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Impact of D-bifunctional Protein Deficiency on Telomere Length and Gene Expression in a Child

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Authors' contributions

This work was carried out in collaboration between all authors. Author MEHC performed microarrays and TRF assays, contributed to the design of the study and drafted the manuscript. Author RCT performed the diagnosis of the patient. Authors DJAA and CMCC participated in the design of the study and author JASR performed the statistical analysis. All authors read and approved the final manuscript.

Case Study

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ABSTRACT

Aim: To explore, in one patient, the possibility that D-bifunctional protein (D-BP) deficiency affects telomere length, and to determine the profile of genetic expression.
Presentation of Case: Due to the symptoms of a newborn and his family background, a peroxisomal panel was performed. There were high levels of very long chain fatty acids and abnormal peroxisomes. At 8 months the patient exhibited other complications, including progressive multi systemic deterioration, and at 15 months died of pneumonia.
Discussion: Analysis of the patient's fibroblasts provided evidence of a defect in the peroxisomes and in the oxidation of fatty acids, leading to a diagnosis of D-BP deficiency. Significant alterations were found in the genetic expression profile, with the

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greatest number of affected genes involved in neuronal functions, two implicated in peroxisomal biogenesis, and some others related to telomere protection and DNA repair. The child had a mixture of very short and normal length telomeres, a condition commonly observed in the elderly and in individuals with chronic degenerative diseases.
Conclusion: The abnormal function of peroxisomes and altered gene expression found in the patient under study could explain the affected telomere length. Further studies are needed to explore this possibility.

Keywords: D-bifunctional protein deficiency;gene expression; telomere length; peroxisomes.

1. INTRODUCTION

D-bifunctional protein (D-BP) is a peroxisomal enzyme expressed mainly in the brain and retina. It is essential for the β-oxidation of fatty acids [1], including the breakdown of intermediate bile acids, C26-0, very-long-chain fatty acids (VLCFA), and 2-methyl-ramified fatty acids [2].

D-BP deficiency is an autosomal-recessive childhood disease [3] with manifestations of abnormal brain development, neonatal hypotonia, seizures, visual and auditory alterations, abnormal craniofacial characteristics, and little or no progress in development [4]. Most affected children die during the first 2 years of life [5]. One of the hallmarks of this disease is a deficiency in β-oxidation of fatty acids.

Although normal β-oxidation of fatty acids yields H_2O_2, and enzymes harbored in peroxisomes produce other free radicals (i.e., superoxide $O_{2\cdot}$ and NO), peroxisomes are capable of detoxifying these reactive oxygen species. A deficiency in fatty acid oxidation can lead to an accumulation of these potentially harmful molecules, causing damage to proteins, lipids or DNA of neuronal cells [6].

It was recently demonstrated that free radicals react with the accumulation of polyunsaturated fatty acids caused by a deficiency in fatty acid oxidation, which in turn liberates lipoperoxide radicals in neurons of the retina and brain and leads to neuronal membrane lysis [7]. Additionally, it is known that this deficiency can damage the telomeric DNA structure [8] that normally protects the end of chromosomes from degradation [9]. To our knowledge, this is the first report that D-BP deficiency affects telomeric length and alters the expression of two genes (*PEX3/PEX16)* participating in the biogenesis of peroxisomes.

2. PRESENTATION OF CASE

The patient was a newborn boy, the product of the third gestation of unrelated parents. Although he was referred for consultation at 16 days of life, the available clinical data was first recorded at the age of two months. At that time he was described as having severe neonatal hypotonia, seizures, craniofacial dysmorphia (dolicocephalia, enlarged fontanelle, prominent frontal, micrognathia, a long philtrum, and epicanthic folds), a high arched palate without evident ophthalmological or auditory alterations and punctiform patellar calcification.
A brother of the patient apparently died from adrenoleukodystrophy, leading to suspicion of metabolic disease in the present case. Hence, a skin biopsy was taken to obtain fibroblasts, which were cultured in three passages and then utilized for quantification of VLCFA content,

plasmalogen synthesis, phytanic acid content and catalase solubility, as well as for peroxisome visualization by immunocytochemistry (for ethical reasons we did not take a biopsy of neural tissue). The diagnosis of D-BP deficiency was founded on accumulation on VLCFA, partial deficiency in phytanic acid oxidation and abnormal morphology of peroxisomes in serum and fibroblasts, which was performed at the Kennedy Krieger Institute's Peroxisomal Disease Laboratory. Data are shown in Table 1. Upon later analyzing the serum VLCFA of the parents and live brother, normal levels of this parameter were observed. Moreover, the mother was not an X-linked adrenoleukodystrophy (X-ALD) carrier.

Table 1. Peroxisomal serum panel and fibroblasts analysis

Peroxisomal panel serum	Units	Reference
C22:0	20.9 umol/L	0.0-96.3
C24:0	48.4 umol/L	0.0-91.4
C26:0	11.49umol/L*	0.0-1.30
C24:0/C22:0	Ratio 2.32*	0.0-1.39
C26:0/C22:0	Ratio 0.551*	0.0-0.023
Pristanic acid	0.51 umol/L	0.0-0.60
Phytanic acid	0.42 umol/L	0.0-5.28
Pristanic/Phytanic acid	Ratio 1.21*	0.0-0.35
VLCFA in fibroblasts		
C22:0	0.766 µg/protein mg	0.90 ± 0.40
C26:0	1.278 µg/protein mg	0.07 ± 0.04
C26:1	1.389 µg/protein mg	0.09 ± 0.07
C26:0/C22:0	1.668	0.08 ± 0.03
Plasmalogen synthesis		
3H/14C	0.750	0.67 ± 0.19
Catalase solubility		
Catalase distribution (soluble %)	39.9	< 10%
Phytanic acid-14C oxidation		
Phytanic acid oxidation (pml/48h/protein mg)	309.3	32.8 (% control mean)
Peroxisomal immunocytochemistry	Abnormal morphology	

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Treatment for D-BP deficiency is very limited. The Kennedy Krieger Institute recommends modification of the diet to avoid the accumulation of phytanic acid, which was in fact implemented. However, the two other recommendations by this institute— a dietary supplementation with docosahexanic acid and therapy with biliary acids— were not possible since they are not available in Mexico. Although these measures do not affect disease evolution, they can forestall its progression.

At 8 months of age, the patient showed severe visual and auditory dysfunction, dysphagia, gastro esophageal reflux, recurrent bronchopneumonia and sensory-motor polyneuropathy. An electroencephalogram showed moderate differentiated background activity, as well as increased right parietal cortical and left front central excitability. The patient showed myoclonic epilepsy, non-cortical lissencephalic dysplasia, delayed myelination, irregular neuronal migration, macrocephaly, dysgenesia of the corpus callosum, pachygyria, developmental delay and progressive multisystemic deterioration. There was multidisciplinary follow-up of the patient until the time of death, which was caused by pneumonia at 15 months of age [10]. Postmortem analysis indicated bilateral atrophy of the

adrenal cortex, demyelination in white matter, an abnormal anatomy (Fig. 1), mixed inflammatory infiltrate, astrocytosis, interstitial pneumonitis, bacterial bronchopneumonia, pulmonary edema, acute esophagitis, accumulation of histiocytes in thymus and lymph nodes, hepatomegaly with central lobular cholestasis, and congestive splenomegaly. All tests were performed with prior informed consent.

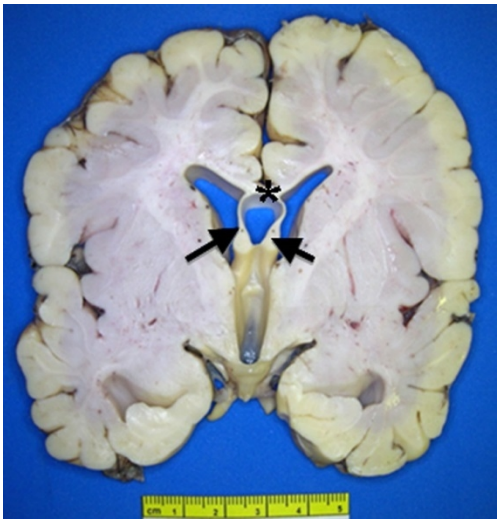

Fig. 1. Gross neuropathology, showing a thick cerebral cortex, thin corpus callosum (*) and duplicated septum pellucidum (arrows).

Fibroblast RNA from patient and healthy controls were used, it was obtained utilizing TRIZOL® (Invitrogen). The criterion for over- or under-expression was a fold change ≥2 (signal log ratio of ≥1 for over-expression and ≤1 for under-expression). The genetic expression profile, determined using Gene system H10KA microarray (MWG-Biotech), was compared to the 10,000 human transcripts available in this data base. The chip was analyzed in ScanArray 4000 (GMI, Inc., USA), finding 348 significantly altered genes, with 210 over- and 138 under-expressed. Two genes are related to peroxisome biogenesis (PEX16 overexpressed; PEX3 under-expressed) and one (ELOVL6 under-expressed) to fatty acid lengthening (Table 2; only selected genes are shown). Under-expression of some genes related with telomere protection and DNA repair was also observed.

For terminal restriction fragment (TRF), we employed the method described by Baur et al. [11]. In brief, 5 μg of each DNA was digested with 20 U of HinfI, HaeIII, and RsaI (New England BioLabs). Restriction products were electrophoresed in 0.8% agarose gels during 20 h at 40 V to obtain appropriate telomere-fragment separation. DNA was transferred onto a Hybond N+ membrane (Amersham Life Science), which was then hybridized against a telomeric ^{32}P probe. Average telomere length was determined by densitometric analysis of autoradiographs using AlphaEase®FC 4.1.0 software (Alpha Innotech Co.). A statistical software package (SPSS 19.0, SPSS, Chicago, IL, USA) was employed for analysis. We found that the patient presented a significant decrease in telomere length compared to the normal value found in healthy children (P= .05) (Fig. 2).

Table 2. Over- and under-expressed genes in a child with D-BP deficiency*

Accession no.	Symbol	Description of possible function	Fold change
Nervous system			
NM_014747	RIMS3	Regulates synaptic membrane exocytosis 3	2.0304
NM_016588	NRN1	Expressed in post-mitotic-differentiating neurons of the developmental nervous system	2.1475
NM_005286	GPR8	An integral membrane protein and G protein-coupled receptor that is primarily expressed in the brain	2.1498
NM_004615	TM4SF2	A cell surface glycoprotein that may play a role in the control of neurite outgrowth	2.2066
NM_003716	CADPS	Encodes a neural/endocrine-specific cytosolic protein and peripheral membrane protein required for Ca2+-regulated exocytosis of secretory vesicles	2.2328
NM_003385	VSNL1	Modulates intracellular signaling pathways of the central nervous system	2.4083
NM_005599	NHLH2	Expressed in the developing nervous system during murine embryogenesis	2.9300
NM_016223	PACSIN3	An up-regulator of signaling for pro-HB-EGF shedding induced by TPA and angiotensin II	2.1859
NM_002442	MSI1	Expressed predominantly in the fetal and adult brain	2.6779
NM_000869	HTR3A	Encodes subunit A of serotonin	2.6959
NM_025236	RNF39	Plays a role in an early phase of synaptic plasticity	2.0809
NM_005096	ZNF261	Expressed most abundantly in the brain	2.7152
NM_003834	RGS11	Increases GTPase activity of G protein alpha sub-units, thus inhibiting signal transduction	2.8108
NM_004877	GMFG	Identified as a growth and differentiation factor acting on neurons and glia in the vertebrate brain	-2.087
NM_001387	DPYSL3	Exclusively associated with neurons, particularly at neuromuscular junctions	-2.110
NM_004495	NRG1	NRG is possibly an axon-associated survival signal for developing oligodendrocytes	-2.666
NM_031500	PCDHA2	Likely plays a critical role in the establishment and function of specific cell-cell connections in the brain	-2.435
NM_007124	UTRN	Located at the neuromuscular synapse and myotendinous junctions, where it participates in maintenance of post-synaptic membranes	-2.056
NM_015678	NBEA	Possible involvement in neuronal post-Golgi membrane traffic	-2.350

Table 2 continues……

NM_018933	PCDHB13	Likely plays a critical role in the establishment and function of specific cell-cell neural connections	-2.143
NM_003269	NR2E1	Tlx is possibly involved in transcriptional control of undifferentiated neuroepithelial cells in the anterior regions of the developing vertebrate brain	-2.101
NM_021728	OTX2	Acts as a transcription factor and may play a role in brain and sensory organ development	-2.250
NM_004177	STX3A	Plays an important role in the growth of neurites and also serves as a direct target for arachidonic acid	-2.131
NM_001525	HCRTR1	A G-protein coupled receptor, expressed in the hypothalamus, that is involved in the regulation of feeding behavior	-2.725
NM_014353	RAB26	An important regulator of vesicular fusion and trafficking that is predominantly expressed in the adult and fetal brain	-2.570
Peroxisome biogenesis			
NM_003630	PEX3	Peroxisomal biogenesis factor 3, essential for the assembly of functional peroxisomes	-2.239
NM_057174	PEX16	Restores the formation of new peroxisomes, suggesting a role in peroxisome organization and biogenesis	3.1460
Fatty acid lengthening			
NM_024090	ELOVL6	ELOVL family member 6, involved in the elongation of long fatty acids chains	-2.009

**The microarray was designed to include a duplicate for each gene.*

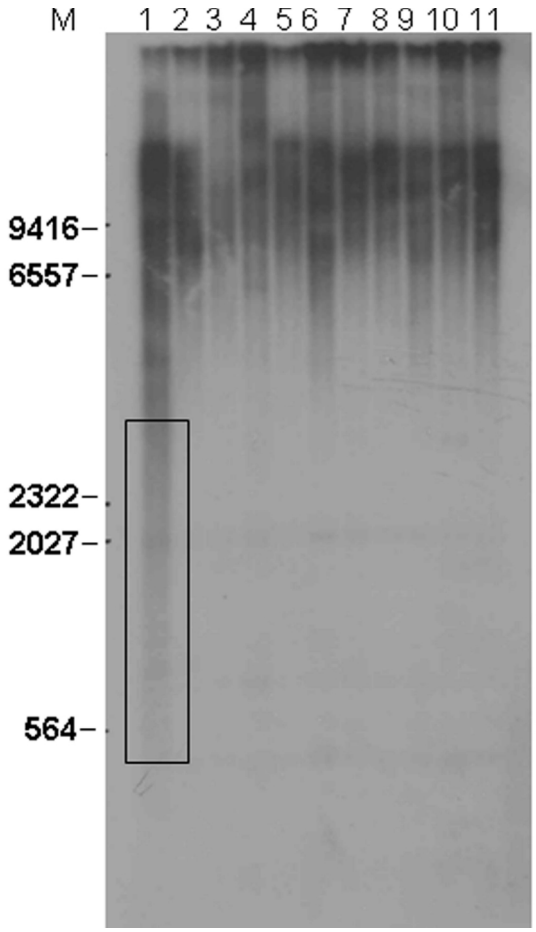

Fig. 2. Autoradiography, representing the mean length of telomeric repeat fragments from genomic DNA. The patient presented a significant decrease in telomere length compared to the normal value found in healthy children (*P* =.05). The molecular weight marker (M) is indicated on the left. Lane 1: D-BP deficiency. Lanes 2-11: Healthy children

3. DISCUSSION

D-BP deficiency is a neurological disease caused by peroxisomal alterations. The characteristics of this disease include delayed neuronal migration, whose most important consequence is the accumulation of VLCFA caused by the abnormal function of peroxisomes in various activities related to lipid metabolism and hydrogen peroxide decomposition [12].

In the patient under study, clinical abnormalities typical of D-BP deficiency were observed. The gene expression profile of this patient showed alterations that could explain such symptoms. 348 significantly altered genes were found (210 over-expressed and 138 under-expressed), including those related to visual function, inflammatory responses, regulation of actin cytoskeleton, cell adhesion, calcium signaling and biosynthesis of steroids (data not

shown). The greatest number of altered genes are implicated in the development and function of the nervous system (neuroactive ligand-receptor interaction and axon guidance pathways), which coincides with the alterations in neuronal growth and development manifested by the patient. Kassmann et al. [13] showed that in oligodendrocytes, peroxisomes serve essential functions for the protection of axons and the maintenance of a noninflammatory environment. Some altered genes, such as GMFB, DPYSL3 and NRG1, may contribute to the development and growth of glia and neurons. Thus, alterations in these processes could account for neocortical dysplasia and neuronal heterotopias that are involved in the deficiency of neuronal migration typical of the disease.

In the patient under study, PEX3 was under-expressed and PEX16 was over-expressed. It is known that *PEX3*, *PEX16* and *PEX19* participate in the peroxisomal protein-import processes and are essential for peroxisome vesicle assembly [14]. *PEX3* is a peroxisomal membrane protein involved in binding to the protein for *PEX19p*. *PEX16* is required for peroxisome membrane protein import. Additionally, *ELOVL6* is needed for LCFA lengthening. Alterations in these genes may have a direct relationship with the abnormal peroxisomal function associated with the disease. It is known that adequate peroxisome function is necessary for brain development, while *PEX*-3 and *-16* participate in importing peroxisomal proteins that are essential for vesicle assemblage [15,16]. Similarly, *PEX19* cannot be recruited without *PEX3,* a deficiency of which would leave the peroxisomes dysfunctional [17]. Also necessary for biogenesis of the peroxisomal membrane is *PEX16*, whose transcript was found to be over-expressed [18]. However, the possible effect of this over-expression on the regulation of the peroxisomal membrane is unknown. On the other hand, one member of the ELOVL family— *ELOVL6*— could be important for fatty acid lengthening. Recently, it was shown in mice that an ELOVL6 deficiency leads to suppression of both synthesis and degradation of fatty acids [19].

Knockout models illustrate that peroxisomes in neurons seem to only marginally contribute to metabolism in the brain, whereas peroxisomes in both astrocytes and oligodendrocytes have an essential role in the same. Peroxisomes are indispensable for the maintenance of myelinated axons in the brain. Among all peroxisomal β-oxidation enzymes, the only one found to cause neurodegeneration and inflammation when inactivated was the D-bifunctional protein [20].

The patient showed a mixture of very short (<600 bp) and normal telomeres, a condition observed in the elderly and in individuals with chronic degenerative diseases. Altered expression was found in genes POT1 and TIN2, which participate in the formation of the telosomal complex that protects telomeres. Some other genes involved in DNA repair, such as APE1, DDB1, DDB2, LIG3 and LIG4, also showed altered levels of expression. The presence of deficient fatty acid oxidation and the under-expression of genes involved in telomere protection and DNA repair could account for the damage to telomeres that are associated with D-BP deficiency.

4. CONCLUSION

As a first report of the analysis of gene expression and telomere length in a patient with D-BP deficiency, the current results provide interesting insights into this disease. The description of the genes with altered expression and the finding of abnormal telomeric length are both consistent with the deficiency in fatty acid oxidation associated with D-BP deficiency. Further studies are necessary with an increased number of patients in order to

confirm the present findings and provide more information on the possible mechanisms involved in this disease.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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