities. Early dysmorphic features included facial coarseness with depressed nasal bridge, hypertrophied gums, thickened alveolar ridges and delayed neuromotor development (**Cathey *et al*., 2010**). This clinical picture is close to what was found in patient 1. This patient had the highest level of β-Hexosaminindase -A enzyme activity in plasma compared to the other 3 patients which reflects a lower level of the phosphotransferase enzyme activity.

It was also reported in the one Chinese patient from East China, in a heterozygous pattern with another heterozygous mutation in exon 2, but the clinical and biochemical data were not available (**Wang *et al*., 2018**).

Exon 19 mutation c.3503_3504delTC (p.L1168QfsX5), was previously reported as the most common MLII causing mutation in Egypt along with other regions worldwide. It was first reported in 2006, then in 2008 it was identified as the MLII-causing mutation in French Canadian population of Saguenay– Lac-Saint-Jean (Quebec, Canada) (**Kudo *et al*., 2006; Plante *et al*., 2008; Essawi *et al*., 2021**). Furthermore, c.3503_3504delTC mutation has been associated with the severe phenotype when found in homozygosity or when found in heterozygosity with nonsense mutations or frameshift mutations (**Cathey *et al*., 2010; Cury *et al*., 2013**). The currently reported female (patient 2) patient's symptoms started at birth with dyspnea and heart disease. She also presented with typical MLII symptoms including short stature, coarse facies, gingival hypertrophy, craniocynostoses, hearing loss. Recurrent detection of this mutation in a new MLII patient ascertains the expected high frequency of it among the Egyptian population.

According to previous studies; the two nucleotides (CT) deletion from a repeating CTCT sequence may cause a mismatch-repair error. The frameshift truncates the enzyme at amino acid 1171 in the β-subunits. Fibroblasts of patients with homozygous mutation showed very low value of ≤ 1% GlcNAc-phosphotransferase activity. Yet, the presence of residual activity of GlcNAc-1-phosphotransferase in some patients was explained by **Steet *et al*., 2005**, who showed that only 3% of normal GlcNAc-1-phosphotransferase activity in patients' fibroblasts is sufficient to phosphorylate 25% of the total lysosomal enzyme oligosaccharides (**Bargal *et al*., 2006; Kudo *et al*., 2006; Plante *et al*., 2008**).

In terms of ethnicity, c.3503_3504delTC mutation in *GNPTAB* gene was reported with diverse ethno-geographic origins including Israeli and Palestinian Arab-Muslim, Turkish, Canadian, Italian, Portuguese, Indian, Argentinian, Bangladesh, Irish traveler, and US patients. A specific French-Canadian founder population was found to be obligate carrier of this mutation (**Encarnação *et al*., 2009; Tappino *et al*., 2009; Aggarwal *et al*., 2014; Singh *et al*., 2017**).

Expanding the diagnostic spectrum of ML in Egyptian patients, we studied the underlying molecular cause in MLIII patients for the first time in Egypt. We identified two different mutations in *GNPTG* gene in two patients

with MLIII. Both patients had mild skeletal symptoms and delayed onset. The splice site mutation detected in patient 3 was associatedwith camptodactyly since birth, generalized progressive joint stiffness and hence limited joints' movement, knock knees and abnormal gait. Yet, the patient facies were not dysmorphic with short stature (- 2.36 SD for age), otherwise were within normal. The mother and a younger brother were carriers for the same mutation and both parents were consanguineous. According to ClinVar database, this mutation was reported once before in 2017 by an institute in USA; however, the clinical data was not available to be compared with our findings.

In *silico* analysis of this mutation using **Alamut Genova** v 1.4 software showed that this mutation created a binding site for extra protein of the serine/arginine-rich (SR) protein family. The SR protein family functions both as general splicing factors and as regulators of alternative splicing. They are essential splicing factors and regulate the selection of alternative splice sites in a concentration-dependent manner. Considering the concentration dependency factor, it will affect the homeostasis of splicing. This suggests that the splice site mutation c.233+1G>A (IVS4+1G>A) is most probably responsible the defect in GlcNAc-1-phosphotransferase through its γ subunit (**Wang *et al*., 2014**).

The other *GNPTG* gene mutation was a novel nonsense mutation, which terminated almost one third of the γ subunit translation producing a truncated polypeptide chain of 220 amino acids protein length instead of 305 amino acids. The α subunit conserved domains' deletion, defined by similarities or no-similarities to known proteins, demonstrated several interesting findings; that both γ subunits and α-subunits are directly bonded, the activity of GlcNAc-1-phosphotransferase is decreased by the loss of γ subunit binding, and that γ subunits residues' 130-238 are implicated in binding to GB domain (**De Pace *et al*., 2015**). Other functions included are the involvement in the recognition of specific lysosomal enzymes, maintaining the stability of the GlcNAc-1–phosphotransferase complex, and might contribute to formation of a binding site for the lysosomal enzymes that is conformational dependent (**Kollmann *et al*., 2013; Qian *et al*., 2015**).

The affected patient (patient 4) has shown symptoms of short stature, joint stiffness, waddling gait, and corneal clouding. Combined with the reduced GlcNAc-1-phosphotrasferase activity, may be attributed to the loss of γ subunits binding site for the GB domain.

CONCLUSION

In this study, we identified ML patients and were confirmed molecularly by the advances of next generation sequencing. Moreover, whole exome sequencing expanding the diagnostic spectrum of this rare disease by detecting the milder form, MLIII patients, through identifying pathogenic mutations in *GNPTG* gene. On the other hand, recurrent detection of c.3503_3504delTC (p.L1168QfsX5) mutation in Egyptian MLII patients in homozygous pattern ascertains the expected high frequency of it among the Egyptian population, which would be due to the high rates of parental consanguinity or as a result of founder effect. More studies are recommended to confirm either of the two concepts.

CONFLICT OF INTEREST

There are no conflicts of interest.

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