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Original article

Atypical *PEX16* peroxisome biogenesis disorder with mild biochemical disruptions and long survival

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Abstract

Background: Mutations in *PEX16* cause peroxisome biogenesis disorder (PBD). Zellweger syndrome characterized by neurological dysfunction, dysmorphic features, liver disease and early death represents the severe end of this clinical spectrum. Here we discuss the diagnostic challenge of atypical *PEX16* related PBD in 3 patients from highly inbred kindred and describe the role of specific metabolites analyses, fibroblasts studies, whole-exome sequencing (WES) and metabolomics profiling to establish the diagnosis.

Methods and patients: The proband is a 12-year-old male born to consanguineous parents. Despite normal development in the first year, regression and progressive spastic diplegia, poor coordination and dysarthria occurred thereafter. Patient 2 (3-year old female) and Patient 3 (19-month old female) shared similar clinical course with the proband. Biochemical studies on plasma and fibroblasts, WES and global metabolomics analyses were performed.

Results: Very-long-chain fatty acids analysis showed subtle elevations in C26 and C26/C22. Global Metabolomics-Assisted Pathway profiling was not remarkable. Immunocytochemical investigations on fibroblasts revealed fewer catalase and PMP70-containing particles indicating aberrant peroxisomal assembly. Complementation studies were inconclusive. WES revealed a novel homozygous variant in *PEX16* (c.859C>T). The biochemical profiles of Patient 2 and Patient 3 were similar to the proband and the same genotype was confirmed.

Conclusion: This paper highlights the diagnostic challenge of *PEX16* patients due to the widely variable clinical and biochemical phenotypes. It also emphasizes the important roles of combined biochemical assays with next generation sequencing techniques in reaching diagnosis in the context of atypical clinical presentations, subtle biomarker abnormalities and consanguinity.

Keywords: *PEX16* gene; Peroxisome biogenesis disorder; Atypical variant; Zellweger syndrome

1. Introduction

Peroxisomes are subcellular, membrane-enclosed organelles that are unique to eukaryotic organisms. These compartments harbor a set of diverse proteins which are involved in a notable array of catabolic and

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anabolic activities [1,2]. β-oxidation of very long chains fatty acids (VLCFA), α-oxidation of branched chain fatty acids, bile acids synthesis, plasmalogens synthesis and detoxification of free radicals are amongst the critical metabolic functions that take place in the peroxisome. The severe multisystem consequences due to disruptions of one or more peroxisomal pathways highlight the essential roles played by these highly conserved organelles [3,4].

Known as peroxisome biogenesis disorders (PBDs) is a heterogeneous group of autosomal recessive conditions characterized by impaired peroxisome functions. Mutations in any of 14 PEX genes can lead to defects in the corresponding peroxins, a coordinated set of proteins that are required for correct peroxisome biogenesis [5,6]. Low plasmalogens and accumulation of VLCFA, pristanic, phytanic, pipecolic and intermediates of bile acids are the biochemical hallmarks of PBDs. Clinically, these disorders are characterized by neurological dysfunction, characteristic craniofacial features, skeletal abnormalities, liver disease, hearing and vision impairment. Severity, age of onset and organ involvement is a continuum with Zellweger syndrome representing the severe end of PBDs spectrum [7–9].

Recently, unusual variant PBDs with relatively mild phenotypes and prolonged survival were reported to have been caused by mutations in *PEX2*, *PEX3*, *PEX10*, *PEX12* and *PEX16*. Patients shared in common subtle biochemical aberrations, slowly progressive neurological deterioration and prolonged survival [10–19]. In this paper we report on atypical PBD in a sib pair and a paternal cousin caused by a homozygous mutation in *PEX16* (Fig. 1). While this gene encodes an integral membrane protein with essential roles in early stages of peroxisome biogenesis [20], fibroblasts studies in our proband showed fewer but import-competent peroxisomes suggesting deviant peroxisomal assembly. This report highlights the role of targeted biochemical studies, whole-exome sequencing (WES), and global metabolomics analyses in the context of atypical PBD presentation in a highly inbred kindred. We also reviewed the clinical and biochemical features of reported *PEX16* cases to compare with our patients.

2. Patients and methods

2.1. Patients

This study was approved by Al Ain Medical District Human Research Ethics Committee. Informed consent was obtained following a full explanation of the procedures and for the publication of the data. The proband is a male born at term to consanguineous parents with a birth weight of 3.2 kg (Patient III-6, Fig. 1). Development in the first year was within normal. Later, he underwent progressive spastic diplegia starting with toe walking at age of 2. At 4 years of age, he had mid-thoracic syrinx treated with syringopleural shunt. Clinically, the proband showed bilateral horizontal nystagmus, head and hand tremor with dysmetria, increased tone in lower limbs, normal sensation, very brisk tendon reflexes at the knees, positive Babinski sign and sustained bilateral ankle clonus. No hypotonia was noticed. At age of 7, he became wheelchair bound with difficulties in coordination, developed joint contractures and hip dislocation which necessitated surgical intervention. Dysarthria and dysphagia became more prominent at 9 and 11 years of age, respectively. His intelligence was preserved despite progressive regression of variable skills. Recently his condition deteriorated further, in a form of inability to sit unsupported and his dysarthria became more obvious. Current examination showed that he is alert and oriented without decrement in mental capabilities. On the other hand, substantial regres-

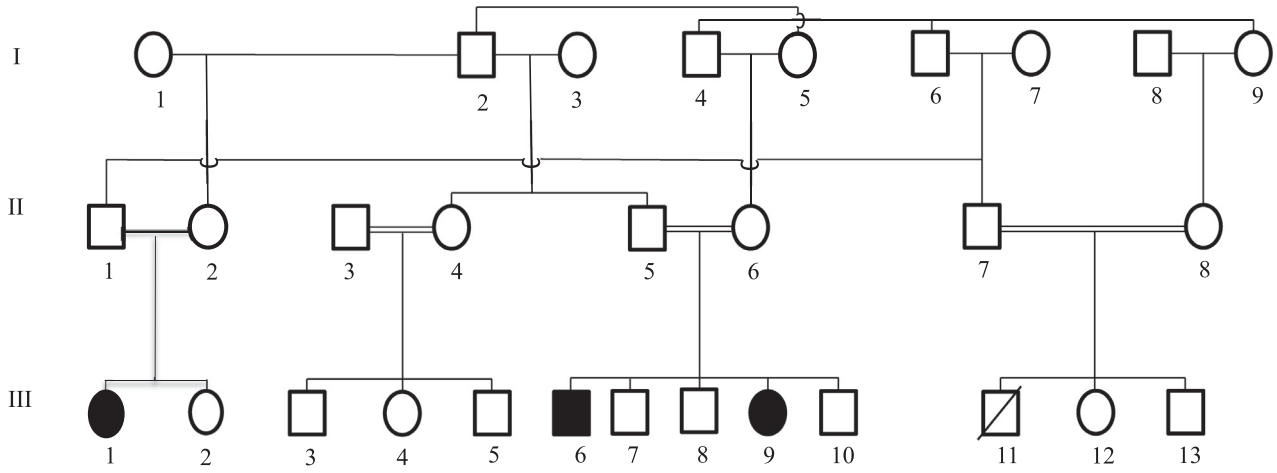

Fig. 1. Family pedigree. Patient III-6 is the proband. Patient 2 and Patient 3 are III-9 and III-1, respectively. Individuals III-3 and III-4 have glutaric aciduria type 1while individual III-13 has Schindler disease. Neonatal death was reported in individual III-11.

sion was noted in his gross and fine motor skills, with significant intension tremor, dysarthria, spasticity, hyperreflexia and progressive scoliosis. Ophthalmological examination revealed refractive error and nystagmus. ECHO and abdominal ultrasound investigations were unremarkable.

Brain MRI was initially performed at the age of 3 and was unremarkable (Fig. 2A). Repeated brain MRI at 7 (Fig. 2B, C) and 12 years of age (Fig. 2D–F) showed progressive bilateral high signal periventricular, subcortical frontal and parieto-occipital white matter on T2W image.

Patient 2 (III-9, Fig. 1), the younger sister of the proband, was born at term with unremarkable antenatal history. At birth, parents noted that she has features similar to the proband with missing phalanges in great toes in addition to broad and deviated thumbs (Fig. 3). Apart from that, physical and neurological examinations were within normal. Developmental milestones at 1 year of age were met. Brain MRI at 8 months was unremarkable (Fig. 2G, H). She continued to attain normal developmental milestones in the second year of life but at 26 month of age parents noticed that she started to lose previously acquired verbal and motor skills. Follow-up MRI at 2 years of age showed increased T2 and FLAIR signal intensity mainly in the right frontal and right periventricular white matter (Fig. 2I–L).

Patient 3 (III-1, Fig. 1) is a paternal cousin of the proband, who was born at term with unremarkable antenatal history. She was able to stand independently at the age of 1 year. At 17 months, her motor skills regressed and currently she is not able to stand without support.

Family history revealed a paternal sister to have two children (III-3, III-4, Fig. 1) with glutaric aciduria type 1 and a cousin from maternal and paternal sides with a child with Schindler disease (III-13, Fig. 1). Neonatal death with no established diagnosis was also reported (III-11, Fig. 1).

2.2. Metabolite studies and metabolomics analyses

Blood samples were collected for biochemical investigations. VLCFAs, phytanic and pristanic acid measurements were performed at a major commercial laboratory. Plasmalogens were determined as previously described [21].

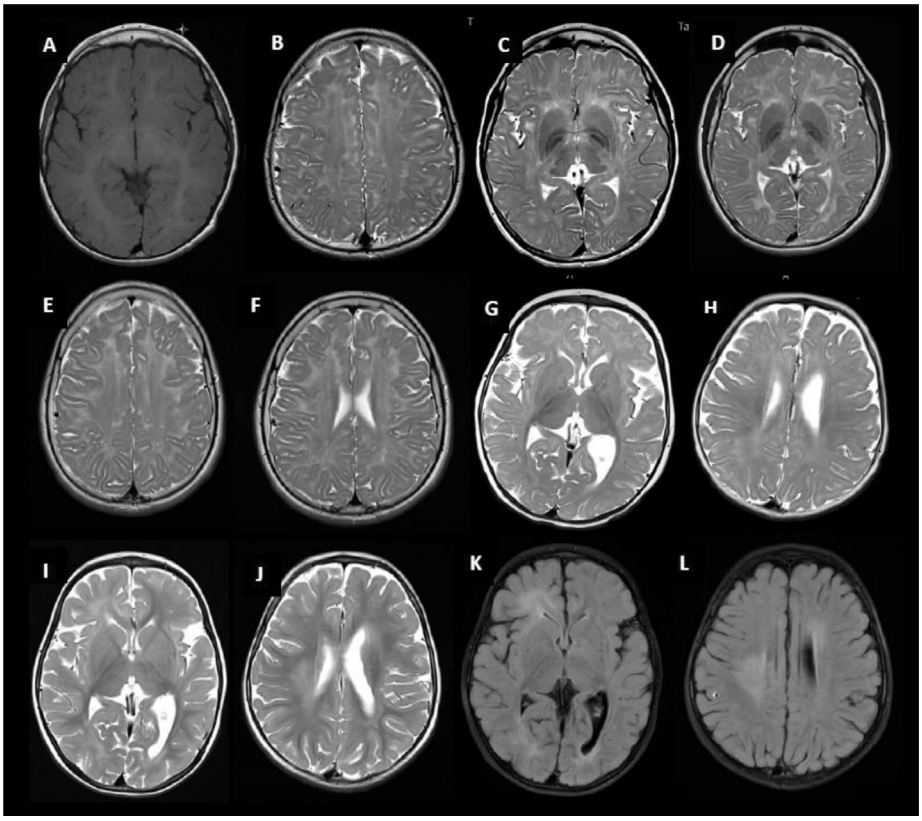

Fig. 2. Illustration of serial MRI pattern of proband (III-6) and affected sibling (III-9). Initial MRI of proband was normal (A, age 3 years), while the follow-up MRI of proband (B, C, age 7 years) and the latest MRI of proband (D-F, age 12 years) showed progressive bilateral high signal periventricular, subcortical frontal and parieto-occipital white matter on T2W image. MR images of sibling (G-H, age 8 months) was normal and the follow-up images of sibling (I-L, age 2 years) showed increased T2 and FLAIR signal intensity mainly in the right frontal and right periventricular white matter.

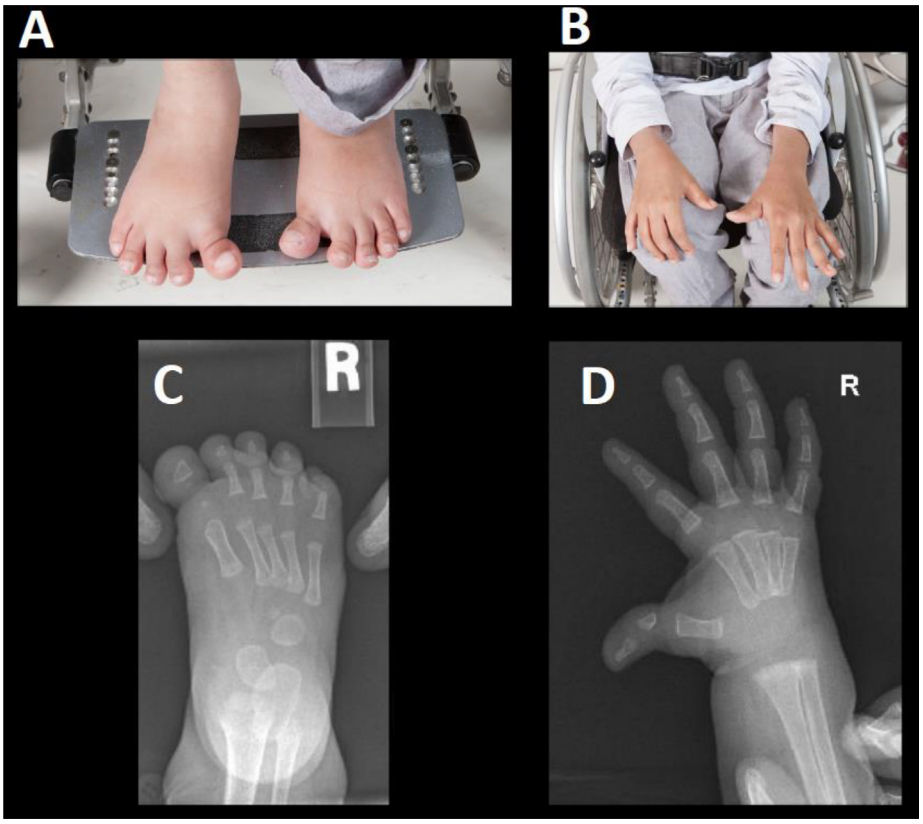

Fig. 3. Photographs of deviated great toe (A) and thumb (B) of patient III-6. Radiograph of patient III-9 at 1 year of age showing absence of proximal phalanx of great toe (C) and deviated thumb (D).

Global Metabolomics-Assisted Pathway Screening (Global MAPS®) was performed at Baylor Genetics (Houston, TX) and Metabolon, Inc. (Durham, NC).

2.3. Fibroblast investigations

Skin biopsy was collected from the proband for metabolites, immunofluorescence and complementation studies. Indirect immunofluorescence microscopy was used to visualize peroxisomes in fibroblasts [22]. Complementation analysis was performed as previously described [23]. In brief, patient fibroblasts were fused serially with fibroblast cell lines that are each deficient of *PEX1* (group E), *PEX13* (group H) and *PEX16* (group D). Peroxisomes were stained using antibodies against peroxisomal integral membrane protein PMP70 and catalase as peroxisomal membrane and matrix markers, respectively. These studies were performed at 37 °C and 40 °C to assess for temperature sensitivity.

2.4. Whole-exome analysis (WES)

WES analysis was outsourced to BCM Genetics Laboratories (Huston, TX, USA) and was performed on Illumina HiSeq sequencing system. Atlas-SNP, Atlas-Indel, HGSC-SNP-anno and HGSC-indel-anno in-house developed software suite were used as tools for variant calls and annotation.

3. Results

In the proband, plasma VLCFA profile showed marginal elevation of C26:0 and the ratio C26/C22. Other studied metabolites and ratios were within normal limits. In fibroblasts, subtle abnormalities in C26:0, C26/C22 ratio and C26:0 β-oxidation rate were observed, while other assessed parameters were within the respective normal ranges (Table 1). Global MAPS® profiling was not remarkable.

Immunocytochemical staining of fibroblasts from the proband revealed catalase (Fig. 4B–D) and PMP70-containing particles (Fig. 4F–H) that are fewer in number compared to controls (Fig. 4A, E). Immunofluorescence evaluation of catalase and PMP-70 was performed semi-quantitatively and no statistical analysis was performed. Complementation analysis with group E (*PEX1* deficiency), group H (*PEX13* deficiency) and group D (*PEX16* deficiency) was equivocal because patient's fibroblasts had the inherent capability to produce catalase and PMP70-containing particles. Peroxisomal assembly in fibroblasts did not attenuate after

Table 1
Biochemical investigations in blood and cultured fibroblasts.

Patient	Plasma						Fibroblasts				
	C26 (μmol/L)	C26/C22	C24/C22	Pristanic (μmol/L)	Phytanic (μmol/L)	Plasmalogens (C16:0 DMA/C16:0)	C26 (μmol/gram protein)	C26/C22	C24/C22	C26 β-oxidation (pmol/(h.mg protein)	Pristanicβ-oxidation (pmol/(h.mg protein)
III-6	1.68	0.027	1.26	0.33	2.66	0.0206	0.40	0.11	2.19	799	893
III-9	2.69	0.039	1.26	0.27	2.30						
III-1	1.77	0.078	1.21	6.48	29.04						
III-10	0.76	0.014	1.35	0.11	1.53						
Controls	≤1.30	≤0.023	≤1.39	≤2.98	≤9.88	0.0122–0.0468	0.18–0.38	0.03–0.07	1.55–2.30	800–2040	790–1690

culture at 40 °C for 3 days indicating no temperature sensitivity.

In the proband, WES revealed a homozygous c.859C>T (p.R287C) variant of unknown significance in *PEX16*. Both parents were heterozygous for this change. WES also showed hemizygous c.394 T>C (p.W132R) variant of unknown clinical significant in the *ABCD1* gene. Defects in this gene are associated with X-linked adrenoleukodystrophy (X-ALD). These findings were confirmed by Sanger sequencing. Using targeted mutation analysis, Patient 2 (III-9, Fig. 1) and Patient 3 (III-1, Fig. 1) were confirmed homozygous for the *PEX16* variant. A newly born sibling (III-10, Fig. 1) who was confirmed heterozygote by targeted analysis of the *PEX16* variant produced a normal VLCFAs profile (Table 1).

4. Discussion

Autosomal recessive PBDs are caused by heterogeneous mutations in any of at least 14 distinct *PEX* genes including *PEX 16* [1]. The overall prevalence of PBDs is estimated at 1:50,000 [24] but only less than 1% is attributed to *PEX16* mutations [25]. Human *PEX16* encodes an integral peroxisomal membrane protein composed of 336 amino acids [26]. This protein, in association with *PEX3* and *PEX19*, is specifically involved in the early steps of peroxisome biogenesis [20]. Due to the critical role it plays in the assembly of peroxisomal membrane, deleterious *PEX16* mutations were reported to cause a lack of peroxisome remnants leading to high morbidity and mortality [17].

We describe 3 patients with a novel homozygous missense mutation c.859C>T in the *PEX16* gene. Our patients shared a similar clinical course of early normal development through the first year of life followed by slowly progressive neurological deterioration thereafter with ataxic presentation and long survival. To date, a total of 14 *PEX16* patients have been reported (Table 2), 5 of whom presented with the severe Zellweger phenotype with dysmorphic features, hepatic dysfunction, early onset seizure and death with complete absence of peroxisomes in their fibroblast [26–29]. On the other hand, majority of the reported *PEX16* patients (i.e. nine out of fourteen) were characterized by atypical phenotype presenting beyond the infancy period, with progressive spastic paraplegia, ataxia and long survival [17–19,30]. Our 3 patients make 12 out of 17 (70%) the fraction of *PEX16* patients reported to date with ataxic presentation and long survival. The observed phenotype in this and other reported studies suggests wide variability in the clinical manifestations of *PEX16* mutations ranging from mild to severe. Although the severe phenotype might be underreported, reported patients to date indicate that more than two thirds of *PEX16* patients have the attenuated phenotype suggesting that genetic

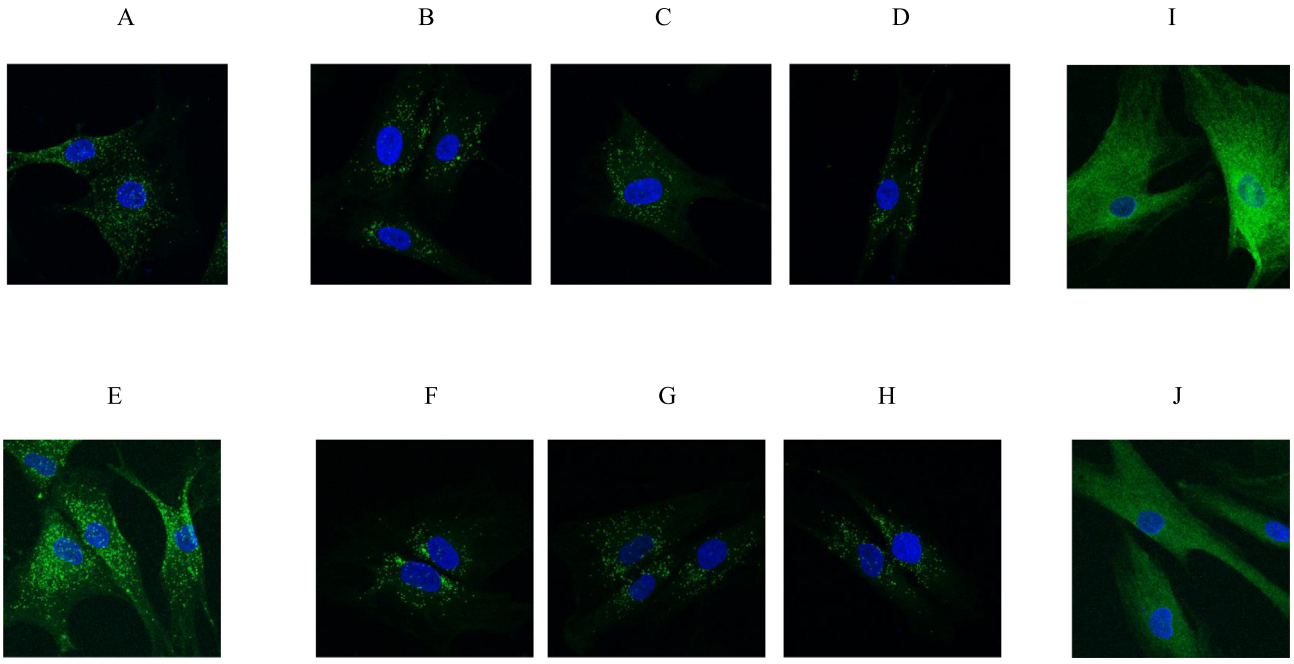

Fig. 4. Catalase immunofluorescence in control fibroblasts (A) and patient III-6 fibroblasts (B–D). PMP70 immunofluorescence in control fibroblasts (E) and patient III-6 fibroblasts (F–H). Note the fewer number of catalase and PMP-containing particles in patient fibroblasts as compared to the control. Panels I and J show catalase and PMP70 immunofluorescence in fibroblasts from a Zellweger patient with *PEX16* mutation IVS10 + 2 T>C as positive control (note the complete lack of peroxisomes in the diffused fluorescence pattern in these fibroblasts).

defects in this gene are more often associated with atypical PBD and long survival. Nevertheless, validation of this conclusion with more patients is required. It is noteworthy that although *PEX3* and *PEX19* also mediate peroxisomal membrane synthesis, hitherto, patients reported with defects in these genes exhibited severe clinical impairment except for the recently reported attenuated phenotypes in two patients with *PEX3* mutations [12,13].

Biochemically, our proband showed subtle VLCFA abnormalities in plasma and cultured fibroblasts, well below levels often seen in classical Zellweger patients. Plasmalogens, pristanic and phytanic acids were within normal limits. Peroxisomes in fibroblasts were detectable by immunofluorescence albeit fewer with atypical localization (Fig. 4). Complementation and temperature sensitivity studies at 40 °C were inconclusive. On Global MAPS® analysis, all known PBD related biomarkers were unremarkable except for slightly elevated phytanic acids in Patient 2 which was interpreted as dietary marker. Abnormalities in C26:0 and C26/C22 ratio were more noticeable in Patient 2 and Patient 3 compared to the proband, nevertheless the levels were well below those usually seen in classical Zellweger patients. Taken together, absolute levels and ratios of VLCFA quantified using specific biochemical assays proved to be indispensable tools to diagnose our three patients and differentiate them from their unaffected relatives. As illustrated in this report, WES also remains a valuable approach to establish the diagnosis of patients who have atypical clinical features and subtle biochemical disturbance.

In the proband, a variant of unknown significance c.394 T>C (p.W132R) in the *ABCD1* gene was detected by WES. Follow up immunofluorescence microscopy of adrenoleukodystrophy protein in cultured fibroblasts was normal. Brain MRI was inconsistent with X-ALD. The father (II-5, Fig. 1) was hemizygous while the mother (II-6, Fig. 1) was heterozygous for this change, both asymptomatic. While defects in *ABCD1* are associated with X-ALD, our results indicate that the c.394 T>C variant to unlikely be pathogenic. It is worth mentioning that in addition to WES, whole genome sequencing was also performed on the proband and no other genes that may explain the biochemical and clinical phenotypes were identified. Taken together, our results suggest that the etiology of the biochemical and clinical manifestation in this family is the mutated *PEX16*. Interestingly, skeletal abnormalities in the form of missing phalanges in great toes with broad and deviated thumbs were what brought the proband initially to medical attention. Similar findings were also noted at birth in the affected sibling (III-9, Fig. 1). At first, this was assessed as a possible, yet unusual consequence of defected *PEX16* in these patients. However, the presence of these abnormalities in an unaffected sibling (III-10, Fig. 1) indicates these are less likely to be related to *PEX16*. This suggests that in highly inbred families,

Table 2
Biochemical, molecular and clinical characteristics of patients with *PEX16* defects.

Patient	Age at diagnosis	Age (Year)	C26:0 (μmol/L)	C26/ C22	Clinical phenotype	Mutation	Ref
1	2 year	NA	NA	NA	Zellweger syndrome	ARG176TER	[27]
2	NA[1]	NA	NA	NA	Zellweger syndrome, no peroxisomal ghost in fibroblast	IVS10 2 T>C	[26]
3	NA	NA	NA	NA	Zellweger syndrome, no peroxisomal ghost in fibroblast	IVS10 2 T>C	[26]
4	9 month	NA	2.78	0.03	Normal development till 9 month. Toe walking at 13 months. At 3 years, normal mental status with spastic ataxia, dysmetria, brisk tendon reflexes. Subsequently, cataract, wheelchair-bound and leukodystrophy.	c.984delG (p.I330SfsX27)	[17]
5	NA	11 year	4.07	0.07	Brother of case 4, lower limb spasticity, unsteady gait at 17 months; wheelchair-bound at 3 years. Rigidity, brisk reflexes, dysmetria and ataxia at 4 years. At 5 years worsening of constipation and difficulty in bladder emptying. Progressive leukodystrophy and brain atrophy.	c.984delG (p.I330SfsX27)	[17]
6	NA	14 year	3.0	0.25	At 2 years, evidence of motor delay at persistent hepatomegaly with micronodular fibrosis on biopsy. Developmental regression at 3 years. Facial dysmorphism, spastic tetraplegia, dysmetria and head tremor with leukodystrophy	c.753_755delTGT (p.V252del)	[17,31]
7	NA	NA	2.83	0.13	Mild peroxisomal biogenesis phenotype	c.865C>A (p.P289T)	[17]
8	2 year	NA	1.93	0.03	Ataxia at 2 yrs. Mild cognitive impairment, dysarthria and nystagmus at 6 year, Leukodystrophy	c.992A/G (p.Y331C)	[17]
9	1.5 year	9 year	2.27	0.04	Developmental regression, ataxia, spasticity and deterioration in cognitive function at 18 months of age. Leukodystrophy	c.952 + 118_1011+80 (p. R318SfsX13, p.R318IfsX38, p. E296DfsX33)	[17]
10	At birth	NA	NA	NA	Typical Zellweger facies, talibes and hypotonia. Elevated Liver enzymes. MRI brain: prominent ventricles, widening of sulci, sylivian fissures and cerebellar vermis hypoplasia. Died at 8 months	c.460 + 5G>A (p.A121WfsX122)	[28]
11	9 year	NA	NA	0.038	Hypotonia, ataxia, mild cognitive delay with retinal pigmentary changes cerebellar and vermis atrophy	c.679C>T/c.526C>T (p.R227W/ p. R176*)	[30]
12	3 year	21	0.44	NA	Ataxia at 3 years, spastic paraparesis 9 years, wheelchair bound at 15 year and hallucination and cognitive impairment at 18 years.	F332del	[18]
13	2 month	NA	7.6	0.252	8-month-old female with typical Zellweger syndrome, hypotnia and leukodystrophy. Mitochondrial pathology on muscle biopsy	c.460 + 5G>A	[29]
14	5 year	6.5 year	NA	NA	Spasticity and ataxia at 2 years; slow speech and cognitive deterioration, elevated transaminase, leukodystrophy	c.953-1_974del23ins5 (splicing defect)	[19]
15	1.5 year	12 year	1.68	0.027	See text	c.859C>T (p.R287C)	This study
16	6 month	3 year	2.69	0.039	See text	Same as above	This study
17	18 month	1.6	1.77	0.078	See text	Same as above	This study

[1] NA: not available.

unusual findings should be approached with caution to avoid false conclusions on disease phenotype expansions.

5. Conclusions

This report underscores the wide phenotypic variability encountered in patients with *PEX16* defects. It also highlights the importance of specific biochemical assays combined with next generation sequencing in diagnosing atypical patients with subtle metabolite disruptions. While no definite treatment is available for PBDs to date, early recognition and identification of underling gene mutations is important to provide appropriate medical care and genetic counseling.

Acknowledgments

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